Chemical Interactions between Drugs Containing Reactive Amines and Acrylates in Aqueous Solutions

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Presented to

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by

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Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of PhD is entirely my own work, and that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: _____ (Candidate) ID No.: 10118420 Date: _____

Dedication

for Paul and Helen

Acknowledgements

I would like to thank my supervisor Dr. Blánaid White for her encouragement, guidance and perseverance throughout my research project. That all of this was managed at a distance was even more remarkable and is testament to both her experience and exceptional organization skills. For direction and insight on all things organic I am indebted to Dr. Kieran Nolan at the School of Chemical Sciences.

I would also like to thank my employer, Allergan for funding this project. I am very grateful for the opportunity to complete my research and their financial support and encouragement is very much appreciated. Thanks especially to a number of people who were instrumental setting up and maintaining the collaboration with DCU; Siobhan, Mary, Ayleen and Lorraine and to the 'students' Derrick, Aidan, Adrian and Tricia who understand the challenges of part-time study and allowed me to keep things in perspective.

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Abbreviations

AA	Acrylic acid
ACN	Acetonitrile
ALC	Alcaftadine
API	Active pharmaceutical ingredient
BADGE	Bisphenol A diglycidyl ether
BMT	Brimonidine tartrate
BUN	Levobunolol Hydrochloride
DABCO	1,4-diazabicyclo[2.2.2]octane
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DOE	Design of Experiment
EA	Ethyl acrylate
EPT	Ephinastine Hydrochloride
ESI	Electrospray ionisation
Et_2O	Diethyl ether
FDA	US Food and Drug Administration
GAT	Gatifloxacin
GSH	Glutathione
HPLC	High performance liquid chromatography
HILIC	Hydrophilic interaction liquid chromatography
ICH	International Conference on Harmonisation
IOP	Intraocular pressure
IPP	1-Phenyl piperazine
LDPE	Low-density polyethylene
MA	Methyl acrylate
MeOH	Methanol
MVK	Methyl vinyl ketone
NMR	Nuclear magnetic resonance
OFL	Ofloxacin
OFAT	One factor at a time approach
PEG	Poly ethylene glycol

PGS	Pregelatinized starch
PHN	Phenylephrine Hydrochloride
PI	Photo-initiators
PTFE	Polytetrafluoroethylene
QbD	Quality by design
RH	Relative humidity
RRF	Relative Response Factor
Rs	Resolution Factor
r.t.	Room temperature
SFC	Supercritical fluid chromatography
SFE	Supercritical fluid extraction
SPE	Solid phase extraction
tG	Gradient run time
THF	Tetrahydrofuran
TIM	Timolol Maleate
TLC	Thin layer chromatography
UHPLC	Ultra High performance liquid chromatography
USP	United States Pharmacopeia
UV	Ultra Violet

Abstract

Chemical Interactions between Drugs Containing Reactive Amines with Acrylates in Aqueous Solutions

Mary Mc Grath

Acrylate monomers are widely used components of inks, varnish and adhesive applied to labels for pharmaceutical packaging. LDPE bottles used to dispense ophthalmic solutions are generally a poor barrier to volatile compounds which may migrate both into and out of the bottle. The mild reaction conditions of the aza-Michael addition of a nitrogen containing drug substance and unreacted acrylic monomers migrating from pharmaceutical packaging mean that this is a feasible mechanism by which unwanted adducts could be formed in prepared drug formulations.

The reaction stoichiometry, temperature and rate of stirring were investigated for conjugate addition of 1-phenylpiperazine to methyl acrylate under solvent free and aqueous conditions. A number of common organic solvents were screened. Significant rate acceleration of this reaction was observed in polar protic compared to aprotic solvents.

Chemical reactions between 1-phenylpiperazine.HCl with methyl acrylate and acrylic acid, in aqueous buffered solutions were investigated. Products were identified by UPLC-Q-TOF/MS. Both acrylic acid and the amine salt were unreactive under nominal reaction conditions. However, the amine salt reacted with both methyl acrylate and acrylic acid on standing for 6 and 12 days, demonstrating that given sufficient time, even the less reactive amines and acrylates will form adducts. A drug-acrylate compatibility screening model was developed to predict potential stability problems due to interactions of amine drug substances with acrylate leachables in ophthalmic buffered solutions. Eight ophthalmic formulations containing various amine drugs (primary, secondary, tertiary and salt counter-ions) were spiked with acrylates and tested for the formation of acrylic adducts.

This case study demonstrates that leachable compounds that migrate into the drug product can react with the active ingredients to form impurities and the results obtained here strongly suggest that formation of amine-acrylate adducts may constitute a significant problem upon long-term storage of ophthalmic solutions in their final packaged configuration.

Chapter 1:

Literature Review

Mechanisms of the aza-Michael Reaction in Pharmaceutical Formulations

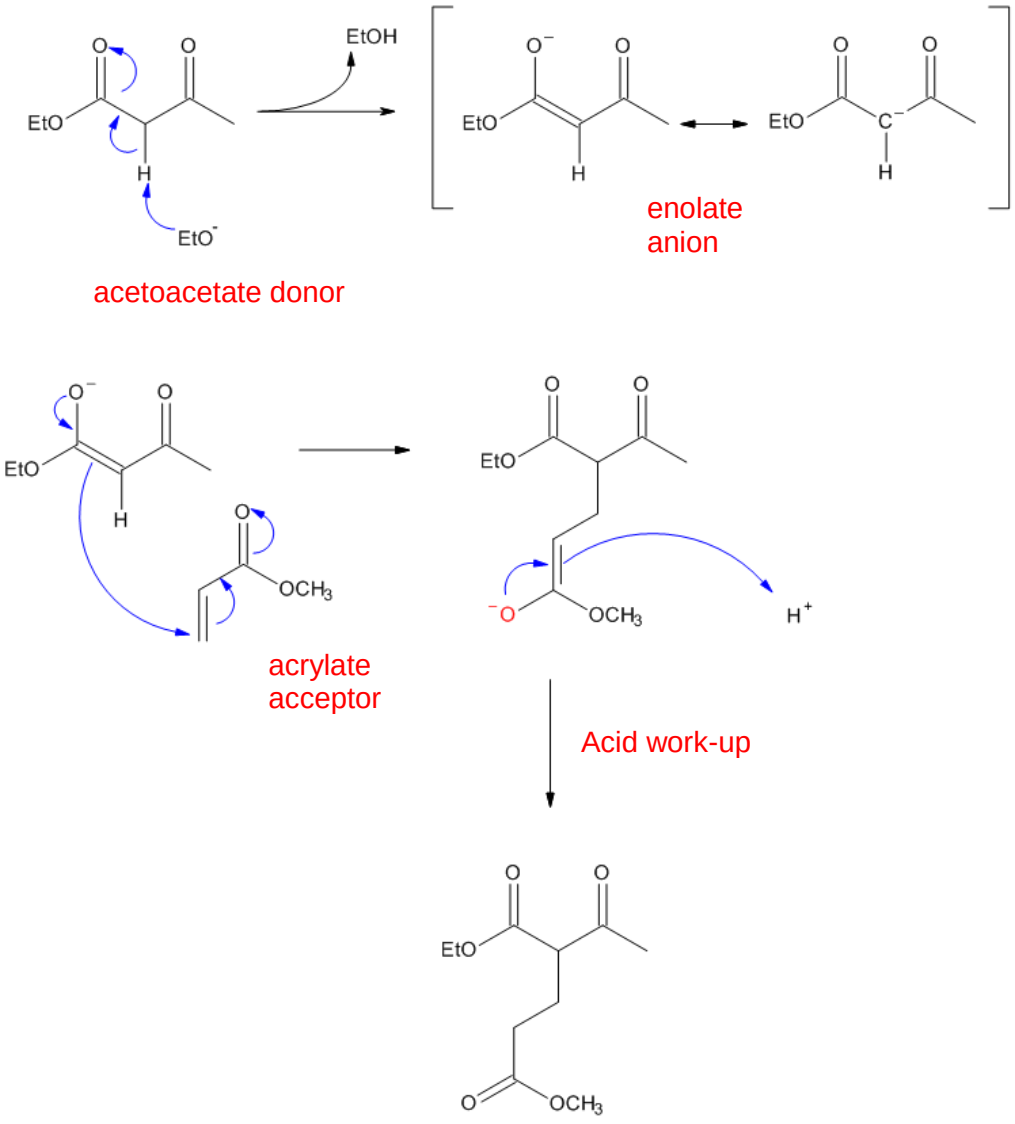
1.0 Introduction to the Michael Addition Reaction

The Michael addition [1, 2] is a conjugate addition reaction, and is one of the most useful ways to create carbon-carbon bonds. It describes the addition of a nucleophile, the Michael donor, to an activated electrophilic olefin (usually an α,β-unsaturated carbonyl compound), the Michael acceptor, resulting in formation of an adduct.[3] The reaction is noted for high yields under mild reaction conditions and its use is widespread in polymerisation reactions such as the anionic polymerisation of alkyl methacrylates and cyanoacrylates.[4] The classic reaction refers to the base catalysed addition of enolate nucleophiles such as acetoacetic or malonic ester to activated olefins, as shown in Section 1.1.[5]

1.1 Mechanism of the Carbon-Michael Addition Reaction

The classic Michael addition reaction consists of three key steps, as illustrated in Scheme 1. A base catalyst is typically used to deprotonate the Michael donor, generating the enolate anion. The α-carbon of the resulting enolate anion is negatively charged and highly reactive towards the acrylate acceptor. The enolate reacts with an activated α,β-unsaturated carbonyl containing compound via 1,4-conjugate addition at the β-carbon. The intermediate product of a conjugate addition is itself a potential donor *i.e.* an enolate anion, and reaction of the product donor with the acceptor must be controlled to avoid Michael polymerization. The last step of the reaction involves rapid proton transfer to produce the final Michael adduct and regenerate the base catalyst. [5] In the classic Michael condensation the product enolate is much more basic than the donor enolate, and is thus rapidly discharged by protonation by the solvent, other proton donors (*e.g.*, by ethanol if sodium ethoxide is used as base) or by the starting β-dicarbonyl compound. Proton

abstraction from the protonated base regenerates the base catalyst. The reaction is terminated by protonation of the adduct.



Scheme 1: General Carbon-Michael Reaction Mechanism using Acetoactate, Ethyl Acrylate and Sodium Ethoxide as Base. [5]

The kinetics of the Michael addition reaction are dependent upon base type and concentration as well as the concentrations of both the Michael donor and the Michael acceptor. Pre-equilibration of the Michael donor with a base catalyst results

in a steady-state concentration of the enolate anion and a rate law which follows pseudo-first order kinetics with respect to the concentration of the Michael acceptor (acrylate).[6]

Michael addition reactions have been conducted in a wide range of molecular solvents; from non-polar solvents toluene and tetrahydrofuran (THF) to polar solvents such as N,N dimethylformamide (DMF), dimethylsulfoxide (DMSO), methanol (MeOH) and acetonitrile (ACN).[7-9] Ranu and co-workers have shown that imidazolium ionic liquids with a hydroxide counter-anion provide both the reaction medium and the catalyst in a self-catalysed Michael addition of methylene compounds to conjugated ketones and esters.[10] The role of the solvent is tied to that of the catalyst; the synthetic utility of the reaction has expanded with the design of chiral catalysts where the choice of solvent has proved to be a controlling factor in the enantioselectivity of the product. [11-13]

A review of the literature would suggest that 1,3 dicarbonyl and nitroalkane compounds are the starting point for most Michael carbon donor selection and α, β-unsaturated carbonyl compounds the predominant choice for Michael acceptors. [9, 14, 15] The synthetic utility of the reaction is due in part to the wide range of donors and acceptors that can be employed in this reaction; the variety in acceptors resulting from the many possible activating groups (ketones, aldehydes, esters, amides, nitriles, nitro).

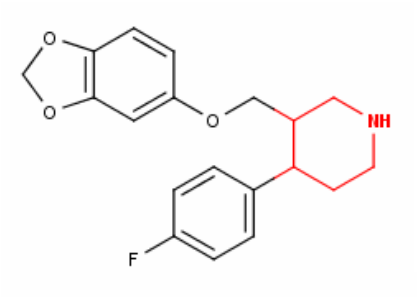
The carbon Michael reaction is driven by base activation of the nucleophile and the literature review has shown that the reaction will not take place in the absence of the base. For example, while extensive work has been carried out by Ballini on the synthetic utility of nitroalkanes as nucleophiles, he has also demonstrated that the carbon Michael reaction cannot occur in the absence of a base.[16] The bulk of the

research carried out relates to developments in the area of organic base catalysts. [6] The limitations of the alkoxide bases saw them replaced by phosphazene and guanidine organobase catalysts and recently by the bi-functional thiourea base catalysts. [17-19]

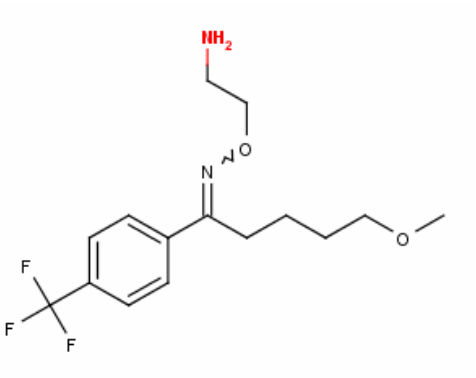
1.2 Aza Michael Reaction

Of particular interest to this study is the amine or 'aza-Michael' reaction. The aza-Michael reaction is a nitrogen-carbon bond forming reaction between a nitrogen nucleophile and an α,β-unsaturated carbonyl compound. Many drug substances contain amines, which are ideal Michael donors (Scheme 2), while pharmaceutical packaging (label ink and adhesives) routinely contains acrylic monomers which could readily act as Michael acceptors. The mild reaction conditions of Michael addition of a nitrogen containing drug substance and unreacted acrylic monomers migrating from pharmaceutical packaging mean that this is a feasible mechanism by which unwanted adducts could be formed in prepared drug formulations.

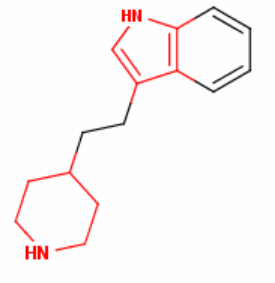
Over the last two decades, the aza-Michael reaction has gained popularity as the mild reaction conditions typically required are in line with the aims of green chemistry *i.e*. the elimination or reduction of volatile solvents in organic synthesis.[20] The reaction is central to the generation of β-amino carbonyl compounds.[21] Among the chemical methods employed in accelerating the aza-Michael reaction are the use of aqueous solutions [22, 23] or hydrogen donor solvents,[24] ionic liquids,[25] highly basic amines [26] and Lewis acid catalysts.[4] Sonication or ultrasound [27, 28] and temperature variation [29] are among the most widely used physical methods.



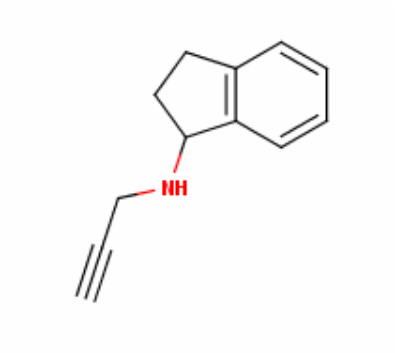
Paroxetine



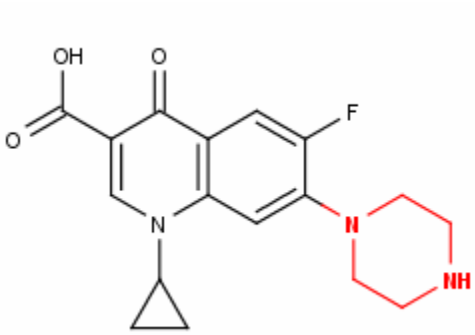
Fluvoxamine



Indalpine



Rasagline



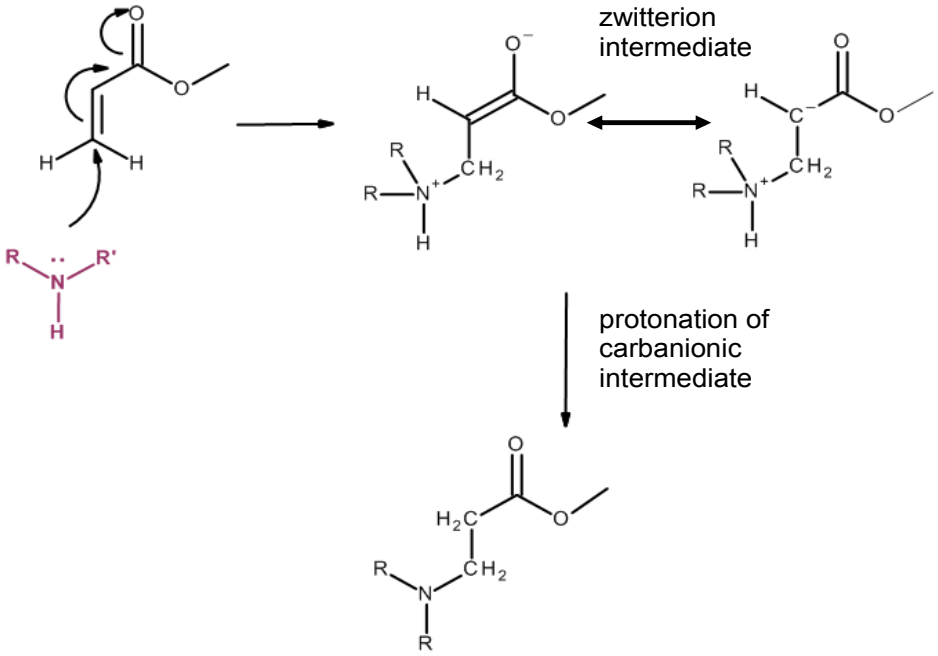
Ciprofloxacin

Scheme 2: Active pharmaceutical ingredients containing potential amine Michael donors, source PubChem 2013.

1.3 Mechanism of the Aza-Michael Reaction

If the amine donor is sufficiently nucleophilic, direct addition will proceed without the addition of an acid or base catalyst, rendering Step 1 of the Michael reaction, which for carbon-carbon bond formation is the rate limiting step (as discussed in Section 1.1), unnecessary. [30] In contrast, the aza-Michael reaction follows second order kinetics based on the concentration of both the amine and the olefin acceptor in

what was Step 2 of the carbon- carbon reaction. The mechanism of the reaction is illustrated in Scheme 3.



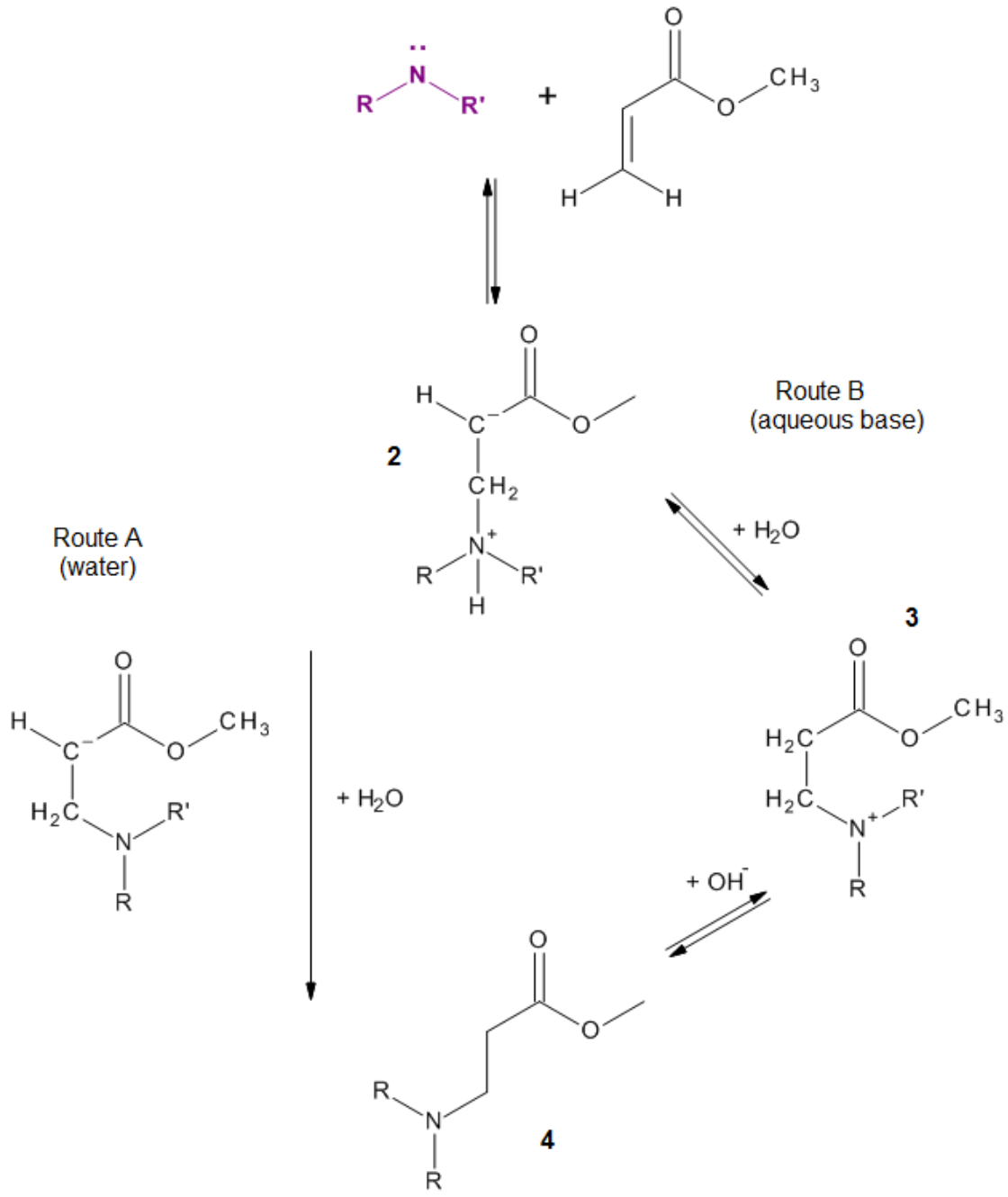
Scheme 3: Mechanism of the Aza- Michael Reaction.[30]

The reaction begins by nucleophilic attack of the secondary amine on the β-carbon of the conjugated alkene acceptor (as before) generating a zwitterionic intermediate. Proton abstraction from the solvent or from the nitrogen of the amine donor is the final step. Once again, the carbonyl group stabilises the resulting anion until proton transfer occurs.

The difference between the reaction equilibrium for primary and secondary amines compared to that of tertiary amines was investigated by Bunting and Heo,[31] and is illustrated in Scheme 4 for primary and secondary amines and Scheme 5 for tertiary amines.

1.3.1 Mechanism for Primary and Secondary Amines

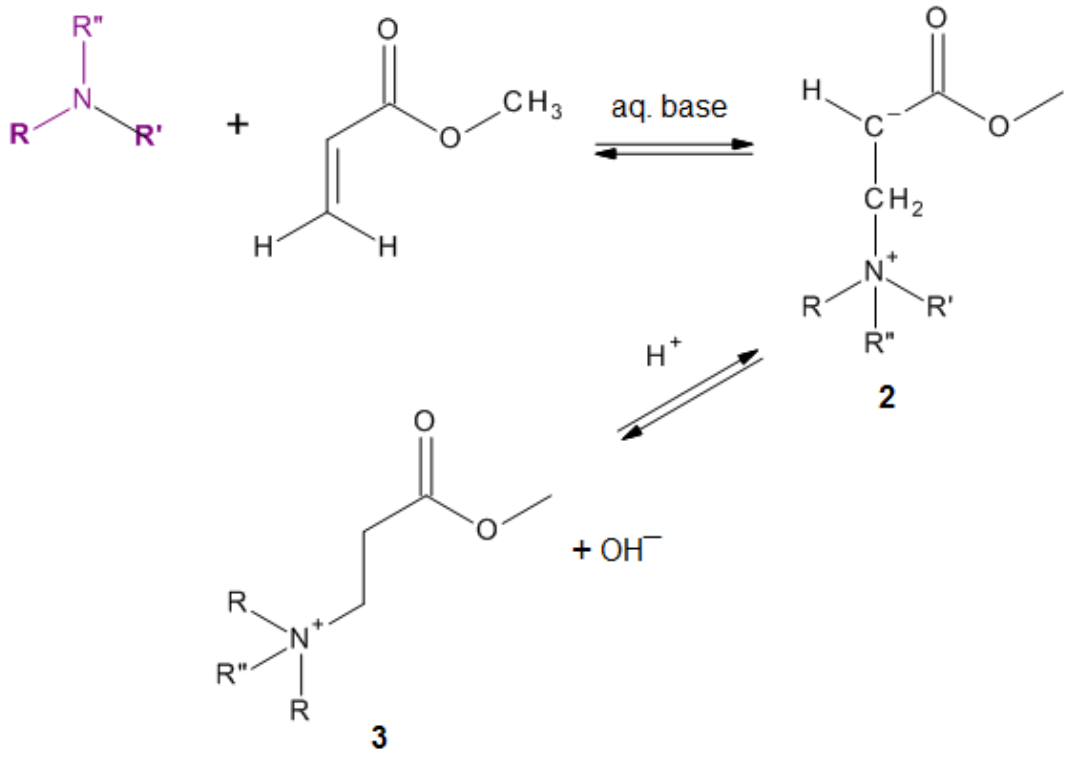
Scheme 4 is a typical aza-Michael nucleophilic addition for primary and secondary amines. Bunting and Heo demonstrated that nucleophilic attack by the amine was the rate limiting step for primary and secondary amines when the reaction was carried out in aqueous base.[31] The scheme involves an additional acid base equilibrium step between **2** and **3** for the deprotonation of the ammonium ion of the carbanionic intermediate **2**. The scheme demonstrates that two possible routes to the protonation of the carbanionic intermediate are available; Route A to **4** via protonation of **2** or Route B to **4** via protonation of **3**. In aqueous base, deprotonation of the ammonium ion first, followed by protonation of the carbanion would be expected to be fast and favour the formation of **3**. Protonation of **3** by water would therefore be expected to be the fastest route. A third option, initial protonation of the negatively charged carbon followed by deprotonation of the amine was dismissed as an unviable pathway.[32]



Scheme 4: Aza-Michael reaction between a secondary amine and methyl acrylate, adapted from Bunting and Heo [31]

1.3.2 Mechanism for Tertiary Amines

For the majority of primary and secondary amines the rate of reaction was determined by nucleophilic attack of the amine. This was not the case for tertiary amines where the rate limiting step proved to be protonation (by a water molecule) of the carbanionic intermediate **2**.[31]. Subsequent protonation needed to be sufficiently rapid (seconds) in order that the intermediate did not revert back to the starting amine. The reaction was carried out in aqueous base (Scheme 5). The product **3** retained the net positive charge of the ammonium ion.



Scheme 5: Aza-Michael Reaction between a tertiary amine and methyl acrylate, adapted from Bunting and Heo [31]

1.4 Amine Nucleophilicity

If the amine donor is sufficiently nucleophilic, no catalyst is required and the driving force behind the reaction is the nucleophilicity of the amine donor. Studies of amine reactivity examine the role of the amine nucleophile rather than its action as a base catalyst, *i.e.* their direct addition to the reference electrophile (R^+) and the stability of the product following protonation of the intermediate [33]

Nucleophilic reaction: $R^+ + R'_2NH \longrightarrow RNR'_2 + H^+$

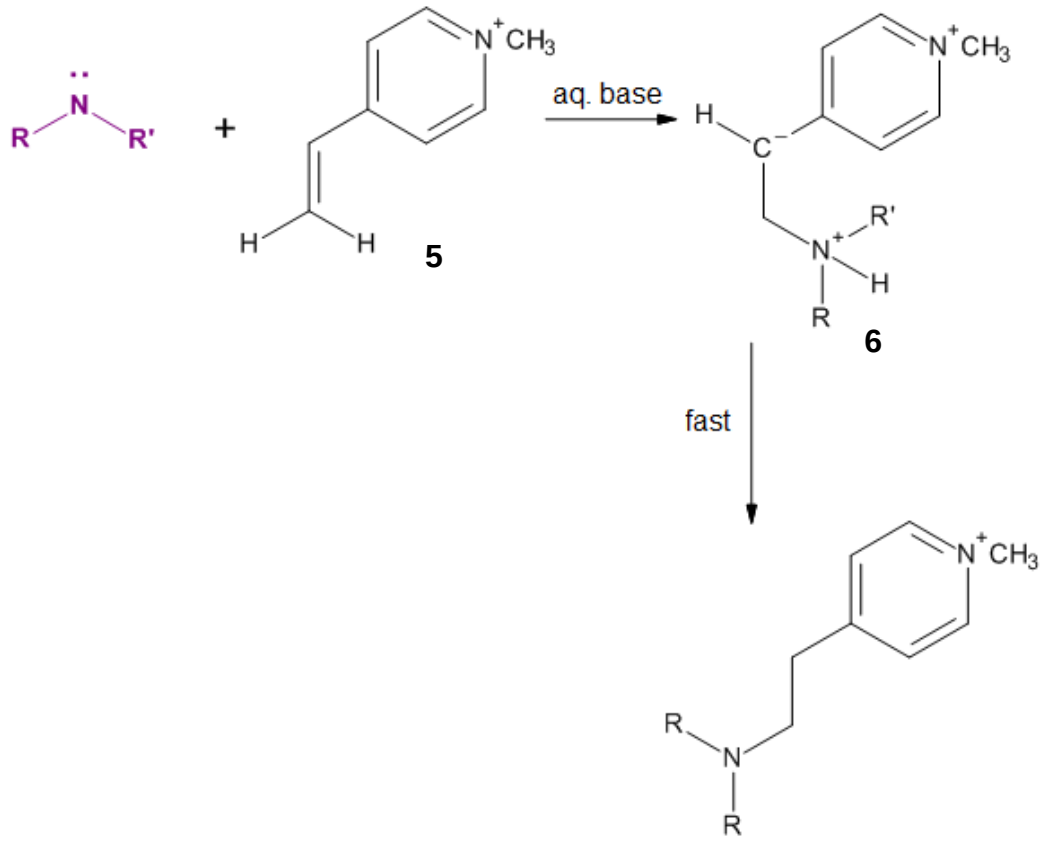
General base catalysis in water: $R^+ + H_2O \xrightarrow{R'_2NH} ROH$

General base catalysis of amine: $R^+ + R'_2NH \xrightarrow{R'_2NH} RNR'_2$

For successful nucleophilic reactions of primary or secondary amines, proton loss from the ammonium ion must be faster than the reverse reaction as illustrated in Scheme 4. Several scales exist, but the order of nucleophilicity (*N*) for primary and secondary amines shows good general agreement using a variety of reference electrophiles.[30, 31, 34-39]

The reactivity of a given nucleophile is dependent on the substrate, solvent and reaction conditions *i.e.* the nucleophilicity of an amine can change from one reaction to the next. [31] As such, it is not possible to determine whether an amine donor requires a catalyst to initiate Michael addition without knowing the solvent and reaction conditions. The most comprehensive scale of amine nucleophilicity was established by Bunting and Heo (1994) using a single acceptor and solvent system.[31] The study investigated the reactivity of 91 amines toward the 1-methyl-4-vinylpyridinum cation **5** (Scheme 6) in aqueous base at 25 °C.

The study demonstrated that the nature of the amine substituent plays a greater role than basicity in the aza-Michael reaction, as discussed below. This poor correlation between nucleophilicity and basicity is in agreement with the earlier work of Richie. [40].



Scheme 6: Aza- Michael reaction for secondary amine with 1-methyl-4-vinylpyridinum cation **5** [30]

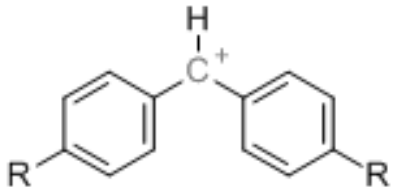
1.4.1 Primary and Secondary Amine Nucleophilicity

Contrary to expectation, the less hindered primary amines are often less reactive than secondary amines of the same basicity. Bunting and Heo divided primary, secondary and tertiary amines into a number of sub-classes based on structural features, *e.g.* substitution at the α and β carbon of the amine donor.[31]

For the primary and secondary amines examined it was noted that reactivity decreased with increasing substitution at the α carbon of the amine.[31] This was attributed to the fact that increasing steric hindrance at the α carbon atom of the amine led to an increase in non-bonded interactions in the carbanionic intermediate (**6)** following nucleophilic attack on the Michael acceptor.

Reactivity increased in the case of primary and secondary amines in which the β carbon of the amine was unsaturated (sp^2 or sp hybridized).[31] The increase in π electron density on the β carbon atom increased reactivity regardless of the electro-negativity of the atom attached *e.g.* carbonyl, vinyl and nitrile all showed an increase in reactivity. The enhanced reactivity was also observed in the aromatic primary amine, benzylamine. In general, secondary amines were found to be more reactive than primary amines of the same basicity. The increase in reactivity is attributed to the role played by the additional alkyl group of the secondary amine in stabilising the positively charged intermediate (**6)** formed following nucleophilic attack on the electrophile. It is this stabilising influence that is thought to be responsible for increased reactivity rather than any role the electron donating alkyl group might play in activating the amine lone pair of electrons prior to nucleophilic attack.

A 2007 study by Mayr supports the earlier findings or Bunting and Heo regarding the general reactivity of primary and secondary amines.[30] Using a variety of benzhydrylium ions (**7**) (Ar_2CH^+) as the electrophile, Mayr noticed a dramatic increase in reactivity when the hydrogens of ammonia were replaced by one and two alkyl groups.



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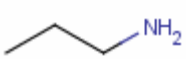
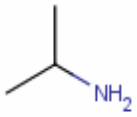
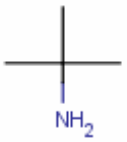
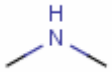
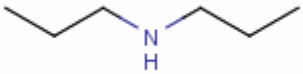
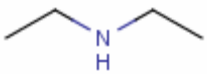
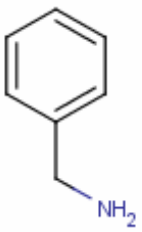
The nucleophilicity parameter (*N*) increased across the series from ammonia (*N* = 9.48) to methylamine (*N* = 10.66) to dimethylamine (*N* = 17.12).[30] The increase in nucleophilicity was attributed to the decrease in hydration energy as each of the hydrogen atoms of ammonia was replaced by a methyl group. [33] The nucleophilicity parameters *N* calculated by Mayr are listed in Table 1.1 alongside the pK_{aH} of the various amines.

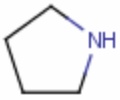
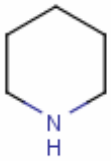
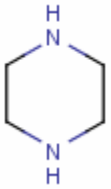
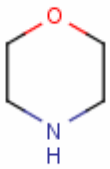
Table 1.1 Nucleophilicity Parameters (*N*) Calculated by Mayr for Primary and Secondary Amines in Water and Acetonitrile [30, 38]

Primary and Secondary Amines		**pK_{aH}** [41]	**N, Water** [30]	**N,ACN** [38]
Ammonia	NH_3	9.21	9.48	
n-Propylamine		10.53		15.11
n-Butylamine		10.59		15.27
Methylamine		10.62	13.85	
Ethylamine		10.63	12.87	
Isopropylamine		10.63	12.00	13.77
t-Butylamine		10.86	10.84	12.35
Dimethylamine		10.64	17.12	
Di-n-propylamine		11.00		14.51
Diethylamine		11.02	14.68	15.10
Benzylamine		9.34	13.44	14.29

Steric factors played a greater role in the nucleophilicity of secondary amines compared to primary amines. For example, the *N* parameter for methylamine and ethylamine are 13.85 and 12.87 respectively whereas those for dimethyl and diethylamine are 17.12 and 14.68 in the Mayr study.[30] Similar differences were observed by Bunting and Heo in the earlier study.[31]

Compared to acylic amines, their cyclic analogs have a higher basicity; the N atom in the ring is less sterically hindered and therefore more easily prontonated. Piperidine and related cyclic unsaturated secondary amines were 1.9 times more reactive than N-Methyl secondary amines and 8.37 times more reactive than N-Ethyl secondary amines.[31] Bunting and Heo observed that reactivity decreased four fold with increasing ring size from a five-membered (pyrrolidine) to an eight-membered ring (perhydroazocine) despite there being very little difference in basicity for the ring amines (pKa in the range 11.00 to 11.27). However, the reverse trend was noted by Mayr.[30] In this case, the 5 membered ring was the least nucleophilic, with nucleophilicity increasing with ring size. The difference in reactivity for the amine rings demonstrates that nucleophilicity is reaction specific *i.e.* attack on the vinylic carbon electrophile generates an ammonium ion which must be stabilised, whereas the benzhydrylium cation used by Mayr yields a neutral adduct. See Table 1.2 for nucleophilicity parameters *N* calculated by Mayr.[30, 38]

Table 1.2 Nucleophilicity Parameters Calculated by Mayr for Cyclic Aliphatic Amines in Water and Acetonitrile [30, 38]

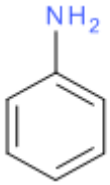
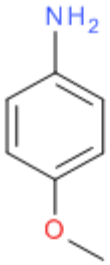
Cyclic Aliphatic Amines		**pK_{aH}** [41]	**Nucleophilicity parameter, N in water** [30]	**Nucleophilicity parameter, N in ACN** [38]
Pyrrolidine		11.27	17.21	18.64
Piperidine		11.12	18.13	17.35
Piperazine		9.72	17.22	n/a
Morpholine		8.36	15.62	15.65

Overall, the Mayr study supports the earlier findings regarding the general reactivity of primary and secondary amines.[30] The results for the reactivity of primary and secondary amines reacting with acrylamine in aqueous solution [40] were also in good agreement with the findings of Bunting and Heo. [31]. Irrespective of the choice of electrophile or solvent, a variety of studies confirm that the correlation between nucleophilicity *N* and basicity (pK_{aH}) is poor for amines, with the *N* parameter providing a better indication of reactivity for several classes of amine.

1.4.2 Aniline Nucleophilicity

Though considerably less basic than ammonia, aniline proved to be a much stronger nucleophile when reacted with the benzhydrylium ions (**7**) in both water and acetonitrile.[30] The nucleophilicity parameter (*N* = 12.99) of aniline was similar to that of primary alkyl amines, as shown in Table 1.1. However, reversibility of the initial attack was noted when less electrophilic benzhydrylium ions were used and a higher excess of aniline was required for the reaction; a linear relationship between the concentration of the amine and the rate of reaction showed that the attack of the amine on the benzhydrylium ion remained the rate determining factor. The β-carbon effect noted by Bunting [31] is evident in the nucleophilicity of benzylamine (*N* = 13.44), which has a pKa_H close to that of ammonia but more reactive than aniline (see Tables 1.1 and 1.3)

Table 1.3 Nucleophilicity Parameters Calculated by Mayr for Aromatic Amines in Water and Acetonitrile [30, 38]

Aromatic Amines		**pK_{aH}**	**Nucleophilicity parameter, N in water** [30]	**Nucleophilicity parameter, N in ACN** [38]
Aniline		4.59 [42]	12.99	12.64
4-Methoxyaniline		5.16 [42]	16.53	13.42

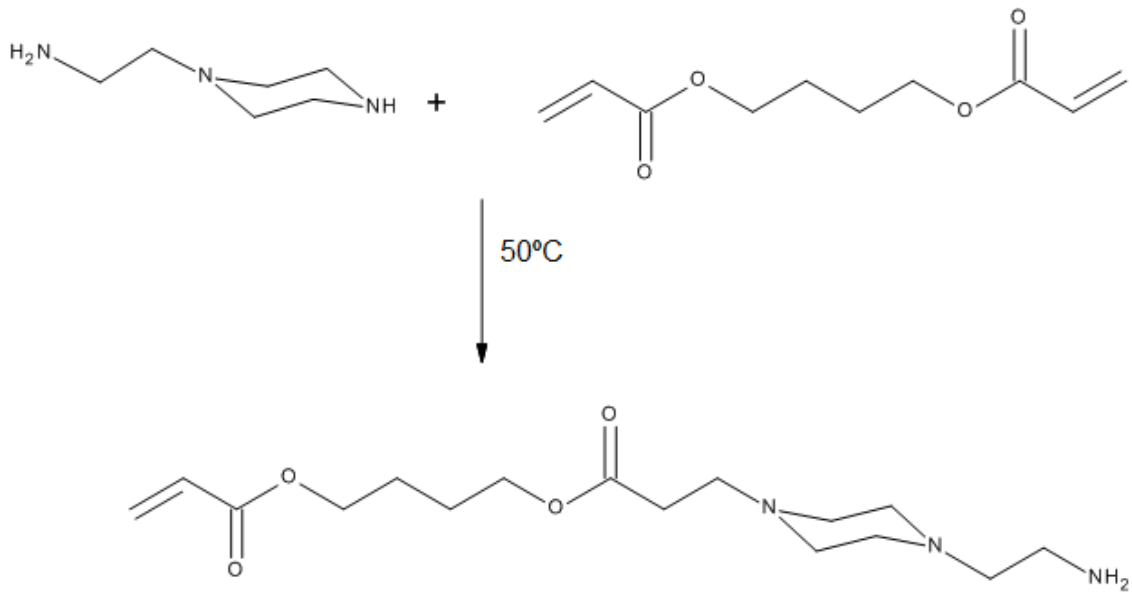
1.4.3 Tertiary Amine Nucleophilicity

The zero order linear relationship noted between nucleophilicity and basicity for groups of primary and secondary amines was not replicated for tertiary amines. There was a poor relationship between the reactivity (nucleophilicity) and basicity of the amines investigated by several groups.[31, 40, 43] In general tertiary amines were less reactive than primary and secondary, with trimethylamine proving unreactive.[31] The N, N-dimethyl amines [$XCH_2CH_2N\text{-}(CH_3)_2$] did react providing that X was an oxygen or nitrogen containing constituent. The most reactive tertiary amine was N-methyldiethanol amine, which was one of the least basic studied.[31] The cyclic amines N-methylpyrrolidine, N-methylpiperidine and N-methylmorpholine all proved unreactive with the vinylic electrophile employed by Bunting.

