Development and Validation of a Rapid Analytical Method and Sampling Methodology for Trace Level APIs in Support of Cleaning Validation

By

Patricia Boyle, BSc.

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School of Chemical Sciences

Dublin City University

Under the supervision of

Prof. Apryll Stalcup

School of Chemical Sciences

Dublin City University

and

Dr. Damian Connolly

Department of Science

Waterford Institute of Technology

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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 **Master of Science** 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.

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List of Abbreviations

AA	Atomic Absorption
ACN	Acetonitrile
API	Active Pharmaceutical Ingredient
APIC	Active Pharmaceutical Ingredient Committee
BMT	Brimonidine Tartrate
BUN	Levobunolol Hydrochloride
CSH	Charged Surface Hybrid
DEX	Dexamethasone
DOE	Design of Experiments
EPDM	Ethylene propylene diene monomer
EtOH	Ethanol
FBP	Flurbiprofen
FDA	Food and Drug Administration
FML	Fluorometholone
HAC	Hydrocortisone Acetate
HPLC	High Performance Liquid Chromatography
HSS	High Strength Silica
IC	Ion Chromatography
ICH	International Conference on Harmonisation
KET	Ketorolac Tromethamine
LC	Liquid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantitation
MAC	Maximum Allowable Carryover

MeOH	Methanol
MS	Mass Spectrometry
OFL	Ofloxacin
P17-AC	Prednisolone 17-acetate
PAC	Prednisolone Acetate
PDA	Photodiode Array
PDA	Parenteral Drug Association
POH	Prednisolone
PTFE	Polytetrafluoroethylene
QC	Quality Control
RAL	Residue Acceptance Limit
RP	Reverse Phase
R_S	Resolution
RSD	Relative Standard Deviation
TIM	Timolol Maleate
TOC	Total Organic Carbon
UPLC	Ultra Performance Liquid Chromatography
USP	United States Pharmacopeia
UV	Ultraviolet

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Abstract

Development and Validation of a Rapid Analytical Method and Sampling Methodology for Trace Level APIs in Support of Cleaning Validation.

By Patricia Boyle

In pharmaceutical industries, the cleaning procedure is one of the most critical processes. It is crucial to prove that the production equipment surfaces are consistently cleaned from product residues to prevent possible contamination and cross-contamination. Therefore, the cleaning procedure must be appropriately validated and robust. The cleaning procedures must be supported by validated analytical methods and related sampling methodologies. The cleaning analytical methods and related sampling methodologies are critical in establishing quality validated cleaning programs, as they quantitatively determine the amount of residual API, if present, on the manufacturing equipment post-cleaning.

The overall aim of this research was to develop a rapid liquid chromatographic method and related sampling methodologies which can be used to verify that production equipment surfaces have been cleaned to an extent that meets existing regulatory requirements. A High Performance Liquid Chromatographic (UPLC) analytical method designed to have a broad applicability for the simultaneous determination of a range of API residues in ophthalmic solutions was developed. The API residues investigated were dexamethasone fluorometholone, prednisolone, prednisolone acetate, ketorolac tromethamine, flurbiprofen, timolol maleate, levobunolol hydrochloride, brimonidine tartrate and ofloxacin. A Design of Experiments (DOE) approach utilising Dry Lab and Minitab software was adopted to facilitate rapid method development and ensure the desired resolution (≥ 2.0) between each peak pair within 15 minutes. The optimised method, using a UPLC reverse phase chromatographic column and gradient elution with an ammonium acetate buffer and acetonitrile provided the desired resolution between all the drug substances in 13 minutes. For evaluation purposes, standard analytical performance criteria were examined for the developed method.

The developed analytical method was used in combination with the developed sampling methodologies to ensure optimal recoveries for each API residue from the various product contacting surfaces. Interferences from the swab and product contacting surfaces that may affect the quantitation of the APIs were examined. A variety of swabs (polyester, cotton, etc), product contacting surfaces (steel, glass, silicone, and teflon), and solvents were included in this study to investigate for interferences and evaluate the recoveries of the APIs from the product contact surfaces. The TX761 polyester swab was found to provide the least amount of extractables and interferences which may interfere with the selected drug substances. In addition, the TX761 swab was shown to adequately remove the majority of the selected API residues from stainless steel, teflon, glass and silicone. It was shown however that flurbiprofen was more difficult to remove from the silicone surfaces than the other APIs.

Chapter 1

A Review of Analytical Techniques used for the Determination of API Residues in Support of Cleaning Validation

1.1. An introduction to cleaning validation

Since the 1990's, cleaning validations have gained a lot of interest within pharmaceutical and bio-pharmaceutical companies. In 1988, the FDA experienced its first major incident with cross contamination traceable to inadequate cleaning and cleaning validation. The finished drug product, Cholestyramine Resin USP, was recalled due to contamination with low levels of intermediates and degradants from the production of agricultural pesticides. The cross-contamination was believed to have come from the use of drums in the production of the active pharmaceutical ingredient (API). These drums had previously been used to store solvents for the manufacture of agricultural pesticides at another plant. The drums were inadequately cleaned leading to low levels of the agricultural pesticides entering the API manufacturing processes. This incident raised the FDA's awareness to the potential impact of cross-contamination from un-validated cleaning processes [1, 2].

The U.S Food and Drug Administration (FDA) issued its *Guide to Inspections – Validation of Cleaning Process* in 1993. Previously, validation was required only for the manufacturing process of drug products and not for the cleaning process of the manufacturing equipment. The objective of cleaning validation is to prove that the equipment is consistently cleaned from product, detergent and microbial residues to an acceptable level, to prevent possible contamination and cross-contamination. The driving force for cleaning validation is patient safety and product quality. Cross-contamination with residues of any kind presents a safety risk to patients consuming the drug product. It threatens to alter the strength, chemical identity, and integrity of the drug substance and formulation. For these reasons, it is essential to have a robust validated cleaning process that is suited to its intended purpose [1,3-7].

An inadequate or incomplete cleaning regime between production campaigns could result in trace levels of APIs remaining on the production equipment with obvious implications for product quality. Therefore, determining what level of API residue is allowed to remain on the manufacturing equipment after the cleaning process has taken place is a crucial part of an effective cleaning validation program. The maximum allowable carryover (MAC) of an API residue is determined by a number of factors such as the therapeutic dose of the product, the defined safety factor, batch sizes of the product and minimum/maximum daily doses of the product. These residue acceptance limits are further discussed in Section 1.2. Generally, residue

acceptance limits are established first, then the analytical method and related sampling methodologies are developed and validated to ensure that the maximum acceptable limits and below can be accurately quantitated.

After a cleaning regime on manufacturing equipment surfaces post-product manufacture, several swabs are generally taken from different equipment surfaces (reactor, centrifuge, dryer, etc.) and are analysed for API residues. The analytical results are required as soon as possible to minimize down-time between product campaigns. If API residue is found above the acceptable residue carryover limit on any of the swabs, production would be delayed by the initiation of a further cleaning/rinse/analysis cycle before the next batch of product is manufactured. It is therefore crucial to employ rapid methods for cleaning validation activities. In addition, it is crucial to have an effective sampling methodology in place [1,3-7].

1.2. Establishing residue acceptance limits

1.2.1. Introduction

An important aspect of a cleaning validation is to establish what level of API residue is allowed to remain on the manufacturing equipment surfaces after the cleaning process has been carried out post-production. This is a critical aspect as any residue remaining on the manufacturing equipment after cleaning will be transferred over into the first batch of the next pharmaceutical product as shown in Figure 1.1 [1-7]. In the past, detection and quantitation of trace level residues was more difficult due to the limited analytical technologies available. Today however, there are many analytical technologies available such as Ultra Performance Liquid Chromatography (UPLC) and Mass Spectrometry (MS) that are capable of detecting and quantitating trace amounts of API residues that may remain on the manufacturing equipment after the cleaning process has been carried out.

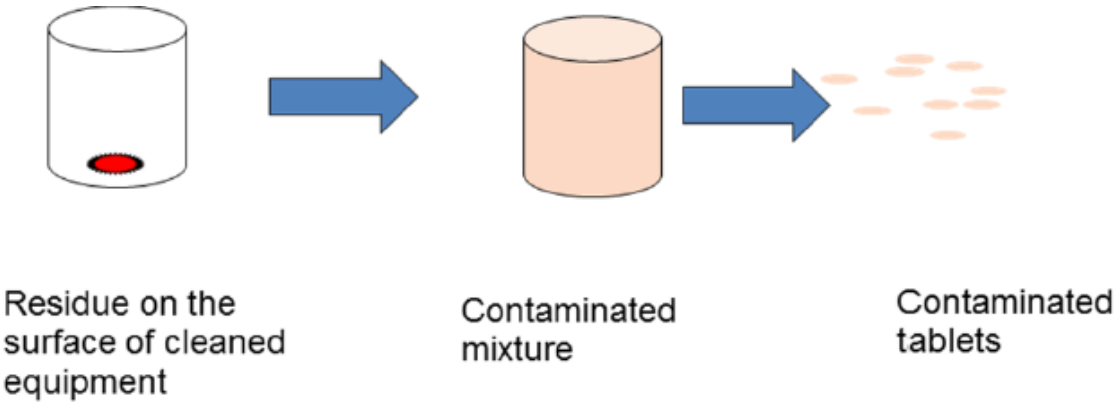

Figure 1.1: Residue contamination of pharmaceutical product, taken from [4].

One of the earliest articles published regarding the validation of cleaning procedures was by Harder in 1984. In the article, Harder discusses different aspects of what may be required for an effective cleaning procedure. With regards to the setting of residual acceptance limits, Harder wrote that limits should be practical and achievable using the defined cleaning procedure and that the residue acceptance limits should be verified by the analytical technique used by the company [2,8]. In many of the guidances today, it states that the establishment of residue acceptance limits must be practical, achievable and verifiable [1,3-6]. In 1993, an article by Fourman and Mullen of Eli Lilly proposed the use of the following combination of residue acceptance limits for cleaning validation [9].

- No more than 0.001 dose of any product will appear in the maximum daily dose of another product.
- No more than 10 ppm of the product will appear in another product.
- No quantity of residue will be visible on the equipment after cleaning procedures are performed.

In recent years, a number of regulators have adopted this approach and have included these limits in their own guidance documents [3-6]. The FDA has not included residue acceptance limits in their guidelines due to the wide variation of equipment and products used from company to company. However, they do state that a company's rationale for establishing residue acceptance limits should be practical, achievable and verifiable, and they have accepted the application of Fourman and Mullen's residue acceptance limits approach as the industry norm. Rather than focus

on the basis for the limits, it is more important to focus on correct implementation of the limits [1].

1.2.2. Dose residue acceptance limit approach

Pharmaceutical companies must demonstrate that the cleaning procedures used for the various pieces of manufacturing equipment are effective and fit for their intended purpose. The cleaning procedures must show that the potential carryover of residue into the next product is limited, and if there is carryover, it is at an acceptable level. The limits established must be based on sound scientific rationale. They must also be practical, achievable and verifiable [1-9]. As stated in the Fourman and Mullen article 'No more than 0.001 dose of any product will appear in the maximum daily dose of another product'. The logic behind the 0.001 ($1/1000^{th}$) dose derives from three factors of 10 [9].

1. 'Pharmaceuticals are often considered to be non-active at 0.1 of their normally prescribed dosages'.
2. 'A safety factor'.
3. 'The cleaning validation program should be robust, i.e. be vigorous enough that it would be considered acceptable for quite some time in a world with ever tightening standards'.

This approach is provided in many of the guidances. In cases where the therapeutic dose in not known, the MAC may be calculated using toxicity data [3-7].

1.2.3. 10 ppm residue acceptance limit approach

An alternative residue acceptance limit criterion stated in the article by Fourman and Mullen is that 'No more than 10 ppm of the product will appear in another product' [9]. Generally in the current guidance documents, if the limit per swab area calculated is above 10 ppm, then the 10 ppm residue acceptance limit approach is used [3-7].

1.2.4. Visible residue acceptance limit approach

The acceptance criterion stated in many of the regulatory and guidance documents is ‘No quantity of residue should be visible on the equipment after cleaning procedures are performed’ [3-6]. Before validated cleaning processes were introduced into pharmaceutical companies, the manufacturing equipment was always visually inspected before any drug manufacturing took place. Since the introduction of cleaning validation programs, analytical testing has been the preferred approach for establishing the amount of residue, if present, remaining on the manufacturing equipment surfaces after cleaning. However, visual inspections are still carried out in pharmaceutical companies as per regulations and industry guidances, but they are carried out in conjunction with the analytical methodologies [10-15].

The general approach to establishing a visible residue limit is to apply a standard solution containing the API residue onto a coupon that is the material representative of the product contacting surface, e.g. stainless steel. The level of residue is applied at approximately the residue limit. The coupon is dried under nitrogen to minimize oxidation which might alter the appearance of the dried API residue. The coupon with the dried API residue is then compared to the manufacturing equipment after the cleaning process has been completed, to establish whether the manufacturing equipment meets the visible residue limit criterion, as shown in Figure 1.2 [10-15].

Figure 1.2: Visual inspection of manufacturing equipment using the visual residue acceptance limit approach, taken from [12].

1.3. Analytical techniques used in support of cleaning validation

1.3.1 Introduction

With the analytical technologies that are available today, quantitation of API residues at very low levels from the manufacturing equipment is possible even after vigorous cleaning procedures have been carried out. The analytical cleaning methods that are used to quantitate API residues should be specific for the residue/residues to be analysed and should be sensitive enough to detect the target API. If the API residue is not detected using the specified analytical method, it does not necessarily mean that there is no residual API contamination after the cleaning process. It only means that levels of API residue greater than the detection limit of the analytical cleaning method are not present in the sample. There are many different analytical techniques available for cleaning validations, but selection of an appropriate analytical technique depends on the parameters to be measured. There are two types of analytical methods that are generally used for cleaning validations, specific and non-specific analytical methods [1,3-7]. Some examples of specific and non-specific methods are listed in Table 1.1.

Table 1.1: Non-specific and specific methods used for cleaning validation.

Non-specific	**Specific**
Total Organic Carbon (TOC)	Liquid chromatography (LC)
pH	Ion chromatography (IC)
Conductivity	Atomic absorption (AA)

1.3.2. Non-specific methods

Non-specific analytical methods measure a general property, such as conductivity or total organic carbon (TOC), which may result from a variety of chemical species or sources. One of the most widely used non-specific analytical techniques for cleaning validations is TOC analysis. TOC is the amount of carbon found in an organic compound and is determined by the oxidation of these organic compounds into carbon dioxide. The oxidation can occur through a number of mechanisms such as UV, persulfate, and combustion. TOC is generally used for the analysis of water soluble drug substances, cleaning agents and excipients with regards to cleaning validations. The advantages of TOC are; low level detection, fast analysis time and low cost. However, the limitation associated with TOC analysis is that it cannot give exact quantitative results for the target residue, therefore making it harder to pass the stringent residue acceptance limit criteria [6,16-20]. The pH and conductivity methods are generally used for residual analysis of cleaning agents. A linear response between the conductometer and the ion concentration in the cleaning agent sample can be established, however, pH can only be used to verify qualitatively the presence of the correct cleaning solution [6,21].

1.3.3. Specific methods

Specific methods can quantitate the exact amount of the target residue present in a sample in the presence of expected interferences. They can simultaneously give quantitative and qualitative results, and therefore they are generally the preferred analytical technique for analysis of API residues in support of cleaning validation [4,6,16,17,21]. Some examples of specific methods shown in Table 1.1 include liquid chromatography (LC), ion chromatography (IC) and atomic absorption (AA).

One of the most widely used specific analytical techniques for quantitating API residues from manufacturing equipment surfaces is LC. Examples of the LC technologies available today that have the advantage of improved speed, sensitivity and selectivity are liquid chromatography-mass spectrometry (LC-MS), and ultra-performance liquid chromatography-mass spectrometry (UPLC-MS). However, high performance liquid chromatography (HPLC) or UPLC methods hyphenated to MS are more expensive than using traditional LC detectors and are not as widespread yet in pharmaceutical companies for the analysis of residual APIs [4,6,16,17,21]. Ion chromatography is another powerful analytical technique that is used for trace analysis in support of cleaning validation. It is generally used for the analysis of anions and cations in the cleaning agents used to clean the equipment trains post-production, and can quantitate cleaning agent residues down to parts per million (ppm) levels [22,23]. Atomic absorption (AA) is typically used for metal ions and is an effective technique for the analysis of rinse samples with trace level quantitation down as far as parts per billion (ppb) levels [24,25].

1.3.4. Overview of Ultra Performance Liquid Chromatography

UPLC was the analytical technique chosen for this research due to its many desirable characteristics. Its advantages over the use of HPLC are increased resolution, increased speed, and increased sensitivity. HPLC typically uses stationary phases consisting of particle sizes from 3 to 5 µm, while UPLC uses columns with particle sizes of 2 µm and less. The Van Deemter plot in Figure 1.3 shows the height equivalent to the theoretical plate versus the linear velocity for different particle sizes. It shows that the higher column efficiencies were obtained when the size of the particle decreased. Therefore, smaller particle sizes allow for increased speed and better resolution between analytes. In addition, lower flow rates are used for UPLC methods. Therefore, there is a reduction in solvent consumption and solvent waste which makes UPLC both cost effective and environmentally friendly [26-28].

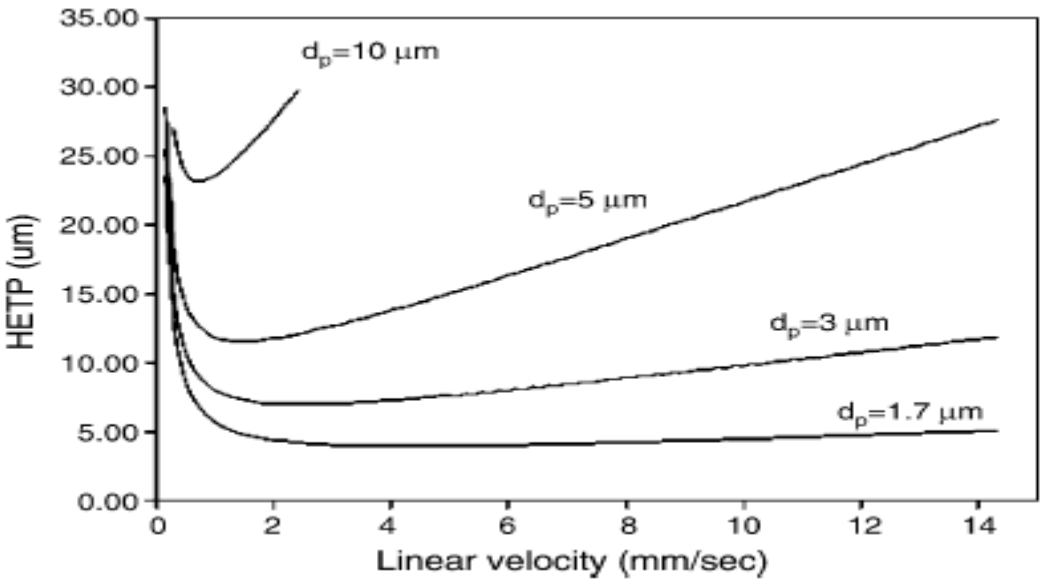

Figure 1.3: Van Deemter Curves for particle sizes, 1.7, 3, 5, and 10 µm, taken from [28].

1.3.5. Validation of analytical methods in support of cleaning validation

An important part of cleaning validation is setting residue limits and then measuring the residues left on the product contacting surfaces after cleaning. Therefore, it is critical that the analytical cleaning method is validated appropriately. There are generally four different types of analytical methods with varying validation requirements which are shown in Table 1.2.

A cleaning method validation is typically carried out using the criteria in International Conference on Harmonization (ICH) guidelines Q2 (R1) for quantitative testing of impurities. For swab sampling, recovery studies may be performed as part of the analytical method validation or they may be performed as separate studies once it is determined that the analytical method can appropriately measure residues in solutions. In addition to the ICH Q2 parameters, swab sample stability as a function of storage conditions (time, temperature, vial for storage, etc.) may be evaluated if there is a significant interval between sampling and analysis [4,6,29].

Table 1.2: Validation of analytical procedures [29].

Characteristic	Type of Analytical Procedure			
		Testing for Impurities		
	Identification	Quantitative	Limit	Assay
Accuracy	-	+	-	+
Precision				
Repeatability	-	+	-	+
Intermediate Precision	-	+[1]	-	+
Specificity[2]	+	+	+	+
Detection Limit	-	-[3]	+	-
Quantitation Limit	-	+	-	-
Linearity	-	+	-	+
Range	-	+	-	+
	- Signifies that this requirement is not normally evaluated + Signifies that this requirement is normally evaluated 1 In case where reproducibility has been performed, intermediate precision in not required 2 Lack of specificity of one analytical procedure could be compensated by other supporting analytical procedure(s) 3 may be needed in some cases			

1.3.5.1. Validation Parameters

1.3.5.1.1 Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present, such as impurities, degradants or the sample matrix [29]. The surface of a production equipment train generally consists of mostly stainless steel (> 95%), but there are critical surfaces, which are made of glass, teflon, and silicone. These critical surfaces may be hard to clean, so it is necessary to sample these areas during the cleaning validation process. With regards to specificity, interferences from the sampling procedure must be taken into account. This should include blank extractions of the swab material, as well as blank swabs from all the sampling surfaces (stainless steel, glass, teflon and silicone). Specificity is demonstrated by lack of interferences or sufficient resolution between the peak of interest and any interfering peaks from the swab or product contact surfaces [4,6,7,29-31].

1.3.5.1.2. Linearity

The linearity of an analytical cleaning procedure is its ability to obtain test results which are directly proportional to the concentration of residue in the sample. A minimum of 5 concentrations is measured across the range of the analytical method. Dilute stock standard preparations of the API residue to be quantitated may be used. The signals are plotted as a function of concentration. A correlation coefficient of ≥ 0.99 is sufficient for residual cleaning analytical methods [4,6,7,29-31].

1.3.5.1.3. Limit of Quantitation (LOQ)

The limit of quantitation of an analytical cleaning procedure is the lowest amount of residue in a sample which can be quantitatively determined with suitable precision and accuracy. For a cleaning method, the LOQ must be at least 10% of the residue limit for the swab sample, so that robustness of the cleaning process can be established [4,6,7,29-31].

1.3.5.1.4. Precision (method and swabs)

The precision of an cleaning analytical method is the closeness of agreement between the results obtained from multiple sampling by applying the analytical method and the sampling methodology under prescribed conditions that may include repeatability, intermediate precision and reproducibility [4,6,7,29-31].

1.3.5.1.5. Accuracy

The accuracy of a cleaning method measures the closeness to the true value. It is measured by recovering the residue from various product contact surfaces such as stainless steel and silicone. The residue is recovered and analysed using the developed sampling methodology and analytical method [4,6,7,29-31].

1.3.5.1.6. Robustness

Robustness is not a validation requirement, however it should be considered during the development of the analytical method. The robustness of an analytical cleaning procedure is a measure of its capability to remain unaffected by small

deliberate variations in methods parameters and provides an indication of its reliability during normal usage. The method parameters that may be altered include column temperature, flow rate, buffer and organic composition of the mobile phase [4,6,7,29-31].

1.3.5.1.7. Swab sample stability

Swab sample stability is not a validation requirement but the stability of the swab samples may be evaluated if there if a significant interval between the swab sampling and the analysis of the swab samples. The swab sample study is conducted under the normal storage conditions, i.e. vial for storage, time and temperature [6,7].

1.4. Sampling methodologies

1.4.1. Introduction

The two most common types of sampling techniques are swabbing and rinsing. These techniques have been addressed by the FDA, and are identified in many publications [1,3-7]. In the 1993 FDA cleaning validation guide, the FDA states that; "the most desirable is the direct method of sampling the surface of the equipment"; swab sampling must therefore be the primary method of sampling in support of cleaning validation [1]. Swab sampling methodologies are a critical aspect in establishing a robust validated cleaning program. They are essential to accurately determine and quantitate API residues remaining on the manufacturing equipment trains. The results of the swab samples that are analysed using the validated analytical cleaning method are compared to the maximum allowable carryover (MAC) limits to determine the robustness and effectiveness of the cleaning program [3-7,32,33].

1.4.2. Swab sampling

Swab sampling involves wiping a surface with a fibrous material saturated with solvent (e.g., water or alcohol) to dissolve residue on the manufacturing equipment. A dimensionally defined surface area and defined technique are required for swab sampling. Swab sampling is adaptable to a wide variety of surfaces (stainless steel, glass, silicone, etc.) and allows for both dissolution and physical removal of residues. Additionally, areas that are hard to clean and which are reasonably accessible can be

evaluated, leading to establishing a level of contamination or residue per given surface area (μg/cm^2). Swab sampling is the sampling technique recommended by the FDA, and it must be supported by recovery studies in which the swabbing technique is demonstrated to remove an acceptable percentage (≥ 50%) of product residue from the manufacturing equipment surfaces [1,3-7].

1.4.3. Swab selection

There is a direct physical interaction between the swab, the solvent, the surface, and the residue to be removed. Therefore, the choice of swab is crucial to the effectiveness of the sampling procedure. The swab material should have an appropriate level of sorptive properties. The material must be sorptive enough to pick up acceptable levels of residue from the surface, but cannot be so sorptive, that it may not release the analyte from the swab for acceptable recoveries. The selection of swab to be used requires an evaluation of the swab properties, such as interferences and shedding properties as the swabbing material may interfere with the analysis of the samples. For example, the adhesive used in swabs has been found to interfere with the analysis of samples [3-7].

Polyester swabs that are specially processed to meet the stringent requirements associated with cleaning validation protocols are often the best choice and are the most commonly swabs used for cleaning validation. An example of a TX761 polyester swab in shown below in Figure 1.4. Polyester swabs offer high absorbency of residues, ultra-low particulates, and minimal extractable interferences. The specially processed polyester material in combination with the analytical cleaning method can provide desirable analytical characteristics that other materials such as cotton, nylon or polyurethane foam cannot meet [6,7,34].

Figure 1.4: A Tex-wipe TX761 polyester swab.

Jenkins et al. [35] screened a number of materials for their suitability as swabbing materials for total organic carbon (TOC) analysis. Some of the materials

tested included 100% polyester, woven cotton, polyurethane foam, a nonwoven polyester/cellulose and quartz wool. The criteria for the materials tested were; minimal interferences from the swab, high recovery rates of the analyte from the selected surfaces and low particle generation from the swab. The 100% polyester materials were the only materials that met all the above criteria. Quartz wool met the criteria for minimal interferences and recovery rates, but its excessive particle generation was deemed unacceptable for surface sampling for TOC analysis. Several materials were tested for non-volatile residues and it can be seen from Figure 1.5 that the 100% polyester swabs feature extremely low non-volatile residues [34,35].

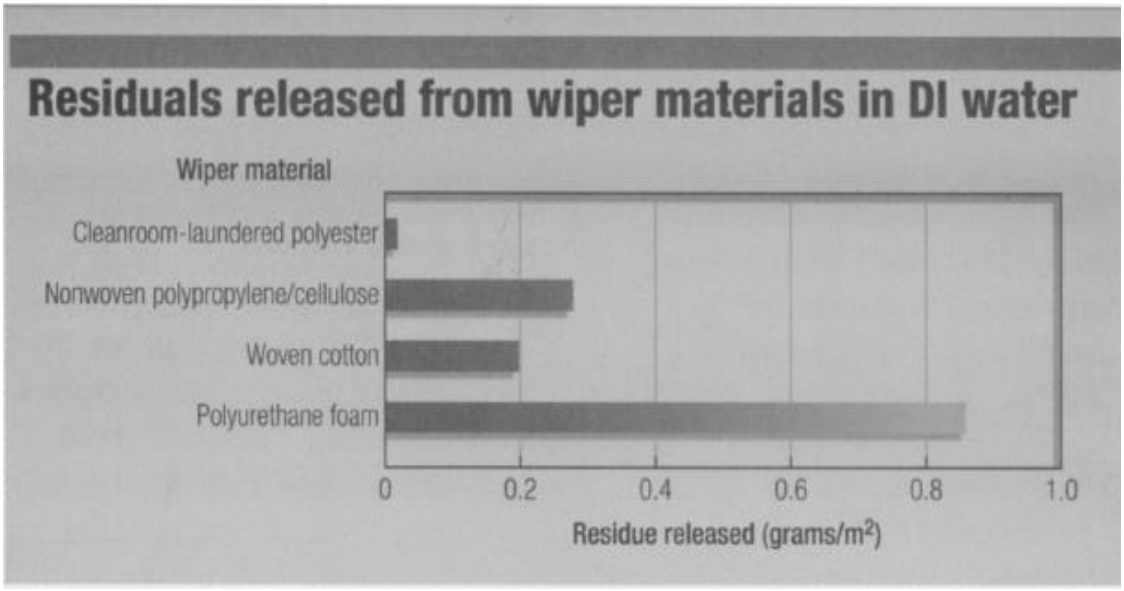

Figure 1.5: The comparison of levels of non-volatile residues from 100% polyester swabs versus other commonly used swab materials reported by Jenkins et al. [7].