Mayr investigated the same tertiary amines using the same methodology that had been previously used to qualify the nucleophilic reactivities of the primary and

secondary amines.[44] The reactions were monitored by a colour change as the benzhydrylium ions were coloured and the reactions with the amines yielded colourless adducts. However, the formation of the quaternary ammonium salts proved thermodynamically unfavourable and the methodology used previously could not be applied successfully. The reaction with triethylamine was highly reversible and could not be measured directly. In addition the reaction was carried out in the aprotic solvents acetonitrile and dichloromethane which would not be expected to promote the reaction as was demonstrated by Bunting.[31]

A ‘real life’ application of the scale of amine reactivity was demonstrated in an investigation into the aza-Michael reaction of trifunctional amines and diacrylates by Wu *et al*.[45]. The reaction was carried out in chloroform and monitored in-situ using NMR. The initial reaction between the diacrylate (1, 4-butanediol diacrylate) and 1-(2-aminomethyl) piperazine took place exclusively at the secondary amine on the piperazine, with an 80% conversion within 2 hours, as shown in Scheme 7. The reaction at the primary amine and subsequent polymerisation was monitored over a period of 50 hours. No reaction took place at the tertiary amine on the piperazine, supporting the contention by Bunting and Heo[31] that nucleophilic attack by the amine is no longer the rate limiting step for tertiary amines; rather the ease at which the carbanionic intermediate is protonated by the reaction medium now determines whether the reaction goes to completion or not.



Scheme 7: Higher reactivity of secondary amines in aza-Michael addition. The reaction took place exclusively at the secondary amine on the piperazine. Adapted from Wu *et al* [45]

1.4.4 Solvent Effect on Amine Nucleophilicity

Mayr also noted that the rates of the reactions of amines with benzhydrylium ions were strongly affected by solvent polarity.[30] Anilines reacted 2 times faster in water than in acetonitrile. The authors reasoned that hydrogen bond stabilisation of anilines in water plays a minor role because of their low basicity.

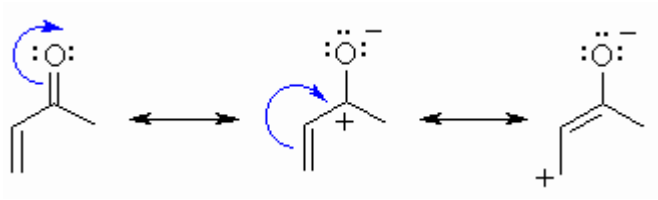
Previously Mayr had determined that the rates of reaction of carbocations with neutral π and σ nucleophiles were only slightly affected by solvent polarity because charges are neither created or destroyed in the rate determining step, but this was found not to be the case for amines.[46] Amine nucleophiles were strongly dependant on the solvent.[38] For amine nucleophiles, the rate of reaction decreased with increasing polarity of the solvent (E_T^N values). For example,

morpholine reacted 72 times more slowly in water than in DMSO.[30] Solvation of amines in acetonitrile is still a significant factor as the intermediate tertiary ammonium ion formed is generally a stronger acid than the corresponding secondary or primary ion.[34] See Tables 1.1, 1.2 and 1.3 for details of nucleophilicity parameters (*N*) determined in acetonitrile and water. As with water, the nucleophilic reactivities of the amines in acetonitrile correlated poorly with their corresponding pK_{aH} values. Aniline was found to be 5 times more nucleophilic in water than n-propylamine despite the higher basicity of the aliphatic amine.[30] In acetonitrile the opposite was observed; primary alkylamines were 10 times more reactive in acetonitrile than in water. This reversal was attributed to the different solvent effects on the aromatic and aliphatic amines; either decreased solvation of aromatic amines or increased solvation of aromatic ammonium ions by water. [34] However, the reactivity of aniline in acetonitrile is still considerably higher than would be predicted on the basis of its basicity.[38].

1.5 Michael Acceptor

Michael acceptors, such as α,β-unsaturated carbonyl compounds, are more stable than non-conjugated carbonyl compounds. α,β-Unsaturated ketones and aldehydes are also more polar than simple ketones and aldehydes. Interaction between the π electrons of the C=C double bond and those of the C=O group leads to a partial delocalization of the π electrons across all four atomic centres. The carbonyl group is therefore crucial to the success of the overall Michael reaction. Without it, the C=C double bond would not be polarised and no transfer of electron from the acetoacetate donor to the acrylate acceptor would occur. The resonance structures

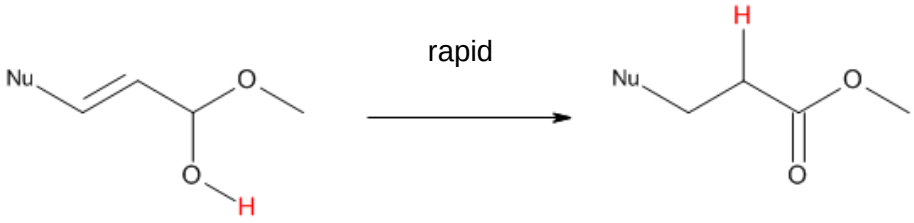
of an α, β-unsaturated carbonyl acceptor (Scheme 8) show that the positive charge is allylic and is shared by the β-carbon, rendering it electrophilic.



Scheme 8: Resonance structures of α, β-unsaturated carbonyl compound [3]

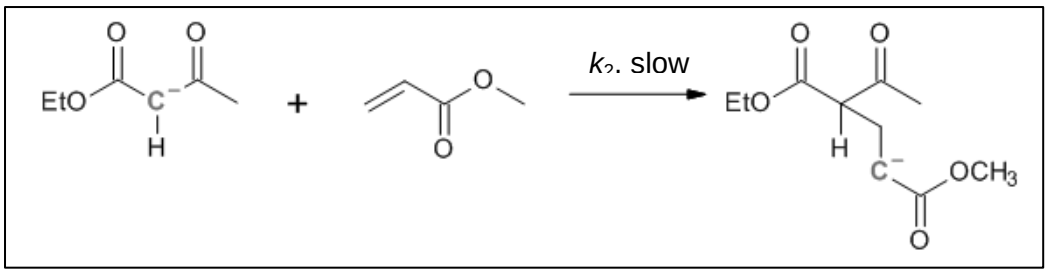
Nucleophilic addition of the donor can take place at either of two sites: at the carbonyl carbon (direct 1,2-addition) or at the electrophilic β-carbon of the acceptor to give the conjugate (1, 4-addition) product. Conjugate addition is favoured over the competing 1, 2-addition of the enolate since the more stable carbon–oxygen π bond is maintained (versus the less stable carbon–carbon π bond). [5]

The 1,4 adduct is almost always thermodynamically more stable, so selecting conditions where the 1,2-addition is reversible will result in formation of 1,4 products. 1,4-addition results in a ketone-enol tautomer. At room temperature the chemical equilibrium of the two forms is thermodynamically driven and favours the keto form, as illustrated in Scheme 9 and Scheme 10.



Enol Tautomer (less favoured) Keto Tautomer (favoured)

Scheme 9: The product of 1,4-addition is an enol that will tautomerize rapidly at room temperature to the more stable carbonyl compound, *i.e*. the thermodynamic product.[3]



Scheme 10: 1,4 conjugate addition of enolate anion to the β-carbon of acrylate

Predicting Michael acceptor reactivity as a determinant of their toxicity has been the subject of a number of studies which use both experimental and computational calculations. The model nucleophile methane thiol, glutathione (GSH), acts as the donor in a buffered aqueous solution. A 10 fold difference in reactivity was observed between acrylates and their methacrylate analogs when reacted with GSH in a buffered non-enzymatic system.[47] The difference was attributed to (i) steric hindrance as a result of α-methyl substitution and (ii) a decrease in the partial positive charge on the β-carbon of the methacrylates. 2-Hydroxy ethyl acrylate was found to be the most reactive ester, with the addition of the hydroxy group leading to enhanced electrophilicity. α,β-Unsaturated aldehydes, ketones and esters

(acrylates) were the subject of a 2011 study, again using the GSH model nucleophile.[48] The acceptors were further divided into sub-groups depending on the level of substitution at the α and β carbon; those with no substitution at either the α or β-carbon, α-carbon only, β-carbon only and both the α and β-carbons. The α and β substitution have distinctive effects on reactivity *i.e.* steric accessibility to the β-carbon has an impact on reactivity. The relative reactivity's are determined by the 2nd-order rate constant of the reaction with glutathione (GSH), with both experimental and predicted values, *k*GSH (L mol^{-1} min^{-1}). Results in Table 1.5 have been reproduced from Mulliner *et al*. [48]

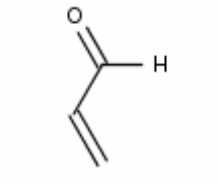
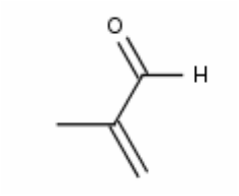
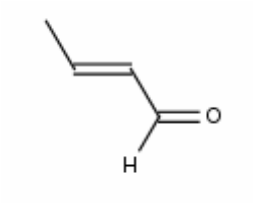
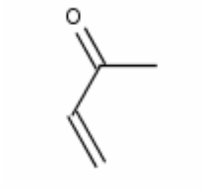
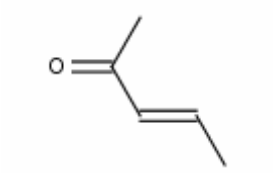
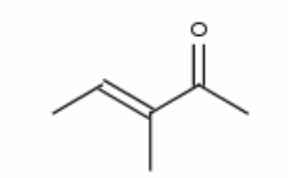
The effect of substitution at the β carbon was shown in a base catalysed (0.025M aq. NaOH,) reaction between nitromethane and various acrylates.[16] Increasing the ester alkyl chain from methyl to ethyl slowed the rate of reaction from 1 to 2 hours but the addition of a methyl group at the β-carbon increased the reaction time to 15 hours with a reduction in yield from 76 to 60% (see Table 1.4). The following reaction times and yields were recorded by Ballini.[16]

Table 1.4 Reaction time and yield for addition of Nitromethane to Various Acrylates [16]

	Time hours	%Yield
Methyl acrylate	1	85
Ethyl acrylate	2	76
n-Propyl acrylate	1	68
Ethyl 2-butenoate	15	60

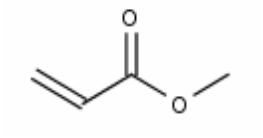
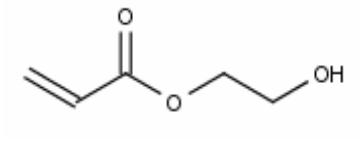
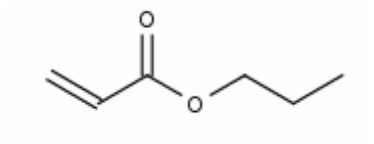
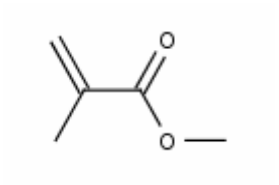
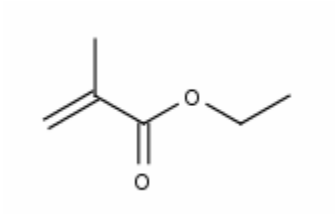
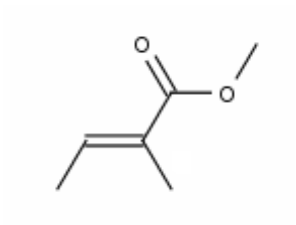
In terms of general reactivity, aldehydes were found to be more reactive than ketones, and ketones more reactive than esters. The polarizing effect of the carbonyl oxygen is responsible for activation of the β-carbon. This partial negative charge is somewhat diluted by the acetate oxygen of the ester. As the electron density is spread between the two oxygen's, the positive charge on the β-carbon is reduced making it less electrophilic. This general acceptor reactivity (aldehyde > ketone > ester) appears to be a feature of the buffered aqueous conditions used in the GSH studies and was not observed in other studies using amine donors as the nucleophile. In a neat reaction using pyrrolidine as the Michael donor, Ranu found methyl acrylate to be highly reactive, producing 92% of the adduct in 30 minutes, whereas the ketone 3-buten-2-one proved very sluggish yielding only 60% after a prolonged reaction time.[49] When an identical reaction was performed in 1 mL water, 3-buten-2-one and methyl acrylate both yielded > 90% in 20 minutes but α, β unsaturated aldehydes were unreactive.

Table 1.5 Michael-Acceptor Reactivity of Various Aldehydes, Ketones and Esters

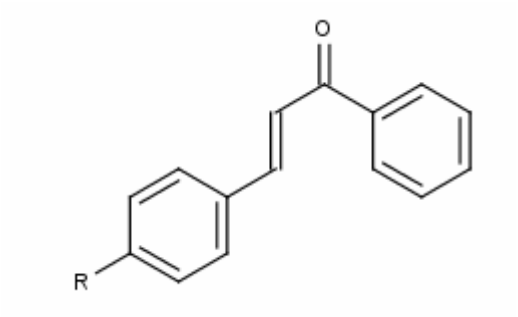
Aldehyde	***Experimental log kGSH (L mol^{-1} min^{-1})***
Prop-2-enal	 4.27 [a]
2 Methyl prop-2-enal	 2.31
(2E)-but-2-enal	 1.70 [a]
Ketones	
3-buten-2-one	 3.51 [a]
3-penten-2-one	 1.43
3-methyl-3-penten-2-one	 -0.11

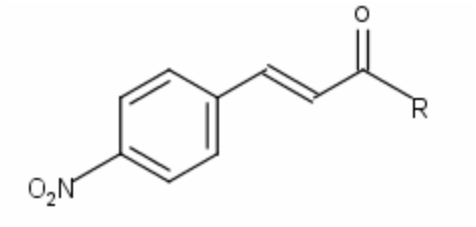
[a] No experimental value available. Predicted log *k*GSH (L mol^{-1} min^{-1}) given

Table 1.5 Michael-Acceptor Reactivity of Various Aldehydes, Ketones and Esters

Acrylates	***Experimental log kGSH (L mol^{-1} min^{-1})***
Methyl acrylate	 1.06
2-hydroxyethyl acrylate	 1.29
Propyl acrylate	 1.01
Methyl methacrylate	 -1.14
Ethyl methacrylate	 -1.24
Methyl (2E)-2-methylbut-2-enoate (methyl tiglate)	 -2.15

The rate constants of the cyclopropanation reactions of Michael acceptors with a sulfur ylide in DMSO, indicated that the enones **8, 9** and **10** showed a moderate increase in reactivity in-line with their electron-withdrawing substituents.[50] Overall the reactivity's of the α, β-unsaturated ketones **8** to **13** illustrated in Scheme 11 differed by less than a factor of 25. The variation of the alkyl group attached to the ketones **11, 12** and **13** had almost no effect on reactivity at the C=C double bond, however the corresponding phenyl compound **8** was 25 times more reactive than **13**.





R = NO_2 **8**

R = CN **9**

R = H **10**

R = t-Bu **11**

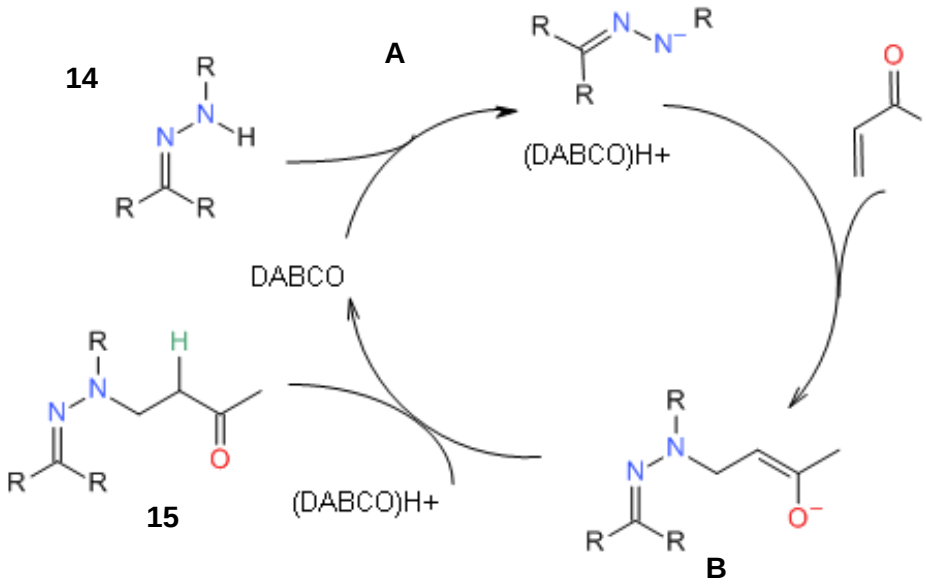
R = i-Pr **12**

R = Me **13**

Scheme 11: The electron withdrawing substituents on enones **8** to **10** had little impact on their reactivity. The phenyl substituted ketone **8** was 25 times more reactive than the methyl substituted ketone **13**. [50]

1.6 Base Catalysed Aza-Michael Reaction

In the event that the amine Michael donor is not sufficiently nucleophilic *e.g*. dibenzylamine, a base catalyst can be used to promote the reaction.[51] In 2005, Shi and co-workers reported high yielding 1,4-diazabicyclo[2.2.2]octane (DABCO) catalysed aza-Michael additions of *N*-tosylated hydrazone with activated olefins, such as methyl vinyl ketone, methyl acrylate, acrylonitrile and phenyl vinyl ketone.[52] The role of the base catalyst in the reaction mechanism was established by deuterium labelling experiments. The tertiary amine catalyst DABCO served as a Brønsted base or 'proton-sponge' rather than a nucleophilic Lewis base as previously reported in the Baylis–Hillman reaction mechanism (the coupling of an activated alkene derivative with an aldehyde).[53] The proposed mechanism is given in Scheme 12. The catalytic cycle begins with DABCO acting as a Brønsted base, directly abstracting a proton from hydrazone **14** to produce nucleophilic intermediate **A**. Intermediate **A** is now a strong enough nucleophile to donate an electron to the Michael acceptor. The subsequent conjugate addition of **A** to methyl vinyl ketone generates enolate **B**. Re-protonation of enolate **B** affords **15** and regenerates DABCO to complete the catalytic cycle.

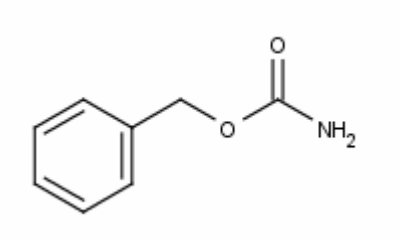


Scheme 12: Proposed reaction mechanism of DABCO catalyzed reaction of hydrazone **14** with methyl vinyl ketone, adapted from [52]

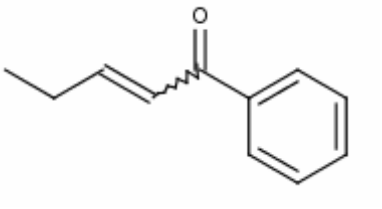
In a further study by Shi *et al* (2010), the *N*-tosylated hydrazone was replaced by *N*-tosylated amines ($TsNH_2$ and $TsNHNH_2$).[54] The product yields were reduced from > 99% for the hydrazone to less than 15% for the amines. The authors suggest that the acidity of the hydrazone N–H proton in plays an important role in the DABCO catalysed reaction. The C=N double bond of the hydrazone renders the alpha hydrogen atom highly acidic and it is readily deprotonated. The nucleophilicity and hence the reactivity of the amine anion towards the acceptor is greater than that of the neutral amine.

1.7 Acid Catalysed Aza-Michael Reaction

In addition to activation of the donor nucleophile via base catalyst as seen in Section 1.6, both Lewis and Brønsted acids have been used to activate the olefin acceptor in an effort to reduce the reaction time and increase the yield of the aza-Michael reaction.[4, 55, 56] Wabnitz and Spencer investigated the idea of using catalytic amounts of Brønsted acid to activate the Michael acceptor by protonation of the carbonyl group. Benzyl carbamate **16** and 1-phenyl-2-penten-1-one **17** were chosen as a model system. [56] Strong acids such as bis(trifluoromethanesulfon) imide ($(CF_3SO_2)_2NH$), triflic acid (CF_3SO_3H) and tetrafluoroboric acid yielded 86 to 98% of the aza-Michael adduct in only 10 minutes.



16

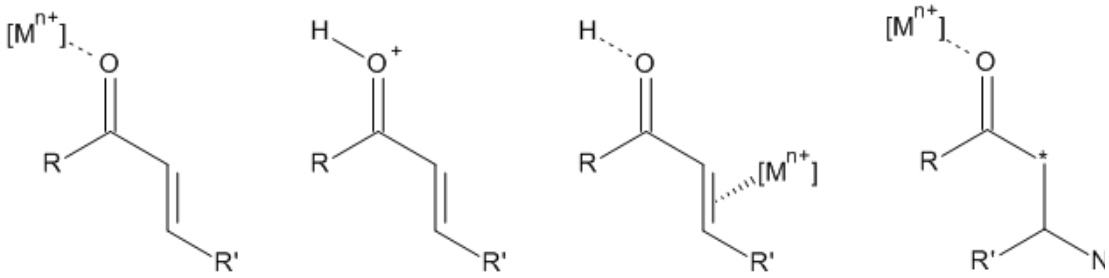


17

Reaction rates were significantly reduced for weaker sulfonic acids and hydrated acids. Conversions were rapid for reactions carried out in dichloromethane, ACN and nitromethane. Solvents with weakly basic oxygen functionalities such as THF, ether, and acetone interfered with carbonyl protonation and gave little or no conversion.[56]

The mechanism of acid catalysis was further explored by Spencer in 2004.[4] The group investigated the role played by the metal ion in a variety of Lewis acid (e.g.

platinum group metal complexes) activated aza-Michael reactions. Four possible mechanisms were investigated, (Scheme 13).



18a **18b** **18c** **18d**

Scheme 13: Four principal mechanisms of Lewis acid catalyst action in conjugate addition reactions to enones under non basic conditions, reproduced from Spencer [4]

Coordination of the metal ion to the carbonyl (**18a**) or to the π-olefin metal complex (**18c**) were ruled out when the reaction proceeded in the presence of a non-coordinating base 2,6-di-tert-butylpyridine. Similarly, co-ordination of the metal ion (**18d**) resulting in a free radical reactive intermediate was ruled out by addition of free radical scavenger to the reaction system as the reaction proceeded in the presence of the scavenger. Finally, activation of the enone can occur via direct protonation of the carbonyl oxygen by Brønsted acids (H^+ donating). The catalytic mechanism was attributed to the ability of certain Lewis acids to liberate hydrogen atoms *i.e.* hydrolyse in organic solvents and behave as a Brønsted acid (**18b**). Authors used ^{1}H NMR to correlate catalytic activity with proton generation in the presence of one or more equivalents of water. The addition of up to two equivalents of water led to a significant increase in reaction rate. However, the addition of four

equivalents of water slowed the reaction rate due the rate limiting effect of waters' Brønsted basic properties. [4]

Encouraged by the work of Spencer,[4] Chaudhuri and his co-workers set about showing that it is this Brønsted acid behaviour that is responsible for the aza-Michael condensation regardless of whether the reaction was catalytic or not.[55] A 10% solution of boric acid in water was used as the catalyst.[55] Boric acid does not disassociate in water as a Brønsted acid, but interacts with the water molecules to form the tetrahydroxyborate ion which liberates the hydrogen atom; $B(OH)_3 + H_2O \rightarrow B(OH)^-_4 + H^+$. [3] As expected, secondary amines reacted faster and gave a higher yield than primary amines. While the results show high yields and fast reaction times for the aliphatic amines, they are no better than those performed in water alone.[22].

1.8 Role of the Solvent in the Aza-Michael Reaction

The studies into amine nucleophilicity [30, 31] and also that of McClelland *et al.* into desolvation of the amine [35] indicate that the choice of solvent is important to success in the aza-Michael reaction.

1.8.1 Aza-Michael in Aqueous Medium

The work of Rideout and Breslow on Diels Adler reactions [57] led to a huge interest in water as an accelerant in reactions between non-polar compounds, with accelerations up to 200 times noted in certain cases. The 'on-water' method ascribed to Sharpless *et al.* [58] describes the rate acceleration observed when an insoluble organic reactant(s) is stirred in an aqueous suspension.

A theoretical investigation of “on water” catalysis postulated that free hydroxy (OH) groups of interfacial water molecules play a key role in catalysing reactions via the formation of hydrogen bonds. Interfacial water molecules with OH groups protruding into the organic phase form stronger hydrogen bonds with the transition state than with the reactants, resulting in acceleration through stabilisation of transition state.[59]

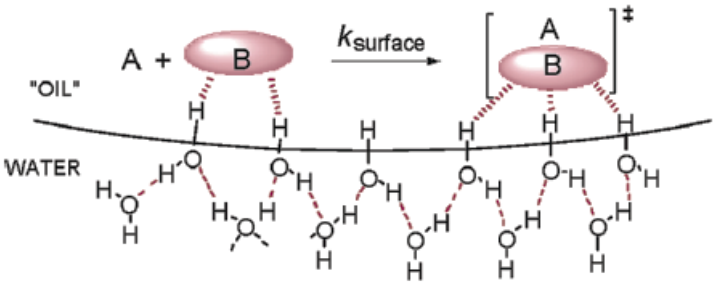
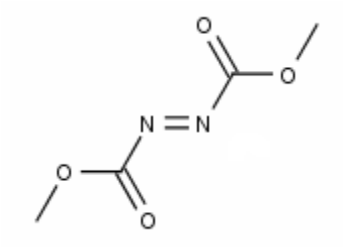


Figure 1: Increased interfacial hydrogen bonding in the transition state resulting in rate acceleration in ‘on water’ reactions. Reproduced from Jung and Marcus [59]

The amount of water used was not considered crucial as long as there was sufficient water to generate an aqueous emulsion.[58] The authors reasoned that the acceleration resulted from the formation of an oil-water interface as substituting perfluorohexane (in which reactants were fully soluble) for water negated the effect and the rate was similar to that of the neat reaction (48 hours). Non-polar liquids that formed a heterogenous mixture resulted in large rate acceleration. In the reaction of quadricyclane (**19**) with dimethyl azodicarboxylate (**20**) in various solvents, a 3:1 ratio of methanol:water resulted in a homogeneous mixture and the reaction time slowed to four hours compared to 10 minutes for the water only reaction.



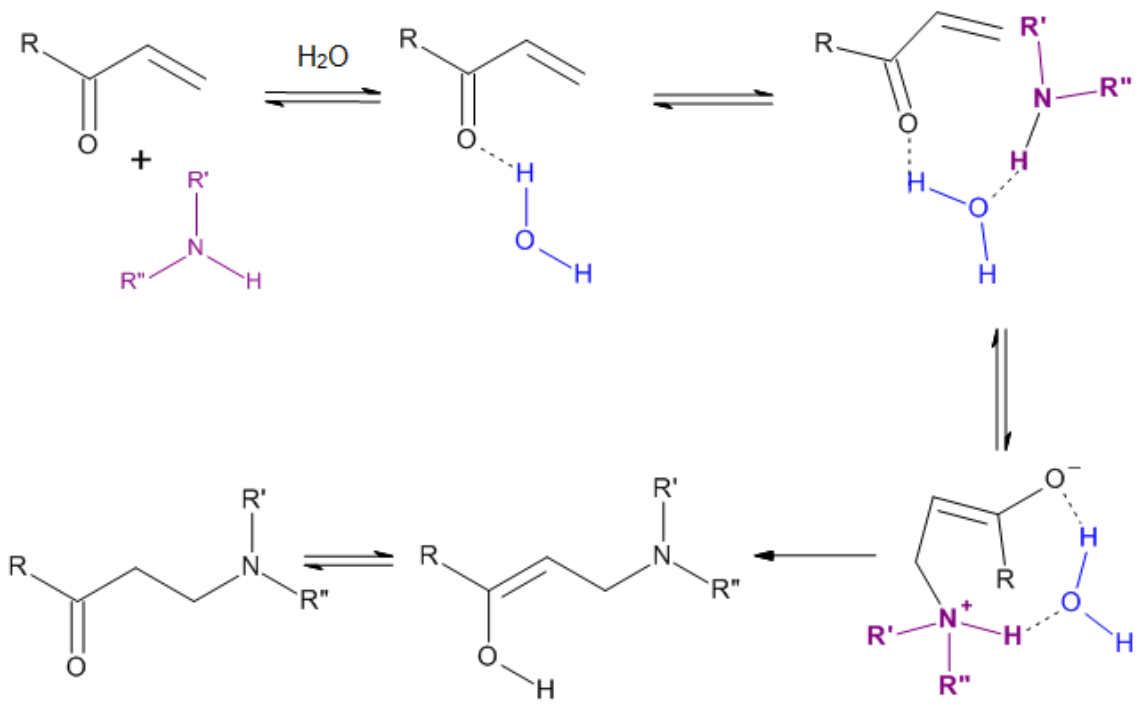
19 **20**

The mechanism of on-water catalysis was examined in a 2010 paper by Beattie, Mc Ellean and Phippen.[60] Again, a Diels-Alder [4+2] cycloaddition reaction (between cyclopentdiene and di-methylfumarate) formed the basis of the study. In order to qualify as a true 'on-water' catalysis the authors propose that the following must apply; the reaction mixture must be heterogeneous *i.e.* there must be an interface between the reactants and the bulk water of the mixture, the interface must be with the aqueous phase and the reaction should be stirred vigorously to create an emulsion. They note that reactions described as accelerated on-water are also subject to acid catalysis. Reactions performed using D_2O could not be described as accelerated with % conversion to product similar to that of the neat reaction, demonstrating a solvent isotope effect. The on-water acceleration was independent of the pH of the aqueous medium and was not affected by the addition of sodium chloride to the water.

The observations made in relation to acceleration of the Diels-Alder reaction on-water find a direct application in the aza-Michael addition of amines and conjugated alkenes in water reported by Ranu and Banerjee. [22] A significant rate acceleration using the on-water method resulted in reaction times of 20 to 50 minutes at room temperature without the use of a catalyst; significantly faster than comparable reactions involving aprotic solvents such as THF and methylene chloride (1 to 15 hours). Primary and secondary aliphatic amines showed

accelerated reaction times in water giving high yields in a short reaction time. However, aromatic and tertiary amines did not react with conjugated alkenes in water using the procedure. α, β-Unsaturated aldehydes were unsuccessful Michael acceptors. In addition, while water has been shown to be a viable solvent for the aza-Michael reaction, it does not provide a route to enantiomerically pure products.

The authors reported that the amount of water used in the reaction did not have any significant impact on the overall rate of reaction or the product yield.[22] The role played by the water molecule in the rate acceleration of the reaction was discussed by the authors and shown in Scheme 14 below. They proposed that hydrogen bond formation involving the oxygen atom of water and the H-atom of the amine increased the nucleophilic character of the N atom of amine. The mechanism in Scheme 14 has elements of the earlier theoretical studies of Bernasconi (1986) and Pardo (1993).[32, 61] For Pardo, the barriers calculated for the addition reaction were found to be significantly reduced by the assistance of a solvent molecule in the intra-molecular proton-transfer process. In the case of the aza- Michael reaction the aqueous solution provides not only a polar medium for the reaction but also a discrete water molecule acts as a shuttle for the proton between the nitrogen and the carbanion of the intermediate. The role of the water molecule in accelerating the reaction is a consequence of the zwitterion intermediate and is not a feature of the classic reaction.



Scheme 14: Dual action of water molecule during the aza-Michael reaction, adapted from Ranu and Banerjee [22]

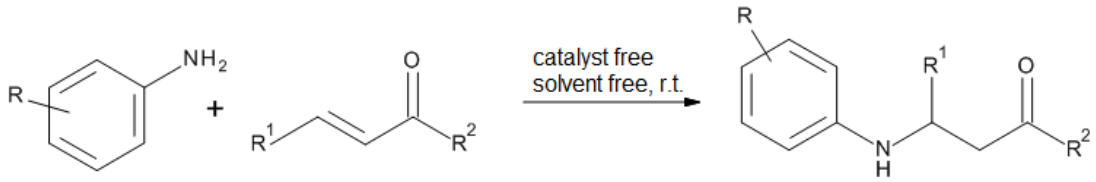
It was noted that the reaction mixture must be stirred continuously. Typical reaction times were 20 to 35 minutes when the mixture was stirred with yields in excess of 85% for the majority of reactants examined.[22] A standing mixture was shown to be only 50% complete after 20 hours. Vigorous mixing was also advocated by Sharpless *et al.* in the 'on water' method described earlier and indicates that the creation of an emulsion is an import feature of the reaction on-water.[58] The reaction was noted to be slightly exothermic but no temperature control was required. Compared to the aliphatic amines, anilines are poor nucleophiles and reaction with methyl acrylate in water at room temperature was unsuccessful even after 40 hours.[22] Aromatic amines and tertiary amines did not react with conjugated alkenes in water using the procedure. This supports the idea that if the amine is sufficiently nucleophilic the reaction will take place under mild reaction

conditions and is second order overall with respect to the concentration of the amine and the olefin. However, α, β-unsaturated aldehydes were unsuccessful as Michael acceptors. This is unexpected as the reactivities of various Michael acceptors with respect to GSH model nucleophile showed that in terms of general reactivity, aldehydes were more reactive than ketones, and ketones more reactive than esters.[48] However, with reference to the earlier study by Pardo, the preferred reaction mechanism for the simple aldehyde acrolein in water proved to be 1, 2 conjugate addition rather than the 1, 4 mechanism.[61]

The poor performance of aniline in water is surprising given that its nucleophilicity is similar to that of other primary amines.[30] The addition of anilines to unsaturated ketones and esters was explored by several groups. Directly referencing the work of Sharpless, a 2010 study by McErleans group had limited success using methyl acrylate as the Michael acceptor.[62] Increasing the reaction temperature from room temperature to 50ºC yielded 35% for the aniline and 94% for the more nucleophilic p-methoxyaniline. Replacing methyl acrylate (MA) with methyl vinyl ketone (MVK) as the acceptor saw the yield for aniline increase to 100%. The authors propose that the underlying mechanism behind the rate acceleration is one of acid catalysis at the oil-water interface rather than 'hydrophobic-driven concentration effects'. To prove that this was the case the neat reaction was carried out and yields compared after a fixed reaction time. After 11 hours the neat reaction between aniline and MVK yielded only 66% compared to the on water result of 100%. Results are contrary to those of Jiang *et al.* detailed below. In this study, the neat reaction yielded 84% (in 6 hours at r.t).[63] The only difference between the two studies is the molar ratios of the reactants. In the 2010 study Phippen, Beattie and McErlean used a 1.1 equivalents of MVK whereas Jiang *et al.* used 1.3 equivalents in their 2011 study.

1.8.2 Solvent Free

The role of the solvent was central to a 2011 study by Jiang *et al.* in the preparation of β-amino ketone compounds (Scheme 15).[63] The challenge of aza-Michael addition of anilines to MVK was taken up and good to excellent yields were reported at room temperature without the addition of catalyst or solvent. In a solvent screening study they observed that protic solvents such as ethanol, water, glycerol and polyethylene glycol (PEG 300) increased the yield of adducts whereas aprotic solvents ACN, DMF and THF performed very poorly with yields of less than 20%. However, the highest yield for the model system was achieved under solvent free conditions. A yield of 84% was achieved for the neat reaction between aniline and MVK after 6 hours at room temperature. This reaction was unsuccessful for Ranu and Banerjee [22] in water, when using methyl acrylate as the electrophile. The choice of substrate may have contributed to the failure of the reaction. For Jiang et al., phenyl vinyl ketone failed to produce the desired adduct.[63]

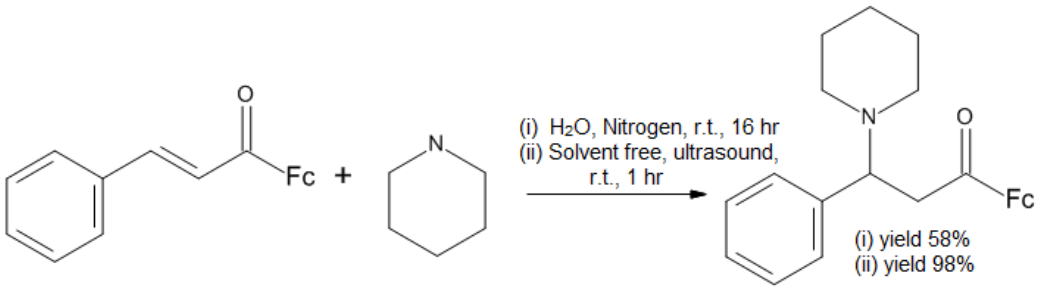


R = H, Me, Br, Cl, I, CN, NO_2,COOH, Ac

R^1, R^2= alkyl or H

Scheme 15: Aza-Michael addition of aromatic amines to α, β -unsaturated ketones[63]

Yang *et al.* (2005) found that a small amount water can promote the Michael addition of secondary amines to α, β-unsaturated ferrocenes.[28] High temperature, acidic media and microwave were all disadvantageous to the reaction rate. At least 10 mol equivalent of amine was required. Contrary to the findings of Ranu, [22] reaction optimisation showed that increasing the amount of water in the system stopped the reaction.



Scheme 16: Water-assisted Michael reaction of amines to ferrocenylenones [28]

Addition of a small amount of water (1 mol equivalent) resulted in a 58% yield of the 1, 4-addition product after 16 hours. [28] When the experiment was repeated under neat conditions but using ultrasound irradiation, the result was a 98% yield of the adduct in 1 hour (Scheme 16). A variety of amines and acceptors were subjected to the ultrasound protocol. Secondary amines were more reactive than primary amines. In all cases, the 1, 4-addition products were observed in good to excellent yield within 2 hours. Once again the aromatic amine failed to produce an adduct when ethyl acetate was used as the acceptor. Compared to the work by Ranu and Banerjee [22] yields were lower and reaction times longer (2 hours as opposed to 20 minutes) for the neat ultrasound reaction with similar amines. The molar ratio of the reactants was significantly different for both systems. Ranu and Banerjee used a 1:1.3 ratio for amine to acceptor whereas Yang *et al.* [28] used a 1:0.1 ratio. The

molar ratio of 1:1.3 was also adopted by Jiang *et al.* [63] in the neat reaction mentioned above.

A single experiment examining the effect of solvent on the aza-Michael reaction of piperidine and methyl acrylate compared the effects of water + ultrasound, water + stirring, and the neat + ultrasound reaction. The water + ultrasound reaction was incredibly fast with 98% yield in 5 minutes, followed by neat + ultrasound, 93% in 15 minutes and finally water + stirring, 96% in 30 minutes.[27] Remarkably, aniline reacted with ethyl acrylate (EA) to yield 92% in only 5 minutes. No side products or bis-adducts were formed. Isolation of products was facilitated by their reduced solubility in the aqueous medium post reaction cooling. The physical acceleration by ultrasound is not fully understood but thought to occur through the formation of gas cavities in the liquid which implode resulting in 'localized transient high temperature and pressures'[27]. Water, with its high energy of activation and heat capacity would be an ideal medium for such a reaction.[64] The molar ratios of the reactants and the amount of water would appear to be significant; the 1:1 ratio of reactants in 1 mL of water would seem to be ideal protocol for reactants that are diffusion controlled and for the formation of the mono adducts.

An early paper by Jenner describes a reaction protocol similar to that of Ranu and Banerjee [22] but with very different results.[23] Using a molar ratio of 1:1 amine to acrylate in 3.5 mL of water, no product was generated for the addition of isopropyl(methyl) amine to MA. While this is not the most nucleophilic of amines the result is still at variance with other studies. For example, Ranu achieved a yield of 85% in 35 minutes for the addition of di-isopropyl amine to MA.[22] In Jenner's experiment, the reaction mixture was not monitored at regular intervals for the formation of product; rather all reactions were run for 24 hours. The anomaly is

interesting because the literature surveyed presents the same protocol of monitoring the rate of reaction and presumably isolating the products as soon as they are formed. Jenner reasoned that the reaction with MA in water is reversible at room temperature as the same reaction with acrylonitrile yielded 72% of product after 24 hours *i.e.* 'the zero yield is simply explained by the fast reversibility of β-aminoesters in highly polar media whereas β-aminonitriles are quite stable in water'[23]. To test this, the β-amino products were stored in both water and acetonitrile under the same conditions as the forward reaction, as shown in Table 1.6. Not unsurprisingly the β-aminoester underwent hydrolysis in water at 50ºC. Therefore, the difference in outcome for the Jenner and Ranu reactions may simply be attributed to the reaction conditions. The Jenner experiments were carried out in a sealed polytetrafluoroethylene (PTFE) tube containing the reactants and 3.5 mL of water (no stirring or mixing of the contents is described) whereas the Ranu reactions were monitored by thin layer chromatography (TLC) and the products isolated after they were formed.

Table 1.6 Occurrence of Reverse Reactions for β-amino Compounds, reproduced from Jenner [23]

	Storage Temperature	**% Residual Amino Compound After 24 Hours**	
	ºC	**Acetonitrile**	**Water**
β-aminoester (iPr)(Me)N-CH(Me)CH_2COOCH_3	50	98	4
β-aminonitrile (iPr)(Me)N-CH(Me)CH_2CN	30	100	100
β-aminoamide $(CH_2)_5N$-CH_2CONH_2	30	98	95

In contrast to the carbon – carbon reaction the solvent is an important component of the aza-Michael reaction and plays a direct role in the protonation of the carbanionic intermediate, either through intramolecular bonding and proton transfer from the nitrogen atom, or through direct protonation of the carbanionic intermediate. Protonation of the carbanionic intermediate by protic solvents such as water and methanol is rapid and makes the reaction pseudo second order. In aprotic solvents (and neat reactions) the nitrogen atom of the intermediate is the source of the necessary proton and without the hydrogen bonds the reaction is slower and more likely to revert to the reactants.[65] . It is unclear from the literature what the optimum solvent conditions are as various studies report using different amounts, with no consensus emerging.