1.4.4. Swab sampling recovery studies

Sampling recovery studies are laboratory studies involving product contacting materials (coupons) that are representative of the actual manufacturing equipment surfaces. The coupons may be constructed of stainless steel, glass, teflon (PTFE, Polytetrafluoroethylene), EPDM (ethylene propylene diene monomer) or silicone. Examples of product contacting coupons are shown below in Figure 1.6. A standard solution containing the API residue to be quantitated is applied onto the coupon. The coupon is swabbed with the defined swab and extraction solvent to extract the residue, and the residue is analysed by the chosen analytical technique [3-7]. These studies demonstrate that if a residue is present on the manufacturing equipment surfaces, it can

be quantitatively recovered by the analytical method in combination with the sampling procedure. The objective is to establish a reproducible and acceptable level of recovery from the equipment surfaces [1,3-7].

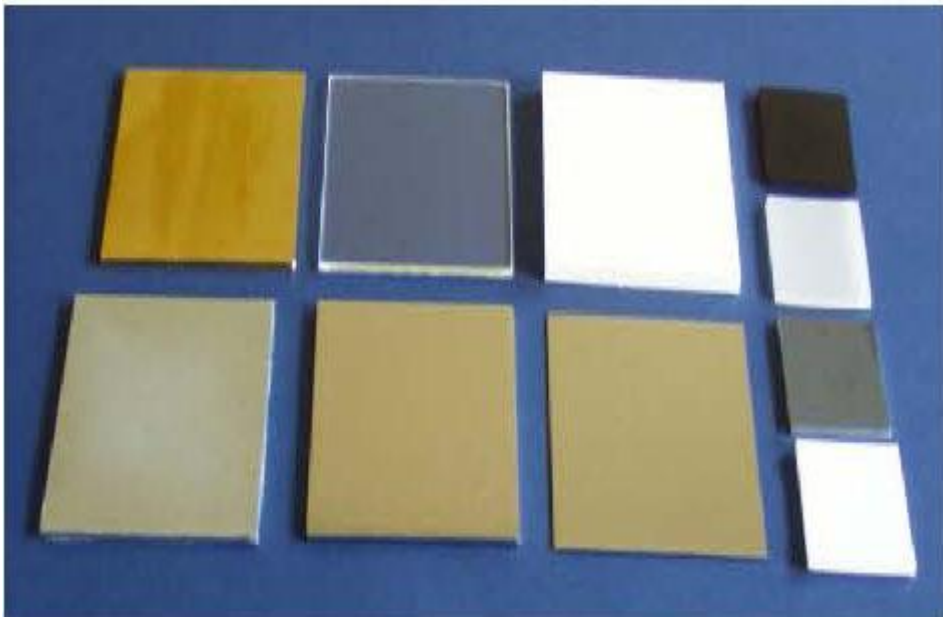

Figure 1.6. Examples of product contacting surface coupons.

Different product contacting materials have different recovery performances. Stainless steel surfaces differ in degree of polish or surface roughness. Polymeric surfaces such as EPDM may have different porosities. The same residue may be highly recovered (e.g., ≥ 90%) from stainless steel but poorly recovered (≤ 20%) from EPDM (ethylene propylene diene monomer), as further discussed in Chapter 3. The selection of swab material and solvent used for swabbing purposes are important factors, since they can have an impact on recovery and influence extractables or interferences. Recovery of residues from the equipment surfaces may also depend on the size and shape of the swab head, as well as the properties (such as flexibility and length) of the swab handle. Another factor which may affect the recoveries is the pressure of the swab head that is applied to the surface during sampling. It is important to apply enough pressure to make the swab head flat, but not so much that movement cannot be controlled, as shown in Figure 1.7 [6,7,32,34]. The steps for performing swab recovery studies are outlined in Section 1.4.4.1.

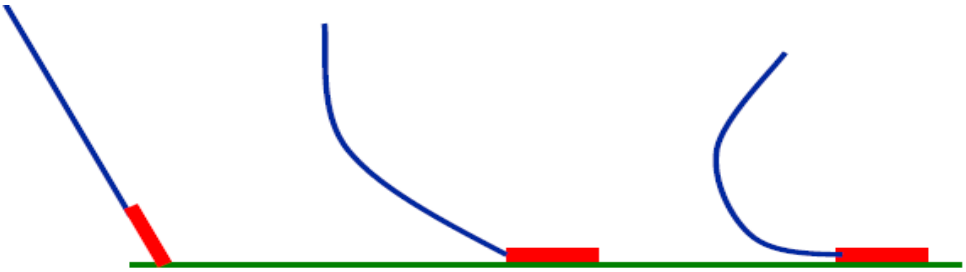

Figure 1.7: Pressure effects of the swab head against the surface.

1.4.4.1. Swab recovery studies, see Figure 1.8.

- A known amount of a standard solution containing the drug substance residue is applied (spiked) onto a defined area of product contacting material. The product contacting material should be equivalent to the actual manufacturing equipment materials to be swabbed in cleaning validation.
- The defined area is carefully swabbed using the defined solvent and technique to transfer the residue from the product contacting material to the swab.
- The swab is extracted with solvent to transfer the drug substance residue from the swab for quantitative analysis.
- The percent recovery of the drug substance residue from the product contacting material is calculated as below;

$$\% \text{ Recovered} = \frac{\text{Actual amount recovered by swabbing} \times 100\%}{\text{Theoretical amount spiked onto the surface}}$$

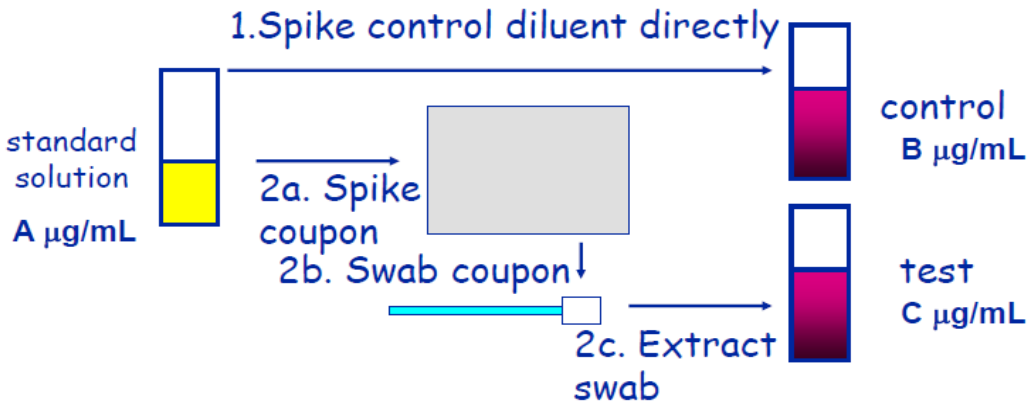

Figure 1.8: Recovery schematic for swab sampling.

1.4.4.2. Correction Factor

A recovery of ≥ 50% from the swab sampling accuracy studies is acceptable if the results are reproducible and a correction factor is applied to the cleaning process. A correction factor is generally applied to the results obtained from the swab sampling studies. This correction factor is used to calculate the 'true' amount of residue that remains on the manufacturing equipment surfaces after the cleaning process has taken place. An example calculation is shown below [3-7].

$$M = \frac{Mres}{C}$$

Where,

M = True value for the amount of residue remaining on the manufacturing equipment after cleaning

Mres = The measured amount of residue on the manufacturing equipment surface, µg/cm^2

C = Correction factor, e.g. for 75%, 75/100 = 0.75

1.4.5. Swab sampling procedure

The swab used for the sampling procedure is typically pre-wetted with water or another appropriate solvent. This removes the drug substance residues from the product contacting surface. The sides of the swab are pressed against the inside of the sampling vial after pre-wetting prior to sampling to remove the excess solvent. It is important to remove the excess solvent as this can serve as a source of residues leading to variable results. Excess solvents may leave extractable substances of interest on the surface of the swab which can reduce the percent recovery or indicate a false positive result for a successful cleaning validation. Also, it is important to ensure that there is not a time delay between pre-wetting the swab and sampling the product-contact surface. A time delay may cause the solvent to evaporate. Therefore, there would be insufficient alcohol on the swab to recover the residue which would potentially cause a false negative result [3-7].

The product contacting surface is sampled with the defined swabbing procedure. The swabs are placed in the sample vial with the extraction solvent. The

extraction solvent releases the drug substance residue from the swab. The swab sample may need to be filtered or sonicated depending on the sample type. These steps may impact the analytical results if leaching occurs from the various materials involved. Therefore, the use of the highest quality suitably engineered swabs can provide assurance that any extraneous contamination observed in the analytical analysis does not originate from the swab. In addition, blank extractions of the swab and blank extractions of the product contacting surfaces used for the swab sampling studies are run to investigate for interferences in the chromatographic analysis of the sample [3-7, 31].

1.5. Thesis Objectives

In pharmaceutical industries, it is crucial to prove that the manufacturing equipment is consistently cleaned from API residues, to prevent possible contamination and cross-contamination of product batched. Therefore, cleaning validation is carried out. The analytical methods used to the detect trace residues are an important aspect of a cleaning validation. With ever increasing regulatory requirements, it is crucial to employ specific, sensitive analytical methods that are capable of quantitating trace level residues, while still retaining the repeatability and robustness necessary for QC laboratories. Swab sampling studies are required to demonstrate that a residue, if present on equipment surfaces, can be adequately quantitated by the analytical cleaning method in combination with the swab sampling procedure. The analytical cleaning method used in combination with the swab sampling procedure should be challenged to show that sufficient amounts of API residues can be effectively removed from the product contacting manufacturing equipment surfaces. An effective swab sampling procedure is an integral, if not dominating part of the cleaning validation method. UPLC is a powerful analytical technique that is widely used for the determination and quantitation of API residues in support of cleaning validation. It advantages over HPLC include shorter analysis times, a decrease in solvent consumption and greater sensitivity.

The aim of this thesis was to develop and validate a specific, sensitive and robust UPLC analytical cleaning method for the simultaneous determination and quantitation of a range of API residues found in ophthalmic solutions. The development of the UPLC method involves the use of columns packed with sub-2 µm

particles for increased sensitivity, speed and better resolution. A Design of Experiments (DOE) approach utilising Dry Lab and Minitab software was adopted to facilitate rapid method development and ensure the desired resolution (≥ 2.0) between each peak pair. In addition, the related swab sampling was also developed and validated using various swabs, solvents and product contacting surfaces.

In Chapter 2, the aim was to develop a relatively fast and robust UPLC analytical cleaning method to enable the simultaneous determination and quantitation of up to 10 different API residues that are found in various ophthalmic solutions. The APIs used for this research included brimonidine tartrate, timolol maleate, ketorolac tromethamine, flurbiprofen, prednisolone acetate, prednisolone, dexamethasone, fluorometholone and ofloxacin. These drug substances are in a wide range of ophthalmic solutions, and some of the ophthalmic solutions may contain the listed drug substances in different combinations. Hence, the analytical method was designed to have a broad applicability.

Chapter 3 details the swab sampling methodology that was carried out in combination with the developed UPLC method to show that the selected API residues can be sufficiently removed from the manufacturing equipment surfaces. A DOE approach was used to choose the most appropriate swab and solvent for adequately removing the residues from the surfaces. An important aspect was to investigate any interferences originating from the swab and the product contacting surfaces.

Chapter 4 summarizes the results obtained in this work and details any potential for future work.

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Chapter 2

Development of an Analytical Method for the Determination of API Residues in Support of Cleaning Validation by Ultra Performance Liquid Chromatography

2.1. Introduction

The aim of the developed analytical method described in this Chapter was to quantitate the residues of a range of drug substances that included brimonidine tartrate, timolol maleate, ketorolac tromethamine, flurbiprofen, prednisolone acetate, prednisolone, dexamethasone, fluorometholone and ofloxacin in support of cleaning validation. These drug substances are in a wide range of ophthalmic solutions, and some of the ophthalmic solutions may contain the listed drug substances in different combinations. Hence, the analytical method was designed to have a broad applicability.

Depending on the class of drug substance, some of these compounds may be grouped together for analysis. For example, the corticosteroids; prednisolone acetate, prednisolone, dexamethasone, and fluorometholone have been determined using isocratic or gradient conditions in different combinations [1-4]. The simultaneous detection of the β-adrenergic receptor blockers, timolol maleate and levobunolol hydrochloride has also been reported [5-6] along with several analysis in the literature of them being quantitated separately [7-9]. There are many analytical methods described for the simultaneous quantitation of fluoroquinolones, one of which is ofloxacin [10-12]. There are also analysis methods described for the detection of brimonidine tartrate [13-15], flurbiprofen [16-18] and ketorolac tromethamine [19-21] quantitated with compounds of the same drug class.

Gradient elution is often used for the separation of analytes in a sample with a wide range of molecular weights and/or polarities. For these types of samples, gradients are useful to screen for appropriate conditions and provide an adequate separation within a reasonable run time [22]. There are numerous gradient analysis analytical methods described in the literature for the separation and determination of brimonidine tartrate, timolol maleate, levobunolol hydrochloride, ketorolac tromethamine, flurbiprofen, prednisolone acetate, prednisolone, dexamethasone, fluorometholone and ofloxacin, as mentioned previously.

This Chapter describes a robust UPLC liquid chromatography analytical method for the separation and quantitation of up to 10 different drug substances, which will cover a broad range of ophthalmic solutions. The separation is for a mixture of acidic, basic and neutral drug substances which is not typical of most pharmaceutical applications as it consists of drug substances with significantly different $pK_{a,}$ Log P

and Log D values. A Design of Experiments (DOE) approach utilising Dry Lab and Minitab software was adopted to facilitate rapid method development and ensure the desired resolution ($\geq$ 2.0) between each peak pair.

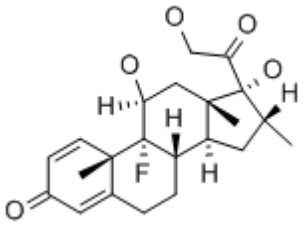
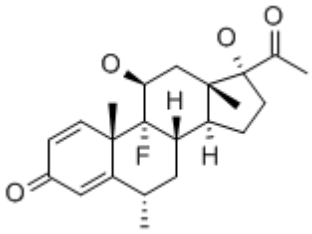
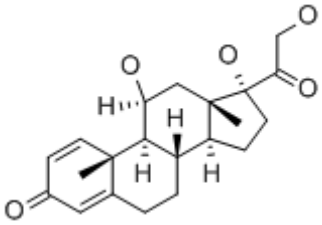
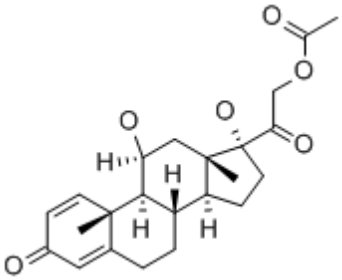
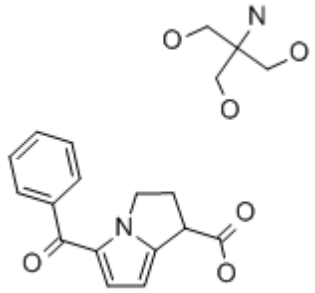
2.2. Experimental

2.2.1. Reagents and materials

Reference materials, dexamethasone (DEX, 99.7%), prednisolone acetate (PAC, 99.4%), and fluorometholone (FML, 99.7%) were purchased from Sanofi Aventis (Paris, France), levobunolol hydrochloride (BUN, 99.7%) and brimonidine tartrate (BMT, 99.9%) from Piramal Healthcare (Andhra Pradesh, India), timolol maleate (TIM, 99.9%) from PCAS Finland OY (Turka, Finland), ketorolac tromethamine (KET, 98.7%) from Recordati (Milan, Italy), ofloxacin (OFL, 99.7%) from Chemo (Madrid, Spain), prednisolone (POH, 98.5%) from Sigma Aldrich (St. Louis, Missouri, US), and flurbiprofen (FBP, 99.6%) from Aesica Pharmaceuticals Limited (Cramlington, UK). The structure, abbreviation, and pharmaceutical class of each of the drug substances in this mixture and their $pK_{a,}$ Log P and Log D values are shown in Table 2.1.

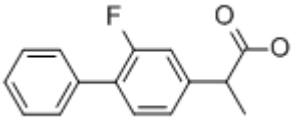
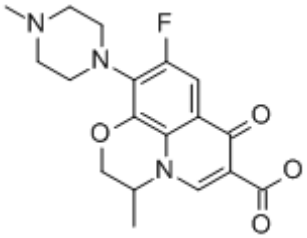
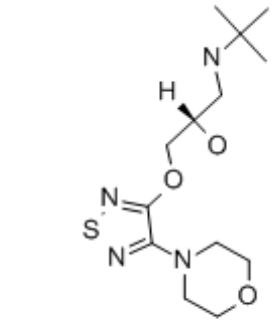
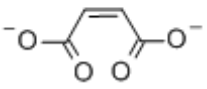
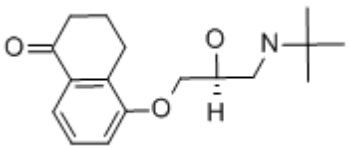
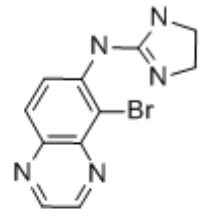
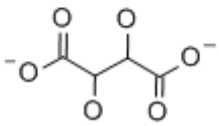
Analytical grade ammonium acetate was purchased from Merck (Darmstadt, Germany) and ammonium hydroxide solution (34%) from Lennox (Cambridge, UK). Phosphoric acid (85%) and acetic acid were purchased from Sigma Aldrich (Arklow, Ireland). HPLC grade methanol (MeOH) and acetonitrile (ACN) were purchased from Labscan (Dublin, Ireland). All chemicals were used as received, without any further purification. Deionised water was obtained from a Millipore Milli-Q water purification unit (Millipore, Bedford, MA, USA).

Table 2.1: The chemical structure, abbreviation, pharmaceutical class, pK_a value, Log P, and Log D values of each drug substance.

Structure[1]	Pharmacologic class	pK_a values	Log P	Log D
 Dexamethasone (DEX)	Synthetic Corticosteroid	12.42 [23]	1.83 [24]	1.83 [24]
 Fluorometholone (FML)	Synthetic Corticosteroid	12.64 [23]	2.00 [24]	2.00 [24]
 Prednisolone (POH)	Synthetic Corticosteroid	12.58 [23]	1.62 [24]	1.62 [24]
 Prednisolone acetate (PAC)	Synthetic Corticosteroid	12.61 [23]	2.40 [24]	2.40 [24]
 Ketorolac tromethamine (KET)	Non-steroidal anti-inflammatory drug	8.95[23] 3.84 [23] -7.8 [23]	2.28 [23]	0.84 (pH 5.5) [25] -0.95 (pH 7.4) [25]

[1] These are the counter ions of the drug substances, i.e tromethamine, hydrochloride, tartrate, and maleate.

Table 2.1 (continued)

Structure[1]	Pharmacologic class	pK_a values	Log P	Log D
 Flurbiprofen (FBP)	Non-steroidal anti-inflammatory ophthalmic	4.42 [23]	4.16 [24]	2.74 (pH 5.5) [25] 0.98 (pH 7.4) [25]
 Ofloxacin (OFL)	Fluoroquinolone	5.45 [23] 6.2 [23]	-0.39 [24]	-1.17 (pH 5.5) [25] -1.41 (pH 7.4) [25]
  Timolol maleate (TIM)	Beta-adrenergic blocking agent	9.76 [23] 14.08 [23] -0.32 [23] 5.91 [26] 3.05 [26]	1.83 [27]	-2.34 (pH 5.5) [28] -1.20 (pH 7.4) [28]
 • HCL Levobunolol hydrochloride (BUN)	Beta-adrenergic blocking agent	9.66 [26]	2.40 [27]	0.84 (pH 5.5) [28] -0.95 (pH 7.4) [28]
  Brimonidine tartrate (BMT)	Alpha-adrenergic receptor agonist	8.32 [26] 4.49 [26] 2.72 [26] 13.03 [26] 14.44 [26]	1.37 [26]	0.84 (pH 5.5) [28] -0.95 (pH 7.4) [28]

2.2.2. Instrumentation

The chromatographic separation was performed using a Waters Acquity H-Class Ultra Performance Liquid Chromatography (UPLC) system, which comprised of a vacuum degasser, a quaternary solvent manager, a sample manager, an auto sampler with temperature control, a column pre-heater and a photodiode array (PDA) detector with ultraviolet (UV) channels. The columns chosen were a Waters Acquity High Strength Silica (HSS) C18 (1.8 µm, 2.1 mm x 100 mm) column (Waters, Santry, Dublin, Ireland) (*Column A*) and a Waters Acquity Charged Surface Hybrid (CSH) C18 (1.7 µm, 2.1 mm x 100 mm) column (Waters, Santry, Dublin, Ireland) (*Column B*). Features of *Column A* and *Column B* are shown below in Table 2.2.

Table 2.2: Features of *Column A* and *Column B.*

Features	***Column A* (HSS)**	***Column B* (CSH)**
Endcapping	√	√
Ligand density	3.2 µmol/m^2	2.3 µmol/m^2
Carbon load	15%	15%
Surface area	230 m^2	185 m^2
Pore Size	100 Å	135 Å

A Branson sonication bath model 8510 (Branson Ultrasonics Corporation, Danbury, USA) was used for dissolving the standard reference materials and the balance used was a Mettler Toledo model XS205 (Manson Technology, Dublin, Ireland). The pH meter used (Orion 3 Star) was purchased from Thermo Electron Corporation (Thermo, PA, USA).

Mobile phase pH, gradient times and solvent strength optimisation was performed using Dry Lab 2000 Plus Chromatography optimisation software (Molnar-Institute Berlin, Germany) and Minitab software was used to predict optimal mobile phase pH and buffer concentration of the mobile phase.

2.2.3. Method

The chromatographic analysis on *Column A* was performed using 50 mmol L^{-1} sodium phosphate buffer (pH 2.5 or 6.0, adjusted with phosphoric acid) or 50 mmol L^{-1} sodium acetate buffer (pH 4.6, adjusted with acetic acid) as mobile phase A and ACN as mobile phase B. The gradient programme used was a linear gradient from 0-20 minutes (15-85% B).

The chromatographic analysis on *Column B* was performed using 40 mmol L^{-1} ammonium acetate buffer (pH 9.85, adjusted with ammonium hydroxide solution) as mobile phase A and 40 mmol L^{-1} ammonium acetate buffer (pH 9.85)/ACN (50/50, v/v) as mobile phase B. The gradient programme was a linear gradient from 0-10 minutes (30-100% B).

Both *Column A* and *Column B* had a 3 minute column re-equilibration time[2]. The flow was set at 0.4 mL min^{-1} and the column temperature was maintained at 30 °C for both columns. Additionally, the injection volume was 5 µL, with detection by UV at 254 nm for BMT, FBP, PAC, POH, BUN, DEX and FML, and 295 nm for OFL, KET and TIM for both *Column A* and *Column B.*

2.2.4. Standard preparation

Stock standard solutions of BMT, KET, FBP, TIM, and BUN were prepared in deionised water; PAC, POH, OFL and DEX in MeOH:water (70:30, v/v); and FML in methanol, each at a concentration of 500 µg/mL. The standards were stored in a refrigerator at 4 °C, for two weeks with no significant loss of API. Working standards (5 µg/mL) were prepared fresh from the stock solutions before analysis in ACN:water (5:95, v/v).

[2] 3 minute column equilibration time; The column was washed for 3 minutes at the end of each chromatographic run with the same mobile phase composition as the start of the gradient. This step was employed to re-equilibrate the column back to the original conditions before each injection.

2.3. Results and discussion

2.3.1. Optimisation of chromatographic conditions

Two different columns were used in optimizing chromatographic conditions; *Column A* and *Column B. Column A*, a general-purpose silica-based C18 column, was primarily used to scout for conditions. Mobile phase pH was investigated in optimizing the chromatographic conditions for *Column A*, and mobile phase pH, buffer concentration, column temperature, and gradient optimisation were investigated for *Column B*. The desired criteria for an optimal separation were a resolution of ≥ 2.0 between every peak pair and tailing of ≤ 2.0 for every drug substance.

2.3.1.1. Optimisation of mobile phase pH on *Column A*

One of the most widely used columns is an octadecyl carbon chain (C18)-bonded silica (USP classification L1) which was the column type chosen for this separation. Initially, the separation was developed using *Column A* which is a general-purpose silica-based C18 column with applicability to a broad range of drug substance classes (as stated by the manufacturer). Another advantage of the high strength silica-based material is that it can withstand high UPLC pressures (15,000 psi), and has a mobile phase pH range of between 1 and 8 (manufacturers recommendation).

The impact of mobile phase pH on the retention of brimonidine tartrate, timolol maleate, ketorolac tromethamine, flurbiprofen, prednisolone acetate, prednisolone, dexamethasone, fluorometholone and ofloxacin were investigated on *Column A* and the results are presented below. For these experiments, a scouting gradient of 15-85% B over 20 minutes was employed to ensure elution of all drug substances.

2.3.1.1.1. Impact of pH on retention

The effect of mobile phase pH on the retention of the ten drug substances was studied in the range of 2.5 to 6.0, as shown in Figure 2.1. The drug substances fall into different groups. For one group (POH, DEX, PAC, and FML), the retention was unaffected by pH which is consistent with their lack of ionisable groups. It is also interesting to note that at all three pH values (pH 2.5, 4.6 and 6.0), the BMT peak exhibited very poor retention. The basic functional groups of BMT were ionised in this acidic pH range which contributed to its lack of retention on a nonpolar stationary

phase as shown in Table 2.3. Even though the pH range was relatively narrow, the influence of the mobile phase pH on the retention of KET and FBP was significant. As can be seen in Table 2.3, an increase in mobile phase pH led to a decrease in retention of FBP and KET, as they became more ionised and less retained.