1.9 Aza-Michael Reaction in Formulated Dosage Forms

1.9.1 Reaction between drug substances and pharmaceutical excipients

Drug formulation compatibility testing is carried out to ensure that excipients used in the formulation do not react adversely with the active pharmaceutical ingredient (API). Excipients used in the formulation of pharmaceuticals should ideally be chemically unreactive. However, since many excipients (sugars, parabens, salts) contain functional groups, reactions with the drug substance are possible. The Maillard reaction of a secondary amine with reducing sugars such as maltose and lactose is one of the most commonly cited examples of a drug excipient interaction.[66-69]

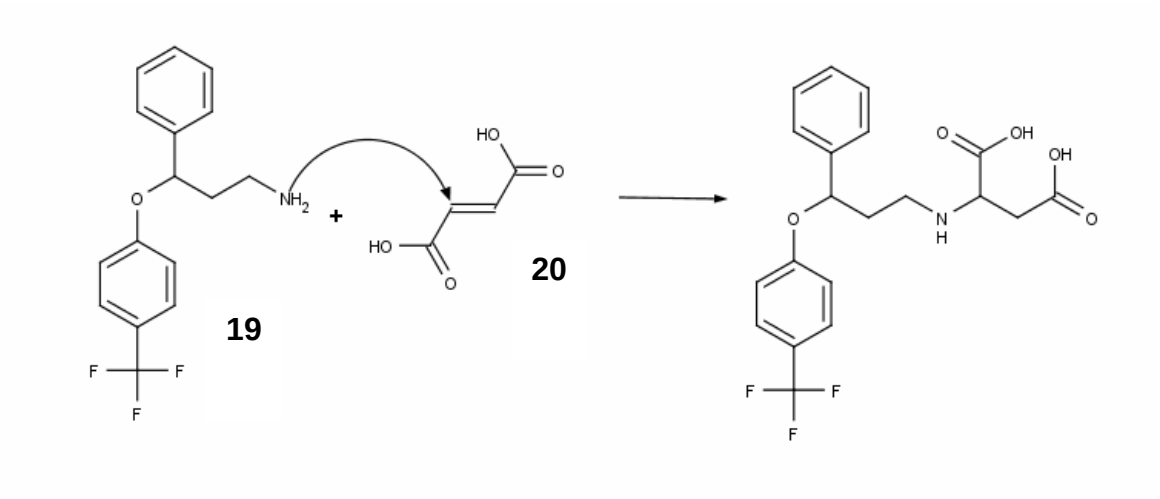
Examples of the aza-Michael reaction, as a consequence of drug-excipient interaction, have been described in the literature, particularly in relation to liquid

dosage forms. The most common aza-Michael addition is that of an API – salt interaction where the molecular weight of the adduct is the sum of API and the counter ion. [70-73]

The Michael addition of seproxetine **19** to its maleic acid **20** counter ion was described by Schildcrout, Risley, and Kleemann (1993).[70] The bulk drug seproxetine maleate hemihydrate (SMH) was found to be stable when stored at 40°C for 1 month. Solutions of the drug were prepared and stored at 40°C to identify potential degradation products. A pH 8 buffered solution stored for 1 month, resulted in the formation of the 1, 4-addition product of seproxetine and maleic acid, see Scheme 17. A range of pH adjusted aqueous solutions indicated that optimum adduct formation occurred in the pH range of 5.5 to 8.5, with no adduct formation below pH 3.0 (when stored at 40°C for 2 weeks). The 1, 4-addition product proved to be stable in the pH adjusted solutions for a further two weeks at both room temperature and 40°C, with no reversal of reaction observed.

Pre-formulation isothermal stress testing was carried out with a number of excipients to determine compatibility in a capsule dosage form. Formulation with pregelatinized starch (PGS) as a 1 and 20 mg free base equivalent gelatine capsule resulted in the formation of the 1, 4 adduct described above, when stored at 25 and 40°C.[70] The free water (7-15%) contained in the starch was thought to contribute to the adduct formation. Stability data generated for two capsule strengths stored at 25 and 40°C showed the 1, 4 adduct to be the sole degradation product. The rate of formation was significantly higher at 40°C; 14 times greater for the 1 mg capsule and 7.4 times greater for the 20 mg capsule. The data (and further testing at 50°C) fits a zero order reaction equation, with 1 month at 40°C corresponding to 1 year at 25°C. The percent adduct formed at 40°C was 17.34% for the 1 mg capsule versus

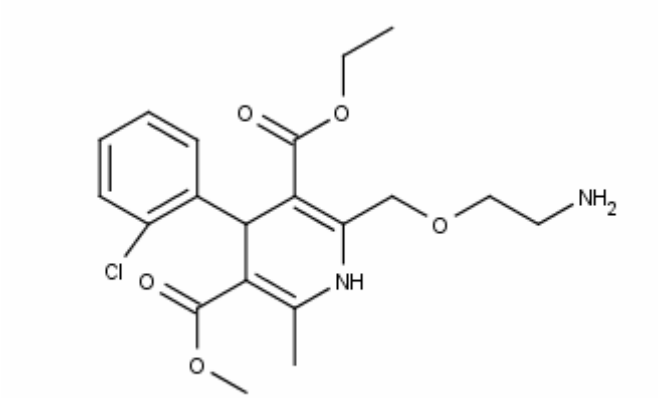
1.57% for the 20 mg capsule. While this was not remarked on, the ratio of SMH to pregelatinized starch would be greater for the capsule containing 1 mg of SMH, and the percentage of free water in the system would also be greater. The higher percentage of water in the system could be responsible for the increase in formation of the adduct.



Scheme 17: 1, 4 addition product of seproxetine and maleic acid counter ion, adapted from Schildcrout.[70]

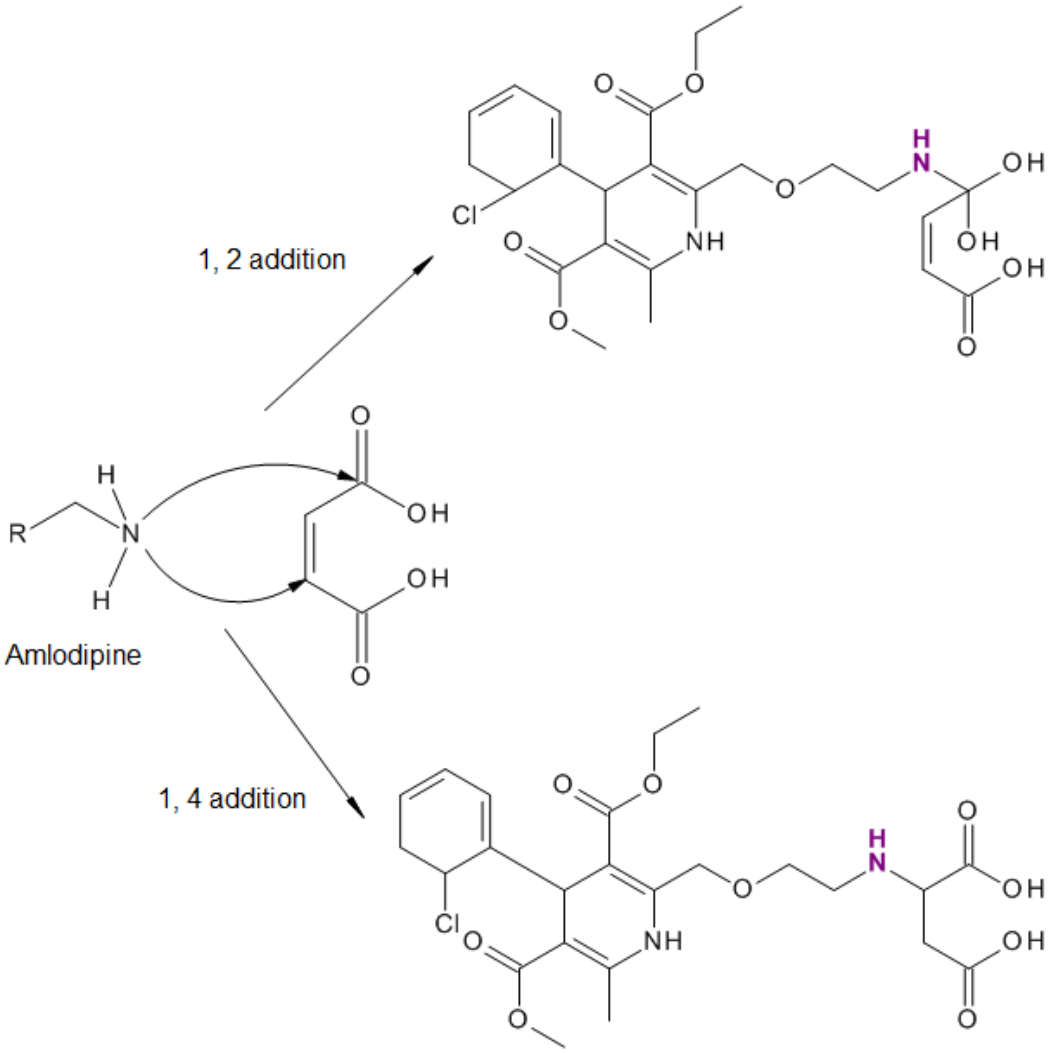
An alternative capsule formulation of SMH and talc was prepared and evaluated under the same isothermal stress conditions as the pregelatinized starch. Talc was selected as it is hydrophobic and contains neither surface water or water of crystallisation. When stored at 50°C, the interaction with maleate salt resulted in exclusive formation of the amide adduct with the subsequent loss of a water molecule.

A Michael addition reaction between the anti-hypertensive drug amlodipine **21** and maleic acid **20** was described by Pan *et al*. (2011) in a review of pharmaceutical impurities in formulated dosage forms. [73]



21

Authors note that two possible reactions could occur between the primary amine in amlodipine and maleic acid; nucleophilic attack by the amine at either the carbonyl carbon or the β-carbon of maleic acid as shown in Scheme 18.[73] There are also two available amine nucleophiles, the primary amine and the secondary amine of the 1,4 dihydropyridine ring. However, the product ratios and reaction rates were not discussed. Nevertheless, potential routes for excipient formation from amine API's were identified.



Scheme 18: Two possible reaction mechanisms for the addition of amlodipine **21** to maleic acid; the 1, 2 addition to the carbonyl or the 1,4 Michael addition to the beta carbon. Adapted from Pan *et al*. [73]

1.9.2 Reaction between Drug Substance and Leachables

Ophthalmic pharmaceutical formulations have been classified as having a high likelihood of packaging component-dosage form interactions (FDA)[74]. Indeed, numerous interactions of plastic container components and drug components have been documented in literature [75]. For this reason, migration of components, in particular phthalates, from polymer containment systems, has been the subject of multiple research projects [76, 77]. Interestingly, low-density polyethylene (LDPE) containers, typically used in ophthalmic formulation packaging, were found to have the highest diffusion coefficient of a range of polymer containment systems investigated, significantly increasing the likelihood of migration of components from outside the container itself [78]. A number of studies have been concerned with the potential migration of components from the adhesive, inks and lacquers used to label the plastic containers. In one study benzophenone was detected, probably as a result of incomplete UV adhesive curing [79], while in an other, a component from the lacquer applied over the label was found to have migrated through to the pharmaceutical formulation (and interacted with a known excipient therein).[80]

While the migration of labelling and adhesive components into liquid pharmaceutical formulations has not been extensively researched, the migration of leachables from these components when utilised in food packaging has been the subject of considerably greater investigation. Each new innovation in food packaging technology is accompanied by the risk of new contaminants migrating into foodstuffs.[81] For example, antioxidants added to new ‘active’ packaging materials to extend the shelf life of packaged food resulted in the migration of non-volatile impurities into a variety of food simulants [82]. The challenge of identification of unknown impurities migrating from food packaging, in particular non-volatile components was discussed in a recent review by Nerin *et al*. [83]. Interaction of the

leachable with the food substance has also been observed. For example, bisphenol A diglycidyl ether (BADGE), a lipophilic monomer used for coating cans and lightweight food containers, formed adducts with the primary amino groups of food proteins following its migration into foodstuffs [84].

The migration of these leachables therefore has a potential to chemically interact with constituents of liquid formulations. For this reason, their migration into ophthalmic pharmaceutical formulations potentially poses a significant risk, particularly if they can initiate or propagate degradation reactions such as oxidation, hydrolysis or Mallaird reactions etc. The presence of acrylate monomers in both adhesives and inks utilised in pharmaceutical packaging labelling is potentially concerning, as if they migrated to the pharmaceutical formulation, they could act as Michael acceptors in aza-Michael addition. Aza-Michael addition reactions have previously been documented between APIs containing amine functional groups and drug excipients in pharmaceutical formulations [85-87]. While they have been described as analogous to classic Michael additions, there is one difference, which is of critical importance – while a catalyst is required in the classic reaction, none is required for the aza reaction. For this reason, the migration of acrylate acceptors into ophthalmic solutions (which are frequently housed in LDPE containers with high diffusion coefficients) which have APIs containing amine groups is of concern.

While there is some consideration of drug excipient interactions as part of quality risk management in pharmaceutical development there is no tolerance for reactions between drug packaging and drug product; as the choice of packaging is entirely the responsibility of the manufacturer [88]. It is expected that packaging components are selected to provide adequate protection of the drug and that they are compatible with the dosage form and the route of administration. The guidelines

for sterile liquids are particularly stringent. Modifications to bottle caps, cap liners and seals and an increases in wall thickness are generally considered minor for non-sterile semisolids and liquids and do not require prior approval. In contrast, almost all packaging changes to sterile drugs (ophthalmic solutions) are considered major, especially those that might affect sterility. Container – content compatibility studies are required as part of the regulatory submission of a new product market authorisation file or for a change relating to the primary product packaging. Changes that have even moderate adverse potential require the submission to the appropriate regulatory agency for prior approval.[89] All ophthalmic solutions fall into the category of sterile drugs, therefore the impact of minor changes to the packaging as a consequence of leachable interactions with the drug substance are of major concern. Regulatory publications are available (Table 1.7), however these guidelines are not precise enough to allow a consistent and standardised approach when interpreting technical requirements. The onus is on the drug product manufacturer to assess the risk to the patient without overestimating the effect of the material on the safe use of the product.

Table 1.7 Regulatory Publications

Regulatory Agency and Publication Title	Details
US Food and Drug Administration (FDA) Center for Drug Evaluation and Research (CDER) Center for Biologics Evaluation and Research (CBER) *Guidance for Industry Container Closure Systems for Packaging Human Drugs and Biologics (1999)*	Guidance on general principles for submitting information on packaging materials used for human drugs and biologics. All forms of packaging, not only plastic are considered. Requirements are based on the protection, safety, compatibility and performance of the packaging.
European Medicines Agency (EMEA) CPMP/QWP/4359/03 EMEA Guideline on Plastic Immediate Packaging Materials December 2005	Introduces the quality aspect in which information on primary packaging has to be provided. Migration studies are included for ophthalmic studies.
World Health Organisation WHO Technical Report Series, No. 902, 2002	Review of the various elements of the packaging of a pharmaceutical product is aimed at ensuring that medicines arrive safely in the hands of the patients for whom they are prescribed.
ICH Harmonised Tripartite Guideline Pharmaceutical Development Q8(R2). International Conference on Harmonisation of Technical Requirements for Registrations of Pharmaceuticals for Human Use (ICH), Geneva, (2009).	Guidance on the contents of section 3.2.P.2 (Pharmaceutical Development) for drug products as defined in the scope of Module 3 of the Common Technical Document (ICH M4: Common Technical Document for the Registration of Pharmaceuticals for Human Use)

1.9.3 Source of Acrylates Migration into Packaged Pharmaceutical Liquid formulations

A typical container closure system of a finished ophthalmic solution contains primary packaging components such as LDPE bottles or vials, and secondary packaging such as pressure sensitive label, ink, foil laminate, paper insert and cardboard boxes. Of the above components, the main sources of acrylate migrants are the pre-printed pressure sensitive label and foil laminate. Both the UV cured ink and the acrylate adhesive used on the label contain low molecular acrylate monomers used as reactive diluents in the UV curing process and in the formulation of the adhesive.

UV cured ink formulations consist of a blend of light sensitive photo-initiators (PI), pigments and variety of acrylic resins. The radiation curing process involves the photo-generation of a radical species that catalyses the polymerisation of acrylates in the coating once it has been applied to the substrate.[90] The use of digital ink is becoming increasing popular as the process is very cost effective for low volume printing. Digital inks are made up of polymer based pigment particles (as small as 1 micron) dispersed in a carrier liquid. The oil based carrier liquid may comprise a combination of different resins in an iso-paraffin liquid solvent. Typical resins used in HP Electra ink include polyethylene methacrylic acid (PEMAA) and polyethylene acrylic acid (PEAA) copolymer. As the composition of both acrylic adhesives and ink formulations are proprietary, with only the major components declared, pharmaceutical companies must establish that the labels are compatible for use with their products. For example, hexane diol di-acrylate migration has been traced to a digital ink formulation by our laboratory during trials for new packaging configurations. The compound was not declared by the vendor. Table 1.8 provides

a list of acrylates commonly found in UV cured inks with reference to both the literature [91] and results of extraction studies performed in our laboratory.

Table 1.8 Acrylate Monomers

Compound	Abv	Source	Property
Hexane diol di-acrylate,	HDDA	UV Cured and Digital Ink	Reactive monomer/diluent
Dipentaerythritol penta-acrylate	DPHPA	UV Cured Ink	
Pentaerythritol tri-acrylate	PETA	UV Cured Ink	
Phenoxy ethyl acrylate	PEA	UV Cured Ink	
Glycerine propoxylate tri-acrylate	Ebecryl OTA-480	UV Cured Ink	
Polyester acrylate oligomer	EB450	UV Cured Ink	Binder
Acrylic acid	AA	Label Adhesive	Reactive diluent
Methyl acrylate	MA	Label Adhesive	Reactive diluent
2-Ethylhexyl acrylate	-	Label Adhesive	
Polyethylene methacrylic acid	PEMAA	Digital Ink	Resin
Polyethylene acrylic acid	PEAA	Digital Ink	Resin
Trimethyl propane tri-acrylate	TMPTA	UV Cured Ink Label Over varnish	Reactive diluent
Dipropylene glycol di-acrylate	DPGDA	UV Cured Ink Adhesive in Laminated foil pouch.	Reactive diluent

1.10 Conclusion

A review of the literature demonstrates ample evidence of the viability of the aza-Michael reaction in water without catalyst. The rates of reactivity and yields obtained are broadly similar for a variety of studies and are in line with the nucleophilicity parameters of the amine donors. The reaction conditions summarized in Table 1.9 demonstrate that nucleophilicity of the amine donor is the driving force behind the reaction. For example, piperidine, one of the most reactive amines is high yielding when reacted with methyl acrylate under both water and neat conditions. Aniline at the lower end of the nucleophilicity scale is only reactive when combined with the more electrophilic methyl vinyl ketone or β-nitroacrylate. Yields of 100% and 84% have been reported for aniline and methyl vinyl ketone (runs 6a and 12b) in both water and for the neat reaction. The ultrasound promoted reaction between aniline and ethyl acrylate in water has a standout yield of 92% in 5 minutes.[27]

In contrast to the classic carbon-carbon Michael reaction described in Section 1.0, the aza-Michael reaction will proceed in the absence of a catalyst. Whereas the effect of the solvent in the classic reaction was closely tied to the choice of catalyst, in the aza reaction the choice of solvent is crucial to the reaction. Much of the literature has focused on this element especially in light of the rate accelerations seen in the Diels-Alder reaction.[57] The solubility of the reactants is no longer essential and one reaction being insoluble in water can be an advantage as it forms an oil-water partition.[58]

Moving from synthetic to analytical chemistry there is evidence of the aza-Michael reaction occurring in both solid and liquid formulated drug products through the interaction of drug substance and excipients. Adduct formation as a result of

interaction between components of packaging and an amine drug substance is equally viable. To date however, in spite of the chemical basis for aza-Michael mediated adduct formation, there are no published studies examining the possibility of adducts being generated from a packaging interaction.

Table 1.9 Key:

MA	= Methyl acrylate
MVK	= Methyl vinyl ketone
a	Ratio of amine donor to acceptor in mmol.
b	Reactions were left stirring for a fixed period of time and were not monitored during that time.
c	Reaction left stirring overnight, yield reported as ‘went to completion’.

Table 1.9 Summary of Aza-Michael Reactions in Water and Solvent Free from Literature

	Ratio a/b [a]	Solvent	(mL)	Amine	Acceptor	Yield %	Time hour	Ref
1	1:1	Water	3.5	Isopropyl(methyl) amine	MA Acrylonitrile	0 100	24 [b]	[23, 92]
2	1:1.3	Water + Boric acid	3	Piperidine Aniline	MA	90 0	1.5	[55]
3	1:1.5	Water	1	Diisopropyl amine Piperidine Aniline	MA	85 92 0	0.5 0.5 24	[22]
4	6:5	Water	20	Aniline Benzylamine	β-nitroacrylate	85 45	4 [b]	[93]
5	1:1	Water	1	*n*-propyl amine	1,4 - naphthoquinone	95	5 mins	[94]
6a	1.2:1	Water	4	Aniline	MA	21	24 [b]	[62]
6b	1.2:1	Water 50C	4	Aniline	MA	35	24 [b]	[62]
6c	1.2:1	Neat		Aniline	MA	0	24 [b]	[62]
6d	0.66:0.6	Water	4	Aniline	MVK	100	11	[62]
7	1:1	Water	1	Piperidine	MA	96	0.5	[27]
8	1:1	Water *Plus* Ultrasnd	1	Piperidine Aniline	MA Ethyl acrylate	98 92	5 min	[27]
9	2.4:2	Neat		Piperidine Aniline	MA	90 0	0.75	[49]
10	2:2	Neat		Aniline Piperidine	β-nitroacrylate	92 80	2 1.5	[95]
11	2:2	Neat		Morpholine Piperidine Aniline	MA	90 60 0	2 3 24	[96]
12a	1:1	Neat		4-chloroaniline	MVK	60	4	[63]
12b	1:1.3	Neat		Aniline	MVK	84	6	[63]
12c	1:1.3	Water	2	4-chloroaniline	MVK	87	10	[63]
13	0.5 - 5:0.5	Neat		Piperidine:	Ethyl acrylate	98	0.5	[28]
14	1:1.5	PEG 400, 2.5 g		N-methylpiperazine	MA	99	0.5	[97]
15	57.5:57.5	methanol	20	Methylbutylamine	MA	100[c]	24 [b]	[65]

1.11 Project Aims

The focus of this project was to examine whether reactions that are viable in mild aqueous conditions are likely to proceed, to explore if the potential interactions between the drug substance and packaging components occur under these conditions and to develop a a platform to screen for these interactions in ophthalmic systems. As per ICH Q3B Guidelines, an adduct yield of between 0.5 and 0.9% of the concentration the active pharmaceutical ingredient in the final dosage form must be structurally identified.[98] For a synthetic chemist this level of yield would be considered 'trace', but for pharmaceutical companies, the costs associated with the identification and qualifications of unknown impurities are considerable.

Several papers attest to the acceleration of the aza-Michael reaction in water when compared to the reaction in organic solvent or indeed neat without solvent. In Chapter 2, the aza-Michael reaction was explored using 1-phenylpiperidine (1PP) and methyl acrylate (MA). 1PP was chosen as a model amine containing drug substance and MA was chosen as a probable packaging constituent. To determine whether water acted as an accelerant for the aza-Michael reaction, an ultra-high performance liquid chromatography (UHPLC) method was developed to accurately monitor reactants and products in situ in order to determine extent of reaction acceleration by water. It was determined in this chapter that the aza-Michael addition reaction with 1PP as the amine donor and MA as the acrylate acceptor was rapid in aqueous solutions, and proceeded without the requirement of a catalyst.

In Chapter 3 the possibility and extent of the aza-Michael addition reaction occurring in ophthalmic solutions was examined. The reaction was investigated using a variety of buffered solutions using multiple acrylate acceptors commonly found in pre-printed labels. The effect of the amine salt was investigated as the free base is

seldom encountered in the final drug product. It was shown that aza-Michael addition adducts were formed in all systems analysed: occurring in buffered solutions of both acidic and basic pH, with both free base and salt drug substances, and with both acrylic acid and esters. The adduct yields varied for different permutations, in agreement with what was expected based on their chemical parameters. Hydrolysis of the ester products was also observed.

Pharmaceutical formulations frequently comprise of more than one active ingredient, and this may impact on the profile of impurity adducts formed during both preparation and storage. Chapter 4 describes the application of a design of experiment (DoE) methodology to the development of a stability indicating UHPLC method for the simultaneous analysis of the APIs brimonidine tartrate and timolol maleate and their related substances. Two of the impurities quantitated using the method were the result of an aza Michael addition reaction. There are currently no accredited methods for ophthalmic drug substances which monitor both APIs and their related impurities.

The UPLC method developed in Chapter 4 was one of several used in the screening platform discussed in Chapter 5. Commercial formulations of eight amines were reacted with packaging components methyl acrylate and acrylic acid and screened for adduct formation over a period of 40 days. By investigating the conditions under which the reaction was most likely to take place, a protocol was developed for screening of products in the early stages of analytical method development and validation. The screening study combined elements of drug-excipient/packaging compatibility studies and forced degradation studies, and comprised immersing the acrylate packaging components directly in the ophthalmic formulation and monitoring for the presence of adducts under accelerated storage

conditions. The role of the acrylate acceptor reactivity was shown to be of particular importance in terms of the nitrogen nucleophile reaction. It was also illustrated that both secondary and tertiary amine APIs undergo the aza-Michael reaction with both methyl acrylate and acrylic acid under pharmaceutically relevant conditions.

1.12 References

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Chapter 2:

Reaction monitoring using UHPLC:

Investigation of the Parameters which Affect the Rate and Yield of the Aza-Michael Reaction

2.1 Introduction

The aza-Michael reaction describes the conjugate addition of amines to α,β-unsaturated carbonyl compounds and nitriles. This important reaction has wide application in the production of β-amino derivatives which serve as useful intermediates in the synthesis of a large number of products with a wide range of biological activity such antibiotics, β-amino alcohols and other nitrogen-containing molecules.[99-102]

Several papers attest to the viability of the reaction in multiple configurations, including neat without any solvent or catalyst, [49, 63] neat with catalyst, [96, 103] and in aqueous solutions such as sodium carbonate [104], boric acid [55] and as a co-solvent with trifluoroethanol.[105] In addition, several simple, catalyst-free protocols demonstrate that the reaction in water is both fast and high yielding.[10, 27, 62, 94]

Initial interest in water as the sole reaction medium stems from Breslow's reports on the remarkable acceleration of the Diels–Alder reaction performed in water.[57] Many more experiments have been reported since then for other types of organic reactions that are accelerated in water and are discussed in a number of reviews. [64, 106, 107] These reactions are described differently in various papers, sometimes as "in water" and sometimes as "aqueous". Sharpless and co-workers described "on water" conditions under which substantial rate acceleration was observed when the organic reactants were insoluble in the aqueous phase.[58]

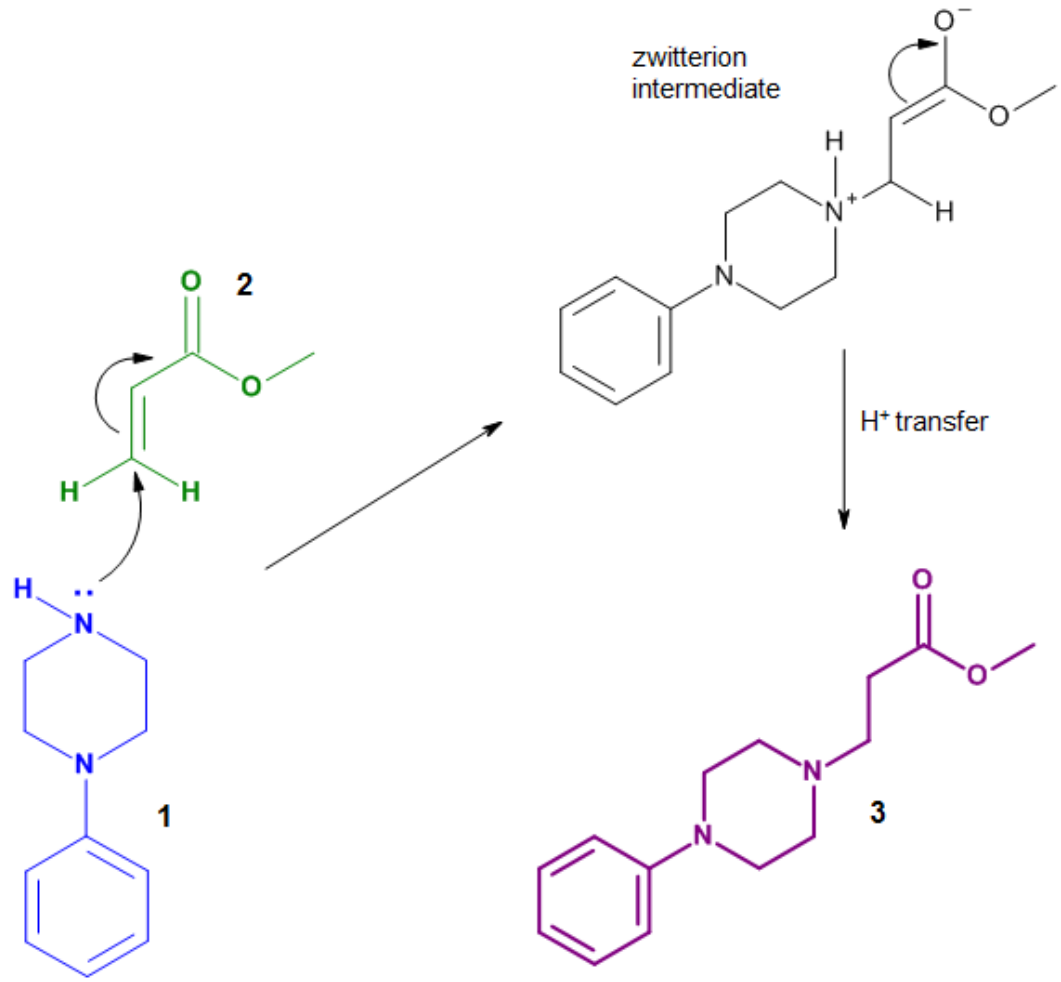
There have been several reports on the aza-Michael reaction using water as the reaction medium.[8, 62, 108] However, there is no firm evidence that the rate in water is greater than for the neat reaction. Jiang *et al*. [63] investigated the rate and

yield of reactions using aniline as the amine using the same the methodological approach as Phippen *et al*. [62] but reported conflicting findings. For the reaction of aniline and methyl vinyl ketone in water, Phippen *et al*. achieved a 100% yield compared to 66% for the neat reaction (6 hrs, r.t.), whereas Jiang *et al.* reported a yield of 84% for the neat reaction. A 2002 paper by Ranu, Dey, and Hajra describes the neat reaction of various amines and acceptors. All reactions were high yielding with reaction times of 30 min to 3 hours.[49] When the experiment was repeated in water several years later by Ranu and Banerjee [22] the authors reported a significant rate enhancement despite the fact that the reaction rates and yields were comparable to those of the neat reactions.

The purpose of this study was to carry out a detailed investigation of the the aza Michael addition of an amine and acrylate using water as the reaction medium to determine whether the reaction can truly be described as accelerated by water. This study considers the effect of solvent type and volume upon reaction rates. A number of common solvents were screened and the effect of variation in solvent compared to that achieved using water as the reaction medium. In addition, the effects of the reaction stoichiometry, temperature and rate of stirring were investigated for the neat reaction.

A key aspect of the 'on-water' rate phenomenon proposed by a number of groups is the chemistry between water and reactants that occurs at an oil-water phase boundary [58, 59, 62]. To achieve this phenomenon, one of the reactants must be insoluble in water. 1-phenylpiperazine (1PP) (**1**) was selected as the model amine nucleophile as it is insoluble in water but soluble in methanol (MeOH); if the reaction was catalyzed by water, no rate enhancement would be expected for the same reaction in MeOH. Methyl acrylate (MA) (**2**) was chosen as the model acceptor

(Scheme 1). The addition to MA was not reversible under the conditions tested and the products therefore resulted from kinetically and not thermodynamically controlled reactions. The rates of reaction were compared by monitoring the conversion of 1PP to methyl 3-(4-phenylpiperazin-1-yl)propanoate (MPP) (**3**) product using varying amounts of reactant for a pre-determined time. The amine loss was quantitatively analyzed by UHPLC.



Scheme 1: Aza-Michael reaction of 1-phenylpiperazine (**1)** and methyl acrylate (**2**) to produce methyl 3-(4-phenylpiperazin-1-yl) propanoate (**3**)

2.2 Experimental

2.2.1 Chemicals and Reagents

1-Phenylpiperazine (P30004) and methyl acrylate (M27301) were purchased from Sigma Aldrich. Potassium phosphate monobasic and ortho-phosphoric acid 85% were purchased from Applichem. HPLC grade acetonitrile and methanol were purchased from Merck. Tetrahydrofuran 'Super purity solvent' was purchased from Romil. Water deionised to a resistance of greater than 18 MΩ was obtained from a Millipore Corporation Milli-Q system, Millipore (Bedford, MA, USA). LC-MS grade acetonitrile was purchased from Merck. All other chemicals were of analytical grade and were used without further purification.

2.2.2 Sample Preparations

All reactions were conducted at room temperature (22 - 24ºC) in 20 mL screw-top round bottomed test tubes that had been washed in distilled water and air dried. Vigorous stirring was performed at 800 rpm on a hotplate stirrer. Room temperature reactions were carried out using a Variomag, Telemodul C (Thermo Fischer Scientific, Waltham, MA). For reactions carried out at 50ºC, a SD162 Stuart stirrer/hotplate (Bibby Scientific, UK) was used.

2.2.3 Standard Conditions for aza-Michael Reactions

The experimental procedure was adapted from Ranu and Banerjee [23] and was as follows for the neat aza-Michael reaction: MA was added to 1PP in a round bottomed test tube containing a magnetic stir bar. The test tube was immediately transferred to the stir plate and stirred vigorously for the prescribed time. After the required time the reaction vessel was removed from the stir plate and 10 mL of tetrahydrofuran (THF) was added directly to

the reaction mixture. The test tube was capped and shaken vigorously by hand 3-4 times. 0.5 mL of sample was removed and added to a 50 mL volumetric flask containing approximately 30 mL of MeOH. The flask was brought to volume with MeOH and shaken vigorously by inversion 10 times. 2 mL of sample was immediately pipetted into a glass vial and placed in the UHPLC autosampler for injection. The entire process from addition of the THF to sample injection took < 90 s.

2.2.4 Determination of the effect of solvent type on yield

1.0 mL of solvent (water, MeOH, ACN or THF) was transferred to the test tube containing 1PP prior to addition of MA. 1PP was not stirred in the solvent prior to addition of the MA. The reaction mixture was stirred vigorously (800 rpm) for 20 mins at room temperature. Samples were prepared for analysis by addition of 9 mL THF to the test-tube. Preparation of solvent samples for UHPLC analysis was analogous to the water preparations described in Section 2.2.3.

2.2.5 Determination of the effect of solvent volume on yield

For reactions in water, 0.5, 1.0, 3.0 or 5.0 mL of water was transferred to the test tube containing 1PP prior to addition of MA. 1PP was not stirred in the solvent prior to addition of MA. The reaction mixture was stirred vigorously (800 rpm) for the prescribed time at room temperature. Samples were prepared for analysis by addition of the appropriate amount of THF to obtain total volume of 10 mL in the test-tube e.g. 9 mL of THF to the test tube containing 1 mL of water. See Table 2.1 for details. Each sample was prepared for analysis as described in Section 2.2.3.

Table 2.1 Reaction Conditions for Variation in Solvent Volume and Type

Molar Ratio 1PP:MA (mmol)	Solvent	Volume mL	Temp. ºC	Reaction Time/min	Amount THF, mL
1:1.5	H_2O	0.5	r.t	0.5	9.5
1:1.5	H_2O	0.5	r.t	1	9.5
1:1.5	H_2O	0.5	r.t	2	9.5
1:1.5	H_2O	0.5	r.t	5	9.5
1:1.5	H_2O	1.0	r.t	5	9.0
1:1.5	H_2O	3.0	r.t	5	7.0
1:1.5	H_2O	5.0	r.t	5	5.0
1:1.5	H_2O	1.0	r.t	20	9.0
1:1.5	MeOH	1.0	r.t	5,10, 20	9.0
1:1.5	THF	1.0	r.t	20	9.0
1:1.5	ACN	1.0	r.t	20	9.0

2.2.6 Determination of the effect of molar ratio of reactants on yield in the neat reaction

The molar ratios of amine to acrylate were adjusted as summarised in Table 2.2. All reactions were conducted at room temperature (22 - 24ºC). Each reaction mixture was stirred vigorously (800 rpm) for 2 mins. The 2 mmol equivalent of amine was analysed at additional time-points as detailed in Table 2.2.

Table 2.2 Reaction Conditions for the Neat Addition of 1PP and MA

Molar Ratio of Reactants 1PP:MA (mmol)	Stirring rate rpm	Temp. ºC	Reaction Time/min
1:1	800	r.t	2
1:1.5	800	r.t	2
1:2	800	r.t	2
1.5:1	800	r.t	2
2:1	800	r.t	2, 5, 10, 20
3:1	800	r.t	2
1:1.5	300	r.t	20
1:1.5	600	r.t	20
1:1.5	800	50	20

2.2.7 Determination of the effect of reaction temperature on yield in the neat reaction

An aluminium beaker containing a mixture of sand and silica beads was placed in an oven at 50ºC for 45 minutes. A 20 mL round bottomed test tube was placed into the vessel containing the heated sand/silica mixture, covered and re-equilibrated to 50ºC on a heated stir-plate. 1PP followed by MA was added to the test tube in a 1:1.5 mmol ratio. The reaction mixture was stirred vigorously (800 rpm) for 20 mins.

2.2.8 Determination of the effect of stirring rate on yield in the neat reaction

A 20 mL round bottomed test tube containing a magnetic stir bar was placed on a magnetic stir-plate set to 300, 600 or 800 rpm. 1PP followed by MA was added to the test tube in a 1:1.5 mmol ratio. Each reaction mixture was stirred at the designated speed for 20 minutes.

2.3 Chromatographic Instrumentation and Conditions

2.3.1 Reaction monitoring using UHPLC

UHPLC analyses were carried out using a Waters ACQUITY UPLC H-Class system (Waters Corporation, Milford, MA, USA) connected to an ACQUITY TUV detector (Waters Corporation, Milford, MA, USA). Data acquisition and integration were performed by using the accompanying Waters Empower software. The analytical column was a Waters ACQUITY CSH C18 (50 mm × 2.1 mm, 1.7 µm particles) from Waters (Waters Corporation, Milford, MA, USA), and was maintained at 30ºC. The autosampler was held at ambient room temperature. The column flow rate was set to 0.4 mL/min, and the injection volume was 1.0 µL. Detection was performed at 240 nm. Chromatographic separations were carried out using isocratic elution. Mobile phase consisted of a mixture of 30 mM potassium phosphate buffer pH 2.8:

acetonitrile (93:7, v/v). The mobile phase was filtered through 0.22 µm Millipore HVLP membrane filters prior to use.

2.3.2 Liquid Chromatography-mass spectrometry (LC-MS)

High resolution mass spectrometry was carried out using an Agilent 1260 Ultra Performance Liquid Chromatography system coupled with an Agilent 6250 Q-TOF (Agilent Technologies, Palo Alto, CA, USA) by electrospray ionization (ESI). The analytical column was a Waters ACQUITY CSH C18 (50 mm × 2.1 mm, 1.7 µm particles) from Waters (Waters Corporation, Milford, MA, USA), maintained at 30ºC. The column flow rate was set to 0.4 mL/min, and the injection volume was 1.0 µL. UV detection was performed at 240 nm. Chromatographic separations were carried out using isocratic elution. Mobile phase consisted of a mixture of 10 mM ammonium formate, pH 2.8: acetonitrile (93:7, v/v). The mobile phase was filtered through 0.22 µm Millipore HVLP membrane filters prior to use. Full scan spectra were taken at a cone voltage of 3500 V using positive electrospray ionisation (ESI).

QTOF Parameters	**Values**
Gas Temp. (ºC)	300
Gas Flow (l/min)	8
Nebulizer (psig)	35
Sheath Gas Temp. (ºC)	350
Sheath Gas Flow (l/min)	11
Capillary Voltage (V)	3500
Nozzle Voltage (V)	1000

2.4 UPLC Method Development

A sensitive and selective reversed phase UHPLC method was developed to allow simultaneous fast reaction monitoring of the reactants and product. All three

compounds (1PP, MA and the product MPP) contain UV active chromophores, enabling UV detection. See Figure 2.1 for photodiode array (PDA) UV spectra of each.

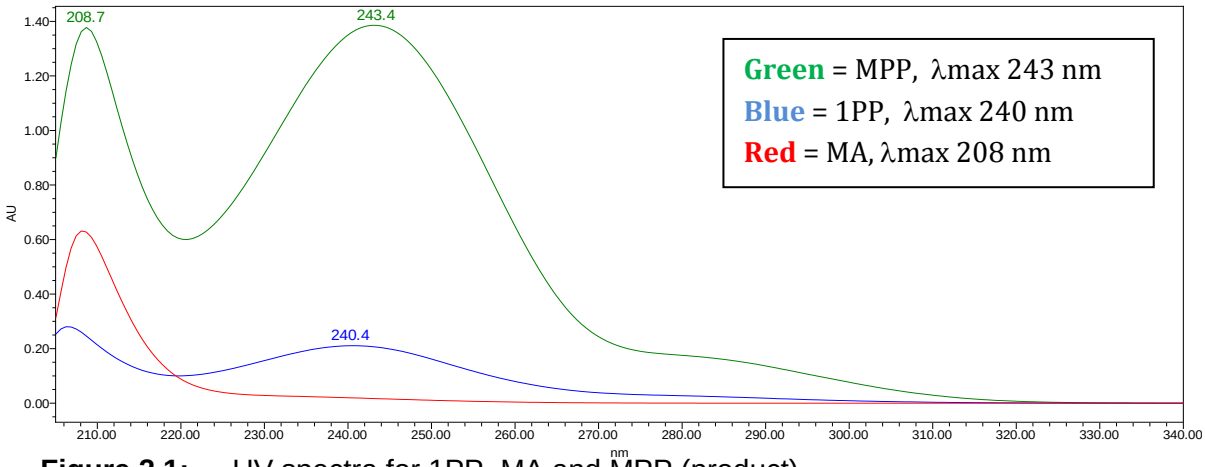


Figure 2.1: UV spectra for 1PP, MA and MPP (product)

2.4.1 HPLC Mobile Phase Optimisation

The chromatographic separation and detection had to first be optimised in terms of runtime, resolution and limit of detection. Sub 5 minute runtimes are common for UHPLC instruments, making it an ideal choice for this type of reaction monitoring where samples are reacted and prepared for analysis within minutes. In this instance the reactants and products eluted within 4 mins, as illustrated in the chromatogram in Figure 2.2.