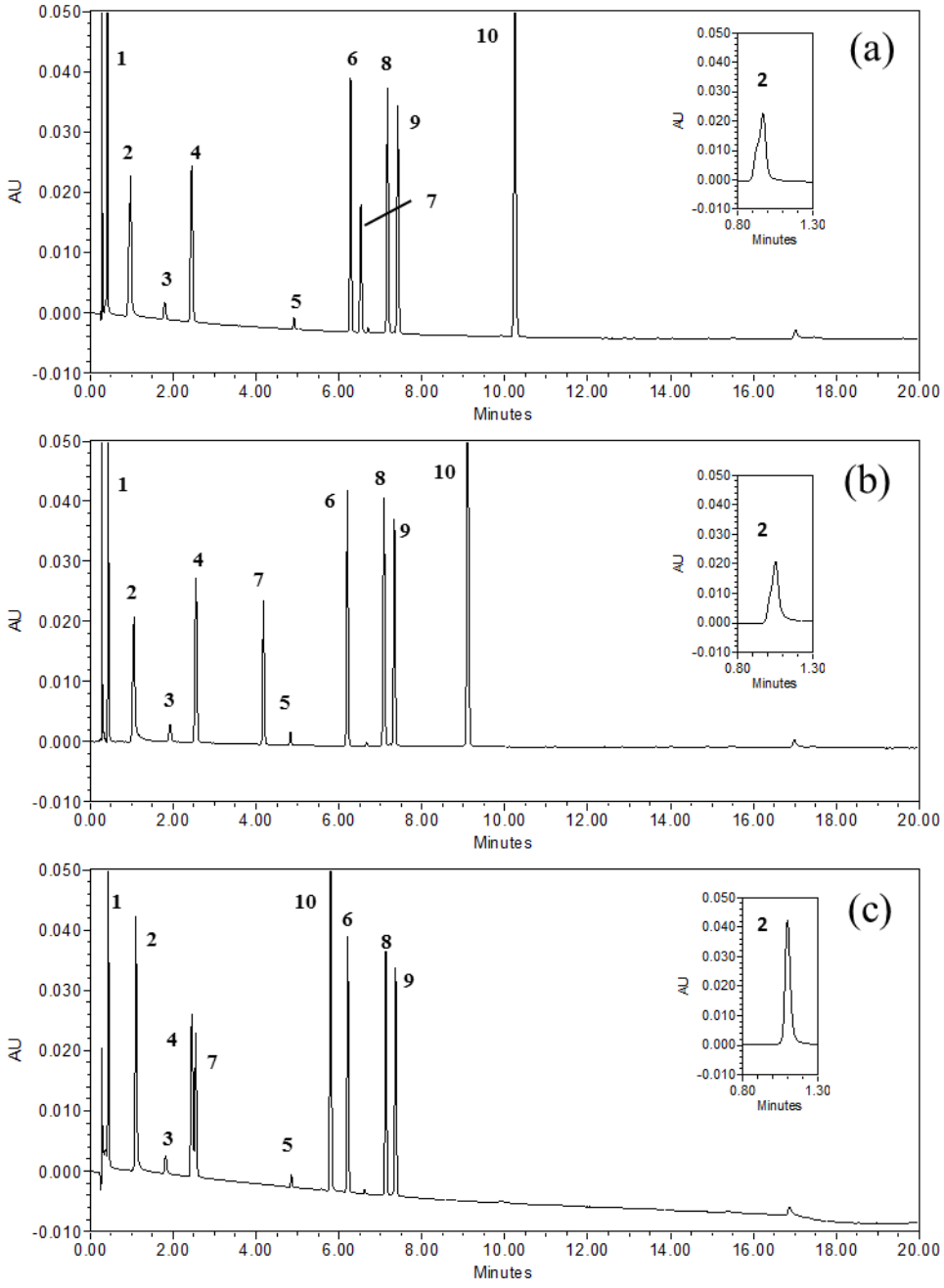


Figure 2.1: The effect of mobile phase pH on the separation of the 10 drug substances at (a) pH 2.5, (b) pH 4.6, and (c) pH 6.0. *Column A*. Mobile phase A: 50 mmol L^{-1} sodium phosphate buffer (pH 2.5), mobile phase B: 100% ACN. Gradient program: 0-20 minutes (15-85% B). Flow rate: 0.4 mL min^{-1}. Column temperature: 30 °C. Injection volume: 5 µL. Detection: 254 nm. Peaks: (**1**) BMT, (**2**) OFL, (**3**) TIM, (**4**) BUN, (**5**) POH, (**6**) DEX, (**7**) KET, (**8**) PAC, (**9**) FML, and (**10**) FBP.

Table 2.3: The effect of acidic mobile phase pH on the retention of BMT, FBP and KET.

Drug Substance	pK_a Value	Retention Time (mins) pH 2.5	Retention Time (mins) pH 4.6	Retention Time (mins) pH 6.0
BMT	8.32 [26]	0.4	0.4	0.4
FBP	4.42 [26]	10.2	9.1	5.8
KET	3.84 [26]	6.5	4.2	2.5

2.3.1.1.2. Impact of pH on resolution

At pH 2.5 and 4.6, the KET peak was significantly resolved from all other drug substances, but at pH 6.0 the KET peak did not achieve baseline resolution[3] from the BUN as shown is Table 2.4. All other peak pairs met the resolution criterion of ≥ 2.0 for all the pH variances. The resolution data between adjacent pairs for all the drug substances in the chromatographic run at pH 2.5, 4.6 and 6.0 are shown below in Table 2.4.

Table 2.4: The effect of acidic mobile phase pH on the resolution between adjacent peak pairs.

Drug Substance/Peak number	R_S pH 2.5	R_S pH 4.6	R_S pH 6.0
BMT (1)/OFL (2)	6.8	8.3	14.4
OFL (2)/TIM (3)	6.9	7.9	8.9
TIM (3)/BUN (4)	6.6	5.6	6.6
BUN (4)/POH (5)	31.2	N/A	N/A
POH (5)/DEX (6)	21.5	21.4	N/A
DEX (6)/KET (7)	3.3	N/A	N/A
KET (7)/PAC (8)	8.4	N/A	N/A
PAC (8)/FML (9)	3.3	3.4	3.2
FML (9)/FBP (10)	33.2	21.7	N/A
BUN (4)/KET (7)	N/A	20.0	1.1
KET (7)/ POH (5)	N/A	10.0	34.6
POH (5)/FBP (10)	N/A	N/A	15.1
DEX (6)/PAC (8)	N/A	12.5	13.1

[3] Resolution (R_S): The resolution is the separation of two components in a mixture, calculated by:

$R_S = 1.18(t_{R2} - t_{R1})/(W_{1,h/2} + W_{2/h/2})$

Where, t_{R2} and t_{R1} are the retention times of the two components; and W_2 and W_1 are the corresponding widths at the bases of the peaks obtained by extrapolating the relatively straight sides of the peaks to the baseline.

2.3.1.1.3. Impact of pH on peak shape

At pH 2.5, 4.6 and 6.0, the tailing[4] of OFL was 0.91, 1.21 and 1.26, respectively, which meets the desired criterion of ≤ 2.0 for tailing, however a tailing value of 0.91 for pH 2.5 indicates fronting of the OFL peak. Additionally, the OFL peak shape is not acceptable at pH 2.5 and 4.6 as shown from the inserts in Figure 2.1.

2.3.1.2. Optimisation of mobile phase pH on *Column B*

The issues with *Column A* were (a) poor retention of BMT, (b) poor peak shape for OFL and (c) poor resolution of BUN/KET. It is interesting to note that BMT, BUN and OFL are basic drug substances under these experimental conditions. Hence, *Column B* was investigated as an alternative stationary phase relative to *Column A*. The best overall separation on *Column A* was achieved at pH 6.0, with respect to the shape of the OFL peak, as shown in Figure 2.1 (c). *Column B* is also a silica based C18 column and it's features include;

- a charged surface hybrid particle platform
- a controlled low-level surface charge to provide enhanced selectivity and peak shape for basic compounds (BMT, BUN, TIM, OFL) at low and high pH
- an expanded mobile phase pH of 1-11

Surface charges have a major impact on the behaviour of ionized analytes. According to the vendor, a new surface modification process was developed that allows the introduction of a reproducible, low level positive charge in acidic mobile phases. In basic mobile phases, ionization of silanol groups creates a negative charge [29]. The CSH technology process is shown in Figure 2.2.

[4] Symmetry factor $(A_S)^2$: The symmetry factor (also known as the tailing factor) is calculated by:

$A_S = W_{0.05}/2f$

Where, $W_{0.05}$ is the width of the peak at 5% height and f is the distance from the peak maximum to the leading edge of the peak, the distance being measured at a point 5% of the peak height from the baseline.

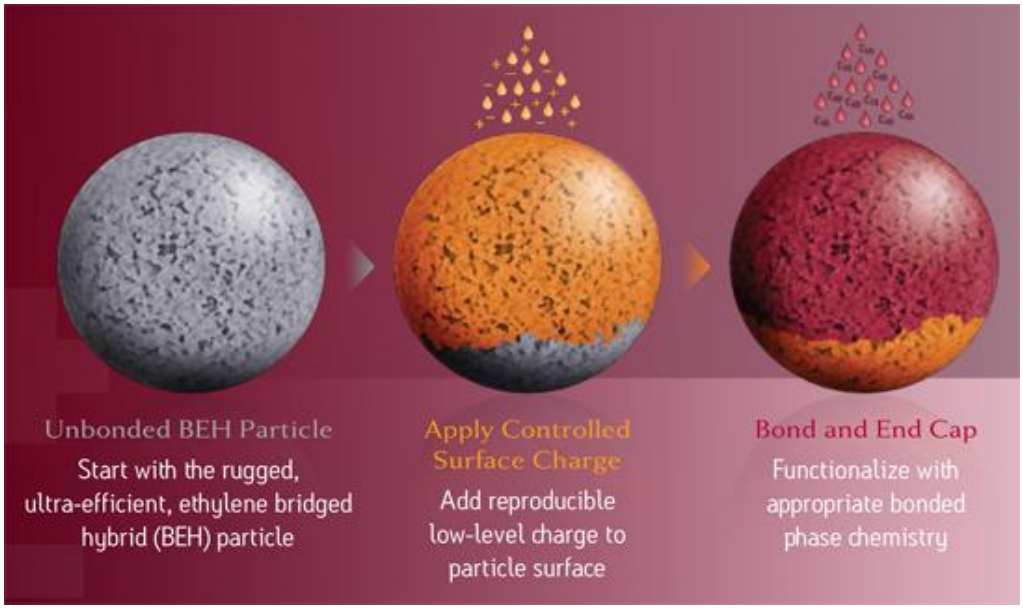


Figure 2.2: CSH Technology Process.

If low mobile phase pH conditions do not aid in the retention of a basic compound (BMT) as shown in Figure 2.1, then a mobile phase with a pH of greater than 8 must generally be used to supress the charge on the basic compound [30]. Firstly, *Column B* was compared to *Column A* using the optimal conditions achieved using *Column A*, which were shown to be a mobile phase of pH 6.0 (Figure 2.1(c)). The comparison of *Column A* versus *Column B* is shown in Figure 2.3.

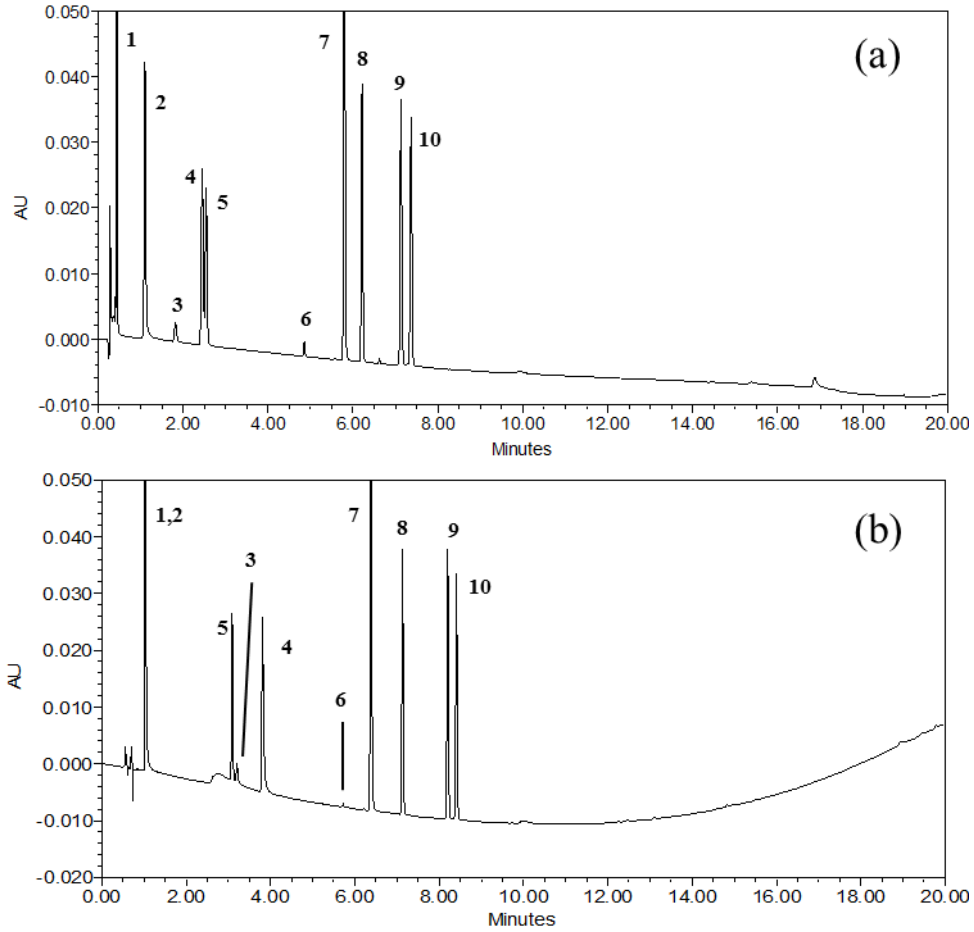


Figure 2.3: A comparison of *Column A* (a) versus *Column B* (b) using mobile phase pH 6.0 on the separation of the 10 drug substances. *Column A/Column B*. Mobile phase A: 50 mmol L^{-1} sodium phosphate buffer (pH 6.0), mobile phase B: 100% ACN. Gradient program: 0-20 minutes (15-85% B). Flow rate: 0.4 mL min^{-1}. Column temperature: 30 °C. Injection volume: 5 µL. Detection: 254 nm. Peaks: (**1**) BMT, (**2**) OFL, (**3**) TIM, (**4**) BUN, (**5**) POH, (**6**) DEX, (**7**) KET, (**8**) PAC, (**9**) FML, and (**10**) FBP.

2.3.1.2.1. Impact of pH on retention

As shown from Figure 2.1, BMT exhibited poor retention with pH 6.0 mobile phase using *Column A*. The retention of BMT improved using *Column B*, however BMT/OFL were now shown to be co-eluted (Figure 2.3 (b)). In order to suppress the charge on BMT which is a basic drug substance, a high pH range was investigated in the approximate range for ammonia buffers of between pH 8.7 to 10.0 using ACN as mobile phase B as shown in Figure 2.4.

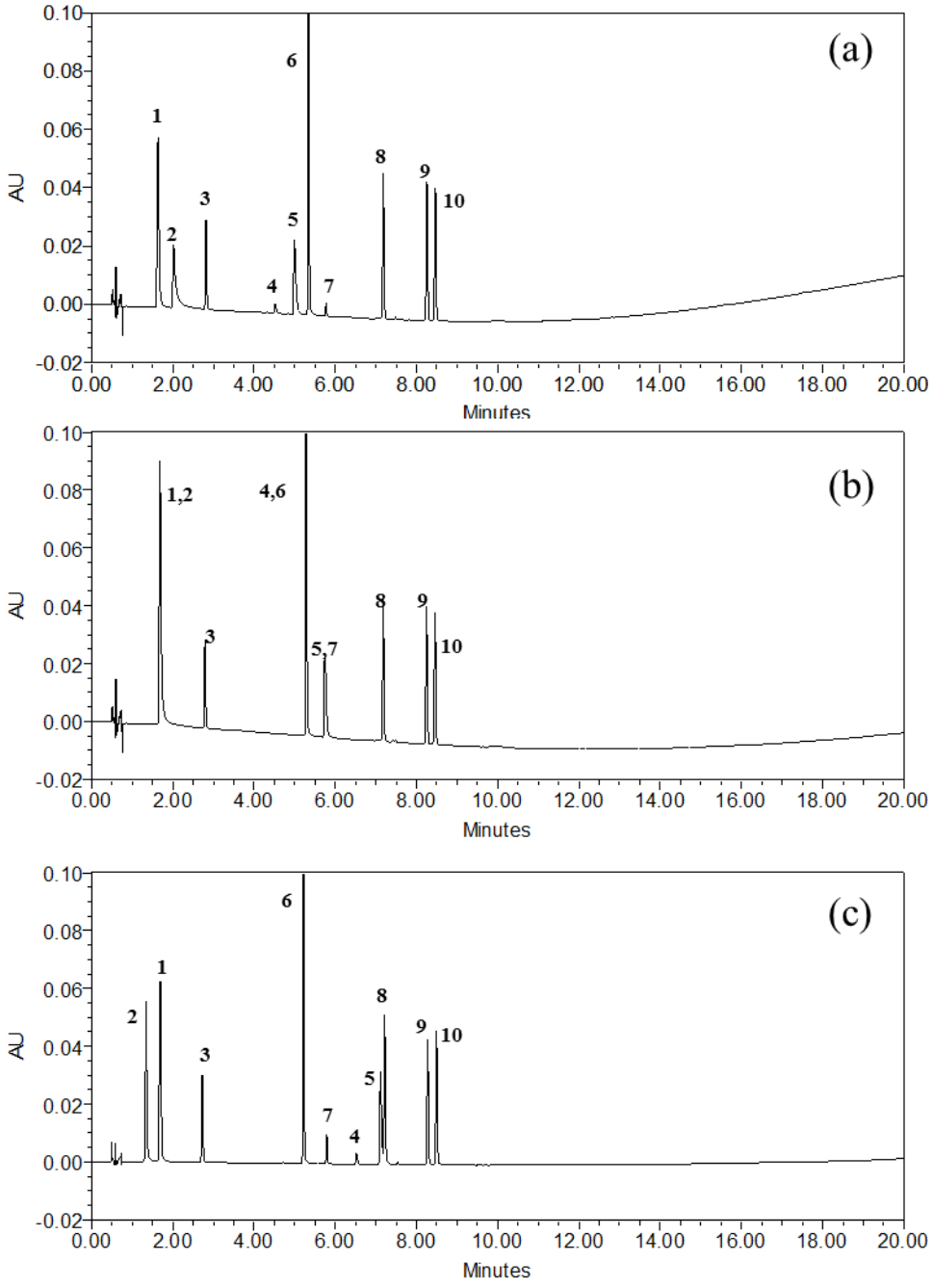


Figure 2.4: The effect of mobile phase pH on the separation of the 10 drug substances at (a) pH 8.7, (b) pH 9.2, and (c) pH 10.0. *Column B.* Mobile phase A: 25 mmol L^{-1} ammonium acetate buffer, mobile phase B: 100% ACN. Gradient program: 0-20 minutes (15-85% B). Flow rate: 0.4 mL min^{-1}. Column temperature: 30 °C. Injection volume: 5 µL. Detection: 254 nm. Peaks: (**1**) BMT, (**2**) OFL, (**3**) KET, (**4**) TIM, (**5**) BUN, (**6**) FBP, (**7**) POH, (**8**) DEX, (**9**) PAC, and (**10**) FML.

2.3.1.2.2 Impact of pH 8.7, 9.2 and 10.0 on retention

At the three high pH values (pH 8.7, 9.2 and 10.0), the BMT peak exhibited better retention relative to the more acidic pH range (Figure 2.1). The change in

selectivity of OFL and BMT using the higher pH range is evident from Table 2.5. The elution order of BMT/OFL reverses from pH 8.7 to pH 10.0.

2.3.1.2.3. Impact of pH on tailing and resolution

Even though the range was narrow, the influence of pH on the resolution between some of the drug substance peak pairs was significant. For instance, there is a significant difference in the tailing of OFL at the high pH range as shown in below in Table 2.5. At pH 8.7, the tailing of OFL is 3.6, and at pH 9.2 BMT/OFL are co-eluting. Additionally to BMT/OFL co-eluting at pH 9.2, this pH range also caused co-elution between TIM/FBP and POH/BUN. At pH 8.7 and 10.0, the resolution between all drug substance peak pairs was ≥ 2.0, except for BUN at pH 10.0 (R_S: 1.5).

Table 2.5: The effect of basic mobile phase pH on the retention/tailing of OFL and BMT.

Parameter	**pH 8.7**	**pH 9.2**	**pH 10.0**
Retention Time OFL	2.0	1.7	1.4
Retention Time BMT	1.6	1.7	1.7
Tailing OFL	3.6	BMT/OFL are co-eluting	1.2

Of all the high pH mobile phases, the best separation was achieved using a mobile phase of pH 10.0. The resolution between all drug substances was ≥ 2.0, except for BUN/DEX (R_S: 1.5), and the tailing of all ten drug substances was ≤ 2.0. BUN/DEX achieved baseline resolution of 1.5, but did not achieve the desired resolution of ≥ 2.0. This drug substance peak pair was therefore the focus of further method optimisation, as described hereafter/in Section 2.3.1.4. Additionally, resolution was not achieved between the impurities of PAC with FML and DEX as described below in Section 2.3.1.2.4.

2.3.1.2.4. Impact of pH on resolution of PAC and it's impurities

Prednisolone acetate contains the impurities, prednisolone 17-acetate (P17-AC) and hydrocortisone acetate (HAC), in addition to POH as shown in Figure 2.5. Baseline resolution (≥ 1.5) was achieved between all ten drug substances at pH 10.0. However, at this pH, there was co-elution between DEX/P17-AC and FML/HAC, as shown in Figure 2.6. The impurities of PAC, P17-AC and HAC are structurally very

similar to DEX and FML (Figure 2.7), which makes the separation particularly challenging. An overlay of a PAC standard containing POH, P17-AC and HAC, and a standard containing all ten drug substances at pH 10.0 is shown in Figure 2.6.

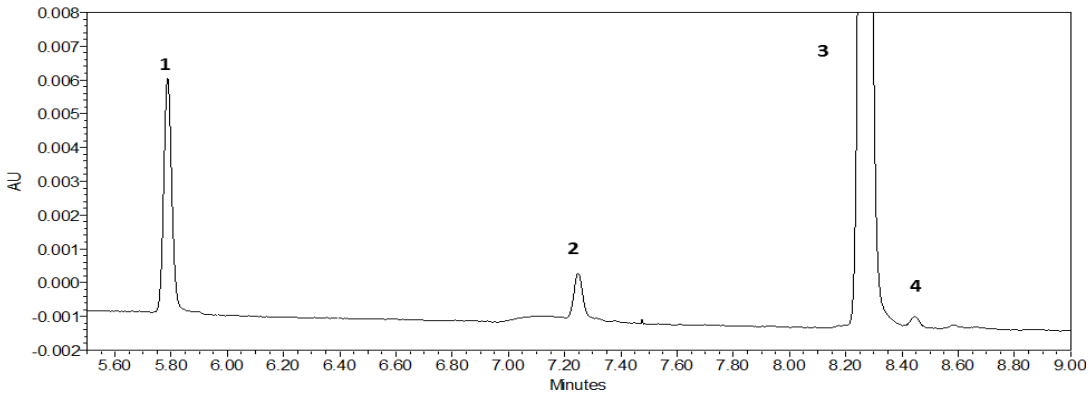


Figure 2.5: The effect of mobile phase pH on the separation of PAC and impurities at pH 10.0 *Column B.* Mobile phase A: 25 mmol L^{-1} ammonium acetate buffer, mobile phase B: 100%. Gradient program: 0-20 minutes (15-85% B). Flow rate: 0.4 mL min^{-1}. Column temperature: 30 °C. Injection volume: 5 µL. Detection: 254 nm. Peaks: (**1**) POH, (**2**) P17-AC, (**3**) PAC, (**4**) HAC. *Note: Chromatogram is zoomed in from 5.5 to 9 minutes to show clearly the PAC and it's impurities.*

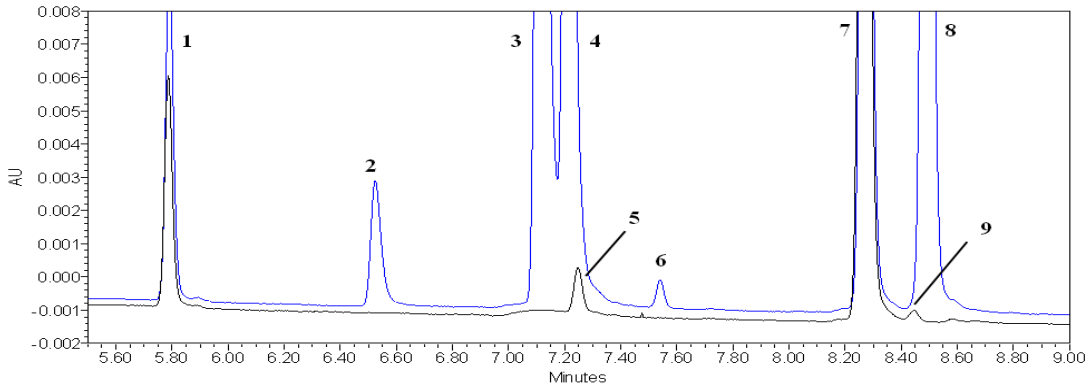


Figure 2.6: The effect of mobile phase pH 10.0 on the separation of PAC and impurities overlaid with a standard containing the ten drug substances. *Column B.* Mobile phase A: 25 mmol L^{-1} ammonium acetate buffer, mobile phase B: 100% ACN. Gradient program: 0-20 minutes (15-85% B). Flow rate: 0.4 mL min^{-1}. Column temperature: 30 °C. Injection volume: 5 µL. Detection: 254 nm. Peaks: (**1**) POH, (**2**) TIM, (**3**) BUN, (**4**) DEX, (**5**) P17-AC, (**6**) system peak, (**7**) PAC, (**8**) FML, and (**9**) HAC. N*ote: Chromatogram is zoomed in from 5.5 to 9 minutes to show clearly the co-elution of the PAC impurities with DEX and FML.*

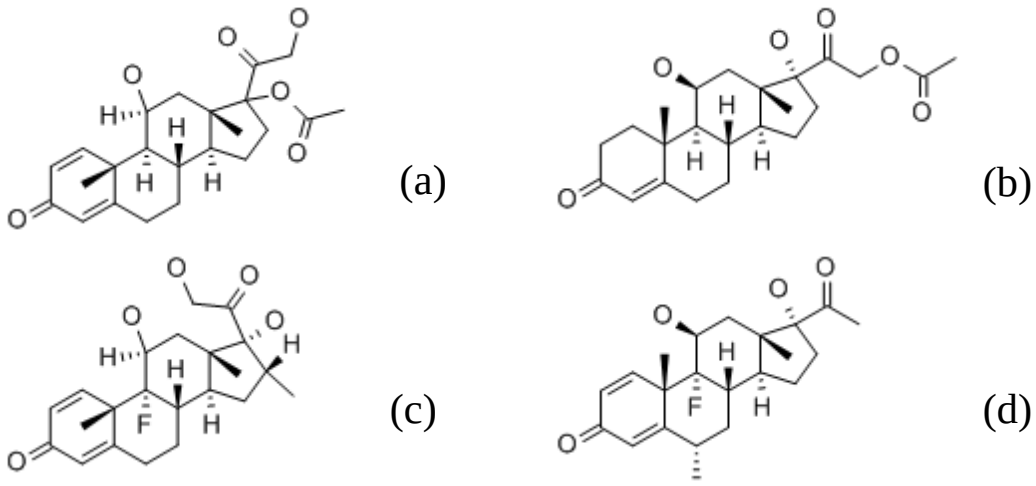


Figure 2.7: Structures of (a) prednisolone 17-acetate, (b) hydrocortisone acetate, (c) dexamethasone and (d) fluorometholone.

2.3.1.3. Separation of the critical peak pairs (DEX/P17-AC, FML/HAC) using Dry Lab Software on *Column B*

Results from the initial screening experiments detailed above indicated that a pH in the region of 10.0 with a linear gradient of 0-20 minutes (15-85% B) provided good retention, resolution and tailing for the ten drug substances as shown in Figure 2.4 (c). However, as shown in Figure 2.6 there was co-elution between DEX/P17-AC and FML/HAC due to their similarities in chemical structures. In an effort to achieve baseline resolution between the two critical pairs, DEX/P17-AC and FML/HAC, Dry Lab software was utilised. DEX, FML, P17-AC and HAC are all neutral drug substances. The retention of neutral drug substances are generally not affected by buffer concentration or mobile phase pH in reverse phase chromatography, therefore column temperature and gradient times were investigated as a means to separate the two critical pairs DEX/P17-AC and FML/HAC.

A design of experiments (DOE) utilising two gradients at two column temperature chromatographic runs were carried out using the ten drug substances to provide data input for the Dry Lab software to predict the optimal column temperature and gradient conditions which would give resolution ($R_S \geq 2.0$) between the critical pairs. These four chromatographic runs were chosen based on the following considerations:

(a) Gradient times should differ by a factor of 3 to 4 for Dry Lab predictions,

(b) Column temperature should differ by at least 15 °C to 20 °C,

(c) A wide gradient range of 15-85% B was chosen for all four chromatographic runs so a broad design space could be modelled by Dry Lab.

The model chromatographic runs are shown in Table 2.6. A 25 mmol L^{-1} ammonium acetate buffer (pH 10.0) as mobile phase A was employed.

Table 2.6: LC-RP Gradient/Temperature mode run parameters.

Run	**1**	**2**	**3**	**4**
t_G	20	60	20	60
Column Temp. (°C)	30	30	60	60

The data from the DOE was imported into Dry Lab using the LC-RP Gradient/temperature (4 runs) mode and the Dry Lab resolution map and chromatogram generated as shown in Figure 2.8 and Figure 2.9. This map represents a plot of predicted resolution for DEX/P17-AC as a function of gradient time (t_G) and column temperature. The two critical pairs are DEX/P17-AC and FML/HAC, but Dry Lab predicted DEX/P17-AC as the pair with the lowest peak resolution as shown from Figures 2.8 and 2.9, therefore DEX/P17-AC was the peak pair selected for further optimisation using Dry Lab.