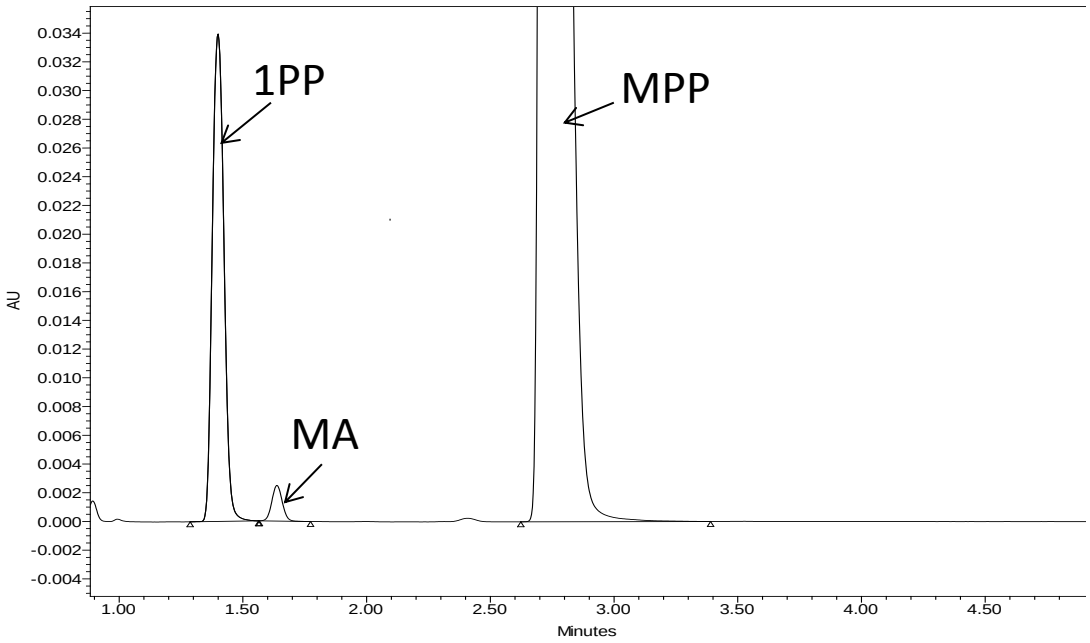


Figure 2.2: Typical UV chromatogram (240 nm) of 1PP, MA and product MPP. All three peaks have eluted within 4 minutes.

Montesano *et al.* detailed a LC-MS screening method for piperazine derived drugs, including 1PP, using a gradient method consisting of 30 mM acetate buffer pH 4.5 and acetonitrile.[109] This mobile phase was chosen as the starting point for method development as it had the added advantage of being LC-MS compatible. While baseline resolution of both reactants and product was achieved, baseline drift and high background noise at the retention time of the 1PP peak meant the method was not sufficiently sensitive for quantitation of 1PP at low concentration. Replacing the acetate buffer with 30 mM potassium phosphate, pH 2.8 had several positive effects; the baseline interference was eliminated and the lower pH reduced tailing factor of the 1PP peak giving better resolution between it and MA. At pH 2.8, the 1PP reactant, which contains both secondary and tertiary amine functional groups (estimated pKa values of the protonated amines are 6.30 and 8.80)[110], would carry a positive charge. Ionisation of the compound reduced the retention time to 1.4 minutes. A number of gradients were investigated but an isocratic elution was

found to be most suitable as it eliminated the baseline drift caused by the mixing of gradient buffers. A mobile phase containing 30 mM potassium phosphate buffer pH 2.8: acetonitrile (93:7, v/v) was found to be optimal for this method.

2.4.2 UHPLC Column Selection

A number of sub 2 micron reverse phase UHPLC columns were trialled as part of method development; Waters ACQUITY CSH (Charged Surface Hybrid) C18 (100 mm × 2.1 mm, 1.7 µm particles) and Waters ACQUITY HSS (High Strength Silica) C18 (50 mm × 2.1 mm, 1.7 µm particles). The CSH column is recommended by the manufacturer for analysis of basic compounds using a low pH, weak ionic strength mobile phase. The HSS column is described as having general purpose silica based C18 chemistry, which may provide increased retention in comparison to the hybrid based C18 columns. The HSS column was trialled to determine if there was any effect on the elution order of the 1PP and MA peaks. While no difference in the elution order was noted, the CSH column gave an improved peak shape for the 1PP amine (due to the presence of positive surface charges) and development continued using the CSH column. The retention time and resolution of peaks obtained with the 100 mm column exceeded requirements and the column was replaced with a 50 mm column to reduce overall runtime.

2.4.3 Sample Preparation for Analysis

Prior to analysis by UHPLC-UV it was necessary to stop or sufficiently slow the reactions to ensure that the reactants did not continue to react during sample preparation. Before injection onto the column it was also necessary to dilute the samples sufficiently to adjust the concentrations of reactants/product to between the linear range of UV absorption (0.1 – 1.0 aufs). Due to the very small volume of reactants used, it was not possible to remove an aliquot of the reaction mixture,

therefore it was necessary to dissolve each reaction mixture completely in a solvent that both stopped the reaction and uniformly dissolved both reactants and products. In addition, sample preparation for injection onto the column required a diluent compatible with the mobile phase.

The aprotic solvents ACN and THF slowed the rate of reaction and resulted in low yields in a number of papers for the aza-Michael reaction [22, 63, 65], and this proved to be the case in this study also. 5 mL of ACN or THF was added to the reaction test tube containing 1PP and MA. The test tube was immediately capped and shaken, followed by immediate dilution with 50% MeOH (1 in 50). The THF sample yielded 9% of product and the ACN sample yielded 17%. Increasing the volume of THF or ACN added to the test tube from 5 to 10 mL and reducing the final sample preparation to a 0.5 mL in 50 mL dilution in 100% MeOH resulted in optimal conditions for sample preparation.

1PP peak shape for the sample prepared in THF + diluted in MeOH was superior to that prepared in ACN + diluted in MeOH and therefore this was chosen as the final preparation. See Figure 2.3. Less than 2% of the 1PP reacted with MA in the time taken to prepare and analyse the samples (< 90 s) when the THF + Methanol protocol was used.

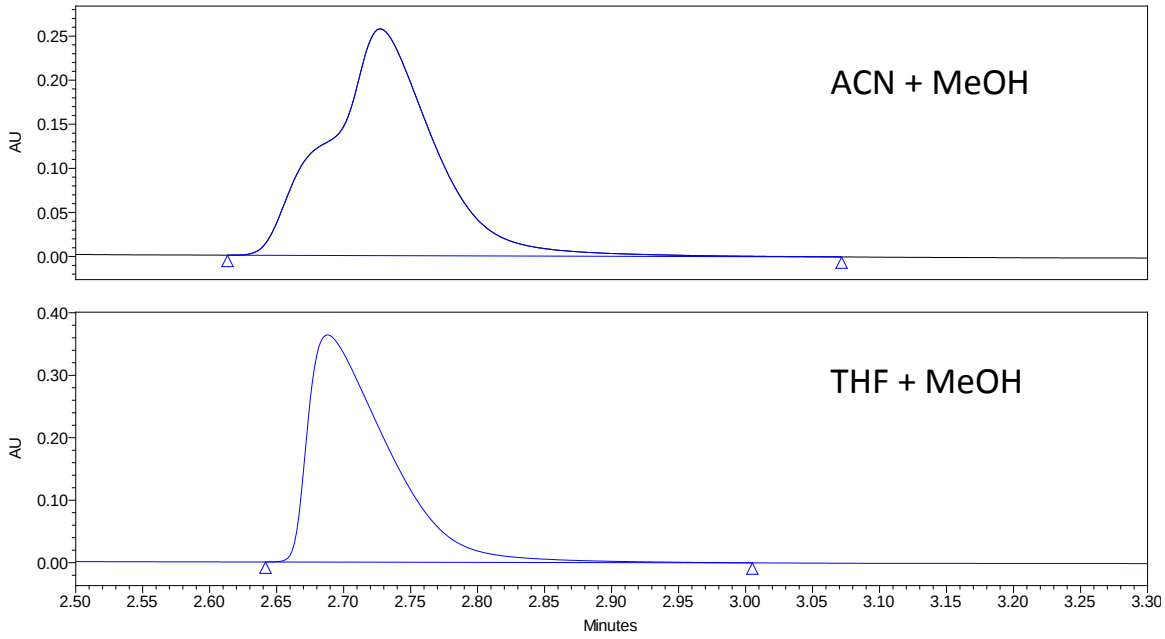


Figure 2.3: Poor peak shape exhibited by reaction sample prepared in ACN followed by dilution in MeOH when compared to sample prepared in THF and MeOH.

2.4.4 Method Verification

Linearity of the detector for 1PP was demonstrated from 0.075 to 300 µg/mL by plotting the peak area of 1PP versus its concentration. Each linearity solution was prepared in duplicate using MeOH as a diluent. A linear least-squares regression was performed, and the correlation coefficient (R) for the regression was 0.999, thus demonstrating a good linear response for 1PP across the concentration range of interest. The linearity plot passed through the origin within 95% confidence interval. Table 2.2 contains the linearity data.

Table 2.2 Linearity Data for 1PP

Parameter	Result
Concentration range, µg/mL	0.075 - 300
Slope, m	8065.4
Y intercept, c	1454.2
Y_{100}, (n = 2)	1228045.9
Y_0/ Y_{100}*100	0.1%
Correlation coefficient, r	0.999

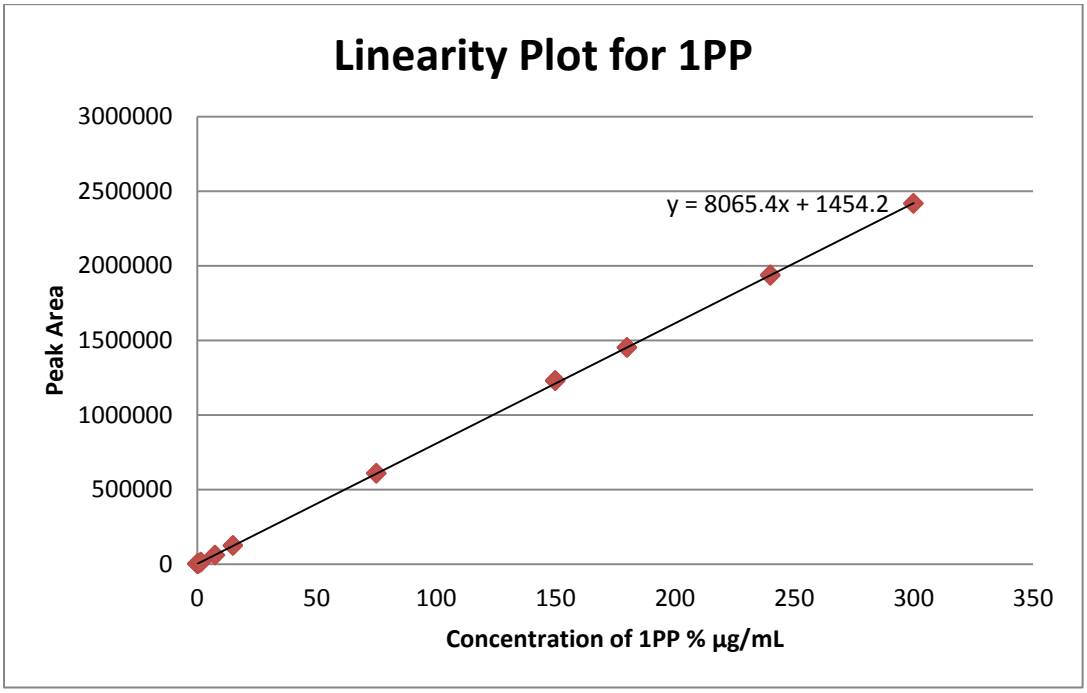


Figure 2.4: Calibration curve for 1PP from 0.075 to 300 µg/mL, n = 2. (error bars are included, but are smaller than the data point icons.)

2.4.5 Internal Standard

The 1PP reagent used in the experiments contained a small impurity. The impurity peak was observed in all 1PP standard chromatograms at approximately 0.4 – 0.6% of the 1PP peak area. The peak area of the impurity with respect to the main 1PP peak proved to be very consistent for each run. As the impurity was unaffected by the reaction with MA, it was possible to use its area to calculate the peak area of

1PP starting material in each reaction. A number of 1PP standard controls were run for each assay and the average peak areas of both the 1PP peak and the impurity peak were calculated. This impurity served as an internal standard throughout the reaction monitoring experiments. The presence of this impurity in every injection allowed calculation of the 1PP starting material for each reaction. Analysis of the 1PP standard using LC/MS yielded a molecular formula of $C_{11}H_{14}N_2O$ and an exact mass of 190.11 m/z and a possible structure 4-phenylpiperazine-1-carbaldehyde.

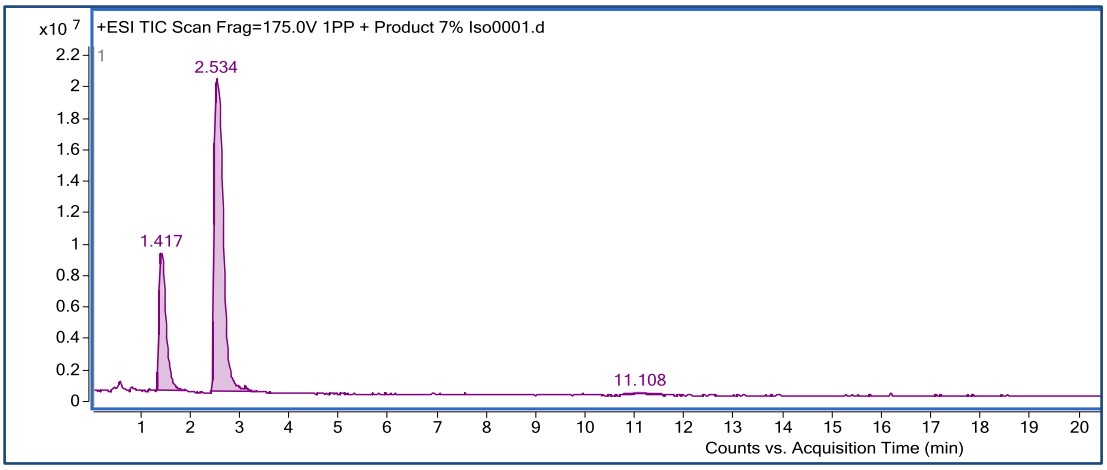


Figure 2.5 TIC for 1PP (1.4 min), MPP (2.5 min) and Impurity (11.1 min)

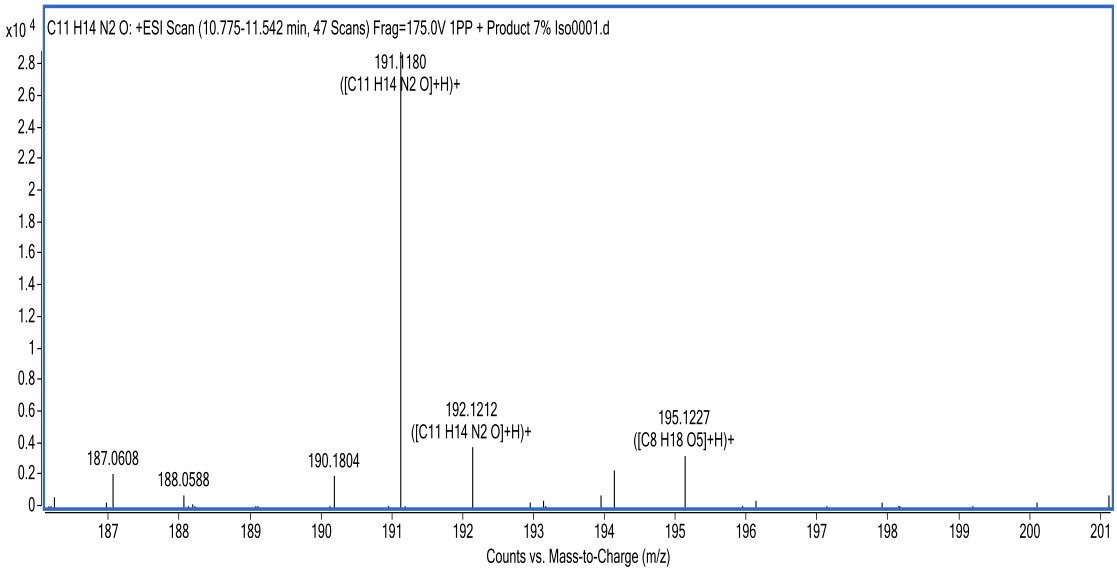


Figure 2.6 ESI scan of impurity peak (which elutes at 11.1 min) showing M+H ion 191.118 m/z

2.4.5.1 Calculation of the Peak Area of the 1PP Starting Material

$$1PP\ Impurity\ as\ \%\ of\ Total = \frac{\text{Peak area 1PP Impurity in Std}}{\text{Peak area of 1PP in Std}} x\ 100$$

Example:

$$1PP\ Impurity\ as\ \%\ of\ Total = \frac{5721}{1264894} x\ 100 = 0.453\%$$

2.4.5.2 Calculation of 1PP Starting Material in Sample using 1PP Impurity as Internal Standard

$$\left[\frac{Peak\ area\ \text{1PP}\ imp.in\ Sample}{1PP\ Impurity\ as\ \%\ of\ Total} x\ 100\right] -\ Peak\ area\ \text{1PP}\ imp.in\ Sample$$

Example:

$$\left[\frac{5664}{0.453} x\ 100\right] -\ 5664 = 1244667$$

2.5 Results and Discussion

2.5.1 Reaction Monitoring by UHPLC

Analysis by UHPLC proved simple and repeatable. Reactions were monitored over a time span of 30 s to 80 min. At time-zero, addition of reactants followed by immediate sample preparation and analysis resulted in a 2% loss of amine. Using the internal standard described in Section 2.4.5, it was possible to accurately quantitate the % loss of 1PP starting material and conversion to product, MPP, and monitor small differences in the reaction yield over time. Figure 2.6 shows that in 0.5 mL of water, initial conversion of 1PP was rapid, with 98% used up in the first 5 min. The reaction slowed after 2 min (90% conversion) as the reactants were consumed. Using the UHPLC technique to investigate the effect of solvent type on the rate of the reaction, it was possible to determine that of the two aprotic solvents examined, the rate in ACN was 1.5 times faster than THF under identical conditions. UHPLC results also demonstrated that the reaction was reproducible for replicates at each time-point.

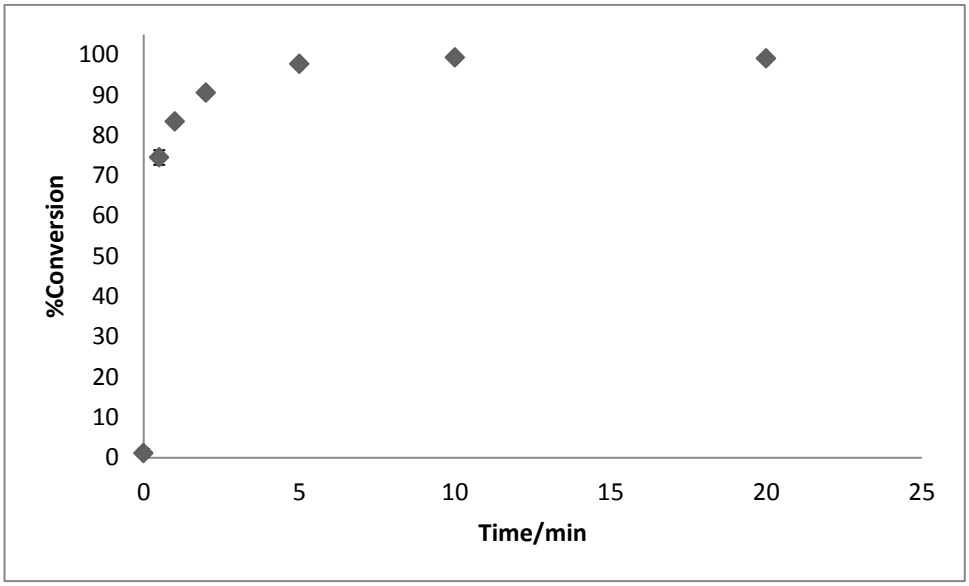


Figure 2.6 % Conversion of 1PP over time for reaction of 1PP and MA in 0.5 mL of water (23ºC). % conversion was determined by UHPLC, using parameters detailed in Table 2.1, n = 2

2.5.2 Effect of Solvent Type on Yield

A comparison of the reaction in protic and aprotic solvents is reported in Table 2.4. The reaction of 1PP with MA was slower with lower yields in aprotic solvents THF and ACN, with only 19 and 28% converted in 20 min compared to 98% conversion in only 5 min for the reaction in water. A 92% conversion was achieved in MeOH after 5 min demonstrating that it is almost on a par with water in terms of its reactivity.

Table 2.4 Reaction Conditions for the Addition of 1PP and MA in Various Solvents

Ratio 1PP:MA (mmol)	Solvent (1 mL)	Temp. ºC	Time/min	Yield %
1:1.5	H_2O	r.t	5	99.0 ±0.6
1:1.5	MeOH	r.t	5	91.8 ± 0.1
1:1.5	ACN	r.t	20	28.1 ± 1.5
1:1.5	THF	r.t	20	19.4 ±1.4

The conjugate addition of secondary amines to α,β-unsaturated carbonyls proceeds through an intermediate state that is accelerated by more polar solvents.[111] The increased reactivity in polar solvents suggests that the intermediate is ionic in nature and more polar than the reactants. In protic solvents such as water and MeOH, protonation of the carbanionic intermediate by the solvent would be rapid (see Scheme 1.3, Chapter 1). Where no proton is available, as is the case with the aprotic solvents, the nitrogen atom of the ammonium moiety provides the proton required for stabilisation.[30]

Systematic investigations into the effect of solvent on the reaction mechanism were carried out by Um and co-workers in the reaction of 1-Phenyl-2-propyn-1-one with alicyclic secondary amines in ACN and water.[112, 113] The amines studied were less reactive in ACN than in water, even though they are more basic in the aprotic solvent by 7-9 pKa units. In contrast to the “on-water” acceleration proposed by Phippen *et al.*, here the authors proposed that the reaction proceeded through a stepwise mechanism, whereby the formation of the addition intermediate remained the rate determining step, and in which proton transfer followed after the rate-determining step. The nature of the transition state was proposed to be responsible for the decreased reactivity in the aprotic solvent; where the proton transfer from the positively charged nitrogen atom to the negatively charged carbon atom occurred

after the rate-determining step. The results are in agreement with the earlier studies of Bernasconi [114].

In the aprotic solvents THF and ACN the reaction has previously been described as 'third order", that is, second order in amine and first order in acrylate [65]. Therefore it can be hypothesised that when the solvent cannot provide the proton required in the transition state, a second molecule of amine is necessary to allow the reaction to occur. To provide insight into the reaction rate, the ratio of amine to acrylate was maintained at 1:1.5 for all experiments. Being pseudo second order, the protic solvents water and MeOH were indeed much faster, as hypothesised. As expected, the rate was depressed in both of the aprotic solvents. In the case of 1PP, MA and MPP, both the reactants and products are neutral (as shown in Scheme 1). As the zwitterionic intermediate is charged, stabilisation of the intermediate favours the polar, protic solvents water and methanol.[115]

2.5.3 Effect of Water Volume on Yield

For practical monitoring of the reaction, the ratio of amine to acrylate was maintained at 1:1.5. In this way, any acceleration noted in the rate of reaction could be attributed purely to the solvent. Initial reaction in water was rapid with 75% of the 1PP converted in just 30 s and 98% in 5 mins. Complete conversion of the reaction was reached after 10 min.

All reactions in water were performed with vigorous stirring (800 rpm) to create an emulsion. The amount of water added to the reaction had no effect on the yield as shown in Table 2.5, entries 4 – 7. Volumes of 0.5 to 5 mL were analysed and an identical result, 98% conversion in 5 min, was achieved in each case. Both Sharpless and Ranu reasoned that the amount of water used was not considered crucial as long as there was sufficient water to generate an aqueous emulsion [20,

23]. While the amount of water used in this study did not have an impact on the yield, the requirement for an aqueous emulsion does not appear to be a feature of the reaction. The results for the reaction in MeOH were almost as high yielding as those in water yet no emulsion was formed in MeOH.

Table 2.5 Reaction conditions for the addition of 1PP and MA

Entry	Ratio 1PP:MA (mmol)	Solvent H_2O (mL)	Temp. ºC	Time/min	Yield/%
1	1:1.5	0.5	r.t	0.5	75
2	1:1.5	0.5	r.t	1	84
3	1:1.5	0.5	r.t	2	91
4	1:1.5	0.5	r.t	5	98
5	1:1.5	1.0	r.t	5	98
6	1:1.5	3.0	r.t	5	98
7	1:1.5	5.0	r.t	5	98

2.5.4 Investigation of "on-water" and neat reaction acceleration

Interest in performing neat reactions is part of an overall trend towards the reduction and elimination of hazardous solvents and catalysts in chemical processes and is often promoted as 'green' in the literature.[28, 63, 116] Of interest to this study were conflicting reports in the literature, often from the same group, describing the aza-Michael reaction as 'accelerated' both in solvent and under solvent free conditions. A 2002 report by Ranu gives a 90% yield in 45 minutes for the solvent free reaction of piperidine and MA.[49] In the highly cited 2007 study by the same author, the reaction was described as accelerated in water for the same reactants, with a 92% yield reported in 30 min.[22] The neat reaction was carried out with a molar excess of the amine (2.4:2), whereas for the reaction in water the opposite ratio was used with the acrylate in excess (1:1.5). As discussed in Section 2.5.1, in the absence of suitable solvent the amine itself can provide the proton necessary to complete the reaction. On both occasions reactions were monitored by TLC. To investigate

whether the reaction was truly ‘accelerated’ in water, 1PP was reacted with MA under the same conditions as those in water (molar ratio 1:1.5). Monitoring the reaction by UHPLC allowed small differences in the conversion of the amine to be tracked.

Results show that the amine was sufficiently nucleophilic, and the neat reaction proceeded smoothly to give 54% conversion in just 2 minutes, as illustrated in Figure 2.7. An initial high reaction rate was expected in the neat reaction due to the high concentration of reactants. The reaction rate slowed considerably thereafter with 90% conversion at 30, 45 and 60 minutes. A 99.5% yield was achieved after 80 min. In the absence of a proton donor (solvent), the nitrogen atom of the zwitterionic intermediate was the source of the necessary proton.[111, 117] This was hypothesised to be the reason why the initial rate of conversion was high, as the amine concentration was greater, but the conversion rate subsequently slowed considerably with the last 10% of the conversion taking almost 1 hour to complete. As the neat reaction proceeded, the test tube was occupied by an excess of product with reducing opportunity for the reactants to collide and dwindling opportunities for protonation of the carbanionic intermediate. The viscosity of the reaction medium changed as the reactants were converted to products, turning from a clear liquid to a yellowish paste and finally to a white crusty residue. The reaction rate and yields of the neat aza Michael addition reaction had previously been reported as ‘inconsistent’ [22]. This was found not to be the case, with a high degree of reproducibility for all the neat reactions performed.

Figure 2.7 also clearly indicates that the reaction did proceed more quickly in water than in the neat reaction. A 90% yield was achieved in just 2 min in the water reaction, whereas it took 20 min for a 90% yield to be achieved in the neat reaction.

As discussed in Section 2.5.2, nucleophilic attack by the amine is rate limiting followed by stabilization of the carbanionic intermediate. In water, protonation of the anion by a water molecule is rapid and the rate of reaction does not depend on the concentration of the reactants. In contrast, for the solvent free reaction the rate of conversion slowed down as the reaction progressed and the amine was used up (in the neat reaction, the amine acts as both the nucleophile and the proton source). This demonstrates that in the case of the neat reaction, the rate of reaction does depend on the concentration of the amine.

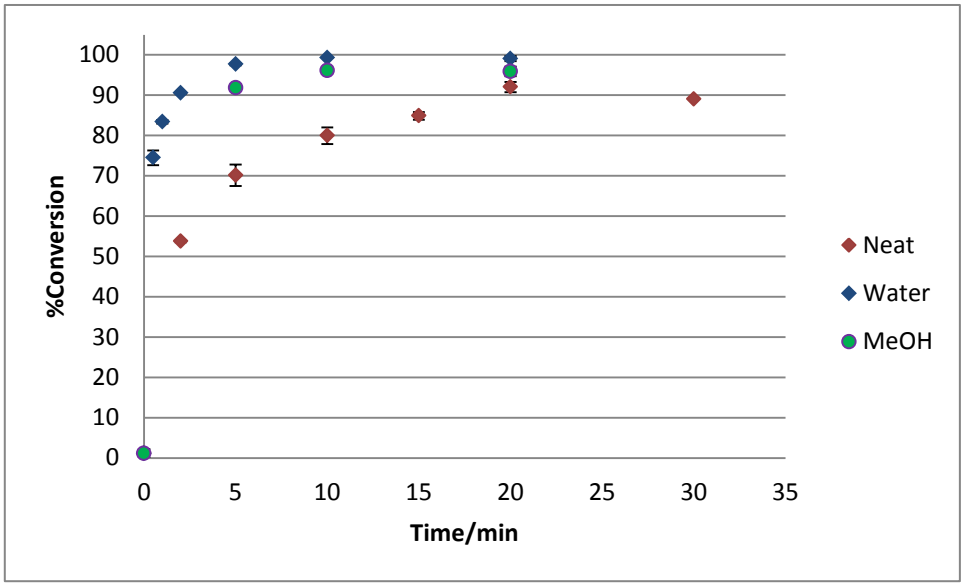


Figure 2.7 Comparison of the rate of conversion of 1PP in reactions carried out in water, methanol and solvent free. All reactions were carried out at room temperature with vigorous stirring (800 rpm).

2.5.5 Effect of rate of stirring and temperature on the neat reaction

The effect of stirring and temperature on the yield of the neat reaction after 20 min is shown in Table 2.6. A small reduction in % conversion was observed for the 300 and 600 rpm rates. This shows that the physical design of the experiment was also

a limiting factor on the conversion; product formed quickly and there was a reduction in the amount of contact between the reactants. As the product formed, the reaction mixture changed from a liquid to a thick paste. Increasing the reaction temperature to 50ºC had no impact on the reaction yield, with 90% conversion achieved after 20 mins, just as was observed for the room temperature reaction.

Table 2.6 Comparison of the effect of stirring rate (rpm value of the stirrer) and temperature of reactants on the yield of the neat reaction

Stirring, rpm	Solvent (mL)	Temp. ºC	Time (min)	Molar ratio	Yield/%
800	Solvent-free	r.t	20	1:1.5	90.6
600	Solvent-free	r.t	20	1:1.5	86.4
300	Solvent-free	r.t	20	1:1.5	85.9
800	Solvent-free	50ºC	20	1:1.5	90.6

2.5.6 Determination of the effect of molar ratio of reactants on yield of neat reaction

Small differences in the ratios of amine to acceptor are reported in various studies reported in the literature. Initially, an experimental design developed from Ranu and Banerjee [23], as detailed in Section 2.2.3, using the 1:1.5 mmol ratio of 1PP and MA was selected to investigate the neat reaction at room temperature. It would appear from several of the published reports, that for the neat reaction an excess of the amine nucleophile had a positive effect on the rate and extent of the reaction [28, 49, 62]. To examine the effect of the amine concentration on the rate of the neat reaction, the ratio of 1PP to MA was altered as per Table 2.2 in the methods section. The results are shown in Figure 2.9. All reactions were carried out at room temperature, with vigorous stirring (800 rpm).

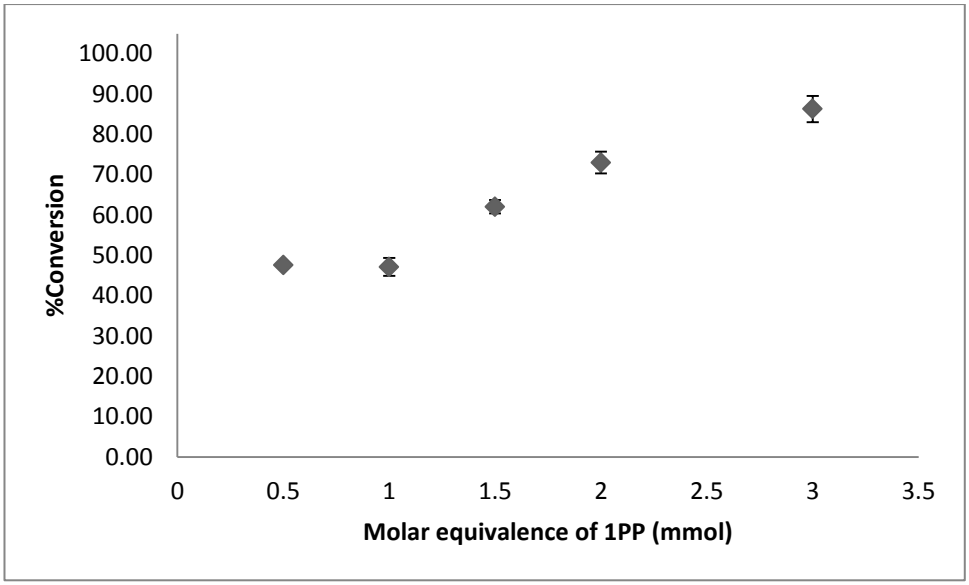


Figure 2.8 Comparison of the effect of molar ratio (1PP:MA) on the yield of the neat reaction. All reactions were carried out at room temperature with vigorous stirring (800 rpm) for 2 min before the samples were analysed by UHPLC.

The neat reaction was expected to behave in a similar way to the reactions in aprotic solvents *i.e.* with second-order dependence on the amine. Increasing the concentration of the acrylate to 1.5 and 2 mmol, while keeping the concentration of the amine constant, had no effect on the rate of reaction. However, the amount of product formed in 2 minutes increased steadily with the increase in amine concentration from 1.5 to 3 mmol, confirming the order is no longer 2nd order overall (1st order in each of the reactants) as is the case for the reaction in protic solvents [111]. Figure 2.8 demonstrates a zero order dependence on the acrylate and a partial order dependence on the concentration of the amine.

2.6 Conclusion

Investigation of the aza Michael addition of 1PP and MA was carried out to examine the effects of solvent type and volume. The effect of molar ratio of the reactants in the neat reaction was also examined. The reaction was monitored using a UHPLC method capable of detecting reactant and products at low levels. The UHPLC method developed for the reaction monitoring demonstrated excellent reproducibility of the sampling and analytical methodologies.

The main focus of this study was to monitor an aza-Michael reaction in such a way as to determine if the reaction could be described as accelerated on-water *i.e.* accelerated by water as has been proposed by other authors. The rate of reaction and % conversion of amine was exceptionally fast for the model reaction in water. MeOH also proved a fast reaction medium. The study demonstrates that while water did have a positive effect on the process, the results of the neat reaction and those of the reaction in MeOH do not support the theory of an oil-water interface. The mechanism of 'on water' catalysis described in Chapter 1 requires a heterogenous mixture to promote acceleration. While the reaction in water is heterogeneous, the reaction in MeOH is not, however the reaction yields were comparable for both solvents.

However, there is a marked difference between the yields of the protic and aprotic solvents. The reaction rate was poor for the aprotic solvents ACN and THF. When compared to the neat reaction (90% yield in 20 min) the results for the aprotic solvents suggest that the neat reaction did not proceed in the same way *i.e.* the amine was not second order in the neat reaction. A difference in the rate reaction was observed between the neat, water and aprotic solvents indicating that the mechanism at work here was not the 'on-water' catalysis proposed by Phippen *et al.*

[62] but rather a mechanism of nucleophilic addition and proton transfer to the carbanion as proposed by Um. [113]

2.7 References

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Chapter 3:

Investigation of the Aza-Michael reactions between amine containing pharmaceutical ingredients and acrylate packaging constituents in ophthalmic solution formulations

3.1 Introduction

Acrylate monomers are widely used components of UV cured ink and adhesives applied to labels for pharmaceutical packaging.[118] For UV inks and adhesives intended for labels applied to low density polyethylene (LDPE) bottles (used for ophthalmic solutions), special care has to be paid to potential migrating species like acrylate monomers not cross-linked in the formed network. [91, 119] The proprietary nature of ink and adhesive formulation makes it difficult for pharmaceutical companies to determine the level of risk associated with each new packaging component. Migration of 'non-intentionally added substances' [120] from food packaging has been the subject of a number of studies by Nerin and co-workers.[83, 121] The group have shown that multilayer packaging materials can be both the source of unwanted impurities and also an effective barrier against migration.[120, 122] The result of a reaction between the active drug substance and a leachable may be a reduction in the concentration of the active component of the drug, but may also result in formation of new impurities of unknown structure and biological effect, which may lead to adverse effects.[66, 123-125] It is therefore important to be aware of such possible reactions during the drug formulation process as well as during drug storage.[126-128] Whilst there is much literature on the aza-Michael reaction in aqueous solutions [55, 129-134], there is little information in the literature on the extent of adduct formation from acrylates and amines migrating from the final packaging into pharmaceutical formulations.

Chapter 2 systematically investigated the rate and yield of the aza-Michael addition of the model amine containing pharmaceutical ingredient 1-phenylpiperazine (1PP) and packaging component methyl acrylate (MA) in various solvents and for the neat reaction. Results indicated that the reaction was indeed optimised by water.

However, it was noted in published literature that Jenner's experiments with MA in water resulted in little or no yield of the ester product when the samples were left standing overnight; presumably as a result of ester hydrolysis.[23] The equilibrium of the ester product relative to the acrylic acid product in aqueous buffered solutions was therefore investigated in a number of standing experiments.

The aim of this research was to investigate the viability of the aza-Michael reaction between amine containing APIs (using 1PP as a model compound) and acrylates from packaging materials under conditions directly applicable to the stability of ophthalmic solutions. The reaction of 1PP and MA was examined in the various buffered solutions commonly used in the formulation of ophthalmic solutions *e.g.* phosphate buffered saline (PBS) and boric acid. The aza-Michael reaction was found to occur in both of these ophthalmic solution formulations. Profiling 1PP-MA formation in appropriate mixtures provided a deeper understanding of the sensitivity of the dynamics of adduct formation, over time, under controlled storage conditions. Many of the amine drug substances used in in ophthalmic solutions are poorly soluble and are formulated as salts to increase the solubility and bioavailability of the API.[135] Additionally, acrylate acids and esters are widely used as reactive monomers and diluents in the adhesives and UV cured ink formulations used in pharmaceutical labels. Therefore a comparison of the reactivity of 1PP to that of its hydrochloride salt (1PP.HCl) was examined in a reaction with both MA and acrylic acid (AA). It was determined that while the reaction was considerably slower, the 1PP.HCl salt did participate in aza-Michael addition, and the corresponding adduct was detected. It was also demonstrated that both the acrylate ester and acrylic acid resulted in the formation of aza-Michael adducts. While adducts were previously observed for acrylate esters, this is the first time the adduct has been reported for the reaction with acrylic acid.

3.2 Experimental

3.2.1 Chemicals and Reagents

1-Phenylpiperazine (1PP), 1-phenylpiperazine hydrochloride (1PP.HCl), methyl acrylate (MA), ethyl acrylate (EA), methyl methacrylate (MMA), methyl vinyl ketone (MVK) and acrylic acid (AA) were purchased from Sigma Aldrich. Phosphate buffer saline powder was purchased from Sigma Aldrich. Boric acid (ACS Grade) was purchased from Applichem. Chemicals for UHPLC and LC-MS analysis were obtained as described in Chapter 2 in Section 2.2.1 .

3.2.2 Buffer Preparation

Phosphate buffered saline (PBS) 0.01 M, pH 7.4 was prepared by dissolving the contents of one pouch of PBS powder (P3813) in 1 L of deionized water. Boric acid 0.3 M solution was prepared by transferring 1.9 g of boric acid into a 100 mL volumetric flask and bringing to volume with deionized water. Sodium phosphate buffer (NaPB), 0.05 M, pH 3.0 was prepared by dissolving 6 g of anhydrous sodium dihydrogen phosphate in 1 L deionized of water and adjusting the pH with phosphoric acid.

3.2.3 Sample Preparations

All reactions were conducted at room temperature (22 - 24ºC) in 20 mL screw-top round bottomed test tubes that had been washed in distilled water and air dried. Vigorous stirring was performed at 800 rpm on a using a Variomag, Telemodul C (Thermo Fischer Scientific, Waltham, MA) stirrer at room temperature.

3.2.4 Standard Conditions for aza-Michael Reactions

The experimental procedure described in Chapter 2 Section 2.2.3 was used to prepare all reaction mixtures of 1PP and acrylates. Sample preparation for UHPLC analysis was as described in Chapter 2 in Section 2.2.3.

3.2.5 Determination of the effect of amine salt on yield

For reactions using the 1PP.HCl amine salt the following was applied; 1 mmol of 1PP.HCl was accurately weighted and transferred to a 20 mL round bottomed test tube containing 2 mL of water. The mixture was vortexed for 30 s to dissolve the amine prior to addition of the acrylate acceptor (1.5 mmol). The reaction mixture was stirred vigorously (800 rpm) or left to stand for the prescribed time minutes at room temperature. All standing experiments were protected from light. After the required time, 8 mL of THF was added directly to the reaction mixture. The test tube was capped and shaken vigorously by hand 3-4 times. Sample preparation for UHPLC analysis was as described in Chapter 2 in Section 2.2.3. Note: The pH of the 1PP.HCl solution (prior to the addition of the acrylate) was 4.76.

Table 3.1 Reactions Conditions for 1PP.HCl with MA and AA

Acrylate (1.5 mmol)	Volume H_2O, mL	Temp. ºC	Stirring (800 rpm) Time/min	Standing, days
MA	2.0	r.t	5, 20, 60	6
AA	2.0	r.t	20	6

3.2.6 Determination of the effect of solvent type on yield

1.0 mL of solvent (water, PBS, boric acid, NaPB) was transferred to the test tube containing 1PP prior to addition of the acrylate (MA or AA). The 1PP was not stirred in the solvent prior to addition of the acrylate. The reaction mixture was stirred vigorously (800 rpm) or left to stand for the prescribed time minutes at room

temperature. All standing experiments were protected from light. Samples were prepared for analysis by the addition of 9 mL of THF to the test-tube. Preparation of the solvent samples for UHPLC analysis was the same as for the water preparations described in Chapter 2 in Section 2.2.3.

Table 3.2 Reactions Conditions for 1PP with MA and AA in buffered and unbuffered solvents

Acrylate	Solvent, (1 mL)	pH	Temp. ºC	Stirring (800 rpm) Time/min	Standing, days
MA	Water	5.6	r.t	20	6
MA	PBS	7.4	r.t	20	12
MA	Boric	4.7	r.t	20	12
AA	Water	5.6	r.t	20	6
AA	Boric	4.7	r.t	20	12
AA	NaPB	3.0	r.t	20	12

3.2.7 Determination of the effect of Michael acceptor on yield

1.0 mL of water was transferred to the test tube containing 1PP prior to addition of the acrylate/ketone (MA, EA, MMA, MVK or AA). The 1PP was not stirred in the water prior to addition of the acrylate/ketone. The reaction mixture was stirred vigorously (800 rpm) for 20 minutes at room temperature. Samples were prepared for analysis by the addition of 9 mL of THF to the test-tube. Preparation of the solvent samples for UHPLC analysis was the same as for the water preparations described in Chapter 2 in Section 2.2.3.