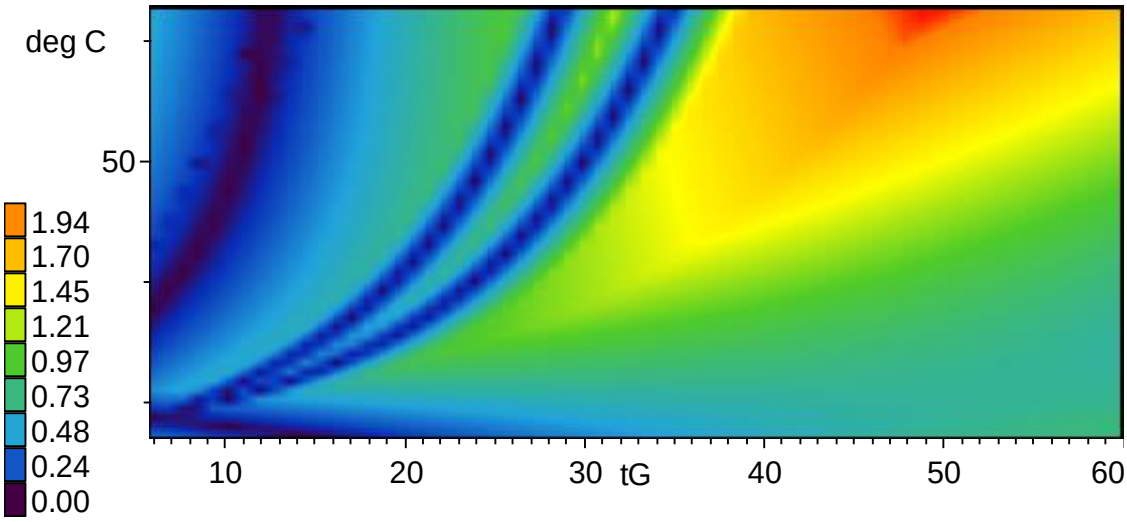


Figure 2.8: Dry Lab resolution map for the separation of DEX/P17-AC using the LC-RP Gradient/temperature mode to predict the highest peak resolution.

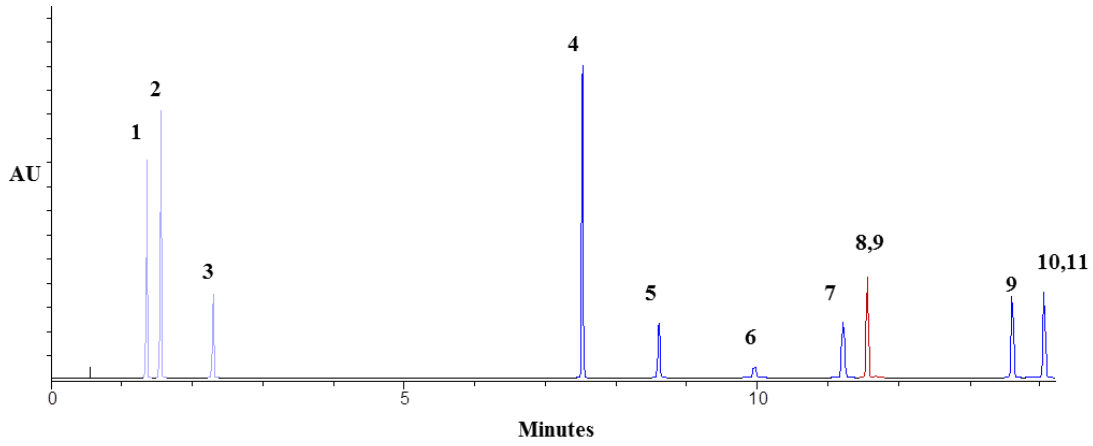


Figure 2.9: Dry Lab prediction for the separation of DEX/P17-AC using the LC-RP Gradient/temperature mode to predict the highest peak resolution. *Column B*. Mobile phase A: 25 mmol L^{-1} ammonium acetate buffer, mobile phase B: 100% ACN. Gradient program: 0-48 minutes (15-85% B). Flow rate: 0.4 mL min^{-1}. Injection volume: 5 µL. Column temperature: 60 °C. Detection: 254 nm. Peaks: (**1**) OFL, (**2**) BMT, (**3**) KET, (**4**) FBP, (**5**) POH, (**6**) TIM, (**7**) BUN, (**8, 9**) DEX, P17-AC (**10**) PAC (**11,12**) FML, HAC.

Calculated peak resolutions of 0.0 to 1.94, in increments of approximately 0.24 are shown as colour coded regions and give a visual representation of the robustness of the separation. A column temperature of approximately 60 °C and a t_G of 48 shows the highest peak resolution for the separation of DEX/P17-AC, (R_S: 1.94, predicted by Dry Lab). Separation of FML/HAC was also achieved at these conditions (R_S: 2.00, predicted by Dry Lab). To verify the Dry Lab prediction, an experiment was carried out with a column temperature of 60 °C and a t_G of 48 minutes. The chromatogram was zoomed in from 0-18 minutes to show the separation of DEX/P17-AC and FML/HAC (Figure 2.10). The predicted Dry Lab chromatogram shows that DEX/P17-AC has the lowest peak resolution (R_S: 1.94), however the experimental run shows DEX/P17-AC to be co-eluted. These results are contradictory, however Dry Lab was only used as an investigatory tool for the separation.

Separation of DEX/P17-AC was not achieved under the experimental conditions (Figure 2.10) as predicted by Dry Lab. Further, a t_G of 48 minutes is not an efficient run time for a cleaning validation method. In addition to this, a column temperature of 60 °C is generally not suitable for a CSH column, as the manufacturer's recommendation for this column when using a high pH mobile phase is a maximum column temperature of 45 ºC. For these reasons, the PAC standard (containing impurities, P17-AC and HAC) was injected separately in future injections. In addition,

PAC contains POH, so for quantitation purposes, the PAC was ran on a separate injection. The remaining nine drug substances (OFL, BMT, KET, FBP, POH, TIM, BUN, DEX, and FML) were selected for further optimisation using Dry Lab.

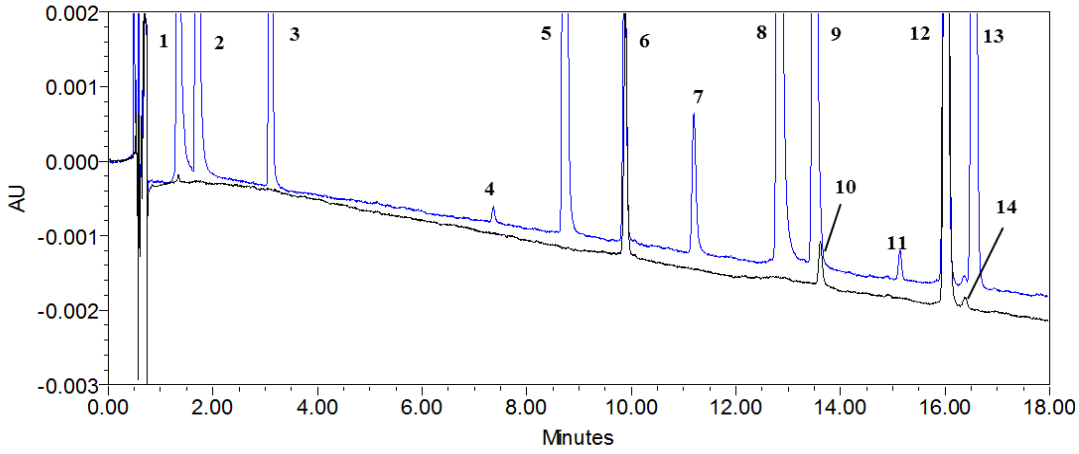


Figure 2.10: The effect of column temperature and gradient time on the separation of DEX/P17-AC and FML/HAC. *Column B.* Mobile phase A: 25 mmol L^{-1} ammonium acetate buffer (pH 10.0), mobile phase B: 100% ACN. Gradient program: 0-48 minutes (15-85% B). Flow rate: 0.4 mL min^{-1}. Column temperature: 60 °C. Injection volume: 5 µL. Detection: 254 nm. Peaks: (1) OFL, (2) BMT, (3) KET, (4) FBP impurity, (5) FBP, (6) POH, (7) TIM, (8) BUN, (9) DEX, (10) P17-AC, (11) system peak, (12) PAC, (13) FML, and (14) HAC. *Note: Chromatogram is zoomed in from 0 to 18 minutes to show clearly the separation of DEX/P17-AC and FML/HAC*

2.3.1.4. Optimisation of the nine drug substances using Dry Lab Software

Based on the results from Section 2.3.1.3, column temperature/gradient optimisation did not achieve separation of DEX/P17-AC, therefore PAC was removed from the standard mixture, leaving nine drug substances to optimise simultaneously. PAC was still included in the study, but was run as a separate injection and therefore co-elution between DEX/P17-AC and FML/HAC was no longer an issue. Dry Lab optimisation was therefore re-visited in light of removal of PAC and associated impurities (notably the impurity P17-AC) which heretofore had co-eluted with DEX. The nine remaining drug substances (OFL, BMT, KET, FBP, POH, TIM, BUN, DEX, FML) are a mixture of acidic, basic and neutral drug substances, so in order to achieve an optimal separation, mobile phase pH, gradient time and % organic of mobile phase B were investigated using Dry Lab. As shown previously in Section 2.3.1.2.3, BUN only achieved baseline resolution of 1.5 at mobile phase pH 10.0.

A DOE using LC-RP Gradient/pH (6 runs) runs were carried out and Dry Lab software was used to predict the optimal pH, organic content and gradient time, which would give resolution ($R_S \geq 2.0$) between the nine drug substances. As in the case of the previous Dry Lab experiment carried out (Section 2.3.1.3), column temperature and gradient times were chosen as the parameters in an effort to separate the two critical neutral pairs, DEX/P17-AC and FML/HAC. For optimal separation of the nine remaining drug substances, the six chromatographic runs were chosen based on the following considerations:

(a) Gradient times should differ by a factor of 3 to 4 for Dry Lab predictions,

(b) A pH range of 9.5 to 10.4,

(1) A reasonable separation was achieved with *Column B* at approximately pH 10.0

(2) The pH recommendation for Dry Lab is no more than 0.5 pH units

(c) A wide gradient range of 15-85% B

(d) A 25 mmol L^{-1} ammonium acetate buffer as mobile phase A and a column temperature of 30 º C was employed.

The DOE are summarized in Table 2.7.

Table 2.7: LC-RP Gradient/pH (6 runs) run parameters.

Run	**1**	**2**	**3**	**4**	**5**	**6**
t_G	20	60	20	60	20	60
pH	9.5	9.5	10.0	10.0	10.4	10.4

The data from the DOE was imported into Dry Lab using the LC-RP Gradient/pH (6 runs) mode and the Dry Lab resolution map and chromatogram generated as shown in Figure 2.11 and Figure 2.12. This map shows that a pH range of between approximately 9.80 and 10.0 at a t_G of 0-10 minutes 15-50% shows a peak resolution of approximately R_S 2.5 to 4.4 for BUN/DEX. In Figure 2.12, there are now 9 drug substances, instead of 10 as shown previously from the Dry Lab chromatogram in Figure 2.9. As discussed in Section 2.3.1.3, PAC was removed from the standard mixture due to PAC impurity co-elution issues and so as a consequence, BUN/DEX then became the critical drug substance peak pair (instead of DEX/P17-AC) as predicted by Dry LAB with the conditions described in Figure 2.12.

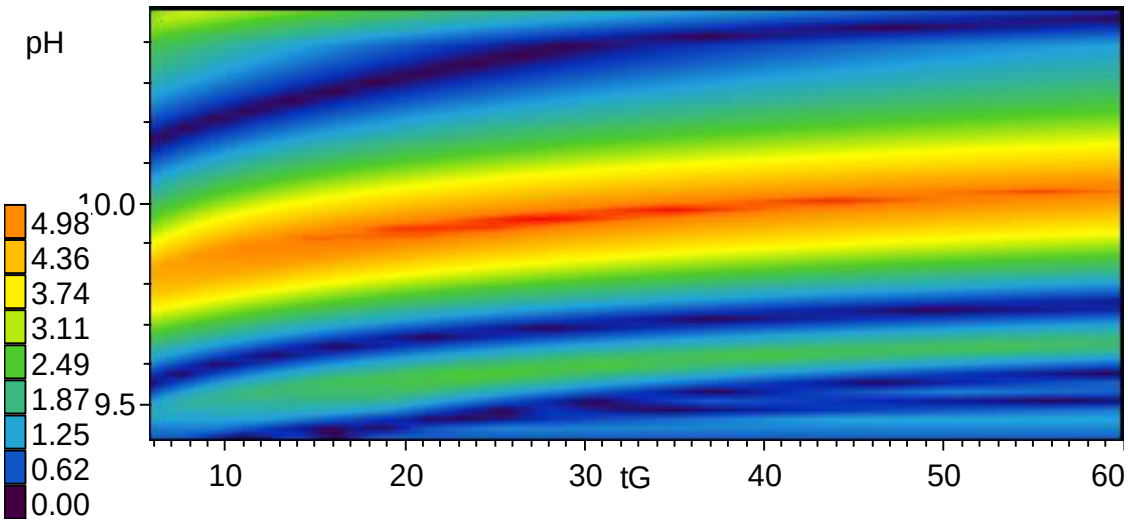


Figure 2.11: Dry Lab resolution map for the separation of BUN/DEX using the LC-RP Gradient/pH mode to predict the highest peak resolution.