3.3 Chromatographic Instrumentation and Conditions

3.3.1 Reaction monitoring using UHPLC

UHPLC analyses were carried out using a Waters ACQUITY UPLC H-Class system (Waters Corporation, Milford, MA, USA) connected to an ACQUITY TUV detector (Waters Corporation, Milford, MA, USA). Data acquisition and integration were performed by using the Waters Empower software. The analytical column was a Waters ACQUITY CSH C18 (50 mm × 2.1 mm, 1.7 µm particles) from Waters (Waters Corporation, Milford, MA, USA), and it was maintained at 30ºC. The autosampler was held at ambient room temperature. The column flow rate was set to 0.4 mL/min, and the injection volume was 1.0 µL. Detection was performed at 240 nm. The chromatographic separations were carried out using an isocratic elution. Mobile phase consisted of a mixture of 30 mM potassium phosphate buffer pH 2.8: acetonitrile (93:7, v/v). The mobile phase was filtered through 0.22 µm Millipore HVLP membrane filters prior to use.

3.3.2 Liquid Chromatography-mass spectrometry (LC-MS)

High resolution mass spectrometry was carried out using an Agilent 1260 Ultra Performance Liquid Chromatography system coupled with an Agilent 6250 Q-TOF (Agilent Technologies, Palo Alto, CA, USA) by electrospray ionization (ESI). The analytical column was a Waters ACQUITY CSH C18 (50 mm × 2.1 mm, 1.7 µm particles) from Waters (Waters Corporation, Milford, MA, USA), and it was maintained at 30ºC. The column flow rate was set to 0.4 mL/min, and the injection volume was 1.0 µL. Detection was performed at 240 nm. The chromatographic separations were carried out using an isocratic elution. Mobile phase consisted of a mixture of 10 mM ammonium formate, pH 2.8: acetonitrile (93:7, v/v). The mobile phase was filtered through 0.22 µm Millipore HVLP membrane filters prior to use.

Full scan spectra were taken at a cone voltage of 3500 V using positive electrospray ionisation (ESI). Full MS conditions can be found in Chapter 2, Section 2.3.2.

3.4 Results and Discussion

3.4.1 Reaction of 1PP with MA in Aqueous Solutions

The formation of acrylate adducts under conditions relevant to the storage of ophthalmic solutions was tested by performing the reaction in phosphate buffered saline (PBS) and boric acid, two of the most commonly used vehicles in the formulation of ophthalmic drug products. PBS (0.01M, pH 7.4) and boric acid (1.9%, pH 4.7) were prepared as per guidelines for the preparation of ophthalmic solutions.[136] There are several reports in the literature of the reaction in both of these mediums and as anticipated the conversion of 1PP was very high in both solvents [55, 133]. The % conversion of amine in the reaction in PBS was identical to that in water after 20 min vigorous stirring. Yield was marginally lower in boric acid (97.7%) but the reaction was faster and higher yielding than those reported by Chaudhuri using 0.1 M of boric acid as the reaction medium, where a yield of 90% for the reaction between piperidine and MA was achieved in 1.5 hours.[55]

Table 3.3 Reactions of 1PP with MA in buffered/unbuffered solvents

Acrylate Acceptor	Solvent, (1 mL)	pH	Temp. ºC	Stirring (800 rpm) Time/min	%Yield
MA	Water	5.6	r.t	20	99.5
MA	PBS	7.4	r.t	20	99.4
MA	Boric	4.7	r.t	20	97.7

3.4.2 Effect of Standing Experiments

To model the conditions under which ophthalmic solution are stored, samples were prepared in PBS and boric acid as described in Section 3.2.6 and left to stand at room temperature for 12 days. See Table 3.4 for details.

Table 3.4 Reaction for the Addition of 1PP to MA in PBS and Boric Acid, 12 Day Standing Experiment

Acrylate Acceptor	Solvent, (1 mL)	pH	Temp. ºC	Standing, days	%Yield	Ratio Ester:Acid
MA	PBS	7.4	r.t	12	100	54:46
MA	Boric	4.7	r.t	12	100	79:21

Complete loss of the 1PP amine was noted for each sample following analysis by UHPLC. However, in addition to the β-amino acid product (MPP) peak, a second peak at the retention time of the acrylic acid adduct was observed in the chromatogram of each sample. The mass of the second peak was confirmed by LC-MS to be the β-amino acid product. The ratio of the ester:acid product was calculated as a % of the total peak area of both peaks.

The reactions in PBS and boric acid were assayed after 12 days and indicated that hydrolysis of the ester product was greater in the PBS solution. The hydrolysis observed was in agreement with the results of a report in 2000 by Lynn and Langer on the degradation of poly–β-amino esters in water and buffer at physiological pH [137]. At pH 7.4, ester hydrolysis was complete in less than 5 hours, whereas a ½ life of 8 hours was reported at pH 5.1. Reaction products were stored at 37ºC. Results of this experiment showed a similar pattern. In PBS, pH 7.4, the acid product accounted for 46% of the total peak area, whereas in the boric acid medium (pH 4.7) this was reduced to 21%. Results indicate that in the long term storage of

ophthalmic solutions, the acid product would dominate in the event of an interaction with acrylate esters.

3.4.3 Reaction of MA and 1PP Hydrochloride

A search of Reaxys (version 2.19790) for structures containing the 1PP moiety resulted in 218 hits, the majority (212) of which were salts with a protonated piperazine ring (by an acid group). This is not surprising as the secondary nitrogen of the piperazine group is basic (estimated pKa 8.30) [110]

In the aza-Michael reaction the nucleophilicity of the amine is a key factor in the viability of the un-catalysed reaction. As the 1PP.HCl salt is already protonated ($1PP^{+}$) it would be expected to perform poorly as a nucleophile when compared to its free base counterpart. To compare the effects on the reactivity of adduct formation of amines and their hydrochloric salts, 1PP.HCl was reacted with MA in water, initially with vigorous stirring and then in a 6 day standing experiment. See results in Table 3.5

Table 3.5 Results for the Addition of 1PP and 1PP.HCl to MA in Water

Amine Donor	Acrylate Acceptor	Water, (mL)	Temp. ºC	Stirring (800 rpm) Time/min	%Yield	Ratio Ester:Acid
1PP.HCl	MA	2	r.t	5	0.5	-
1PP.HCl	MA	2	r.t	20	1.2	-
1PP.HCl	MA	2	r.t	60	2.4	-
				Standing, days		
1PP	MA	1	r.t	6	99.5	81:19
1PP.HCl	MA	2	r.t	6	43.4	99:1

As expected, the 1 hour experiment resulted in a very low yield, but small increases were noted when samples were tested after 5, 20 and 60 min. Reaction mixtures of

1PP and 1PP.HCl with MA were prepared left to stand at room temperature for 6 days. A modest yield of 43% was observed for the reaction of 1PP.HCl with MA.

The pH of the reaction mixture of 1PP.HCl in 2 mL water was 4.7. Since 1PP and $1PP^{+}$ are in equilibrium in water, it might be suggested that the small amount of un-ionized 1PP present at any one time would be sufficient to undergo the conjugate addition reaction. A 1994 study by Domb *et al.* compared the effects on the reactivity of amide formation in amines and their hydrochloric salts in PBS.[6] The pH of the reaction medium proved instrumental in the reactivity of the amines. The Domb study showed that the hydrochloric salts were just as reactive when the pH was maintained at 7.4 but at pH 5.0 no reaction took place for either the salt or the free base.

Results from this study illustrated that given sufficient time, enough of the amine was available to undergo the reaction with MA. After 6 days 43% of the product has converted to the MA adduct. Only 1% of the acid hydrolysis product was observed in the 1PP.HCl sample in sharp contrast to the free base sample, where almost 20% of the ester product had hydrolysed. Results demonstrated that drug product formulation using the HCl salt was no barrier to the reaction of amines in ophthalmic solutions.

3.4.4 Reaction with Acrylic Acid

Reports on conjugate addition to acrylic acid acceptors are rare. In an enzyme catalysed Michael addition of imidazoles and acrylates no products were detected in the reaction with acrylic acid in pyridine.[138] α,β-Unsaturated acids were used instead of esters in a reaction with thiol nucleophiles in THF at -78C. The resulting reaction products were a mix of alpha (*anti*-Michael) and beta substituted products.[139] With a pKa of 4.25, AA is predominantly ionized in water and the

activation of the carbon-carbon double bond would be supressed when compared to the esters and ketone acceptors [140, 141]. However, the occurrence of acrylic acid adducts in our in-house laboratory investigations are not uncommon. It is hypothesised that the acrylic acid adducts observed are in fact the result of a reaction with an acrylate ester followed by hydrolysis of the ester product as discussed in section 3.4.2. Further investigation into the reaction with acrylic acid over extended periods of time was carried out and results are given in Table 3.6. To investigate whether AA was more reactive at lower pH where the un-ionized form was more prevalent, samples were prepared in boric acid and in a sodium phosphate buffered solution (pH 3.0).

Table 3.6 Reactivity of 1PP and lPP.HCl with Acrylic Acid in Various Aqueous Solutions

Amine Donor	Acrylate Acceptor	Solvent	Solvent (mL)	Stirring (800 rpm) Time/min	%Yield
1PP	AA	Water	1	20	4.8
1PP	AA	PBS	1	20	3.7
1PP	AA	Boric	1	20	2.2
				Standing, days	
1PP	AA	Water	1	6	90.4
1PP.HCl	AA	Water	2	6	25.8
1PP	AA	Boric	1	12	86.3
1PP	AA	pH 3.0	1	12	86.0

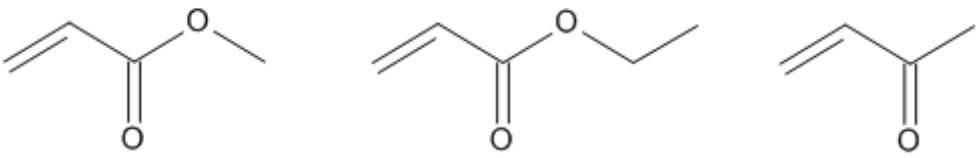
As seen in Table 3.6, for the 20 minute experiments, a poor yield for the reaction with acrylic acid in water was observed, which was not unexpected as acrylic acids are generally less active Michael addition partners [142]. The results indicate that of

the aqueous solutions examined, water gave the highest yields for the 20 min stirring experiment, though yields were extremely low when compared to the reaction with MA. However, over extended time periods, a significant yield of adduct was observed for all 1PP interactions, with the 6 day standing experiment in water yielding 90% AA adduct. The reactions in boric acid and phosphate buffer pH 3.0 both yielded slightly less, with 86% loss of amine after 12 days standing. This was surprising as at the lower pH, it was supposed that a greater proportion of AA would be in the un-ionized form and therefore more reactive. For aza donors, it would appear that the impact of acidic catalysis is double edged; while acid may favour the activation of the carbonyl, an excess might completely consume the nucleophilic amine, by protonation. Once again, the effect of equilibration on standing was evident with 25% loss of amine in the reaction with the 1PP hydrochloride salt. The identical reaction using MA as the acceptor yielded 43%. For amines stored with AA for periods of approx. one week and longer, it can be concluded that aza-Michael addition is a realistic possibility.

3.4.5 Effect of Michael Acceptor on Yield

Schultz and Yarbrough proposed that the different configurations of the of α,β - arrangement of the olefin and the carbonyl moieties can affect their reactivity with nucleophiles [143]. Reaction kinetics and the role of the acceptor in the nucleophilic addition of amines to a variety of olefins were discussed in two early studies by Friedman and Wall [125, 144].

A comparison of the reaction of 1PP with a number of acrylic esters, MVK and AA is reported in Table 3.7. Their structures are illustrated in Figure 3.1. All reactions were performed at room temperature in 1 mL of water. The reaction of 1PP with MMA and AA gave low yields, with less than 5% of the amine converted in 20 mins.



Methyl Acrylate (MA) Ethyl Acrylate (EA) Methyl Vinyl Ketone (MVK)

Methy methacrylate (MMA) Acrylic Acid (AA)

Figure 3.1 Chemical Structures of α, β-unsaturated compounds investigated to examine the effect of Michael acceptor on yield.

Table 3.7 Reactivity of 1PP with Various α,β-Unsaturated Compounds in Water

Acceptor	Time, min	% Yield
Methyl acrylate (MA)	20	99.06 ± 0.6
Methyl vinyl ketone (MVK)	20	98.73 ± 0.2
Ethyl Acrylate (EA)	20	96.06 ± 0.8
Methyl Methacrylate (MMA)	20	4.96 ± 1.4
Acrylic Acid (AA)	20	4.80 ± 0.6

There was no significant difference noted between the reactivity of 1PP with MA and MVK. These results are in agreement with those of Ranu and Banerjee; who using a similar protocol reported 90% yield for MVK and 95% yield for MA in the reaction with pyrrolidine in water [22]. Several studies into the reactivity of olefins rate MVK as being more reactive than its acrylate counterpart, MA [128, 144, 145]. It is proposed that the polarising effect of the carbonyl oxygen is responsible for the activation of the β-carbon, and dilution of this charge by the second oxygen of the acetate group can lower the reactivity of the esters[48]. In the enzyme catalysed Michael addition of imidazoles with acrylates and acrylic acid in pyridine, Cai *et al.* reported that the yield decreased with increasing chain length of acceptor; methyl, ethyl and n-butyl acrylate gave yields of 76, 65 and 62% respectively[138]. However, in this present study, increasing chain length of the alkyl group of the ester from did not change the reactivity significantly with yields of 99 and 96% recorded for the methyl and ethyl acrylates respectively.

3.4.5.1 Substitution at the α-carbon

In the aza-Michael reaction, the reactivity of the electrophile depends on the ability of the carbonyl group to stabilise the negative carbanionic intermediate by either inductive or resonance effects.[143] Substitution at the α-carbon as in the case of a methacrylate results in a tertiary carbanionic intermediate. Since tertiary carbanions are known to be less stable than their secondary counterparts, the reactivity of MMA would be expected to be slower than the corresponding rate with MA on purely electronic grounds.[144] In the case of the reaction with amine nucleophiles such as pyrrolidine and morpholine, the zwitterionic nature of the intermediate is the same for all intermediates *i.e.* following addition of the nucleophile the β-carbon is cationic for all electrophiles in the series, therefore it is the stability of the

carbanionic intermediate that governs the reaction order, with resonance effects more important than inductive.[117] Results of this experiment are in agreement with previous studies. As illustrated in Table 3.7, a 20-fold difference in reactivity between the slowest (methyl methacrylate) and the fastest reacting ester (MA) was observed.

3.5 Conclusions

The reactivity of 1PP with various olefins, typically found in packaging components, in aqueous solutions was examined. Results were in agreement with the literature for MVK, EA and MMA. MA, MVK and EA all resulted in over 95% adduct yield after just 20 minutes. In contrast, MMA and AA resulted in less than 5% adduct formation after 20 min. Nonetheless, this result was highly significant, as the reaction between 1PP and AA has not previously been reported in the literature. AA was poorly reactive in water at room temperature, however 90% conversion of the 1PP amine was observed in a 6 day standing experiment using water as the reaction medium. The same experiment carried out in boric acid and sodium phosphate buffer (pH 3.0) over 12 days was less reactive than in water, but nonetheless resulted in 86% loss of amine.

Results of the investigation into the reactivity of the 1PP.HCl salt showed a similar pattern to that of AA; initial reaction was slow with only 2.2% yield after 1 hour but after standing for 6 days at room temperature a 43% loss of the amine was observed in the reaction with MA and 26% loss in the reaction with AA.

Investigation of the aza-Michael addition of 1PP and MA was carried out in aqueous solutions of boric acid and PBS to examine the effect of pH on the stability of adducts at different pH. As expected, the β-amino ester products were highly

susceptible to hydrolysis at higher pH. Hydrolysis of the ester adduct in PBS was double that of boric acid when stored at room temperature for 12 days.

Overall, the results demonstrate that the viability of the aza-Michael reaction between acrylates migrating from drug packaging and amines present in ophthalmic solutions. Both of the ophthalmic buffers investigated proved to be an excellent reaction medium. Neither the amine salt nor the poorly reactive acrylate acid prevented the formation of adducts under conditions of longer term storage. Therefore, this study raises a concern regarding the interaction between amine drugs and acrylate leachables in ophthalmic solutions.

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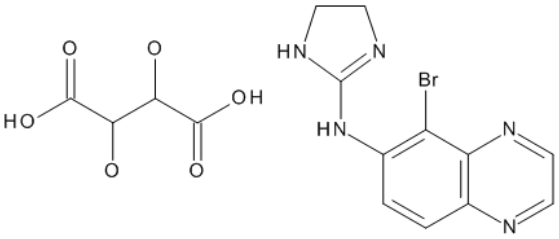
Chapter 4:

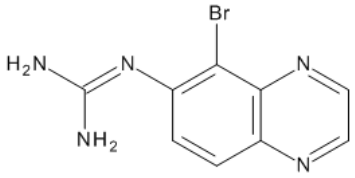
Application of statistical experimental design to the development of a UHPLC method for the simultaneous quantification of brimonidine tartrate, timolol maleate and related substances in ophthalmic solution

Abstract

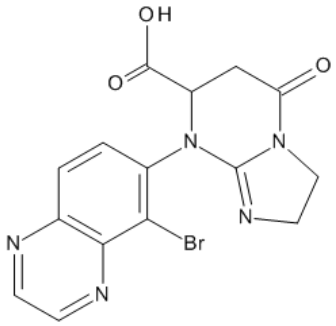
It cannot be assumed that the impurity profile of a combined drug product is simply the sum of the impurities of the individual active pharmaceutical ingredients (APIs). HPLC analysis of APIs brimonidine tartrate (BMT) and timolol maleate (TIM) in samples of a new combined drug product highlighted the appearance of an aza-Michael adduct; formed by an interaction between the amine BMT and the maleate salt of TIM under routine stability storage. An accelerated stability study was carried out at elevated temp and humidity (40ºC/75RH) to investigate the new impurity profile. A second adduct was also detected during the accelerated study which was identified as an acrylic acid adduct of the BMT amine. The two new aza-adducts eluted at the same retention time and were poorly resolved using the existing HPLC drug product method. Accurate quantification of the new adducts required development of a new stability indicating method that was capable of resolving the known impurities of the API and the new adducts. Simultaneous quantitation of BMT, TIM and their impurities in a single method was desirable from a quality perspective as the combined product is a top tier product in our laboratory. The existing HPLC assay has a runtime of 65 min per injection and as the mobile phase contains an ion-pairing reagent, column equilibration can take several hours before system suitability can be established. The aim of this chapter was to develop a fast UHPLC method for the simultaneous quantitation of BMT, TIM and their related substances. Relative response factors (RRF) values for the aza-Michael adducts (RS5 and RS9) were calculated using qualified reference standards by calculating the ratio of the responses of the impurities to the response of the BMT drug substance at 264 nm.



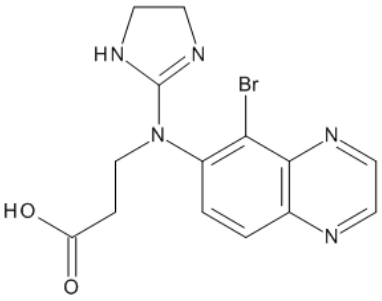
Brimonidine Tartrate (BMT)



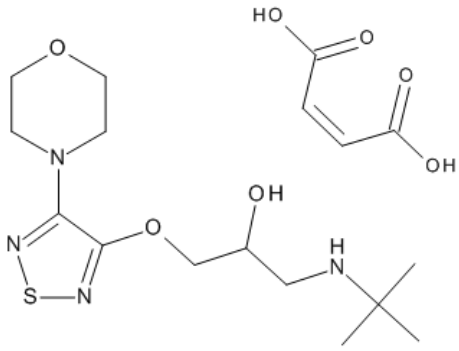
Related Substance 1 (RS1)



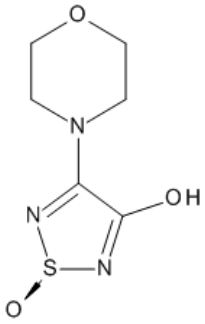
Related Substance 5 (maleate adduct)



Related Substance 9
(acrylic adduct)



Timolol Maleate (TIM)



Related Substance TIM2

Fig. 4.1 Chemical structures of the investigated substances.

4.1 Introduction

4.1.1. Combined Ophthalmic Drug Products

The eye contains a clear, watery liquid (aqueous humor) that nourishes the inside of the eye. Glaucoma occurs when an imbalance in production and drainage of fluid in the eye leads to an increase in intraocular pressure (IOP). Left untreated, glaucoma results gradual loss of the visual field, and can permanently damage the optic nerves and eventually lead to blindness.[146, 147] A 1996 study by Quigley estimated that glaucoma is the second leading cause of blindness globally, with 50% of sufferers in the developing world unaware that they are suffering from the condition. [148]

Treatment of glaucoma frequently requires self-administration of multiple medications at different times during the day. Due to a lack of symptoms, especially in the early stages of the disease, successful treatment of glaucoma requires a high level of commitment on the part of the patient as there is little positive feedback for adhering to the daily dosing regimen. Studies into patient compliance in glaucoma have shown that a simple regimen with less frequent administration could promote compliance. [149, 150] For patients who require more than 1 medication for IOP control, fixed combinations of drugs are more convenient than the concomitant use of the separate components. Other advantages include elimination of potential wash out effects, cost savings and the use of a combination product can also decrease the patient's daily ocular exposure to preservatives. Multi-dose ophthalmic solutions require the addition of antimicrobial preservatives such as benzalkonium chloride, which can be poorly tolerated by some patients.

Combigan (brimonidine tartrate/timolol maleate) 0.2%/0.5% was approved by the FDA in 2005 for the reduction of elevated IOP in patients with glaucoma or ocular

hypertension who require adjunctive or replacement therapy due to inadequately controlled IOP. A twice daily dose of the combined drug formulation was found to be superior to an individual daily dosing regimen of BMT and TIM and was better tolerated than BMT alone.[151] Use of the combination product twice daily results in a daily ocular exposure to preservative that is one third of that associated with the use of both component drugs twice daily.[152]

Timolol Maleate (TIM) (-)-1 - (tert-butylamino)-3- [(4-morpholino-l, 2, 5-thiadiazol-3-yl)-oxy]-2- propanol. Maleate, (Fig. 4.1) is a beta-adrenergic blocker that reduces IOP by decreasing the production of aqueous humour by the ciliary epithelium.[153] Brimonidine tartrate (BMT) [5-bromo-6-(2-imidazolidinylideneamino) quinoxaline L-tartrate] (Fig. 4.1) is an alpha-adrenergic receptor agonist, that reduces IOP by both decreasing aqueous production and increasing outflow. [154, 155]

Several analytical methods have been reported in the literature for determination of BMT and related substances. In European Pharmacopeia, HPLC chromatographic methods have been described for individual determination of both BMT and TIM and their related substances in the drug substance.[156, 157] A stability indicating method using hydrophilic interaction liquid chromatography (HILIC) was developed to assay BMT bulk drug powder. No degradation of BMT was observed following acid, base, peroxide and photo-stressing of the drug powder.[158] However, a forced degradation study of BMT in ophthalmic solution by Ali *et al*., showed BMT to be susceptible to acid, base, peroxide oxidation and photolysis. In solution the drug was particularly vulnerable to light stress with rapid degradation observed.[159] However, no details were given of the nature of the degradation products observed in either study.

An supercritical fluid chromatography (SFC) method for the quantitation of the (R)-enantiomer in (−)-(S)- timolol maleate raw material resolves TIM enantiomers from the impurities specified in the European Pharmacopeia.[160] Several HPLC methods are reported for the simultaneous determination of TIM in both pharmaceutical and physiological fluids.[161, 162] A combined assay and related substance method quantitates TIM, latanoprost, benzalkonium chloride and related substances in the presence of their degradation products in ophthalmic solution. The method uses a gradient elution to separate the two APIs, preservative and 3 known impurities in 55 min.[163] An extensive survey of literature revealed that there is only one HPLC method reported for the simultaneous analysis of BMT and TIM.[164] The method is applied only for the analysis of drug substance in a nanoparticle formulation, is not stability indicating and does not quantitate the related substances of either API or additional impurities arising from the combination product. Therefore there is a need to develop a method to meet these requirements.

4.1.2 Design of Experiment

Conventionally, method development is carried out by varying one parameter while keeping the rest at a target level and then examining the effect of this single change on method performance. The one factor at a time approach (OFAT) is often sufficient when developing methods for a single analyte with one or two well characterised impurities. This approach becomes very time consuming and therefore costly as the number of factors increase. In addition, OFAT does not allow for interactions between different factors.[165] The simultaneous analysis of combination products containing two or more API's of differing polarities, their respective impurities and potential reaction products places a high demand on the developer and requires a more controlled approach.

Design of Experiments (DOE) describes a range of experimental techniques in which the process is 'experimented on' in a controlled manner and the results observed and analysed. The aim is to identify the most important inputs to the process and to understand their effect on the process output. A good design enables all the factors to be investigated at the same time with the minimum number of trials. [165] DOE is closely tied to the quality by design (QbD) approach championed by regulatory agencies such as the FDA, and ICH.[89, 166] Several overviews of the application of DOE and QbD principles to analytical method development and validation have been provided by Rozet *et al*.[167] Molnar [168] and Wang *et al.* [169] The DOE approach is very useful when applied to elements of a method that are subject to a great deal of variation such as sample preparation and pre-concentration using microextraction, solid phase extraction (SPE) supercritical fluid extraction (SFE) techniques.[170-173] Recent examples of the use of DOE to screen for main effects and optimisation using chromatography simulation software include the separation by HPLC of lercanidipine and its three impurities using Drylab [174]; screening of 12 columns using factorial design followed by method optimisation to separate 16 peaks in a UHPLC method for multiple API's.[175]

The aim of this chapter was to develop a simple, rapid and precise UHPLC method for the simultaneous determination of BMT, TIM and their impurities; including two newly identified aza-Michael adducts, one of which was generated due to the combination of BMT and TIM in a single solution. A two-step strategy for method development and optimisation was used and involved the use of Minitab statistical software to determine the factors which had the greatest effect on the resolution of critical pairs of impurities and any possible interactions between factors. Once initial

method conditions were found, Drylab modelling software was used to optimise the final method, which also resulted in an 88% reduction in runtime.

The method was validated in accordance with the current USP Category I for BMT and TIM and Category II for impurities.[176] The validation also meets all the requirements under the ICH Q2 (R1) guidelines.[177]

4.2 Materials and Methods

4.2.1 Materials

Ammonium acetate anhydrous (ACS reagent grade) and glacial acetic acid (HPLC Grade) were purchased from Sigma Aldrich. HPLC grade acetonitrile and methanol were purchased from Merck. Deionized water, purified to a resistance of greater than 18 MΩ, was obtained from a Millipore Corporation Milli-Q system, Millipore (Bedford, MA, USA). Brimonidine Tartrate (BMT), Timolol Maleate (TIM), and all related substances were provided by the Allergan Reference Standard Laboratory, Westport, Ireland. Combigan aged samples were provided by Allergan, Westport, Ireland. All other chemicals were of analytical grade and were used without further purification.

4.2.2 UHPLC Equipment and Chromatographic Conditions

UHPLC analyses were carried out using a Waters ACQUITY UHPLC H-Class system (Waters Corporation, Milford, MA, USA) connected to an ACQUITY TUV detector (Waters Corporation, Milford, MA, USA). Data acquisition and integration were performed by using the Waters Empower software. The analytical column was a UHPLC Column: ACE, Excel 2 C18, Column Part# EXL-101-1002U (100 mm × 2.1 mm, 2 µm particles) and Ace Excel UHPLC Pre-column Filter (Waters Acquity System Compatible) Part # EXL-PCF10/ACQ from ACE (Advanced

Chromatography Technologies Ltd., Aberdeen, Scotland), and it was maintained at either 30ºC or 40ºC. The auto-sampler was held at room temperature. UV Detection was performed at 264 nm. The chromatographic separations were carried out using a gradient elution. Mobile phase A consisted of 50 mM Ammonium Acetate Buffer, with pH adjusted to 4.0, 4.5 or 5.0 (as per Table 4.1). Mobile phase B: methanol (MeOH) or ACN (ACN). The mobile phase was filtered through 0.22 µm Millipore HVLP membrane filters prior to use. A linear gradient was run from 4 to 44% mobile phase B in 10 minutes, followed by a 5 minute equilibration at 4% mobile phase B. Flow rates were 0.4, 0.45 and 0.5 mL/min (as per Table 4.1).

4.2.3 Methods

4.2.3.1 Standards and Sample Preparation

A standard solution containing BMT and TIM was prepared with water as solvent and protected from light by use of amber glassware. Final dilution was made in water and contained 0.008% (w/v) BMT and 0.02% (w/v) TIM (as free base). This working standard solution was used in all screening and optimization experiments. In addition, an aged Combigan sample spiked with approximately 0.2% (w/w) of BMT Related Substance 1 (RS1) and 0.2% (w/w) BMT acrylic adduct (RS9) was prepared by diluting 2 mL of sample into a 50 mL amber volumetric flask. The aged sample contained BMT maleate adduct (RS5), BMT Related Substances 8 (RS8) and 10 (RS10) and TIM Related Substance 2 (TIM2), at concentrations high enough for quantitation. These solutions were used in the method optimization experiments. Studies on the stability of the APIs and impurities found that the standard and sample solutions were stable for 7 days when stored at ambient laboratory temperature in amber glassware.

4.2.3.2 Fractional Factorial Design Experimental Design

A half fractional factorial design experimental design was employed to evaluate the effects of five independent factors, namely flow rate of the mobile phase, mobile phase pH, column temperature, injection volume and the organic component of mobile phase B (MeOH or ACN) on the peak resolution between critical pairs and the symmetry factor of TIM (USP tailing). Table 4.1 shows the different levels for the selected chromatographic factors and their studied responses.

Table 4.1 Chromatographic factors and response variables for Fractional Factorial Design experimental design.

Chromatographic factors	Levels Used		
	Low	Mid	High
Flow Rate, mL/min	0.4	0.45	0.5
pH	4.0	4.5	5.0
Column temperature, ºC	30	35	40
Injection volume, µL	3	4	5
Organic Solvent	MeOH	-	ACN
Response	**Goal**		
USP tailing TIM	Minimise	< 1.5	
USP Resolution of Critical Pair(s)	Maximise	> 1.5	
1. RS1 & RS5 (maleate adduct)			
2. RS5 & TIM2			
3. RS9 (acrylic adduct) & BMT			
4. RS8 & RS10			

For the four continuous variables, experimental conditions were established on two levels (low and high) according to the half fractional factorial design. The fifth component, organic solvent in mobile phase B is a categorical input and is entered

as a text ‘methanol or acetonitrile’ variable. A centre point was entered for each of the numerical factors (pH, temperature, flow rate and injection volume) at the mid-point level to detect non-linear effects. As the organic solvent is a categorical input (either MeOH or ACN) and it is not possible to set the midpoint between 2 different types, Minitab created 2 different centre points (where all the factors are at their mid-points) – one for each type of solvent.

A set of 2^{5-1} = 16 experiments was carried out to determine the factors that made the most important contribution to the selectivity and resolution of the related substances and to the tailing factor of the TIM peak. The central points added another 2 experiments to the design. Table 4.2 gives details of all 18 runs. The following conditions were fixed for each of the experiments:

gradient run time (tG) = 10 minutes, concentration of aqueous buffer, 50 mM.

Table 4. 2 Fractional Factorial Design to identify significant experimental parameters affecting resolution of critical pairs and tailing factor of TIM

Run #	MP B	pH	Column Temp, ºC	Flow Rate, mL/min	Injection Volume, µL
1	MeOH	5.0	30	0.4	3.0
2	MeOH	5.0	30	0.5	5.0
3	MeOH	5.0	40	0.5	3.0
4	MeOH	5.0	40	0.4	5.0
5	ACN	5.0	40	0.5	5.0
6	ACN	5.0	40	0.4	3.0
7	ACN	5.0	30	0.5	3.0
8	ACN	5.0	30	0.4	5.0
9	ACN	4.0	30	0.4	3.0
10	ACN	4.0	30	0.5	5.0
11	MeOH	4.0	30	0.5	3.0
12	MeOH	4.0	30	0.4	5.0
13	MeOH	4.0	40	0.4	3.0
14	MeOH	4.0	40	0.5	5.0
15	ACN	4.0	40	0.4	5.0
16	ACN	4.0	40	0.5	3.0
Centre points					
17	MeOH	4.5	35	0.45	4.0
18	ACN	4.5	35	0.45	4.0

The screening experiments were conducted using the Waters H-Class UHPLC system with a quaternary solvent manager, which can be programmed using Waters Empower software to control up to 4 different mobile phase combinations. In this study, samples were screened with MeOH/buffer and ACN/buffer as mobile phases. Because the compounds of interest are ionic, pH control of mobile phase buffers was essential. For each mobile phase combination, a 10 min short scouting gradient was run. Temperature, injection volume and flow rate are all programmable; the first 16 runs were queued on and run sequentially by Empower, followed by the final two runs for the mid-point buffer pH 4.5. Total analysis time for the 18 injection sets was 2.5 days.

4.2.3.3 Optimisation by Drylab Response Surface Design

The screening experiments were followed by optimization of chromatographic performance using DryLab modelling software. The peak retention times and areas from the pH and column temperature experiments were exported from Empower into DryLab for analysis. Based on the retention data, the software simulates and predicts separations for a large number of variations in chromatographic conditions such as gradient, column dimension, and flow rate without running additional experiments.[178]

4.2.4 Software

Statistical analysis was performed using Minitab 15, purchased from Minitab Inc. (State College, PA, USA). DryLab, version 4, chromatography modelling software package, purchased from Molnar-Institute (Berlin, Germany) was used for screening and optimization of gradient time, temperature, and pH.

4.3 Results

4.3.1 Screening of method factors using Fractional Factorial experimental design

A half-fraction factorial design was used in a preliminary screening study to determine the main effects of five independent factors affecting the peak resolution of 4 critical pairs of impurities and USP tailing of the TIM peak (Table 4.1). Centre points were selected for each of the continuous variables resulting in an 18 run-experimental set (Table 4.2). The experiments were run in a single 'block' using the same column, UHPLC system and mobile phases to minimise the effect of variation over time. The results for each experiment are shown in Table 4.3. USP resolution and tailing factors were generated using Waters Empower software. A result of zero was recorded for impurity peaks that had either fully co-eluted or where more than half of one peak was no longer visible.

Analysis was performed using Minitab statistics software to determine which factors are statically significant *i.e.* there is a relationship (correlation) between the factor and the response. From the results, a main effects plot, interaction plot and Pareto chart (not shown) were generated for each of the five pre-defined responses. The main effect and interaction plots (disussed in Sections 4.3.1.1 and 4.3.1.2) provide an immediate visualisation of the factors that have a significant effect on the response.

To determine if the pattern is statistically significant, multivariate regression analysis was performed using Minitab and a summary of the effect and probability values (*p*-value) for each factor are shown in Tables 4.4.-1 and 4.4-2. The effect value is the average effect of moving a particular factor from its low to high setting. P-values range from 0 to 1; the smaller the *p*-value the greater the statical significance of the

effect. For example, *p*-values of < 0.05 indicate that there is a 95% probability that the effect is genuine *i.e.* not a chance or random event.[179]

Table 4.3 Fractional Factorial Design and Response Results

						Resolution				Tailing
#	MP B	pH	Temp (ºC)	Flow (mL/min)	Inj. Vol (µL)	**RS9 /BMT**	**RS5 /RS1**	**TIM2 /RS5**	**RS8 /RS10**	**TIM**
1	MeOH	5.0	30	0.4	3.0	3.3	1.4	12.9	4.6	1.4
2	MeOH	5.0	30	0.5	5.0	3.2	0	13.5	4.5	1.5
3	MeOH	5.0	40	0.5	3.0	2.6	1.8	14.7	3.5	1.4
4	MeOH	5.0	40	0.4	5.0	2.7	1.4	16.3	3.5	1.7
5	ACN	5.0	40	0.5	5.0	5.0	1.2	12.8	1.1	1.8
6	ACN	5.0	40	0.4	3.0	6.7	1.2	13.5	1.1	1.5
7	ACN	5.0	30	0.5	3.0	5.5	0	9.2	1.2	1.5
8	ACN	5.0	30	0.4	5.0	5.5	0	9.2	1.2	1.7
9	ACN	4.0	30	0.4	3.0	0	0	2.5	0	1.4
10	ACN	4.0	30	0.5	5.0	0	0	2.4	0	1.7
11	MeOH	4.0	30	0.5	3.0	0	6.9	0	1.5	1.4
12	MeOH	4.0	30	0.4	5.0	0	5.4	1.4	1.5	1.5
13	MeOH	4.0	40	0.4	3.0	0	5.0	0	0	1.5
14	MeOH	4.0	40	0.5	5.0	0	4.2	0	0	1.6
15	ACN	4.0	40	0.4	5.0	0	0	2.0	1.1	1.8
16	ACN	4.0	40	0.5	3.0	0	0	1.5	0	1.5
17	MeOH	4.5	35	0.45	4.0	0	4.8	2.5	1.3	1.47
18	ACN	4.5	35	0.45	4.0	1.8	2.6	0	0	1.61

4.3.1.1 Main Effect Plots

The main effect plots for each of the responses are shown in Figures 4.2 to 4.6. A summary of the effects is provided for each plot. The effect of an input factor is calculated by subtracting the average result for when the factor is low from the average result when the same factor is high. For each plot the slope of the line indicates whether or not there is main effect present. When the line is not level, then there is a main effect present. The steeper the slope of the line, the greater the magnitude of the effect. When the line is horizontal there is no main effect present; each of the high and low levels of the factor affect the response in the same way and the response mean is the same across all the factor levels.

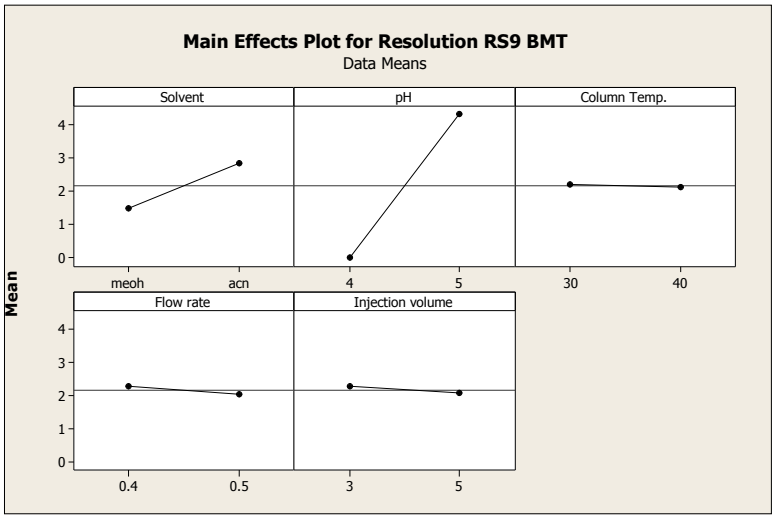


Figure 4.2

The average resolution between RS9 and BMT increased from 0 to 4 when the **pH** was increased from 4.0 to 5.0. The steep slope indicates that pH is the most significant factor. Resolution also increased from 1.46 to 2.82 when ACN was used as the **solvent**. The temperature, injection volume and flow rate were not significant factors in the resolution of RS9 and BMT.

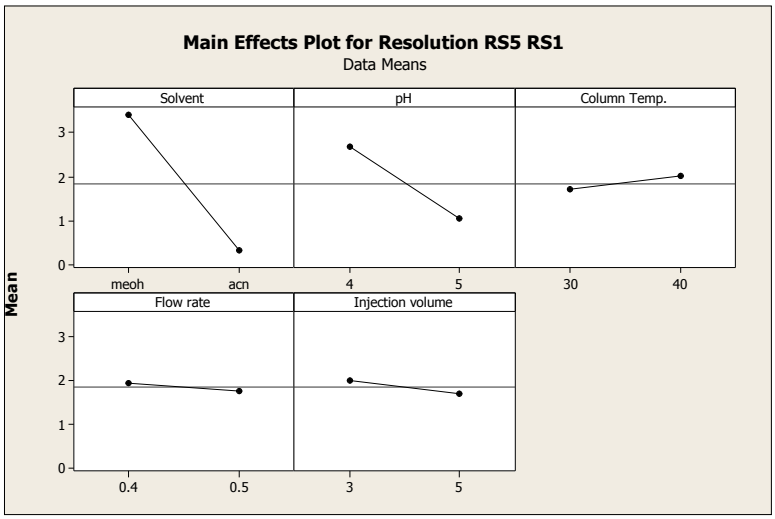


Figure 4.3

The average resolution between peaks RS5 and RS1 increased from 0.3 to 3.2 when ACN was used as the **solvent**. In contrast to the other impurities, the main effect plot shows the average resolution decreased from 2.7 to 0.8 when the **pH** was increased from 4.0 to 5.0. The temperature, injection volume and flow rate were not significant factors in the resolution of RS5 and RS1

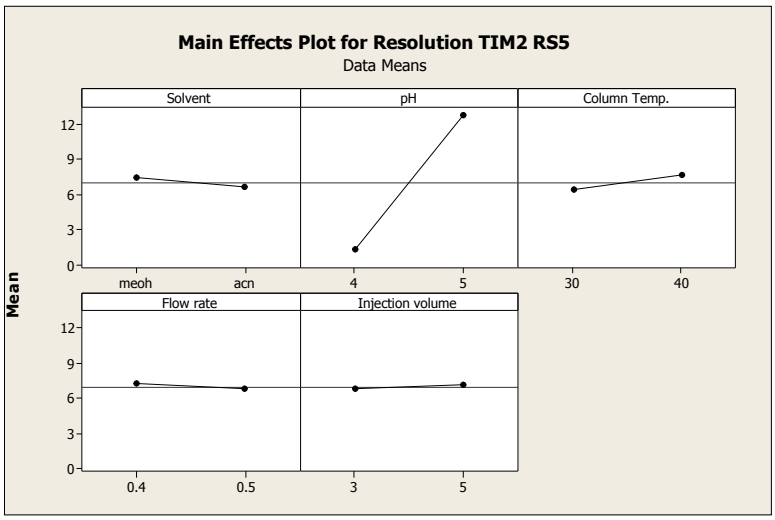


Figure 4.4

The average resolution between peaks TIM2 and RS5 (maleate adduct) increased from 0 to 12 when the **pH** was increased from 4.0 to 5.0. The solvent, temperature, injection volume and flow rate were not significant factors in the resolution of TIM2 and RS5. The mean resolution for all factors bar pH is high at approx. 7.

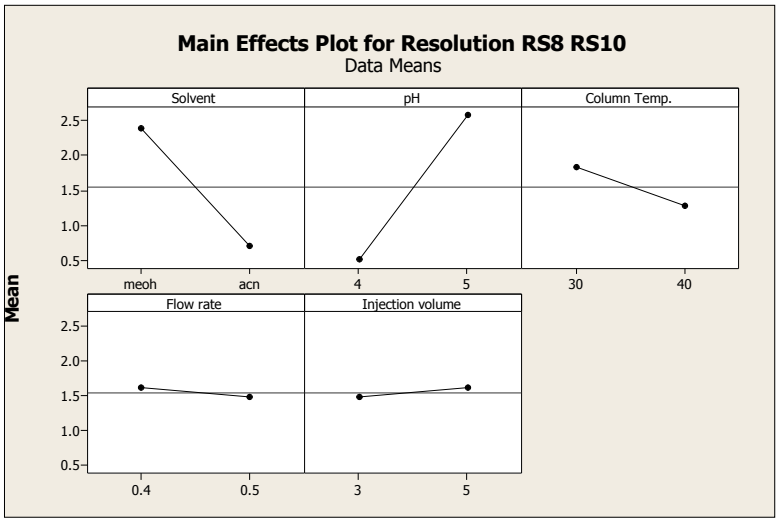


Figure 4.5

The average resolution between RS8 and RS10 increased from 0.6 to 2.4 when the **pH** was increased from 4.0 to 5.0. Resolution decreased from 2.4 to 0.6 when ACN was used as the **solvent** and was reduced to a lesser extent when the temperature was increased from 30 to 40 ºC. The injection volume and flow rate were not significant factors in the resolution of RS8 and RS10

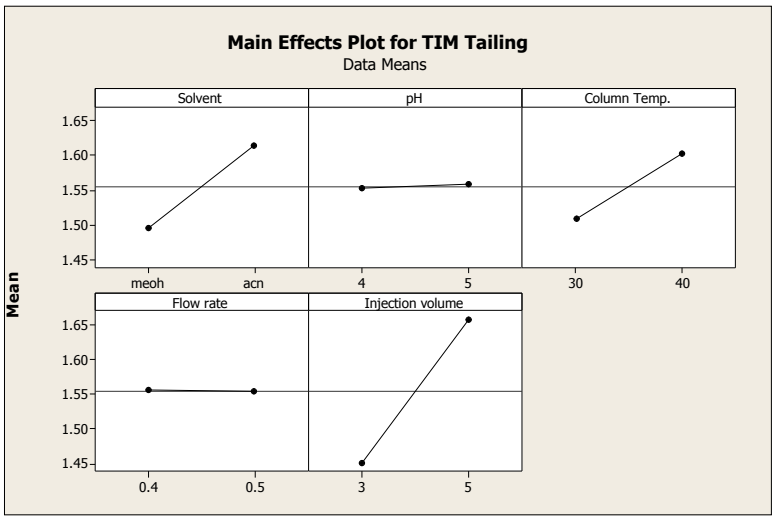


Figure 4.6

The average tailing for TIM increased from 1.45 to 1.65 when the injection volume was increased from 3 to 5 µL. Tailing also increased from 1.45 to 1.6 when ACN was used as the solvent and increased to a lesser extent when the temperature was increased from 30 to 40 ºC. The pH and flow rate were not significant factors in TIM tailing.

4.3.1.2 Interaction Plots

The interaction plot is used to view the interactions between factors. If the lines on the interaction plot are not parallel, this indicates that an interaction exists. The greater the difference in slope between the lines, the higher the degree of

interaction. The interaction plots for the resolution of 3 pairs of impurities are shown in Figures 4.7 to 4.9. No interactions were observed for the resolution of TIM2/RS5 and TIM tailing.

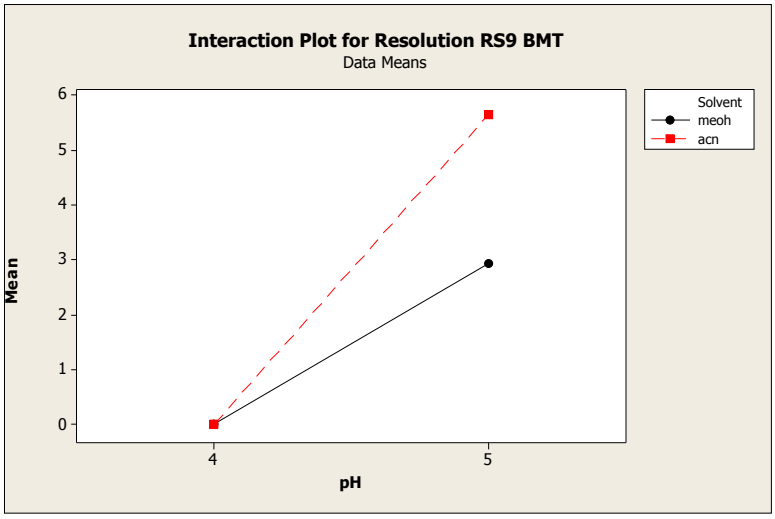


Figure 4.7

The plot for RS9 and BMT indicates an interaction the pH and the choice of solvent. The resolution is higher for both solvents when the pH is increased from 4.0 to 5.0. However, it appears that the difference in resolution between runs is more pronounced for ACN at the higher pH. In order to get the highest resolution for this critical pair, results suggest that the pH is set to 5 and ACN is used as mobile phase B.

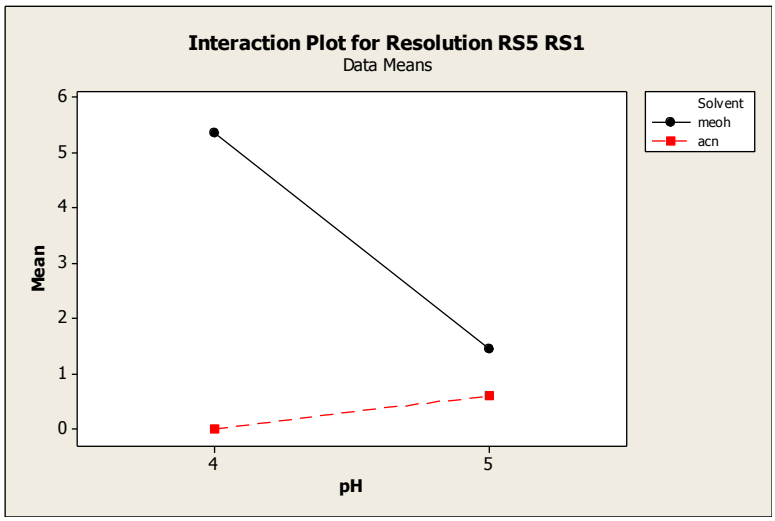
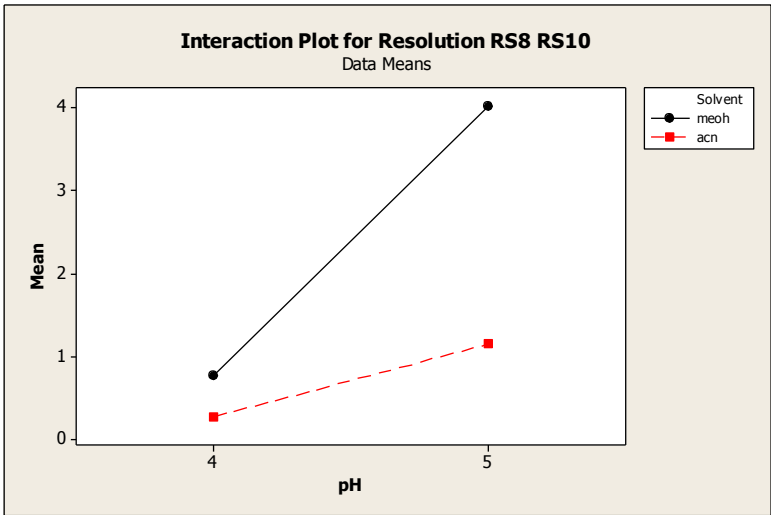


Figure 4.8

The plot for RS5 and RS1 indicates an interaction the pH and the choice of solvent. The resolution is higher for MeOH for both pH 4.0 and 5.0. ACN does not reach minimum resolution for either pH. MeOH does achieve minimum resolution at pH 5.0 but it is not fully resolved and further optimisation will be required.

Figure 4.9



The plot for RS8 and RS10 indicates an interaction the pH and the choice of solvent. The resolution is higher for both solvents when the pH is increased from 4.0 to 5.0. However, the difference in resolution between runs is more pronounced for MeOH at the higher pH. In order to get the highest resolution for this critical pair, results suggest that the pH is set to 5 and MeOH is used as mobile phase B.

4.3.1.3 Statistical Significance of the Main Effects and Interactions

While the main effect and interaction plots provide an immediate visualisation of which factors are important in the study, they do not show whether the effect is significant or not. Multivariate regression analysis was performed for each response using Minitab. Probability values (*p*-values) of less than 0.05 indicate that the effect is 'significant' or true and not simply a matter of chance. The effect for each of the significant results is also shown. Effects are either positive or negative. Results are provided in Tables 4.4-1 and 4.4-2 for each of the 5 responses.

Table 4.4-1 Regression coefficients and associated probability values (p-value) for peak resolution

Term	RS9 and BMT		RS5 and RS1		TIM2 and RS5	
	Effect	*p*-value	Effect	*p*-value	Effect	*p*-value
pH	4.29	**0.019**	-1.81	0.092	11.57	**0.032**
Temperature	-	0.695		0.685	-	0.283
Flow rate	-	0.325		0.934	-	0.567
Injection volume	-	0.360		0.316	-	0.610
Solvent	1.40	**0.055**	-2.85	**0.055**	-	0.350
pH x temp.	-	0.695		0.181	-	0.190
pH x flow	-	0.325		0.576	-	0.960
pH x Inj. vol.	-	0.360		0.829	-	0.962
pH x solvent	1.36	**0.061**	2.40	0.070	-	0.150
Temp. x flow	-	0.363		0.869	-	0.752
Temp. x Inj. vol.	-	0.390		0.551	-	0.962
Temp. x Solvent	-	0.305		0.335	-	0.603
Flow x Inj. vol.	-	0.305		0.454	-	0.606
Flow x Solvent	-			0.893	-	0.857
				0.307	-	0.567

Table 4.4-2 Regression coefficients and associated probability values (p-value) for peak resolution and USP Tailing of Timolol

Term	RS8 and RS10		TIM Tailing	
	Effect	*p*-value	Effect	*p*-value
pH	2.07	**0.036**	-	0.540
Temperature	-	0.132	0.09	**0.048**
Flow rate	-	0.436	-	0.889
Injection vol.	-	0.476	0.20	**0.022**
Solvent	-1.64	**0.043**	0.12	**0.035**
pH x temp.	-	0.670	-	0.540
pH x flow	-	0.496	-	0.889
pH x Inj. vol.	-	0.452	-	0.433
pH x solvent	-1.19	**0.063**	-	0.262
Temp. x flow	-	0.476	-	0.690
Temp. x Inj. vol.	-	0.425	-	0.168
Temp. x Solvent	-	0.101	-	0.262
Flow x Inj. vol.	-	0.253	-	0.690
Flow x Solvent	-	0.523	-	0.303
Injection vol. x Solvent	-	0.464	-	0.358

4.4 Discussion

4.4.1 Minitab screening study

Main effect plots were generated by Minitab for each response (Figs. 4.2 to 4.6). The plots immediately highlighted conflicting trends in the resolution of the critical pairs. Resolution was increased for three of the four pairs at the higher pH (5.0). One pair was more resolved using ACN as the organic modifier whereas resolution of two other critical pairs and the TIM tailing were improved by the use of MeOH. One pair of impurities (TIM2/RS5) were unaffected by the choice of solvent. Interestingly, with regard to the flow rate, the design showed that it was not a significant variable for all responses. This was surprising, as using HPLC, particularly on a gradient, tailing of a peak is typically lower at the higher flow rate. This was one of the many anomalies noted between the HPLC and UHPLC for gradient runs.[180] Likewise, temperature was not a significant variable for the resolution of impurities, but registered as significant factor for TIM tailing. Across the range studied, the pH of the mobile phase is not a factor for TIM tailing. With a pKa of 9.2, timolol will be fully ionised between pH 4 and 5, and so the absence of pH correlation in this range was expected. The injection volume had no effect on the resolution of the impurities yet proved to be the factor that had the greatest effect on the TIM tailing.

4.4.1.1 Resolution of Critical Pairs

The primary goal of developing a UHPLC stability indicating method is to separate the API (s) and impurities (resolution Rs > 2.0) so that accurate reliable data can be recorded over the shelf life of the product and to ensure the quality of the pharmaceutical formulation.

Resolution between a set of peaks is calculated using the general equation

$$Rs = 0.25*N^{1/2}[(\alpha-1/\alpha)](k/1 + k)$$

The selectivity parameter 'α' has the greatest impact on resolution. Selectivity can be changed by modification of the mobile phase composition, column chemistry and temperature. [181]

Multivariate regression analysis using Minitab revealed that for the resolution of 4 critical pairs of impurities, the factors that had the greatest impact were the mobile phase pH and the choice of organic solvent. The direction of the slope indicates whether the factor has a direct or inverse effect on the response. The *p*-values and the condition that gave the highest resolution of each critical pair are summarized in Table 4.5. The injection volume, temperature and flow rate were not significant variables in the resolution of impurities.

Table 4.5 Summary of Main Effects and associated probability values (*p*-value) for peak resolution and USP Tailing of Timolol

	Solvent		pH		Injection Vol.		Temp.		Flow
	p-value	Max.	*p*-value	Max	*p*-value	Max.	*p*-value	Max	
RS9 and BMT	0.05	ACN	0.01	5.0	-				-
RS5 and RS1	0.05	MeOH	-	-	-		-		-
TIM2 and RS5	-	-	0.03	5.0	-		-		-
RS8 and RS10	0.04	MeOH	0.03	5.0	-		-		-
TIM Tailing	0.03	MeOH	-	-	0.02	3 µl	0.04	30	-

Resolution of RS9 (Acrylic Acid Adduct) and BMT

The main effects plot (Fig. 4.2) indicated that most significant factor for resolution of RS9 and BMT was buffer pH and that increasing the pH resulted in an increase in resolution. The effect value (Table 4.4-1) of 4.29 shows that the RS9 impurity co-eluted with BMT at pH 4.0 and was fully resolved at pH 5.0. Choice of solvent was also significant with an increase in resolution observed for ACN. The interaction plot (Fig 4.7) shows that maximising the response by combining the pH and solvent preferences for RS9/BMT would lead to a resolution of 5.7, which was in excess of what was required. As RS9 was the lone impurity for which ACN was preferred, optimisation for all responses meant that MeOH was selected. The interaction plot indicates that a resolution of > 2.0 could still be achieved using MeOH as the solvent. However, the results table (Table 4.3) shows that the pH must be > 4.5 when using MeOH as the solvent as the centre-point trial resulted in co-elution of the peak.

Resolution of RS5 (Maleate Adduct) and RS1

This was the only critical pair for which pH was not the most significant factor. The main effects plot (Fig. 4.3) indicated that greatest resolution would be achieved using MeOH as Mobile Phase B. Table 4.4-1 gives a negative effect value of -2.85 for the solvent. The value is negative because MeOH was input as the 'lower' value in Minitab, even though the solvent does not have a numerical value. It is the size of the effect that is important. As resolution decreases with an increase in pH from 4.0 to 5.0, the effect for pH is also negative, -1.81. This was the only critical pair where the lower pH resulted in higher resolution. The interaction plot (Fig. 4.8) shows that combining pH 4.0 and MeOH would result in a resolution of > 5; again this was

more than was needed. Using MeOH and pH 5 would result in a max Rs of 1.4. This is below target and meant that further optimisation of the mobile phase pH was required. The results table (Table 4.3) shows that at pH 4.5 when using MeOH as the solvent in the centre-point trial, the resolution improved dramatically to 4.8.

Resolution of TIM2 and RS5

The main effects plot (Fig. 4.4) indicated that most significant factor for resolution of TIM2 and RS5 was buffer pH and that increasing the pH resulted in an increase in resolution. Table 4.4-1 confirms this with a single large effect (11.57) noted for the pH factor. The pH was the only significant effect with a strong preference for the higher pH (5.0). This was the only critical pair for which the solvent choice was not a significant factor. TIM2 is the main degradant impurity of Timolol maleate and is structurally very different from the BMT impurity RS5. The differences in ionisation and polarity ensure that the risk of co-elution was lower than for other impurities. However, co-elution was observed (Table 4.3) for the central point result using ACN as Mobile Phase B at pH 4.5. When the same mid-level conditions were run using MeOH as the solvent, resolution of 2.5 was achieved. As MeOH was optimal for two sets of critical pairs and for TIM tailing, it was therefore selected as Mobile Phase B. There were no statistically significant interactions ($p < 0.05$) between factors for the resolution of TIM2 and RS5.

Resolution of RS8 and RS10

The main effects plot (Fig. 4.5) indicated that most significant factor for resolution of RS8 and RS10 was buffer pH and that increasing the pH resulted in an increase in resolution. The plot also shows that the highest resolution achieved was 2.5 using pH 5.0. Choice of solvent was also significant, with an increase in resolution observed for MeOH. An interaction between pH and solvent was observed (Fig.

4.9) that indicates that maximum resolution could be achieved by combining the effects of using MeOH as Mobile Phase B and setting the pH of the buffer to 5.0.

4.4.1.2 USP Tailing for Timolol Peak

USP tailing is a measure of the asymmetry of a peak. A chromatographic peak with a tailing factor of 1 means that it is symmetrical. A tailing factor greater than 1 means peak tailing while a tailing factor less than 1 means peak fronting.[181] The results table (Table 4.3) shows that TIM tailing was present across all conditions. Having this information on a single chart is one of the reasons why a DOE is so worthwhile. A great deal of time can be wasted fine tuning a single response, whereas knowing what factors are not significant and can therefore be eliminated from future trials is a huge benefit. The main effects plot (Fig. 4.6) indicated that most significant factor for tailing was the injection volume and that increasing the injection volume resulted in an increase in tailing. The combined drug formulation contains 0.2% BMT and 0.5% TIM. As the method is designed to quantitate the API's simultaneously the concentration of the sample injected needs to be sufficient for BMT without overloading the column with TIM. Naturally, the higher the injection volume, the greater the concentration of TIM loaded on the column. The accuracy and precision of TIM was determined for each condition trialled. The average recovery for TIM was 100.3% and the %RSD was 0.09 for the 18 runs. This clearly demonstrates that the tailing does not impact on the accuracy and precision of the method. As the injection volume had no impact on the resolution of critical pairs, but significantly affected tailing, it was set to 3 µL, minimising tailing. Choice of solvent and temperature also had an impact on tailing. As the lower temperature reduced tailing from 1.6 to 1.5 it was selected for optimal conditions. In summary, to reduce

tailing for TIM, results suggest that the injection volume be set to 3 µL, MeOH be used as Mobile Phase B and the temperature of the column be maintained at 30ºC. There were no statistically significant interactions ($p < 0.05$) between factors for the TIM tailing response.

4.4.1.3 Response Optimisation

The DOE results (discussed in Sections 4.4.1 and 4.4.2) show that further optimisation of the method conditions were required. Results of individual injection sets show that all five requirements were only met for run 3 (Table 4.3). This is unsurprising as the main effect plots showed conflicting responses for both solvent and pH for different pairs of impurities. Response Optimizer is a function of the Minitab software which can identify the combination of factor settings that jointly optimize a single response or a set of responses. A weighing was attached to each response based on experience with the assay. For example, resolution of the acrylic acid adduct RS9 from the BMT main peak was given a high weighting as this was one of the primary aims in developing the assay. Likewise resolution of the RS1 and RS5 maleate adduct was also of importance. A target value of Rs = 2.5 was set for the critical pairs containing RS5 and RS9, whereas lower target of Rs = 2 was set for the other two pairs. Impurity peaks RS8 and RS10 are similar in size and observed at low levels (< 0.2% (w/w) of BMT LS). As a result they are fully resolved at Rs = 2. By way of contrast, while it is desirable to limit the tailing factor of TIM to below 1.5, results illustrated that the tailing did not have an impact on the accuracy or precision of the result. Table 4.6 details the target values and weight assigned to each of the five responses.

Table 4.6 Factors and targeted criteria used in Minitab optimization

Response	Goal	Lower	Target	Upper	Weight
RS9 and BMT	Target	1.5	2.5	3	1
RS5 and RS1	Target	1.5	2.5	3	1
TIM2 and RS5	Target	1.5	2	4	2
RS8 and RS10	Target	1.5	2	3	2
TIM Tailing	Target	1.0	1.2	1.6	3

The Response Optimizer was used to generate the factor optimization plots (Fig. 4.10 and 4.11) along with the optimized method condition for the five responses. Fig. 4.10 is an example of the optimal conditions suggested to reduce TIM tailing based on a target of 1.2. The results show that under optimal conditions, a tailing factor of 1.3 was the best that could be achieved. When the response for TIM tailing was combined with the resolution criteria (Fig. 4.11) trade-offs were required to achieve optimal results for all responses, with the tailing factor increased to 1.4. when the temperature of the column was increased to 40ºC. The optimisation plot shows the effect of each factor (columns) and the responses or composite desirability (rows). Minitab allows the user to manually adjust the settings (red lines) and displays the change in target response.

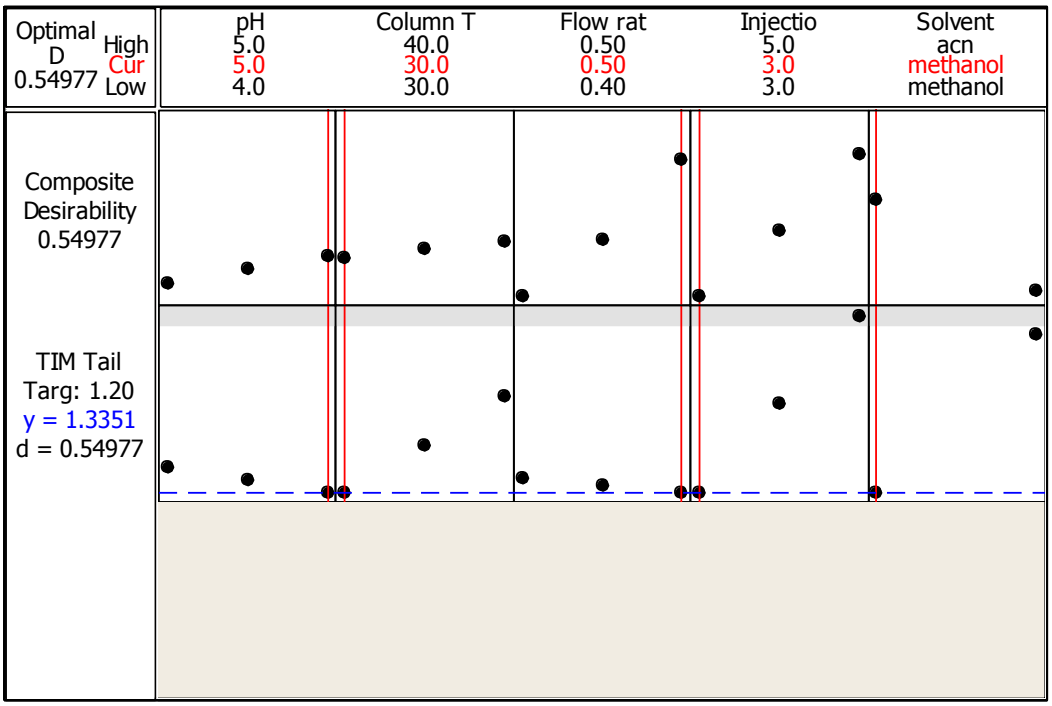


Fig. 4.10 Factor optimization plot for TIM Tailing generated using Minitab.

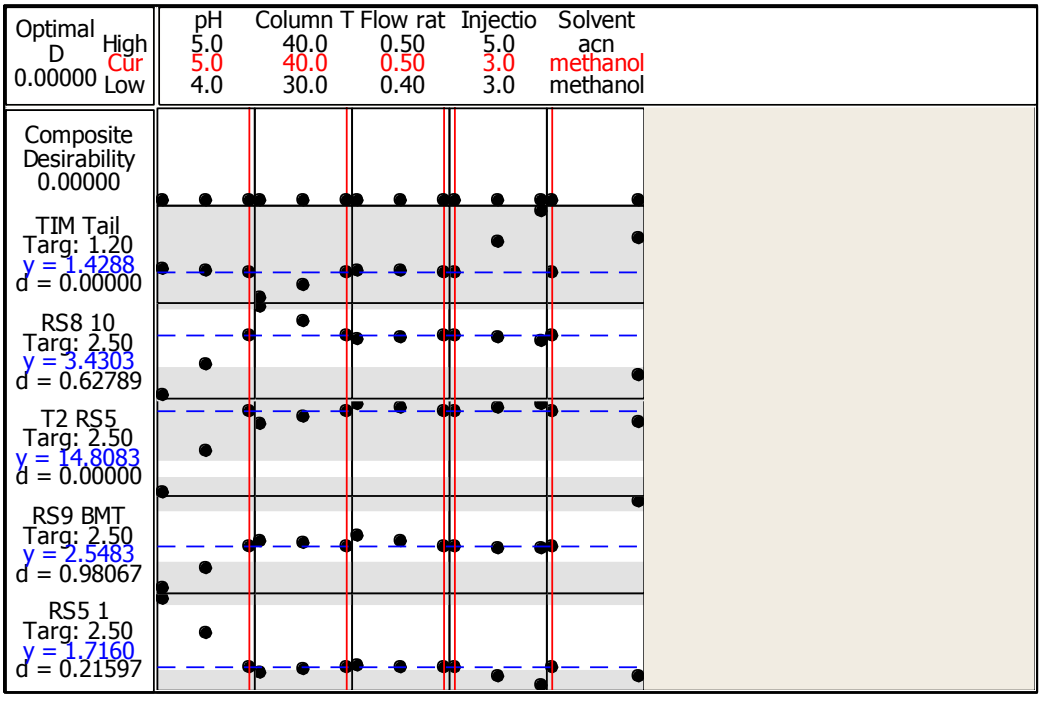


Fig. 4.11 Factor optimization plots for Resolution of 4 Critical Pairs and TIM Tailing generated using Minitab.

As a starting point for further optimisation using Drylab, the following conditions were selected as nominal, based on this section.

Table 4.7 Nominal Conditions for Method Optimisation

Parameter	
Mobile Phase A:	50 mM Ammonium acetate buffer, pH 5.0
Mobile Phase B:	MeOH
Column Temperature:	30ºC
Flow rate	0.5 ml/min
Injection volume	3 µL

4.4.2 Method Optimisation Using Drylab

Following the recommendations of the screening study in Section 4.4.1, MeOH was selected as Mobile Phase B; injection volume was set to 3 µL and the flow rate to 0.5 mL/min. It is critical to select a pH at which the method is robust, to ensure that minor changes in the buffer preparation will not have a negative influence on the resolution of peaks. Based on the DOE screening study, a pH of 5.0 with a 50 mM acetate buffer was found to be optimum for three of the four sets of impurities. The centre-point results (rows 17 and 18, Table 4.3) indicated that further work was required to establish the acceptable limits of the pH of the mobile phase. Investigation of the variation of the aqueous eluent between pH 4.6 and 5.2 was carried out using the fast/slow gradients described in Table 4.8. Chromatograms were integrated using Empower software and the results were exported directly to the Drylab modelling software for analysis. A 2-D resolution map of pH and gradient time (tG) was generated and is shown in Fig. 4.12. The warm orange colour shows the parameter region in which the resolution of the four critical peak pairs was

higher than 2.0 (Rs,crit > 2.0). This region is surrounded on all side by green and blue bands which show that resolution for all critical pairs was not achieved at pH below 4.6 and above 4.9. The map shows the pH was both critical and limited.

Table 4.8 Additional Drylab Sets

	pH	Gradient (min)	Column Temperature (ºC)	Flow rate (mL/min)
1	pH 4.6	10	30	0.5
2	pH 4.6	26	30	0.5
3	pH 5.0	10	30	0.5
4	pH 5.0	26	30	0.5
5	pH 5.2	10	30	0.5
6	pH 5.2	26	30	0.5
7	pH 4.8	10	30	0.45
8	pH 4.8	26	30	0.45
9	pH 4.8	10	45	0.45
10	pH 4.8	26	45	0.45

The colour code in the resolution map represents the value of the critical resolution, with warm “red” colours representing large resolution values (Rs > 2.0) and cold “blue” colours representing low resolution values (Rs < 0.5).[168] It can be seen from the resolution map that the method was robust in the pH range between 4.7 and 4.9 and a gradient time of 6 and 10 min and provides the highest peak resolution (Rs > 2.0). The flow rate of the assay was adjusted using the Drylab software and demonstrated that the resolution was achieved for flow rate from 4 to 5 mL/min. Based on this map, the nominal pH of the mobile phase was set to 4.8 for all subsequent experiments.

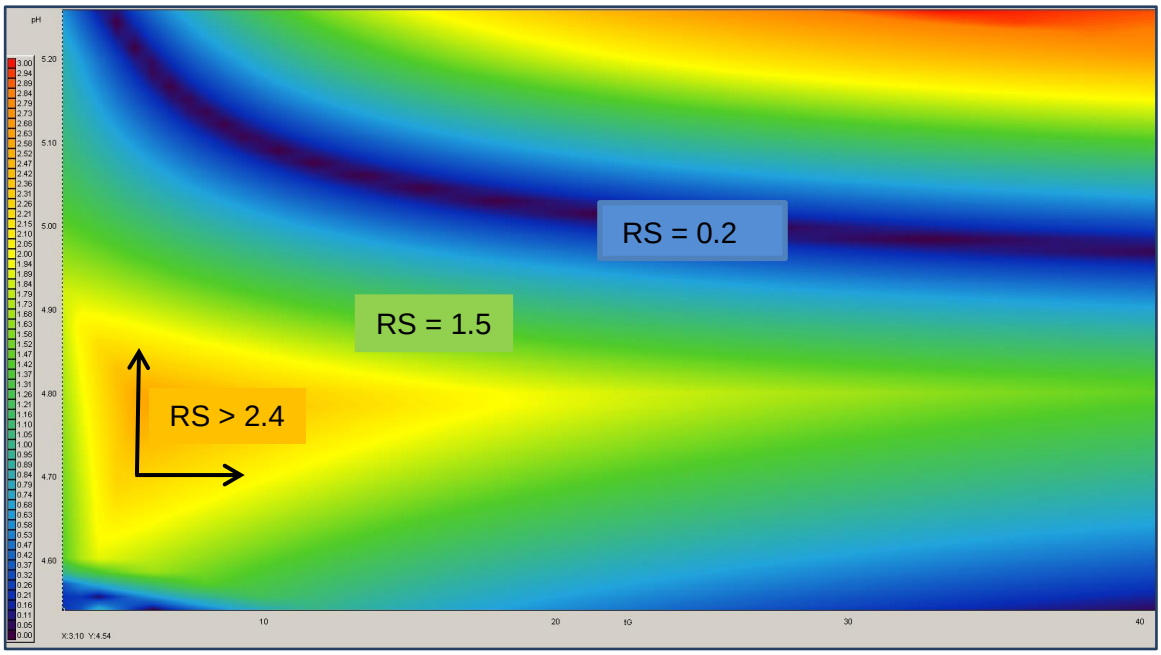


Fig. 4.12 tG and pH model at 30ºC using MeOH as Mobile Phase B; the warm orange colour shows the parameter region in which the resolution of the four critical peak pairs was higher than 2.0 (Rs,crit > 2.0). This region is surrounded on all side by green and blue bands indicating regions where all critical pairs were not achieved.

Once the pH was established, a final set of experiments were carried out to examine the influence of the column temperature (runs 7 – 10 in Table 4.8). The Response Optimisation charts showed a conflicting set of responses for the column temperature, with the USP tailing of TIM favouring the lower temperature and the resolution of impurities favouring a higher temperature. Investigation of the variation of the temperature between 30 and 45ºC was carried out using the fast/slow gradients described in Table 4.8. The aqueous mobile phase consisted of a 50 mM ammonium acetate buffer at pH 4.8. A 2-D resolution map of temperature and gradient time was generated and is shown in Fig 4.13. Results showed a greater range for temperature than for pH, with a 'sweet spot' below 35 ºC. The temperature

was robust between 28 and 32 ºC a gradient time between 6 and 10 min and providedthe highest peak resolution (Rs > 2.0).

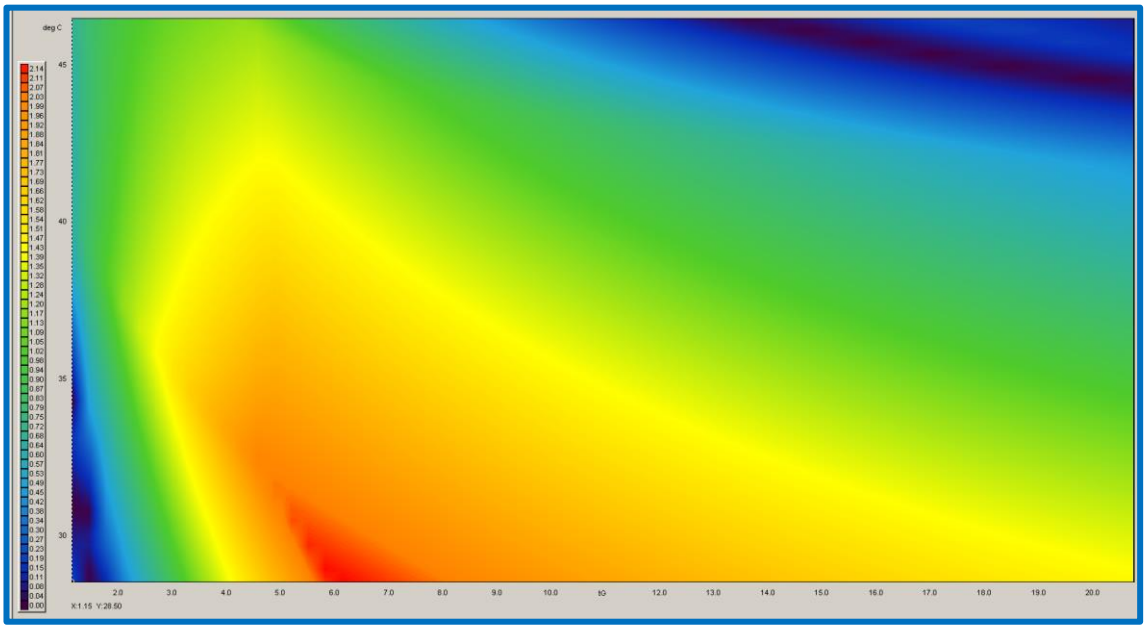


Fig. 4.13 tG and temperature using a pH of 4.8 and MeOH as Mobile Phase B; the red/orange colour showed the parameter region in which the resolution of the four critical peak pairs was higher than 2.0 (Rs,crit > 2.0).

Results from the Drylab study indicated that the method was capable of withstanding adjustments to mobile phase pH, temperature and flow rate using a 10 minute linear gradient. A robustness study was performed by varying chromatographic parameters and the nominal conditions were validated in compliance with ICH Q2R1 guidelines. The final method conditions are shown in Table 4.9

Table 4.9 UHPLC Method Conditions for the Analysis of BMT, TIM and Impurities in Finished Drug Product Ophthalmic Solution

Parameter	Nominal	Robustness Tests
Mobile Phase A:	Ammonium Acetate 50 mM	45mM, 55 mM
Mobile Phase A:	pH 4.85	4.80, 4.90
Column Temperature:	30ºC	28, 32
Flow rate	0.45 ml/min	0.4, 0.5
Mobile Phase B	MeOH	-
Injection volume	3 µL	

Figures 4.14 and 4.15 illustrate the predicted and experimental retention times for 11 peaks (BMT, TIM and 9 impurities).

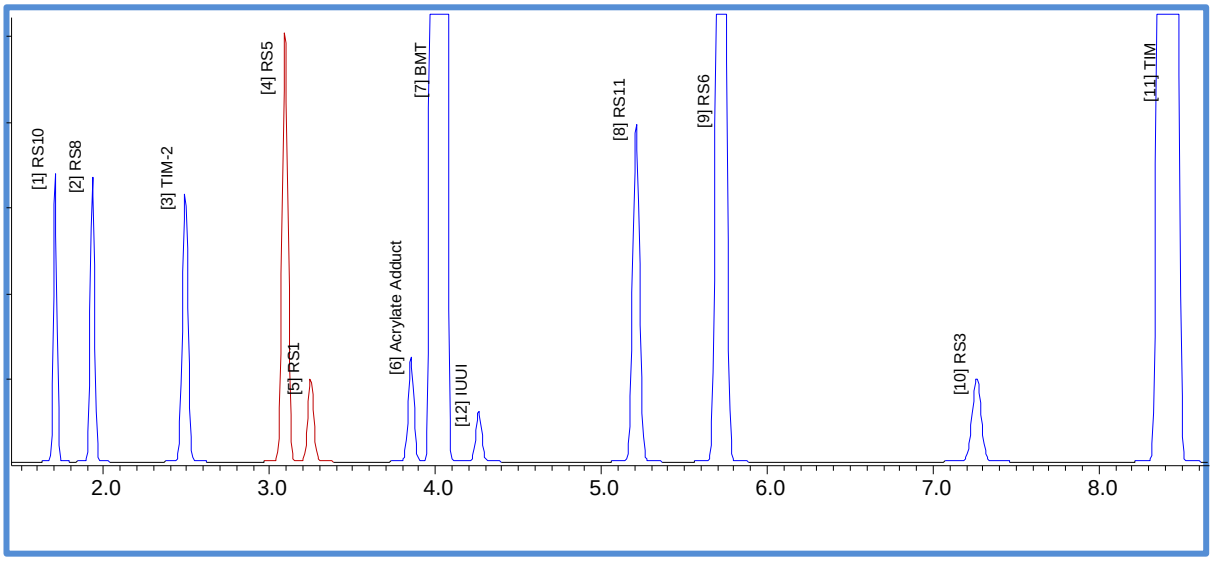


Fig. 4.14 Drylab predicted chromatogram for conditions at pH 4.8, tG = 10, column temperature 30ºC

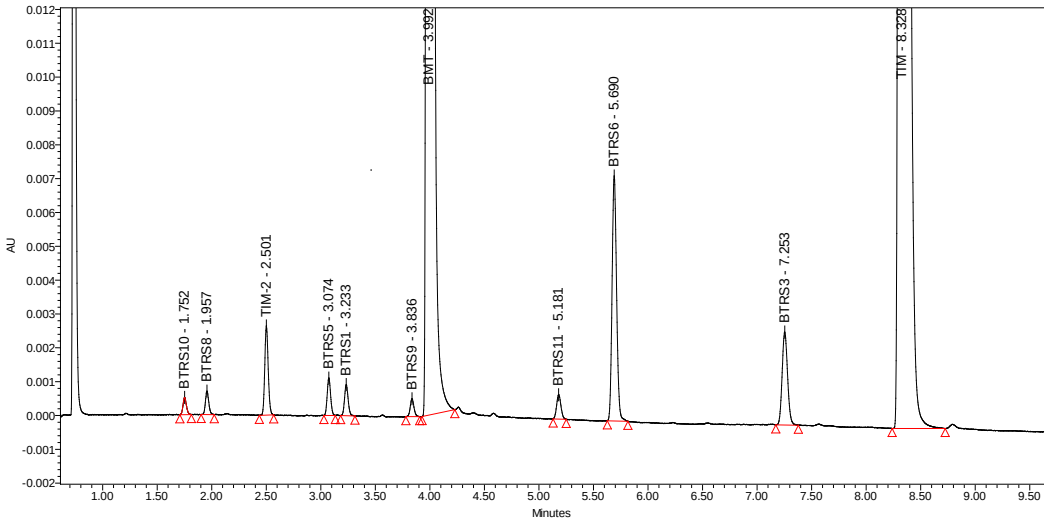


Fig. 4.15 Experimental chromatogram of a drug product sample for conditions at pH 4.8, tG = 10, column temperature 30ºC

4.5 Conclusion

The goal of this chapter was to develop a fast UHPLC separation of BMT and TIM (in combined drug product) and 11 potential impurities. Of particular interest was the resolution of two aza-Michael adducts that were only found in the combined drug product. The UHPLC method developed here will be used in Chapter 5 for the quantitation of acrylic adducts formed by the reaction of BMT and TIM with both methyl acrylate and acrylic acid.

Despite assumptions of scientific impartiality, method development involves a certain amount of personal bias. Based on past experience (good and bad) scientists tend to favour certain brands of column, buffer salts or combinations of organic solvents. Using a DOE strategy allows the developer to remove themselves from the study and let the results speak for themselves. This is especially useful

when preliminary scouting gradients show that there is little difference between the conditions selected and there is no clear ‘best set’ of conditions.

In this chapter a two-step strategy for method development and optimisation was used. Due to the complicated nature of a combined drug product the first step was to screen the main effects of factors that significantly affected the resolution of critical pairs of impurities and tailing of the TIM peak. Multivariate regression analysis was successfully employed to establish which factors had the greatest impact. Graphical methods were used to analyse the results by generating the main effect and interaction plots. Optimized method conditions were obtained by analysing the response data using the Minitab Response Optimizer feature. The most significant factors for the resolution of impurities were mobile phase pH and choice of organic solvent. MeOH was selected as the optimal solvent, which greatly reduced the number of experiments required for the final optimisation step. All experiments (DOE screening and Drylab optimization study) were completed within one week.

Step two involved the use of chromatography modelling software, to find a suitable separation (Rs,crit > 1.5) for 11 impurities, by proper adjustments of gradient, temperature and pH, while maintaining analysis time lower than 15 min. The final method resulted in the baseline separation of 13 peaks in 10 min. Compared to the current HPLC assay, the runtime has been reduced from 65 to 15 minutes, the preparation time has been reduced so that overall the method can be completed in 12.5% of the previous time and the volume of organic waste produced has been reduced by 90% for each injection.

4.6 References

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Chapter 5:

Investigation of aza-Michael reaction in formulated drug product: amine- acrylate screening study

5.1 Introduction

Analytical monitoring of impurities in new drug substances is a key component of the recent guideline issued by the International Conference on Harmonization (ICH). As per ICH guidelines for impurities in new drug substances, for a maximum daily dose ≤ 2 g/day of a drug substance, the reporting and identification thresholds are 0.05% and 0.10%, respectively.[98] Therefore it is a mandatory requirement from regulatory authorities to identify and characterize any unknown impurity present at or above 0.1% level in drug substance.

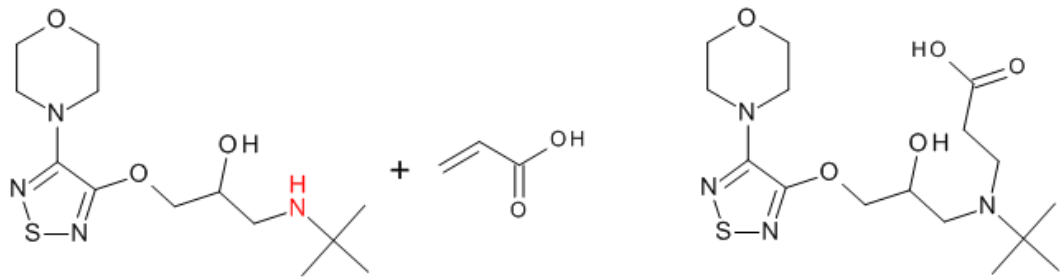
Forced degradation studies provide data to support the identification of potential degradation products that may form during formal stability studies conducted on drug substances and drug products.[182, 183] Occasionally, impurities are detected in stability studies that were not observed in the forced degradation studies. These impurities are often the result of a potential interaction of the drug substance with formulation excipients, packaging or shipping materials and will not be evident unless studies are designed to look for these potential interactions. In the majority of cases, migration of components of the packaging into drug substance or product will result in simple contamination. However, migratory species may also be reactive and form new drug-related impurities.[184] In a recent example, acrylic acid was detected as a leachable in pre-filled syringes containing therapeutic proteins. A direct Michael addition reaction between the acid and proteins resulted in impurities of between 0.02 and 0.3%.[185]

The search for reliable reaction indicators was reported in a number of drug-excipient compatibility tests carried out by Bruni *et al.* The objective was to develop a simple methodology with high predictive ability that could be applied during pre-formulation of solid dose drugs.[186] Ester formation was highlighted in a number

recent drug excipient compatibility reports. [123, 187, 188] A liquid formulation containing the carboxylic acid cetirizine, formed approximately 1% of the monoester impurity after 1 week in the presence of the excipients sorbitol and glycerol. The ester was unstable at higher temperatures (40, 60 and 80ºC) and degraded over time.[188] Schou-Pedersen *et al.* repeated this experiment examining the esterification of cetirizine but employed the use of microwave technology in place of a conventional oven for accelerated stability testing of drug excipient reactions.[187] The typical time taken for esterification was reduced from weeks to hours when two liquid formulations and one solid dose were investigated. Results for the liquid formulations were in agreement with the earlier study but caution was advised in using microwave for studies of solid dose forms.

Active pharmaceutical ingredients (APIs) containing secondary and tertiary amine moieties have been shown to react with olefins such as maleate and tartrate salts and with acrylates migrating from packaging. A major degradation product resulting from an interaction between phenylephrine hydrochloride (PHN) and the maleate salt of chlorpheniramine was identified by Marin *et al.* in a 2005 study.[85] The maleate adduct was present in several commercial formulations tested by the authors. A follow up study contested the structure of the adduct reported by Marin but confirmed the degradant as a Michael addition product of phenylephrine and maleic acid. [189] Phenylephrine was the subject of another excipient interaction in a later study by Trommer *et al*. This time the adduct was formed by an interaction with its own bi-tartrate salt.[86] Results of all three studies indicate that phenylephrine is very susceptible to conjugate addition, especially in liquid formulations.

There are no reports in the literature of interactions between reactive amines and acrylates migrating from drug-packaging. Yet, both methyl acrylate and acrylic acid have been detected as leachables in ophthalmic solutions during an in house evaluation of digital labels from potential vendors at our laboratory (data not published). In a recent example acrylic acid was found to have reacted with the drug substance (timolol) in the product under evaluation. Even though a small amount (from 0.05% to 0.3%) of the timolol drug substance was found to be modified by acrylic acid, the modified drug could potentially be harmful due to the toxicity of acrylic acid. After being modified by acrylic acid, the properties of the drug substance may change due to charge and hydrophobicity variations. Scheme 5.1 shows the proposed formation of an acrylic adduct **3** (2-[tert-butyl-[(2S)-2-hydroxy-3-[(4-morpholino-1,2,5-thiadiazol-3-yl)oxy]propyl]amino]acetic acid) via the aza-Michael reaction of timolol **1** with acrylic acid **2**.

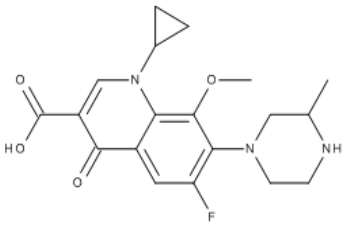


1 **2** **3**

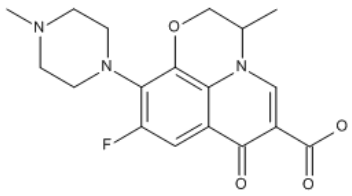
Scheme 5.1 Aza-Michael addition of timolol to acrylic acid

In Chapters 2 and 3 the secondary amine 1-phenylpiperazine was reacted with a number of acrylates, including methyl acrylate and acrylic acid, in a variety of aqueous buffered solutions. The results show that given sufficient time, even the less electrophilic acrylic acid will yield an acrylate adduct. The salt, 1-phenyl piperazine hydrochloride also reacted with both methyl acrylate and acrylic acid when left standing to 6 and 12 days.

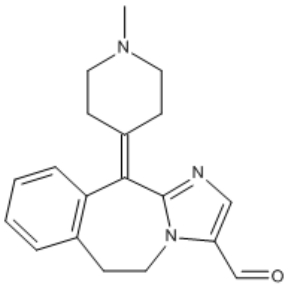
The aim of this chapter was to investigate the extent to which the aza-Michael reactions explored in earlier chapters could occur in pharmaceutical formulations with APIs containing amine groups. A number of ophthalmic solutions containing a variety of amine drug substances in aqueous buffered solutions were spiked with acrylates to determine the levels of acrylate adduct impurities generated under conditions that mimicked the routine storage of drug products. Test APIs included primary, secondary and tertiary amines, with both aliphatic and aromatic compounds included. A protocol was designed to pre-screen the packaging material-drug substance compatibility. All methods used were validated stability indicating methods used for the quantitation of the API and its related substances. Formation of acrylate adducts was observed for all secondary and tertiary amines. No reaction was observed for the primary amine epinastine hydrochloride (EPT). Amine reactivity was governed by structure, with yields significantly reduced for secondary amines with alkyl substitution at α-carbon. With the exception of PHN, hydrolysis of the β-amino ester product was evident for all amines. Tertiary amines (N-methyl substituted) produced stable adducts in the reaction with acrylic acid.



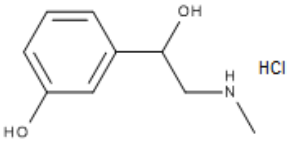
1. Gatifloxacin (GAT)



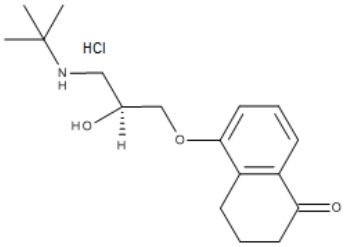
2. Ofloxacin (OFL)



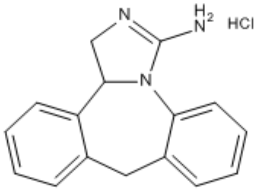
3. Alcaftadine (ALC)



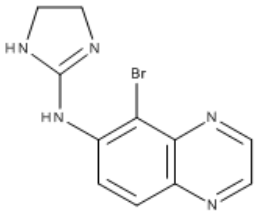
4. Phenylepherine.HCl (PHN)

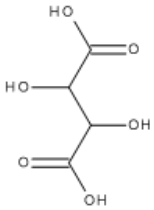


5. Levobunolol.HCl (BUN)

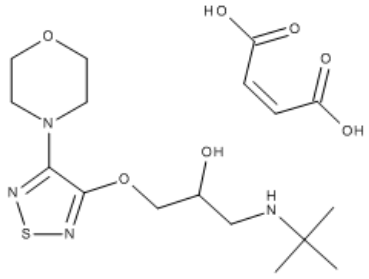


6. Epinastine.HCl (EPT)





7. Brimonidine Tartrate (BMT)



8. Timolol Maleate (TIM)

Figure 5.1 Structures of the compounds related to this study

5.2 Materials and Methods

5.2.1 Materials

Methyl acrylate and acrylic acid were purchased from Sigma Aldrich. HPLC grade acetonitrile and methanol were purchased from Merck. Tetrahydrofuran 'Super purity solvent' was purchased from Romil. Ammonium acetate anhydrous (ACS reagent grade), ammonium phosphate monobasic (ACS reagent grade) and glacial acetic acid (HPLC Grade) were purchased from Sigma Aldrich. Heptane sulfonic acid sodium salt and sodium phosphate dibasic (ACS reagent grade) were purchased from VWR. Triethylamine (ACS Grade), potassium phosphate dibasic and ortho-phosphoric acid 85.0 % min (ACS grade,) were purchased from Applichem. Deionized water, purified to a resistance of greater than 18 MΩ was obtained from a Millipore Corporation Milli-Q system, Millipore (Bedford, MA, USA). Ophthalmic solutions containing Phenylephrine Hydrochloride (PHN), Gatifloxacin (GAT), Ofloxacin (OFL) Alcaftadine (ALC) Brimonidine Tartrate (BMT), Timolol Maleate (TIM), Levobunolol Hydrochloride (BUN) and Ephinastine Hydrochloride (EPT) were provided by the Allergan Stability Testing Laboratory, Westport, Ireland. All samples had exceeded the manufacturer's expiry date by a minimum of 14 months at the time of use. All aged samples had been stored at 25ºC/RH 60%. All other chemicals were of analytical grade and were used without further purification.

5.2.2 HPLC/UPLC Equipment and Chromatographic Conditions

HPLC analyses were carried out using a Waters Alliance 2695 system (Waters Corporation, Milford, MA, USA) with a quaternary solvent manager and auto-sampler connected to a Waters 2489 UV/Visible UV detector (Waters Corporation, Milford, MA, USA). UV spectral analysis was carried out using a Waters 2998 photo diode array (PDA) detector. UHPLC analyses were carried out using a Waters

ACQUITY UPLC H-Class system (Waters Corporation, Milford, MA, USA) connected to an ACQUITY TUV detector (Waters Corporation, Milford, MA, USA). Data acquisition and integration were performed by using the Waters Empower software. The auto-sampler was held at room temperature. Details of the column, instrument parameters and mobile phase used to assay each of the ophthalmic solutions are provided in Appendix A.

5.2.3 Methods

5.2.3.1 Sample Preparation

Refer to Appendix A for details of the sample diluent and final concentration of the API in each solution tested. Class A volumetric pipettes and flasks were used for each preparation.

5.2.3.2. Generation of Reaction Products

Approximately 150 mL of each ophthalmic solution was pooled into a sample jar. Using a Class A glass pipette, 10 mL of pooled sample was transferred into 10 separate 15 mL amber glass sample jars. Each sample was spiked with either methyl acrylate or acrylic acid using a micropipette. Details of the molar concentration of each amine in 10 mL of solution and the amount of acrylate added are provided in Table 5.2.

Each sample was prepared in duplicate, tightly capped and stored in climatic chambers under the following conditions of temperature and relative humidity (RH); 25 ºC/60% RH and 40 ºC/75% RH for a period of up to 8 weeks. A control sample from the same pooled sample was stored under identical conditions and tested at each time-point.

Table 5.1 Molar Concentration of Acrylates (µL) in 10 mL of Ophthalmic Solution

Acrylate	MW g/mol	Density g/cm^3	10 µL/10 mL (mmol)	20 µL/10 mL (mmol)	40 µL /10 mL (mmol)
Methyl Acrylate	86.09	0.9535	11.0	22.1	44.3
Acrylic Acid	72.06	1.051	14.5	29.1	58.34

Table 5.2 Molar Concentration of Amines in 10 mL of Ophthalmic Solution and Amount of Acrylate Added

Drug Substance	Conc. mg/mL	MW g/mol	mmol in 10 mL	µL MA	µL AA
GAT	5	375.4	13.3	40	40
BUN	5	327.9	15.2	40	40
TIM	6.8	432.5	15.7	40	40
OFL	3	361.4	8.3	20	20
ALC	2.5	307.4	8.1	20	20
PHN	1.3	203.7	6.4	20	20
BMT	2	442.2	4.5	20	20
EPT	0.5	285.8	1.8	10	10

5.2.4 Quantitative analysis

With the exception of the BMT-acrylic acid adduct, no reference compounds of the aza-Michael impurities were available. The quantitative measurements of the acrylate adduct formed in the reaction studies were therefore performed using the reaction mixtures obtained as described in Section 5.2.3.2, Table 5.2.

A number of sample controls were run for each assay and the average peak area of both the amine peak and the impurity peaks were calculated. The extent of each

reaction was calculated by comparing the peak area of each reacted sample to that of the control sample. The % loss of amine for each replicate was calculated. The reaction products were quantitated as a % peak area with respect to the loss of amine in the control samples.

5.3 Results and Discussion

The main objective of this research was to establish whether the reaction between an API containing an amine functional group and a component of the packaging (acrylate) was feasible under conditions of routine and accelerated storage. The APIs chosen for this study were Gatifloxacin (GAT), Ofloxacin (OFL), Alcaftadine (ALC), Phenylepherine. HCl (PHN), Levobunolol.HCl (BUN), Epinastine.HCl (EPT), Brimonidine Tartrate (BMT) and Timolol Maleate (TIM), shown in Figure 5.1. These compounds were chosen to enable a comprehensive investigation into the chemical parameters which impacted upon the reaction, and ranged from primary to secondary to tertiary amines, and included both salts and free bases, as detailed in Table 5.3. Two acrylates were chosen, acrylic acid (AA) and methyl acrylate (MA), as both are have been found to be present in packaging components, and both were shown in Chapter 3 to act as acrylate donors in the aza Michael reaction.

The design of this study combined elements of drug-excipient/packaging compatibility studies and forced degradation studies. A forced degradation study subjects drug products and substances to conditions more severe than accelerated conditions to determine the stability of the molecule and to generate degradation products in greater amounts for further study. The stressed samples can be used to develop stability indicating analysis methods and to produce a degradation profile similar to that of what would be observed in a formal stability study under ICH

conditions.[182] Drug packaging compatibility studies often involve extraction of components of the packaging by direct immersion in the finished product solution at elevated temperatures [190]. Borrowing from the forced degradation study example, the reaction was accelerated by direct addition of the acrylate rather than immersion of the components in sample. The reaction of 1-Phenylpiperazine (1PP) and methyl acrylate (MA) in Chapter 3 demonstrated that the aza-Michael reaction was feasible in buffered solutions when samples were left standing at room temperature, and resulted in the formation of unanticipated products. Hydrolysis of the ester product was noted also. To get a more complete picture of the feasibility of the reaction, all reactions in this study were carried out at both 25 and 40ºC. The study consisted of three main components:

- determination of impurity adduct formation;

- evaluation of impact parameters on impurity adduct formation;

- characterisation of impurity adducts formed.

5.3.1 Determination of impurity adduct formation on reaction with Methyl Acrylate

All amines with the exception of EPT reacted with MA to form an impurity adduct. Reaction was calculated by comparing the concentration of the amine in the spiked sample to that of the un-spiked control. Table 5.3 ranks the reactivity of the amines based on the greatest % loss observed at the first time point. The time point selected was dictated by the laboratory schedule and it was not possible to assay each product on the same day. As a result there is some variation between the days for different products. The concentration of the impurity as a % of the amine in the control was also recorded. Tables of results for individual amines are provided in Appendix B.

The relative response factor (RRF) for each impurity has not been established and therefore the % loss of amine is not equivalent to the % increase in the impurity for all amines, with the exception of PHN. The area of the PHN-MA adduct is equal to the % loss of PHN. An RRF of 2.4 has been determined and applied to the BMT-MA adduct.

Table 5.3 Reactivity of APIs on incubation with MA at 40ºC

Reactivity	API	% Loss Amine	% IMP	# Days incubated	Amine classification
High	PHN	100	100	11	2º salt HCl
High	BMT	85	88	7	2º salt Tartrate
Medium	ALC	34	14	5	3º free base
Medium	OFL	32	26	7	3º free base
Medium	GAT	33	17	11	2º free base
Low	TIM	10	11	11	2º salt Maleate
Low	BUN	3	6	19	2º salt HCl
Nil	EPT	2	0	7	1º salt HCl

PHN was most reactive amine, in terms of the impurity yield on reaction with MA at both temperatures. Complete conversion of PHN to the MA adduct was observed after 11 days storage at 40ºC. BMT, another secondary aliphatic amine, also proved highly reactive with 85% loss of amine after 7 days storage at 40ºC. Of medium reactivity were three cyclic amines, the fluoroquinolones GAT and OFL and ALC, an antihistamine. All three achieved approximately 30% loss after 5-11 days storage. TIM showed moderate/low reactivity with 10% amine loss after 11 days. This was not unexpected, as during an earlier accelerated study of TIM in a combination formulation, a small percentage (< 1.0%) of an acrylic adduct was

detected. The % loss of BUN did not correspond to any increase in impurity at the initial time-point (19 days) with a small amount of the MA adduct detected after 40 days. Finally, the primary amine EPT was unreactive over the course of the study. A small reduction in peak area of the amine was detected at the second time-point but this was within the day to day variation of the method.

5.3.2 Evaluation of Impact Parameters on Impurity Adduct Formation

5.3.2.1 Effect of Amine Structure on Reactivity

Of interest to this study was whether the structure of given amine could predict its reactivity in the aza-Michael reaction with an acrylate ester. While in no way comprehensive, the results of the study broadly support the contention of Bunting and Heo and that it is the structure of the amine and not its basicity that plays a greater role in its nucleophilicity.[31, 36] Substitution of the α and β carbons to the nitrogen atom were shown to have the greatest impact on the nucleophilicity of the amine. Two features were identified as key; steric hindrance as a result of increasing substitution at the α carbon led to a reduction in reactivity whereas sp^2 hybridisation or the presence of a hydroxyl group at the β carbon led to a significant enhancement in amine reactivity. The full structure of each compound is shown in Figure 5.1. Figure 5.2 focuses on the groups next to the amine.

Figure 5.2 row 1, contains the amines PHN, TIM and BUN. PHN, a secondary N-methyl amine, was the most reactive of the 8 amines tested. Its amine backbone is identical to that of TIM and BUN. All three are unhindered at the α carbon and contain a hydroxyl group on the β carbon. However, the steric hindrance afforded by the *t*-butyl group attached to the nitrogen of TIM and BUN completely negated

any enhanced reactivity and they proved to be the two most unreactive secondary amines examined.

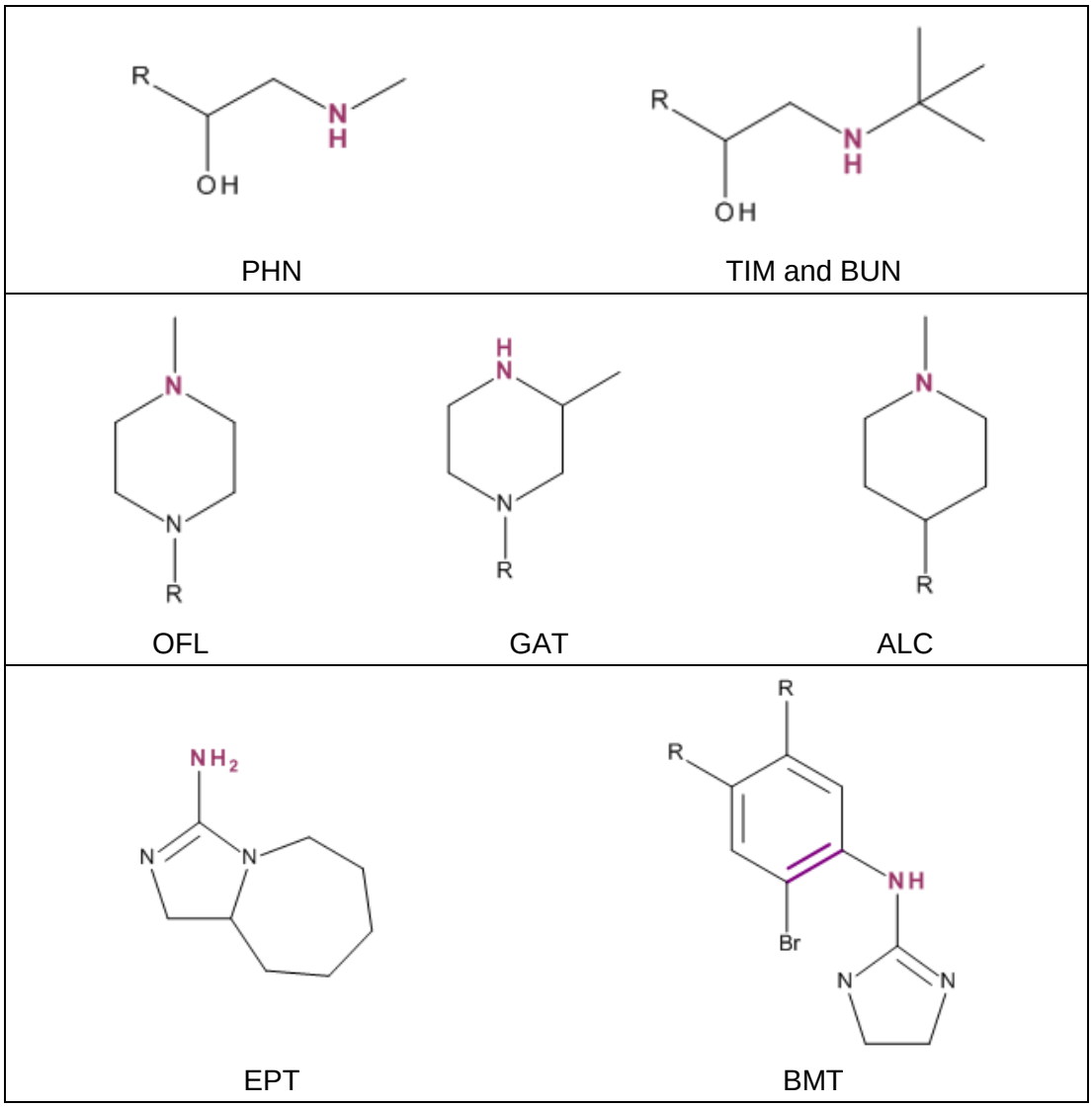


Figure 5.2 Chemical structures of amines investigated in this study. EPT is a 1º amine, PHN, TIM, BUN, GAT and BMT are 2º amines and ALC and OFL are 3º amines.

TIM resulted in 10% loss of amine and BUN in a 3% loss after 11 and 19 days storage respectively. TIM conversion did increase over time at 40ºC where a very small increased was noted in the reaction with MA after 32 days. Results for BUN after 40 days showed 11% loss of amine in one replicate at 40ºC and a

corresponding increase in the MA adduct to 6.8%. Results for BUN were not reproducible in either of the replicates or at either temperature.

Steric hindrance came into play once more when comparing the reactivity of the three cyclic amines, OFL, GAT and ALC. Based on the previous nucleophilicity studies (of Mayer, Heo, Brotzel), the GAT secondary amine was expected to be far more reactive than its tertiary counterpart, OFL. [30, 31] GAT and OFL are almost identical in structure, consisting of a phenyl piperazine ring system. Based on the results of Chapter 2 and 3 which used the model amine 1-phenyl piperazine, the reaction of GAT with MA was expected to be extremely fast at both temperatures. Instead, all three amines studied here yielded very similar results for % loss of amine. Even though GAT is a secondary amine while ALC and OFL are tertiary. The low reactivity of GAT could be ascribed to the steric hindrance of the methyl group adjacent to the nitrogen.[33] While N-methyl substitution of the tertiary amines was not a barrier to their reactivity, the results show that amine loss and generation of products is less predictable and reproducible than for the secondary amines. Stability of the tertiary amines is discussed in Section 5.3.4.1.

The primary amine EPT was unreactive after 7 and 34 days. Some loss of amine was noted (approx. 4%) but no subsequent increase in adduct was observed. The presence of two nitrogen's beta to the primary nitrogen and an α-β unsaturated bond would lead to build up of electron density in the vicinity of the nucleophilic centre, making approach to the electrophile difficult. Interestingly, the same moiety is present as one of the groups on BMT, one of the most reactive amines studied. The second group is a bromo-benzene ring. Overall, the results are in agreement with the amine nucleophilicity scales discussed in the literature review; secondary amines are more nucleophilic than their primary counterparts, with the additional

alkyl group on the secondary amine lending increased stability to the positively charged ammonium intermediate. Results for the tertiary amines are in agreement with the contention of Bunting and Heo [31]that nucleophilic attack by the amine is no longer the rate limiting step, rather the ease at which the carbanionic intermediate is protonated by the reaction medium determines reactivity.

5.3.2.2 Effect of Acrylate Donor – Methyl Acrylate versus Acrylic Acid

As previously discussed, all amines with the exception of the primary amine, EPT reacted with MA. While it was not expected that AA would be as effective an aza-Michael donor, as seen in Chapter 3, a reaction with AA will take place over time if the amine is sufficiently nucleophilic. As seen in Table 5.4, reaction with AA did result in the formation of impurity adducts for a number of APIs investigated, though not with the same trend as observed with MA. While the reaction was similar for some amines, e.g. for the reaction of PHN with AA, 71% loss of amine was observed after 32 days at 40ºC, the less reactive secondary amines GAT, BMT, TIM and BUN showed no significant reaction with AA.

Table 5.4 Reactivity of APIs on incubation with AA at 40ºC

API	Reactivity with MA	% Loss Amine	Reactivity with AA	% Loss Amine	Amine classification
PHN	High	100	High	71	2º salt HCl
BMT	High	85	Low	3	2º salt Tartrate
ALC	Medium	34	Medium	36	3º free base
OFL	Medium	32	Medium	16	3º free base
GAT	Medium	33	Low	6	2º free base
TIM	Low	10	Low	3	2º salt Maleate
BUN	Low	3	Nil	0	2º salt HCl
EPT	Nil	2	Nil	0	1º salt HCl

Where previously, GAT, OFL and ALC all reacted similarly with MA, for the reaction with AA, GAT did not show any significant reaction while the two tertiary amines, OFL and ALC, reacted with AA at both temperatures and formed stable impurity adducts. The reaction of OFL with AA over 32 days resulted in the formation of adducts amounting to 3 and 10% at 25ºC and 40ºC respectively. The results were more pronounced for ALC with 6 and 18% of the ALC-AA adduct formed after 32 days. See Tables 5.5 and 5.6 for details. In this instance therefore, the basicity of the amine as well as the structure would appear to have affected the formation of the impurity adduct. In every scenario, there was a lower reactivity for the API with AA than with MA. This was expected, as AA is not as efficient a donor as MA.

5.3.2.3 Effect of Temperature

With the exception of PHN, the % loss of amine API was greater at higher temperature for secondary amines. The reaction of PHN with MA was complete

after 11 days at both temperatures. The model amine 1PP reacted with MA in Chapter 2 showed a similar effect i.e. if the amine was sufficiently nucleophilic the reaction was rapid and temperature did not have an effect. When PHN was reacted with AA however, the effect of temperature was evident; at 40ºC the rate of formation of the acid adduct was greater at both time-points tested. At 40ºC the % loss of amine increased from 50 to 71%. Over the same period at 25ºC, the increase was 10% to 24%.

The same overall trend was noted for GAT, BMT, TIM and BUN with higher concentrations of adduct formed in the reaction with MA at 40ºC. However, the % loss of amine and subsequent increase in adduct was not as straightforward as with the PHN reaction. GAT for example, demonstrated good reproducibility between replicates for the % impurity formed at both temperatures and time-points, but the % loss of amine was out of trend. Initially, at 25ºC, a 21% loss of amine resulted in 4% of MA adduct whereas at 40ºC a loss of 25/34% for replicates A/B resulted in 17% of impurity. After a further 22 days, the loss of amine was similar across both temperatures (29-38%) but the 25ºC impurity increased from 4 to 13% whereas the 40ºC impurity only increased from 17 to 20%. No additional impurities were observed. A similar anomaly between % loss of amine and increase in impurity was observed for OFL, ALC and BUN.

5.3.3 Stability of the Adducts Formed

5.3.3.1 Ester Hydrolysis

While outside the scope of this study, an interesting observation was made in relation to the rate of hydrolysis of the β-amino ester products resulting from the aza-Michael addition of an amine and an ester. In Chapter 3, the model amine 1PP was reacted with both AA and MA and left standing. Hydrolysis of the ester was

identified in the MA reaction mixture. After 6 days, the ratio of ester:acid product was 80:20, which increased to 45:55 after 12 days in PBS at room temperature. The hydrolysis observed is in agreement with results of a study by Carlise *et al.* who investigated the effects of pH and temperature on the hydrolysis of β-amino esters and acids. A significantly increased rate of hydrolysis was reported for the β-amino esters across a range on temperatures and pH. The half-life of the tertiary amine esters was very fast at both temperatures (37ºC and 80 ºC) and over the range of pH investigated (3 to 9). [191] The same acceleration was not observed in esters lacking the neighbouring nitrogen atom *i.e.* the presence of the nitrogen greatly enhanced the rate of hydrolysis. The results for hydrolysis of the carboxylic acid products were unpredictable and did not follow the expected trend. Further studies carried out by McCoy *et al.*, on the acceleration effect of the amine on ester hydrolysis were in agreement with the finding of Carlise and was termed neighbouring group effect (NGE) by the authors.[192]

In this study, a single adduct was observed at the same retention time for the secondary amines BMT, TIM, GAT when the amines were reacted with either MA or AA. The MA adduct peak formed in the reaction with BMT has been positively identified by LC-MS and NMR as the brimonidine-acrylic acid adduct. The MA adduct of TIM has also been identified as the timolol-acrylic acid adduct. As the AA monomer did not react with either product, it was assumed that complete ester hydrolysis occurred in the PBS solution (pH 7-8).

5.3.3.2 Stability of Adducts - Tertiary Amine Adducts

As shown in Figure 5.3, the product of the aza-Michael addition of a tertiary amine to an acrylate acceptor is a positively charged quaternary ammonium compound. These charged amines are features of many antimicrobial compounds. A study into

the spontaneous hydrolysis of antimicrobial betaine esters was carried out by Lindstedt *et al.* in 1990. [193] Unlike the β-amino esters where hydrolysis was rapid across a range of pH values and at high and low temperatures, Linstedt reported a more complex temperature and pH dependence for the quaternary ammonium esters. For example, a small reduction in the storage temperature from 30 to 25ºC doubled the half-life of the betaine ester studied. A reduction in the free activation energy for alkaline hydrolysis as a consequence of the positively charged nitrogen atom led to increased instability of the ester bond. At low pH the bond was stable whereas increasing the pH of the buffer had the opposite effect, with 50% hydrolysis taking place within 5 hours at pH 7.

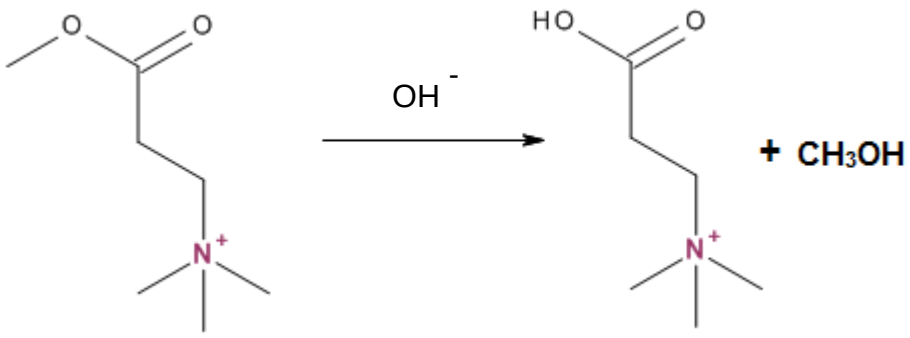


Figure 5.3 Quaternary ammonium ester hydrolysis [193]

The complex dependence of ester adduct formation with temperature was also evident in this study, in reactions of the tertiary amines ALC and OFL with MA. In contrast to the secondary amines, the % amine loss for both ALC and OFL reacted with MA was greater at 25ºC than at the higher temperature. This was not observed for any of the other amine APIs investigated. Instead, for these amines the observed trend was for a higher %adduct formation at higher temperatures, as expected. Moreover, the trend for lower % amine loss at higher temperature seen with MA was not observed for the reaction of ALC and OFL with AA. When AA was used as

acrylate donor, a higher %adduct formation was observed with higher temperature, in keeping with the other amines investigated. Tables 5.5 and 5.6 provide a summary of the results for the reaction of OFL and ALC with MA and AA.

The difference in adduct formation relationship with temperature was not the only difference between the MA and AA studies for ALC and OFL. Two impurity peaks were observed at each time-point for the reaction with MA, whereas only impurity peak was observed at each corresponding time point for the reaction with AA. The impurity observed with AA was deemed to be the acid adduct, with RRT (0.35). The two impurities observed with MA eluted at RRT (0.35) and RRT (0.42). The impurity at RRT (0.35) was found to be the same as the AA impurity, the acid adduct. The impurity at RRT (0.42) was deemed to be the ester adduct. Hydrolysis to the acid adduct was observed in all samples reacted with MA at both temperatures. By comparing the chromatograms of the MA and AA spiked samples it was possible to determine the RRT of the AA adduct as it was the only impurity present in the AA spiked samples. For example, a HPLC chromatogram of OFL samples stored at 25C and 40C for 32 days is shown in Figure 5.4. The main MA adduct was observed at RRT (0.42). Results show a decrease in the MA adduct RRT (0.42) at the higher temperature and a concomitant increase in the AA-adduct RRT (0.35) in the same sample. The acid adduct increased from 1.3% to 5.5% in the 25 ºC sample and from 7 to 9.5% in the 40ºC sample.

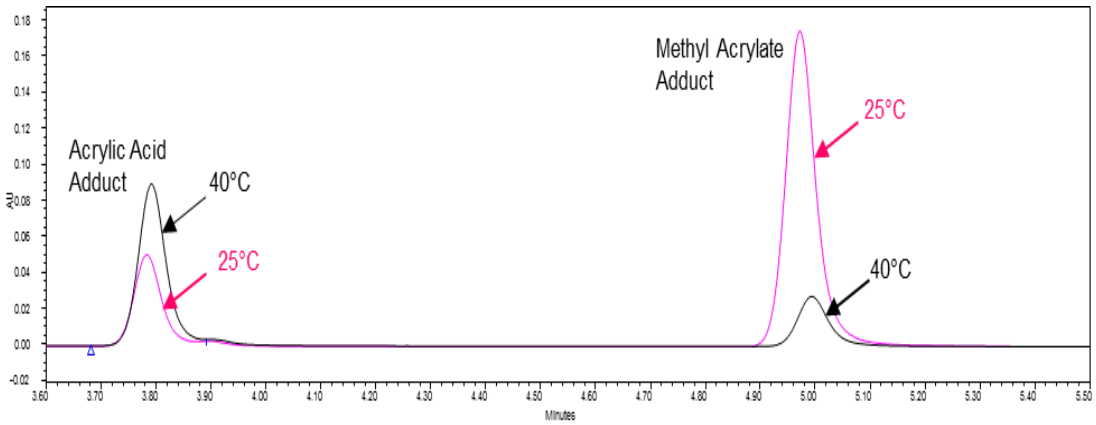


Figure 5.4. HPLC chromatograms illustrating adducts formed , with UV detection at 294 nm of OFL samples overlaid reacted with MA stored at 25 ºC and 40ºC for 32 days. HPLC separation conditions as per section xx.

As discussed above, the results for the reaction of MA and tertiary amines confirm that the MA adduct was not stable over time. However, for the corresponding reactions with AA, a steady loss and subsequent increase in the AA adduct was observed at the two time-points under both conditions. For example, for OFL, compared to the levels of the MA impurity detected, results for level of AA adduct formed (RRT (0.35)) were identical in the A and B replicates under both conditions. This shows that once formed the AA adduct was remarkably stable over time.

Table 5.5 Ofloxacin (OFL) Reactivity, monitored in terms of % API loss, and % impurity formation, monitoring at both 0.35 and 0.42 relative retention times (RRT).

		7 Days			32 Days		
		% API loss	% IMP	% IMP	% API loss	% IMP	% IMP
Methyl acrylate			RRT (0.35)	RRT (0.42)		RRT (0.35)	RRT (0.42)
25°C	A	32.0	1.3	25.5	34.3	5.8	22.2
	B	31.2	1.3	26.2	22.1	5.4	14.2
40°C	A	20.1	7.2	11.0	16.2	9.9	3.5
	B	17.0	7.0	8.9	12.8	9.5	2.6
Acrylic Acid			RRT (0.35)			RRT (0.35)	
25°C	A	0.0	0.7		6.8	2.8	
	B	0.0	0.6		1.2	2.8	
40°C	A	6.2	4.1		13.2	9.7	
	B	4.4	4.0		13.9	9.7	

In the reaction with MA results for ALC were in line with those of OFL. Hydrolysis was greater at the higher temperature with no ester adduct detected after 32 days in sample 40°C. However, for the MA sample at 40°C, sample A had a 9% amine loss with only 1.7% AA adduct detected, while sample B had full recovery of the amine yet a 23% AA-adduct was detected. This is despite the fact that at the first time point, comparable results were obtained from both samples. This highlighted the unpredictability of the adduct formation under these conditions. As with the OFL sample, reaction with AA was viable and showed steady increase over time, especially at 40°C.

Table 5.6 Alcaftadine (ALC) Reactivity, monitored in terms of % API loss, and % impurity formation, monitoring at both 0.34 and 0.64 relative retention times (RRT).

Acrylate Donor		5 Days			32 Days		
		% API loss	% IMP *(0.34 RRT)*	% IMP *(0.64 RRT)*	% API loss	% IMP *(0.34 RRT)*	% IMP *(0.64 RRT)*
Methyl acrylate							
25°C	A	21.0	14.0	9.4	23.3	14.8	16.0
	B	19.1	15.1	16.5	19.4	13.9	9.5
40°C	A	27.0	3.2	15.3	9.2	1.7	0.0
	B	34.2	3.3	14.3	0.0	23.2	1.6
Acrylic Acid			*(0.34 RRT)*			*(0.34 RRT)*	
25°C	A	8.6	1.1		23.2	5.8	
	B	8.0	1.0		21.1	5.6	
40°C	A	29.9	6.7		34.9	18.6	
	B	30.6	6.5		36.0	18.1	

5.3.3.3 UV Spectra of Adducts

All samples were assayed at the wavelength specified by the relevant method. A UV spectrum for each impurity was generated using photodiode array. Both AA and MA contain UV chromophores and can be observed in chromatograms acquired at low wavelengths. PDA was used to identify and eliminate responses resulting from unreacted MA and AA in samples assayed at low wavelength. In addition, the UV spectrum of each adduct was generated. The UV chromatogram and the accompanying UV spectra for the amine and corresponding adducts are shown in Appendix C.

In a report into the interaction of cetirizine and alcohol excipients, the authors used the drug substance for quantification of the formed ester reaction products on the assumption that the esters had the same UV molar absorptivity as cetirizine. [187]

While this is a practical approach to quantitation during screening, results of this study demonstrate that accurate quantitation of impurity adducts requires synthesis of the impurity standard.

5.4 Conclusion

Chapter 3 indicated that the aza-Michael reaction between amines and acrylates in buffered ophthalmic solution was viable and rapid conversion was expected for the reaction of secondary cyclic amines and methyl acrylate. This chapter has shown that substitution next to the nitrogen atom has a marked effect on the amine's reactivity and the aliphatic secondary amines were more reactive than expected. Likewise, the two cyclic tertiary amines studied were highly reactive, forming stable adducts when reacted with acrylic acid. The β-amino and quaternary ammonium esters formed were highly susceptible to hydrolysis.

It has been clearly illustrated here that drugs which are tertiary amines (not just secondary amines as sometimes reported) undergo the aza-Michael reaction with both methyl acrylate and acrylic acid under pharmaceutically relevant conditions. This work demonstrated the complexity of reactions between secondary and tertiary amines and acrylate components migrating from packaging in to liquid formulations. While evaluation of the reaction kinetics in pharmaceutical formulations containing amine drug substances is beyond the scope of this research, the results obtained here strongly suggest that formation of acrylate adducts may constitute a significant problem upon long-term storage of ophthalmic solutions in their final packaged configuration. Companies need to mitigate against the migration of acrylate products through careful selection of low-migration packaging components.

Previous label extraction studies have identified the acrylic acid adduct of BMT, TIM, and GAT as impurity adducts in pharmaceutical formulations (data not published) . While acrylic acid is listed as a component of certain ink formulations and label adhesives, the results of this study indicate that the acrylate donor was most likely an acrylate ester and the impurity detected was the acid hydrolysis product of that ester. The acrylic acid adduct of ALC has also been detected in compatibility study for digital labels under accelerated conditions (40/75 RH). The results of the present study show that the reaction was viable with both the ester and acid acrylate. As both are components of the digital label, migration of either impurity could be the source of this impurity.

This case study demonstrates that leachable compounds that migrate into the drug product can react with the active ingredients to form active ingredient related impurities, and shows clearly the need for compatibility studies when selecting secondary container closure components. However, in many cases, the development scientist will not know whether a specific nitrogen-containing drug will be compatible with a particular packaging configuration or not, usually due to significant structural variations such as inclusion of the nitrogen within rings or the presence of functionality which would greatly diminish the nucleophilicity of the amine drug. The present findings suggest a relatively simple experimental design to probe this question, namely, direct addition of an acrylate ester and acrylic acid to the drug product and storage at pharmaceutically relevant conditions. The protocol, as detailed in section 5.2, would be a worthwhile addition to the forced degradation stress studies carried out as part of method development. Use of the stability indicating method specific to the formulation is also recommended.

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Chapter 6:

Conclusion

6.0 Conclusion

This study set out to explore the issue of drug packaging interactions between an API containing an amine functional group and a component of the packaging (acrylate) in ophthalmic solutions and to identify the conditions under which such reactions are accelerated. Through systematic reaction monitoring of the mechanism underpinning the interactions (the aza-Michael reaction) a better understanding was gained of the role of each of the reactants, amine and acrylate. While the general theoretical literature on the aza-Michael reaction is substantial, there are currently no reports in the literature of interactions between reactive amines and acrylates migrating from drug-packaging. Several of the reactions performed in aqueous buffered solutions and those using acrylic acid as an acceptor have not been reported previously.

The study sought to answer a number of key questions;

1. Is the aza Michael addition of an amine and acrylate viable using water as the reaction medium, and does water have a unique solvent property?

2. What are the factors that impact on impurity adduct formation when the reaction is performed under conditions relevant to ophthalmic solutions?

In the literature, two hypotheses regarding the aza-Michael reaction mechanism have been proposed, one detailing an “on-water” acceleration of the reaction in aqueous solution, and the other detailing nucleophilic addition and proton transfer to the carbanion. In Chapter 2 the question of whether this reaction was ‘accelerated’ using water as the reaction medium was explored. The “on-water” hypothesis required a heterogenous mixture to promote acceleration. While the reaction in water was heterogeneous, the reaction in methanol was not, however the reaction

yields were comparable for both solvents. These results show that the while the rate of reaction in water was exceptionally fast, it was the nature of the solvent itself (polar-protic) rather than any 'unique' property of water that was responsible for the acceleration. This work therefore supports the hypothesis that the aza Michael reaction proceeds via a stepwise mechanism, with the formation of an addition intermediate followed by a proton transfer. Supporting this hypothesis, in Chapters 2 and 3 the secondary amine 1-phenylpiperazine (1PP) was reacted with a number of acrylates, including methyl acrylate and acrylic acid, in a variety of aqueous buffered solutions. Both of the aqueous ophthalmic buffers investigated (PBS and boric acid) were analogous with water in terms of reactivity. In answer to the first key question therefore, yes, the aza Michael addition of an amine and acrylate is viable using water as the reaction medium, but water does not have a unique solvent property. Instead it is water's polar-protic property which results in its accelerative effects, and other solvents which also exhibit these properties also impart these accelerative effects.

The second key question in this thesis explored which factors impacted on impurity adduct formation when the reaction was performed under conditions relevant to ophthalmic solutions, with the nature of the solvent and the structure of both the amine donor and acrylate acceptor identified as being of key importance.

As discussed above, the role of the solvent in the acceleration of the aza-Michael reaction was determined by its ability to protonate the carbanionic intermediate. The poor reactivity of the aprotic solvents and the solvent free reaction in Chapter 2 was consistent with the proposed mechanism in the reaction of a secondary amine and acrylate. With respect to the neat reaction, the ease of purification of the reaction product (no solvent to remove) is often cited as an example of its green

credentials.[20] However, in Chapter 2 it was shown that in order to perform the reaction successfully an excess of the amine reactant is required. As the amine is typically the more expensive reactant, the solvent free route may not be the best option for this reaction.

In the conjugate addition of amine nucleophiles to neutral olefin acceptors the reactivity of the amine is predicated on its structure. [31] Chapter 5 examined the reactivity of eight amines, primary, secondary and tertiary, with a variety of substituents. The reactivity of the amines with methyl acrylate and acrylic acid was in agreement with the reactivity described in the literature; with amine structure having a major impact on reactivity in the case of the secondary amines PHN, TIM and BUN. It was found that the structure of both the amine donor and acrylate acceptor had a profound effect on adduct yields. This is of significant potential concern to the pharmaceutical industry. Critically, in this research, neither the amine salt nor poorly reactive acrylic acid prevented the formation of adducts under conditions of longer term storage, raising a concern regarding the interaction between amine drugs and acrylate leachables in ophthalmic solutions.

In addition to the key questions that this thesis sought to answer, this work has added to our understanding of the impurity products themselves. The hydrolysis of the ester in product observed in Chapters 3 and 5 was in agreement with several reports on the increased rate of hydrolysis of β-amino esters compared to esters lacking a neighbouring nitrogen group [191]. This is supported by the fact that the methyl acrylate ester spiked into the ophthalmic solutions in Chapter 5 did not hydrolyse over the course of the standing experiments. It had been thought in our laboratory that the acrylic acid adducts detected in a number of samples were the result of hydrolysis of an ester acrylate migrating from the packaging and that it was

acrylic acid that reacted with the amine drug substance. This study shows that it more likely that the reaction was with the ester followed by hydrolysis of the β-amino esters. Furthermore the effect of temperature on the hydrolysis on quaternary ammonium compounds, such as OFL and ALC in Chapter 5 was consistent with and adds to that presented by Lindstedt *et al.* in 1990 on the hydrolysis of betaine esters.[193] The results of this study show a similar pattern of hydrolysis in quaternary ammonium compounds that are also β-amino esters.

Reports on conjugate addition to acrylic acid acceptors are rare. However, the poor yield for the reaction of 1PP and acrylic acid in water was expected as acrylic acids are generally less active Michael addition partners [142]. What was surprising was the yield of the same reaction when the reactants were left standing for 6 days. The 90% yield shows that even the unreactive acid will form adducts given sufficient time. The result was evident in Chapter 5, where several amine drug substances (PHN, ALC, OFL) reacted with acrylic acid over time.

The screening platform developed in this research in Chapter 5 provided a great deal of insight into merit of performing a controlled study and several aspects of what was discovered would inform future studies. Firstly, performing the reaction at two temperatures provided insight into how the amines would perform under routine (25ºC) and accelerated storage (40ºC), particularity in relation to the rate of hydrolysis of the ester adducts and the reactivity of acrylic acid. Similarly, the addition of both the ester and acid acrylate is to be recommended. Information from the use of both acrylates and the low/high temperatures are a good indication of how reactive an amine is likely to be. Amines like PHN, BMT and ALC reacted with both acrylates at both temperatures during the study; something that is backed up by packaging compatibility studies in our own laboratory. Whereas no adducts have

been detected in unreactive amines such as BUN and EPT during routine stability storage. One of the objectives of forced degradation study is to generate sufficient amounts of impurity for identification and tracking. To this end, it is not recommended that the study be performed at elevated temperatures. The same principle would apply to a direct addition study as described in Chapter 5. In reality, the level of adducts formed as a result of migration from drug packaging is extremely low and catastrophic degradation of the amine or the generation of additional degradation products is not desirable.

Finally, where possible samples should be stored for a number of weeks and examined at two time-points. The results of the case study show that 1 week and 1 month would provide representative samples for impurity analysis. Considering the simplicity of the reaction which consisted of the addition of an acrylate to an amine in an aqueous solution, the complexity of the results was surprising. The equilibration effects noted in Chapter 3 whereby the unreactive amine hydrochloride salt and acrylic acid both generated significant yields of product on standing, were also evident in Chapter 5. Again, this steady upward trend in the concentration of impurities is the same effect observed during long-term storage of packaged drug products.

This case study demonstrates that leachable compounds that migrate into the drug product can react with the active ingredients to form active ingredient related impurities and the results obtained here strongly suggest that formation of amine-acrylate adducts may constitute a significant problem upon long-term storage of ophthalmic solutions in their final packaged configuration.

The present findings suggest that the relatively simple experimental design described in Chapter 5 for the direct addition of a reactive species to the drug product could be applied to other types of drug product interactions such as the direct interaction between the drug substance and excipients or with impurities of excipients, and interaction of the drug substance with impurities from packaging materials. For example, ethylene glycol is a reactive impurity formed through the hydrolysis of ethylene oxide (ETO) in buffered saline products following ETO sterilisation. Ethylene glycol once formed can react with a carboxylic acid containing drug substance to form an ester impurity. The reaction of drug substances with added antioxidants (either as excipients or from packaging) such as sodium metabisulfite would also be suited to the protocol described in Chapter 5.

As discussed above, there are currently no reports in the literature of interactions between reactive amines and acrylates migrating from drug-packaging. The work presented here provides a detailed investigation of potential aza-Michael addition mediated impurities in ophthalmic formulations between amine containing drug substances and acrylate compounds migrating from pharmaceutical packaging. The mechanisms responsible for these impurities have been investigated, and the critical parameters elucidated. The resultant screening platform provides a useful tool to determine the extent to which potential leachables can interact with drug substances and generate impurities. This thesis may therefore provide a valuable tool as part of drug excipient interaction investigations in quality risk management in pharmaceutical development.

6.1 References

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Chapter 7:

Appendices

7.1 Appendix A - Analysis Parameters for Amines: PHN, GAT, OFL, ALC, BMT, TIM, BUN and EPT

Phenylephrine Hydrochloride (0.13%)

HPLC Conditions		Column
Column Temperature	25ºC	Lichrocart 100, C8, 250 mm, 4.6 mm x 5µm
Flow Rate	1 mL/min	
Injection Volume,	30 µL	
Runtime	20 mins	Mobile Phase:
Wavelength,	272 nm	0.3 mM HSA/ACN/TFA (85/15/10 v/v/v)
Sample Diluent	Water	
Conc. Sample % (w/v)	0.0026%	

Gatifloxacin (0.5%)

HPLC Conditions		Column
Column Temperature	40ºC	YMC Basic, C8 Mixed, 100 mm, 4.6 mm x 3µm
Flow Rate	1 mL/min	
Injection Volume,	25 µL	
Runtime	60 mins	Mobile Phase:
Wavelength,	325 nm	75mM KH_2PO_4, 1% TEA, pH 5.1/ACN (92/8 v/v)
Sample Diluent	0.1% Phosphoric Acid	
Conc. Sample % (w/v)	0.02%	

Ofloxacin (0.3%)

HPLC Conditions		Column
Column Temperature	37ºC	Waters Xbridge, C18, 150 mm, 4.6 x 3.5 µm
Flow Rate	0.9 mL/min	Mobile Phase A:
Injection Volume,	10 µL	50 mM Na_2HPO_4, pH 6.4/MeOH (85/15 v/v)
Runtime	27 mins	
Wavelength,	294 nm	Mobile Phase B: MeOH
Sample Diluent	0.05N HCl	Gradient: % B
		0 – 40% in 16 mins
Conc. Sample % (w/v)	0.006%	

Alcaftadine (0.25%)

HPLC Conditions		Column
Column Temperature	257ºC	Phenomenex Gemini, C18, 250 mm, 4.6 x 5 µm
Flow Rate	1.0 mL/min	Mobile Phase A:
Injection Volume	5 µL	5mM K_2HPO_4, pH 8.4
Runtime	55 mins	Mobile Phase B:
Wavelength	286 nm	ACN/5mM K_2HPO_4, pH 8.4 (70/30 v/v)
Sample Diluent	50% MeOH	Gradient: % B
		25 – 50% in 30 mins
Conc. Sample % (w/v)	0.05%	50 – 100% in 42 mins

Brimonidine Tartrate (0.2%) and Timolol Maleate (0.5%)

UHPLC Conditions		Column
Column Temperature	30ºC	ACE Excel, C18, 100 mm, 2.1 x 2µm
Flow Rate	0.45 mL/min	
Injection Volume	3 µL	Mobile Phase A:
Runtime	15 mins	50 mM(NH_4)Acetate, pH 4.85
Wavelength	264 nm	
		Mobile Phase B:
		50 mM(NH_4)Acetate, pH 4.85/MeOH (50/50 v/v)
Sample Diluent	Water	Gradient: % B
		8 – 88% in 10 mins
Conc. Sample % (w/v)	0.008% BMT	
	0.02% TIM	

Timolol Maleate (0.5%)

HPLC Conditions		Column
Column Temperature	30ºC	Waters Sunfire, C18, 75 mm, 4.6 x 3.5 µm
Flow Rate	1.5 mL/min	
Injection Volume	10 µL	Mobile Phase A:
Runtime	22 mins	0.01% TFA in Water
Wavelength	295 nm	
		Mobile Phase B:
		0.1% TFA in MeOH
Sample Diluent	Water	Gradient: % B
		10 – 35% in 10 mins
Conc. Sample % (w/v)	0.02%	35 – 90% in 15 mins

Levobunolol Hydrochloride (0.5%)

HPLC Conditions		Column
Column Temperature	44ºC	Waters Sunfire, C18, 150 mm, 4.6 x 3.5 µm
Flow Rate	1.0 mL/min	
Injection Volume	5 µL	Mobile Phase:
Runtime	20 mins	0.43 mM HSA
Wavelength	254 nm	Buffer/MeOH/Acetic Acid (54.5/45/0.5 v/v/v)
Sample Diluent	50% MeOH	
Conc. Sample % (w/v)	0.02%	

Epinastine Hydrochloride (0.05%)

HPLC Conditions		Column
Column Temperature	50ºC	Inertsil ODS-3, C18, 150 mm, 4.6 x 5 µm
Flow Rate	1.5 mL/min	
Injection Volume	25 µL	Mobile Phase:
Runtime	20 mins	43 mM $(NH_4)H_2PO_4$
Wavelength	210 nm	Buffer,pH4.5/ACN/THF (80/19/1 v/v/v)
Sample Diluent	Mobile Phase	
Conc. Sample % (w/v)	0.01%	

7.2 Appendix B - Reactivity of Amines with Methyl Acrylate and Acrylic acid

Table 7.1 Phenylephrine Hydrochloride (PHN) Reactivity as determined by monitoring % loss of the initial API, and % formation of impurity adduct, observed at two distinct relative retention times (RRT), with MA and AA used as acrylate donors

Acrylate Donor		11 Days			32 Days		
		% API loss	% IMP formed	% IMP formed	% API loss	% IMP formed	% IMP formed
Methyl acrylate			(0.31 RRT)	(1.24 RRT)		(0.31 RRT)	(1.24 RRT)
25°C	A	99.1	78.9	21.2	99.8	99.9	0.0
	B	99.2	79.7	20.5	100.0	98.1	0.0
40°C	A	100.0	112.6	0.0	100.0	124.6	0.0
	B	100.0	112.3	0.0	100.0	121.2	0.0
		11 Days			32 Days		
		% API loss	% IMP formed	% IMP formed	% API loss	% IMP formed	% IMP formed
Acrylic Acid			(0.29)	(0.34)		(0.29)	(0.34)
25°C	A	11.0	7.7	1.0	26.1	29.6	1.8
	B	9.8	6.9	1.0	24.6	28.0	1.9
40°C	A	50.4	41.2	3.7	71.1	58.0	3.3
	B	53.0	43.4	3.7	74.8	58.0	3.3

Table 7.2 Brimonidine Tartrate (BMT) Reactivity as determined by monitoring % loss of the initial API, and % formation of impurity adduct, observed at two distinct relative retention times (RRT), with MA and AA used as acrylate donors

			BMT+TIM 7 days		BMT only 20 Days	
			% API loss	% IMP formed	% API loss	% IMP formed
Methyl acrylate						
	25°C	A	46.3	20.3	26.8	9.0
		B	45.0	20.7	26.4	8.9
	40°C	A	85.1	37.7	82.2	30.8
		B	83.9	35.5	78.2	29.5
Acrylic Acid						
	25°C	A	1.4	0.05	1.2	0.0
		B	2.4	0.05	1.3	0.0
	40°C	A	1.7	0.21	2.9	0.3
		B	0.0	0.23	2.3	0.3

Table 7.3 Alcaftadine (ALC) Reactivity as determined by monitoring % loss of the initial API, and % formation of impurity adduct, observed at two distinct relative retention times (RRT), with MA and AA used as acrylate donors

Label			5 Days			32 Days	
		% API loss	% IMP formed	% IMP formed	% API loss	% IMP formed	% IMP formed
Methyl acrylate			*RRT (0.34)*	*RRT (0.64)*		*RRT (0.34)*	*RRT (0.64)*
25°C	A	21.0	14.0	9.4	23.3	14.8	16.0
	B	19.1	15.1	16.5	19.4	13.9	9.5
40°C	A	27.0	3.2	15.3	9.2	1.7	0.0
	B	34.2	3.3	14.3	0.0	23.2	1.6
Acrylic Acid			*RRT (0.34)*			*RRT (0.34)*	
25°C	A	8.6	1.1		23.2	5.8	
	B	8.0	1.0		21.1	5.6	
40°C	A	29.9	6.7		34.9	18.6	
	B	30.6	6.5		36.0	18.1	

Table 7.4 Ofloxacin (OFL) Reactivity as determined by monitoring % loss of the initial API, and % formation of impurity adduct, observed at two distinct relative retention times (RRT), with MA and AA used as acrylate donors

			7 Days			32 Days	
		% API loss	% IMP formed	% IMP formed	% API loss	% IMP formed	% IMP formed
Methyl acrylate			*RRT (0.35)*	*RRT (0.48)*		*RRT (0.35)*	*RRT (0.48)*
25°C	A	32.0	1.3	25.5	34.3	5.8	22.2
	B	31.2	1.3	26.2	22.1	5.4	14.2
40°C	A	20.1	7.2	11.0	16.2	9.9	3.5
	B	17.0	7.0	8.9	12.8	9.5	2.6
Acrylic Acid			*RRT (0.35)*			*RRT (0.35)*	
25°C	A	0.0	0.7		6.8	2.8	
	B	0.0	0.6		1.2	2.8	
40°C	A	6.2	4.1		13.2	9.7	
	B	4.4	4.0		13.9	9.7	

Table 7.5 Gatifloxacin (GAT) Reactivity as determined by monitoring % loss of the initial API, and % formation of impurity adduct, observed at an the same relative retention times (RRT), with MA and AA used as acrylate donors

Acrylate			11 Days		33 Days	
			% API loss	% IMP formed	% API loss	% IMP formed
Methyl acrylate						
	25°C	A	21.2	4.4	29.2	12.7
		B	21.2	4.4	32.0	12.8
	40°C	A	25.3	17.1	28.9	20.3
		B	33.5	17.1	38.5	21.2
Acrylic Acid						
	25°C	A	5.4	0.5	2.3	1.4
		B	6.7	0.5	2.6	1.4
	40°C	A	2.2	3.3	5.7	7.6
		B	3.4	3.3	6.6	7.3

Table 7.6 Timolol Maleate (TIM)

Reactivity as determined by monitoring % loss of the initial API, and % formation of impurity adduct, observed at an the same relative retention times (RRT), with MA and AA used as acrylate donors

			TIM only 11 Days		TIM only 40 Days	
			% API loss	% IMP formed	% API loss	% IMP formed
Methyl acrylate						
	25°C	A	2.7	1.8	3.6	1.5
		B	2.5	1.6	1.3	1.3
	40°C	A	9.9	11.1	13.0	9.1
		B	9.8	11.2	13.4	9.1
Acrylic Acid						
	25°C	A	1.2	0.0	0.7	0.0
		B	1.1	0.0	0.0	0.0
	40°C	A	0.1	0.2	2.5	0.1
		B	0.0	0.2	2.3	0.1

Table 7.7 Timolol Maleate (TIM) Reactivity in Combined Drug Product as determined by monitoring % loss of the initial API, and % formation of impurity adduct, observed at an the same relative retention times (RRT), with MA and AA used as acrylate donors

			TIM+HTL 11 days		TIM only 11 Days	
			% API loss	% IMP formed	% API loss	% IMP formed
Methyl acrylate						
	25°C	A	4.0	3.2	2.7	1.8
		B	3.8	3.4	2.5	1.6
	40°C	A	7.7	9.0	9.9	11.1
		B	8.1	9.5	9.8	11.2
Acrylic Acid						
	25°C	A	0.9	0.0	1.2	0.0
		B	0.6	0.0	1.1	0.0
	40°C	A	0.1	0.0	0.1	0.2
		B	0.0	0.0	0.0	0.2

Table 7.8 Levobunolol (BUN) Reactivity as determined by monitoring % loss of the initial API, and % formation of impurity adduct, observed at an the same relative retention times (RRT), with MA and AA used as acrylate donors

		19 Days		40 Days	
		% API loss	% IMP formed	% API loss	% IMP formed
Methyl acrylate					
25°C	A	6.7	0.9	8.1	1.8
	B	3.1	0.9	3.6	1.9
40°C	A	0.0	2.4	11.2	6.8
	B	3.9	5.6	1.3	3.0
Acrylic Acid					
25°C	A	0.8	0.0	2.0	0.0
	B	0.4	0.0	0.0	0.0
40°C	A	0.0	0.1	2.8	0.1
	B	0.0	0.1	0.0	0.1

Table 7.9 Epinastine (EPT) Reactivity as determined by monitoring % loss of the initial API, and % formation of impurity adduct, observed at two distinct relative retention times (RRT), with MA and AA used as acrylate donors

		7 Days		34 Days	
		% API loss	% IMP formed	% API loss	% IMP formed
Methyl acrylate					
25°C	A	2.2	0.1	3.0	0.5
	B	2.9	0.1	5.6	0.9
40°C	A	1.5	0.0	2.4	4.0
	B	1.9	0.0	5.9	7.2
Acrylic Acid					
25°C	A	1.5	0.0	4.1	0.0
	B	1.4	0.0	4.4	0.0
40°C	A	1.8	0.0	1.1	0.0
	B	1.3	0.0	0.8	0.0

7.3 Appendix C - UV Chromatograms and Spectra

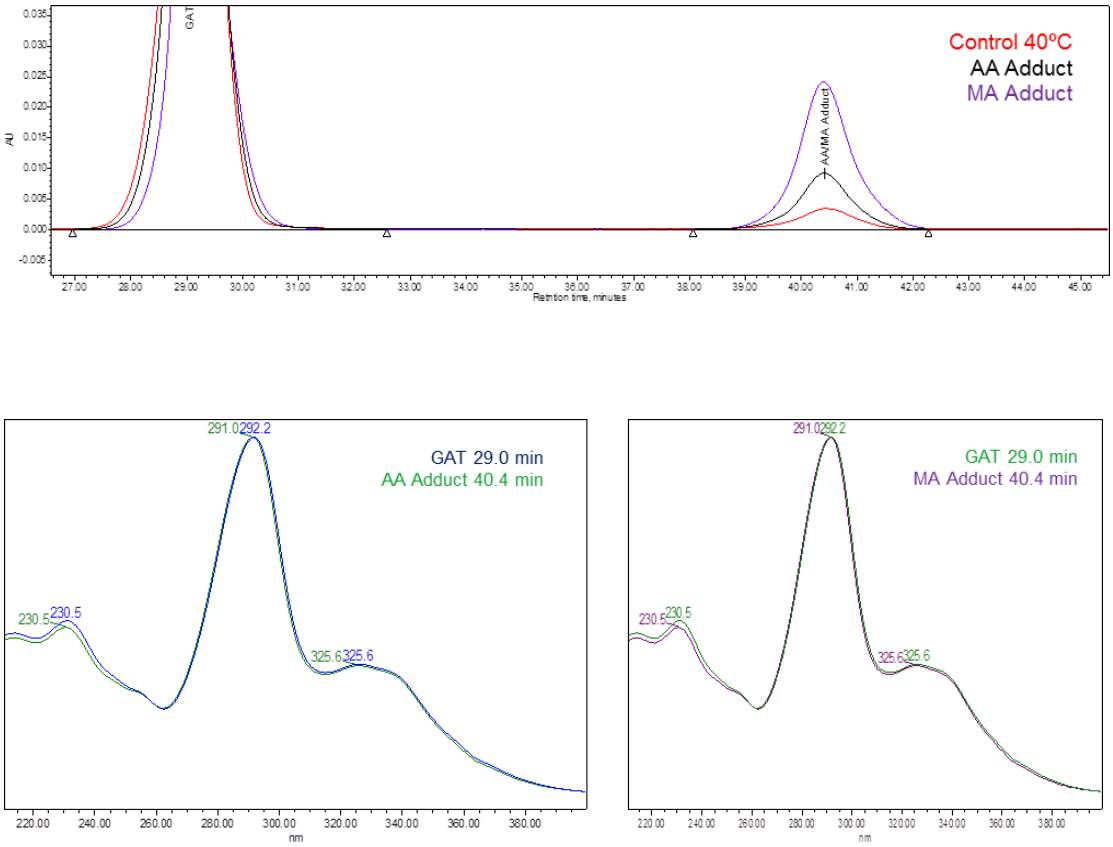

Figure 7.1 (Top) HPLC chromatogram with UV detection at 325 nm of GAT samples reacted with MA and AA stored at 40ºC/75% RH for 32 days. The samples are overlaid with the GAT 40ºC/75% control sample, which also contains the methyl acrylate adduct.

(bottom) UV spectrum of GAT samples reacted with AA (left) and MA (right) stored at 40ºC/75% RH for 33 days. A single peak was observed for both the MA and AA adducts. The UV spectrum was identical for both the MA and AA adduct with a maximum absorbance at 291.02 nm. The max absorbance of the GAT peak was 292.2 nm in both samples.

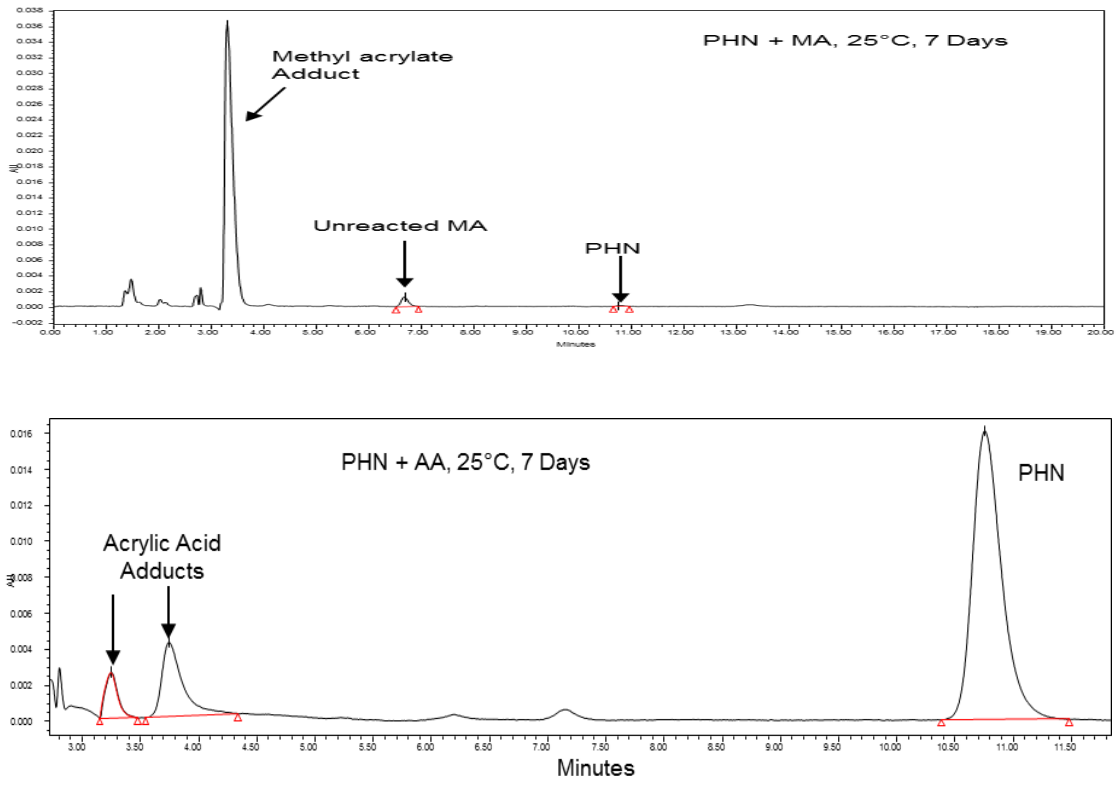

Figure 7.2 HPLC chromatograms with UV detection at 210 nm of PHN samples reacted MA (top) and AA (bottom) stored at 25ºC/60% RH for 11 days.

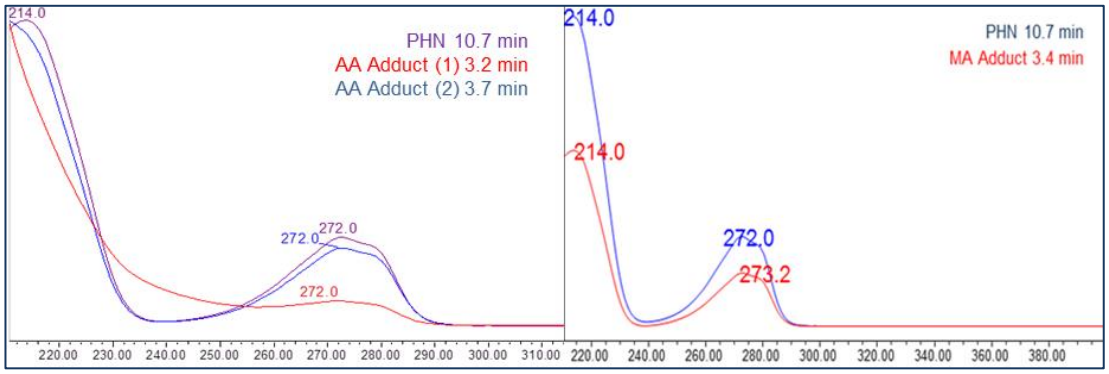

Figure 7.3 UV spectrum of PHN samples reacted AA (left) and MA (right) stored at 25ºC/60% RH for 11 days.

The BMT-AA adduct was the only one for which a reference standard was available. As mentioned in Section 5.2, a UV response factor of 2.4 was established for the BMT-AA adduct. An overlay of the UV spectra of MA, BMT, BMT-MA and BMT-AA is given in Figure 7.4. A small shift in λ max from 246.5 nm (BMT) to 241.0 nm (MA and AA adducts) can be seen in the overlay. The overlay also includes the spectrum for unreacted MA in the sample. The UV detector was set to 264 nm in the UHPLC assay for BMT and a small peak was detected in the samples at 3.7 min. As the MA-adduct peak eluted at 3.8 min, the PDA was used to good effect when identifying the impurity adducts. The spectrum of the unreacted MA was easily distinguished from the BMT sample and adducts.

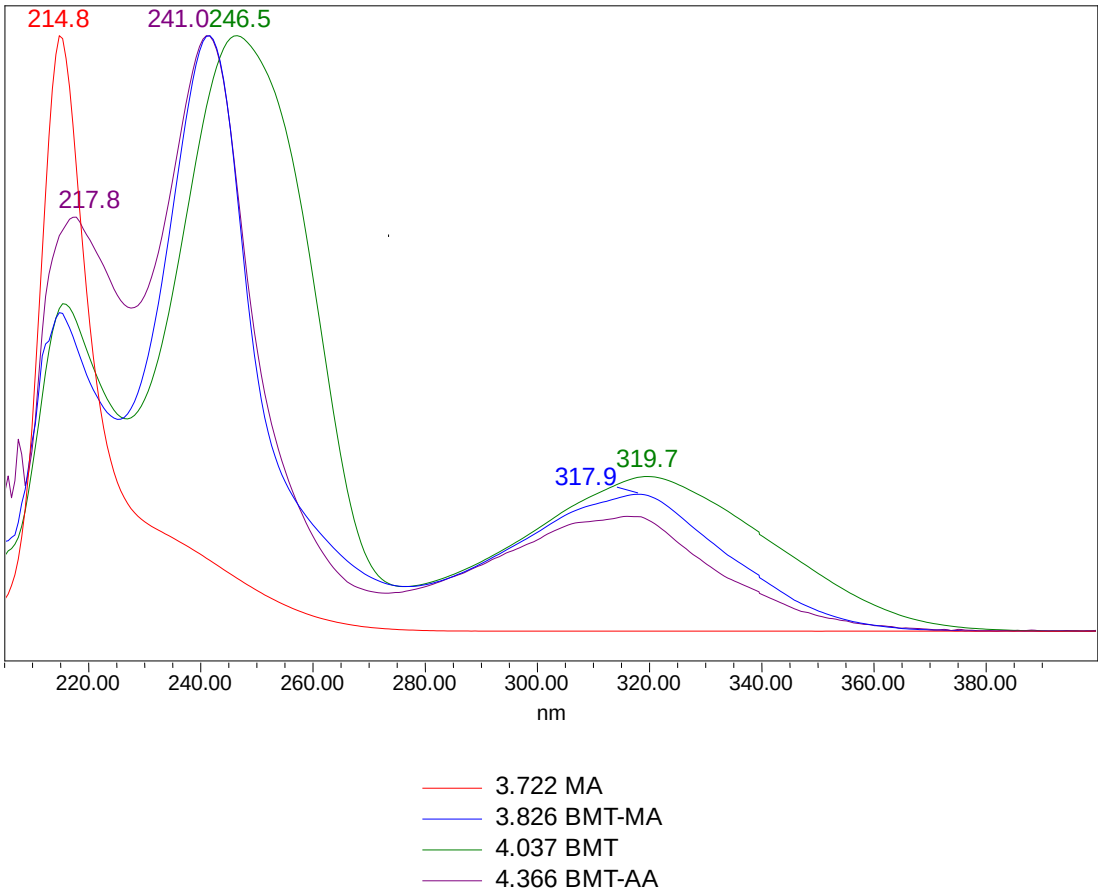

Figure 7.4 UV spectra of BMT samples reacted AA and MA stored at 25ºC/75% RH for 7 days. A shift in the maximum absorbance for BMT (246.5 nm), AA adduct and MA adduct (241.0 nm) was noted. The UV spectrum for unreacted MA remaining in the sample is included and has a maximum absorbance of 214 nm.

The HPLC chromatograms and UV spectra for OFL, TIM and BUN are given in Figures 7.5 to 7.8. The HPLC-PDA chromatograms were processed using the 'MAX PLOT' as the derived channel in Empower, which is the maximum absorption of the spectrum at every given time point. Using this feature, the unreacted MA peak was visible in the OFL and BUN chromatograms.

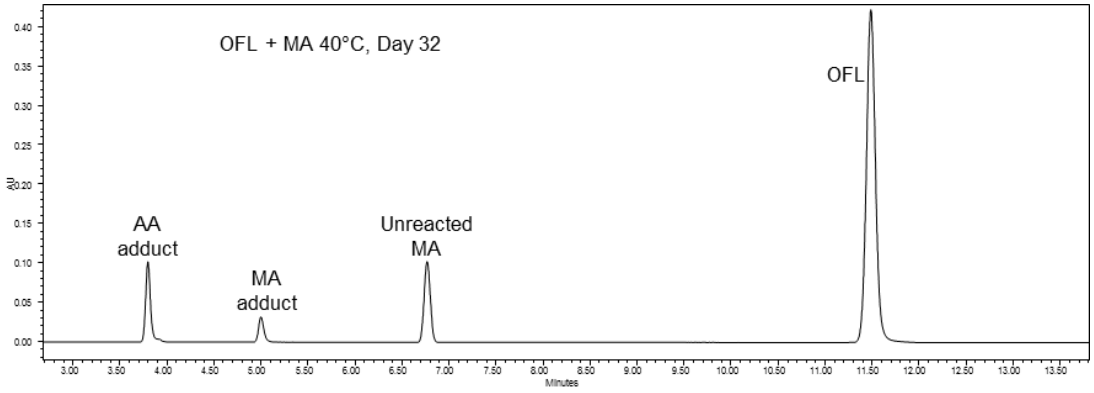

Figure 7.5 (Top) HPLC chromatogram with UV detection at 294 nm of OFL samples reacted with MA stored at 40ºC/75% RH for 32 days.

Figure 7.6 UV spectra of OFL samples reacted AA and MA stored at 40ºC/75% RH for 32 days. A slight shift in the maximum absorbance is noted for OFL (289.5) nm, AA adduct (288.9 nm) and MA adduct (288.2 nm). The MA and AA adduct peaks were baseline resolved chromatographically.

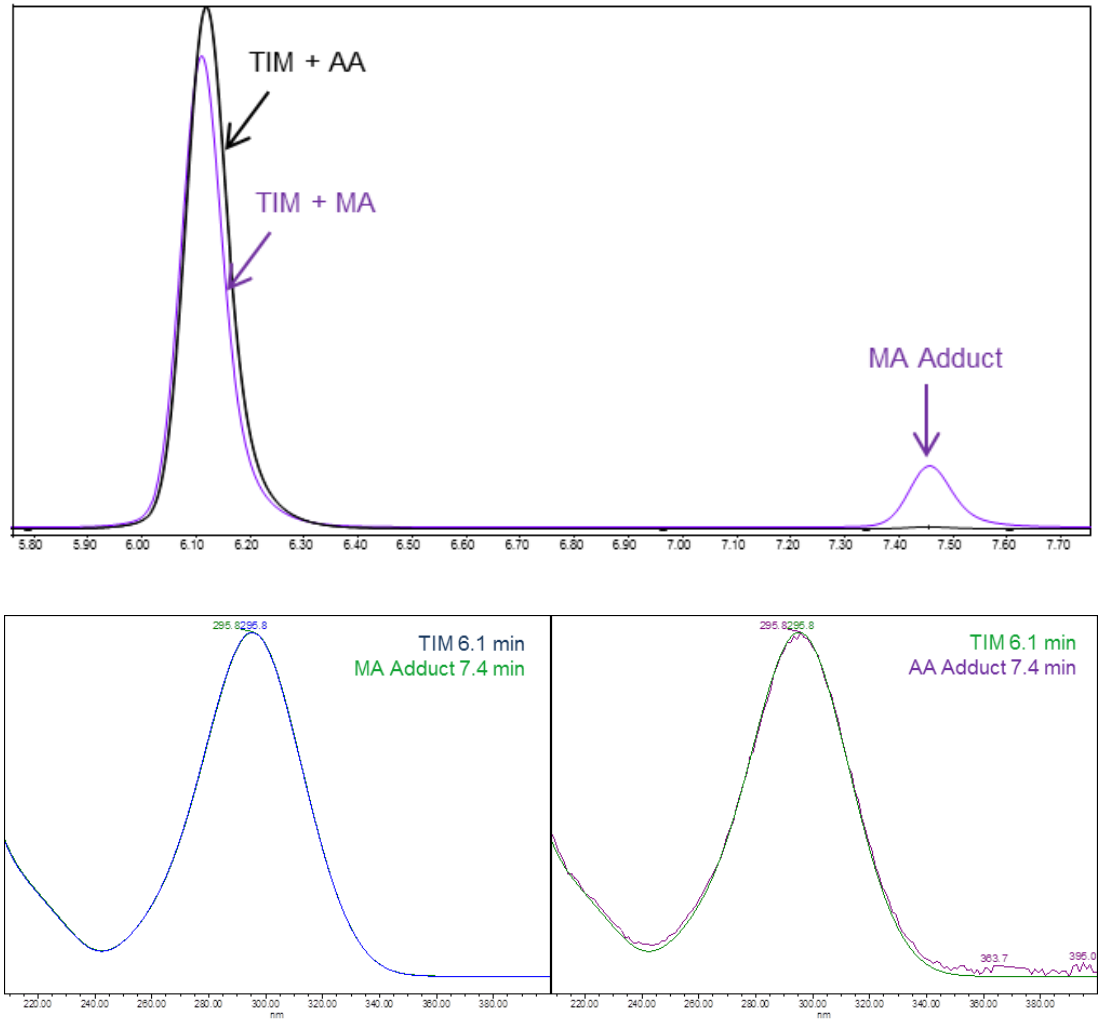

Figure 7.7 (Top) HPLC chromatogram with UV detection at 295 nm of TIM samples reacted with MA and AA stored at 40ºC/75% RH for 32 days.

(bottom) UV spectrum of TIM samples reacted with MA (left) and AA (right) stored at 40ºC/75% RH for 32 days. A single peak was observed for both the MA and AA adducts. The UV spectrum is identical for TIM and the MA and AA adducts with a maximum absorbance at 295.8 nm.

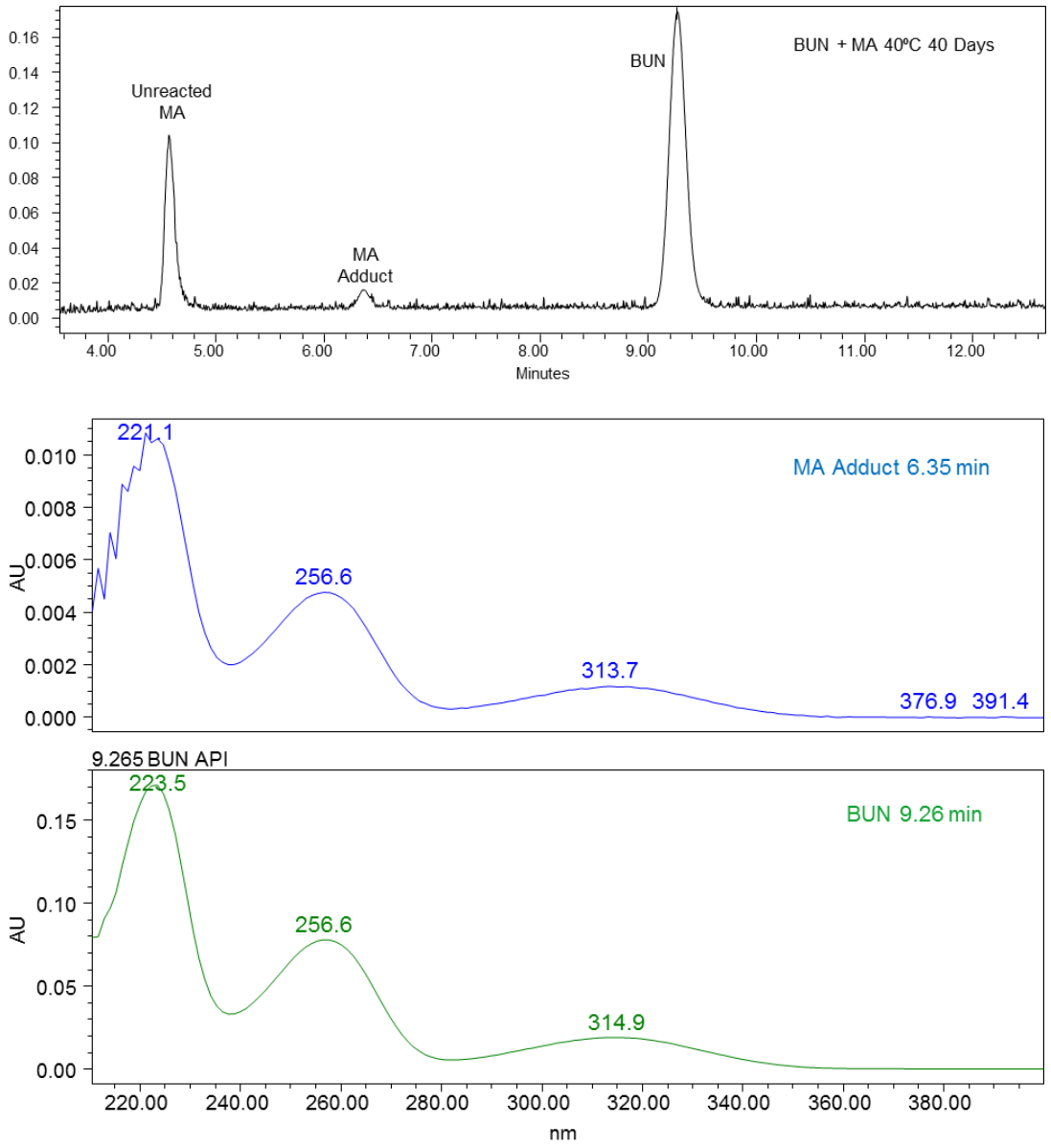

Figure 7.8 (Top) HPLC chromatogram with UV detection at 254 nm of BUN samples reacted with MA and AA stored at 40ºC/75% RH for 40 days. Unreacted MA is detected at 254 nm. The AA adduct was not detected in samples.

(bottom) UV spectrum of BUN sample reacted with MA stored at 40ºC/75% RH for 40 days. The MA adduct spectrum closely correlated with that of the BUN drug substance.