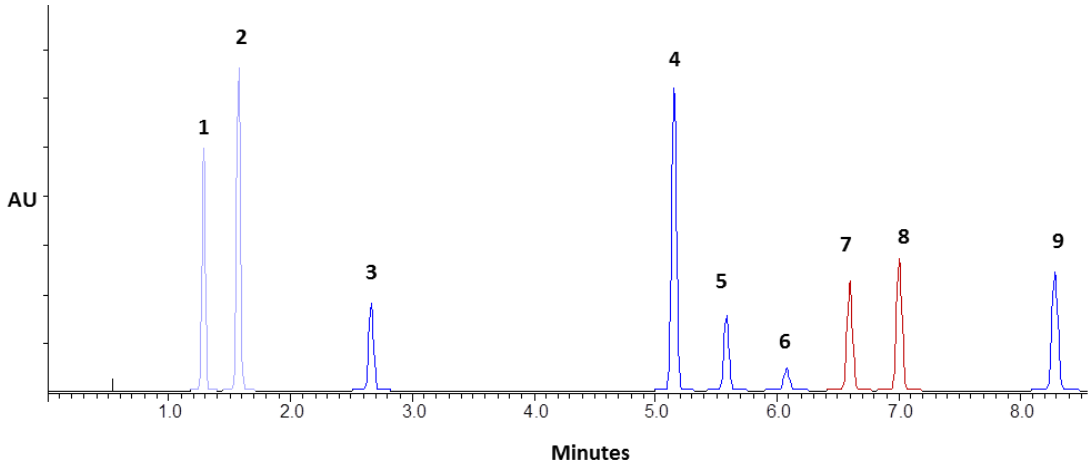


Figure 2.12: Dry Lab chromatogram for the separation of the nine drug substances using the LC-RP Gradient/pH mode to predict the highest peak resolution. *Column B*. Mobile phase A: 25 mmol L^{-1} ammonium acetate buffer (pH 9.9) mobile phase B: 100% ACN. Gradient program: 0-10 minutes (15-50% B). Flow rate: 0.4 mL min^{-1}. Injection volume: 5 µL. Column temperature: 30 °C. Detection: 254 nm. Peaks: (**1**) OFL, (**2**) BMT, (**3**) KET, (**4**) FBP, (**5**) POH, (**6**) TIM, (**7**) BUN, (**8**) DEX, (**9**) FML

To verify the Dry Lab prediction, an experiment was carried out with a mobile phase of pH 9.9 and a t_G of 10 minutes (15-50% B) as shown in Figure 2.13. The predictive ability of the Gradient/pH model was evaluated by comparing the predicted and experimentally obtained retention times as shown in Table 2.8. The compared retention times were in good agreement, with all the % retention time errors being ≤ 10%. In the case of the resolutions obtained, the predicted Dry Lab chromatogram (Figure 2.12) shows that BUN/DEX has the lowest peak resolution (R_S: 4.7), which is

in agreement with the experimental run (Figure 2.13), which also shows BUN/DEX to have the lowest peak resolution (R_S: 2.6). The results for resolution between BUN/DEX show some variability between the experimental and Dry Lab run, however Dry Lab was only used as an investigatory tool for the separation.

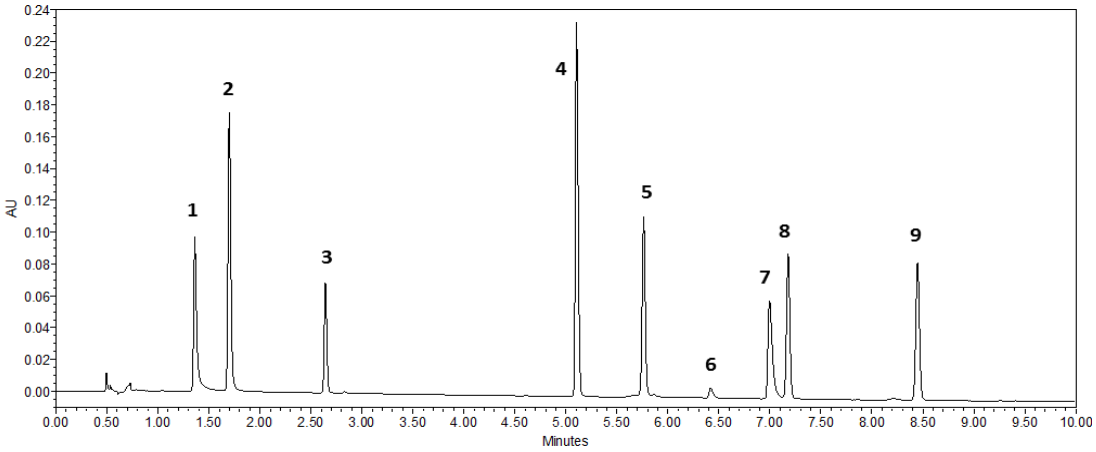


Figure 2.13: The effect of mobile phase pH 9.9 on the separation of the 9 drug substances. *Column B*. Mobile phase A: 25 mmol L^{-1} ammonium acetate buffer, mobile phase B: 100% ACN. Gradient program: 0-10 minutes (15-50% B). Flow rate: 0.4 mL min^{-1}. Column temperature: 30 °C. Injection volume: 5 µL. Detection: 254 nm. Peaks: (**1**) OFL, (**2**) BMT, (**3**) KET, (**4**) FBP, (**5**) POH, (**6**) TIM, (**7**) BUN, (**8**) DEX, and (**9**) FML.

Table 2.8: Dry Lab predicted and experimental retention times values.

Peak name	**Predicted retention time (min)**	**Experimental retention time (min)**	**Difference between retention times (min)**	**% error**
OFL	1.28	1.36	0.08	6.25
BMT	1.57	1.69	0.12	7.64
KET	2.66	2.64	0.02	0.75
FBP	5.15	5.10	0.05	0.97
POH	5.58	5.76	0.18	3.22
TIM	6.07	6.42	0.35	5.76
BUN	6.59	6.99	0.40	6.06
DEX	7.00	7.17	0.17	2.42
FML	8.28	8.44	0.16	1.93

2.3.1.5. Optimisation of mobile phase buffer concentration on *Column B*

As shown in previous Sections 2.3.1.1, 2.3.1.2, 2.3.1.3 and 2.3.1.4, mobile phase pH, column temperature and gradient time were investigated. In this section the effect of buffer concentration was investigated. Buffer concentration usually has only a minor effect on relative retention of ionic samples for separations at low pH on modern (type-B) alkylsilica columns. However, for separations at pH > 6, protonated bases can be retained by ion exchange as a result of interaction with ionised silanols of silica-based column packings. Ion-exchange retention decreases as mobile phase ionic strength increases, with the result that an increase in buffer concentration will tend to decrease the retention of protonated bases [25].

The effect of ammonium acetate buffer concentration on the separation was investigated in the range between 5 and 45 mmol L^{-1} as shown below in Figure 2.14.

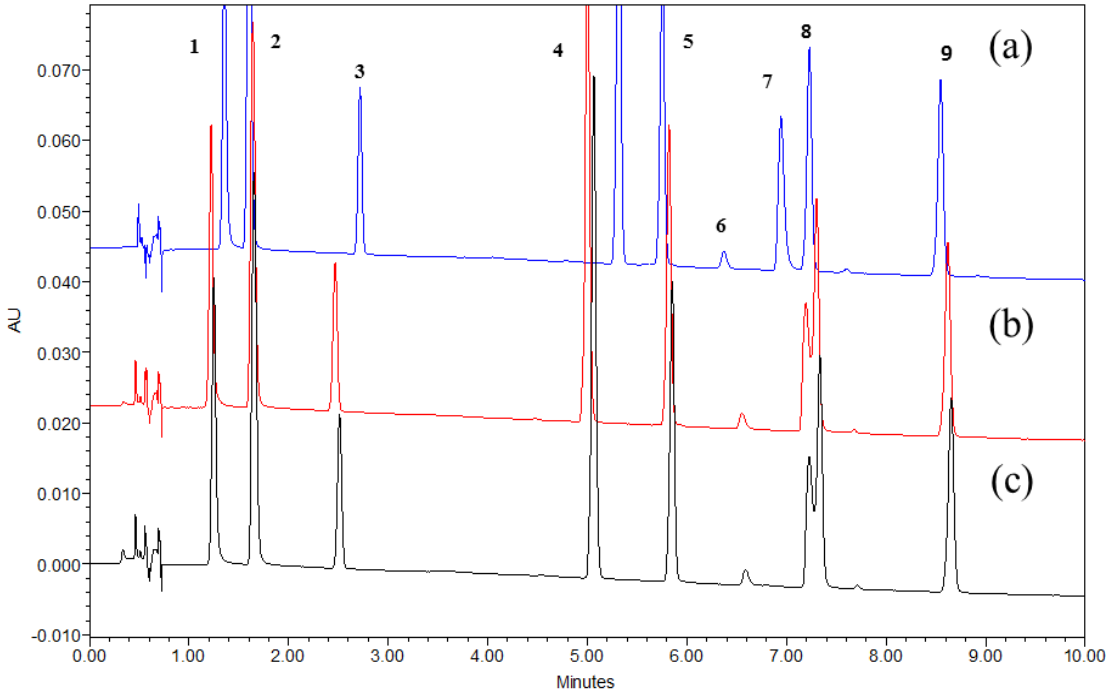


Figure 2.14: The effect of mobile phase buffer concentration on the separation of the 9 drug substances at (a) 45 mmol L^{-1}, (b) 15 mmol L^{-1} and (c) 5 mmol L^{-1} ammonium acetate buffer, pH 9.9. Mobile phase B: 100% ACN. *Column B*. Gradient program: 0-10 minutes (15-50%). Flow rate: of 0.4 mL min^{-1}. Column temperature: 30 °C. Injection volume: 5 µL. Detection: 254 nm. Peaks: (**1**) OFL, (**2**) BMT, (**3**) KET, (**4**) FBP, (**5**) POH, (**6**) TIM, (**7**) BUN, (**8**) DEX, and (**9**) FML.

There was no significant influence of the buffer concentration on the separation of the drug substances, except for BUN/DEX, OFL/BMT and FBP/POH. At 5 and 15 mmol L^{-1}, baseline resolution for BUN/DEX (R_S: 1.3) was not achieved, whereas at 45 mmol L^{-1}, the resolution between BUN/DEX was 2.7. A plot of buffer concentration versus resolution is shown below in Figure 2.15. Resolution between BUN/DEX was highest at 45 mmol L^{-1} (R_S: 2.7), however the resolution between OFL/BMT (R_S: 3.2) was the lowest at this buffer concentration and highest at 5 mmol L^{-1} (R_S: 5.1), as shown in Figure 2.15. As the buffer concentration increased from 5 to 45 mmol L^{-1}, the resolution of FBP/POH decreased from 9.4 to 5.5, respectively as shown below. However, the resolution of FBP/POH meets the desired resolution of $\geq$ 2.0 at all buffer concentration variations.

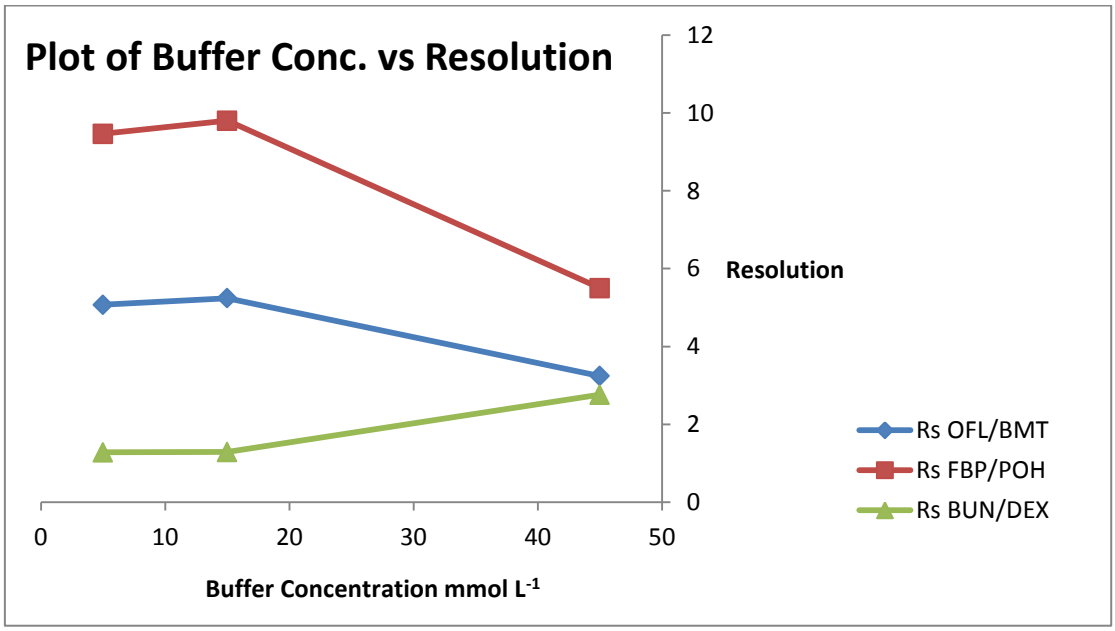


Figure 2.15: Plot of buffer concentration of the mobile phase versus resolution between OFL/BMT, POH/FBP and BUN/DEX.

In terms of retention, an increase in buffer concentration caused a decrease in retention for BMT, BUN, and TIM as these drug substances are protonated bases under the conditions used in these experiments. There was little or no effect on the retention of DEX, POH, and FML as these are neutral compounds and are generally not affected by buffer concentration in RP-HPLC. And finally, for OFL, FBP, and KET, there was an increase in retention as the buffer concentration increased as these are weakly acidic drug substances. Overall, the buffer concentration at 45 mmol L^{-1} achieved the best separation.

2.3.1.6. Optimisation of mobile phase buffer concentration and pH using Minitab

To ensure method robustness, a DOE [31, 32] using Minitab was employed to define the parameters which affect the separation and resolution of the drug substances under investigation and to optimise each of those conditions. The purpose of the DOE was to generate a method which separates the drug substances as efficiently as possible within a robust design space. The method parameters which affect the separation were defined as: (1) mobile phase pH as shown from Section 2.3.1.1, 2.3.1.2, 2.3.1.4 and (2) buffer concentration of the mobile phase as shown from Section 2.3.1.5. A ten minute gradient time was considered optimum in order to elute the nine drug substances within ten minutes.

A two level fractional factorial DOE using Minitab was applied to the UPLC process. Factorial experimental designs investigate the effects of several different factors by varying the factors simultaneously instead of changing only one factor at a time. Factorial designs allow estimation of the sensitivity to each factor and also the combined effect of two or more factors. A DOE approach using Dry Lab and Minitab are very similar in that they are both software packages used for method development optimization. For both Dry Lab and Minitab, a designed experiment is created to investigate the effects of input variables (factors) on the output variable (response). A series of chromatographic runs are carried out, and the collected data is used to determine the factors settings that give the optimal results. Dry Lab results are visually analyzed using a 2D color resolution map and 3D color resolution cube, whereas Minitab results are statistically analyzed using analytical and graphing tools.

For this DOE using Minitab, buffer concentration and mobile phase pH were identified as the input variables as discussed previously and resolution between OFL/BMT and BUN/DEX were examined as the output. The DOE parameters and resolution results for OFL/BMT and BUN/DEX are outlined in Table 2.9. Buffer concentrations of 25 to 55 mmol L^{-1} were chosen as the lowest and highest concentrations. As shown previously (Section 2.3.1.5), baseline resolution between BUN/DEX was not achieved at the low buffer concentrations of 5 and 15 mmol L^{-1}. The highest buffer concentration used in Section 2.3.1.5 was 45 mmol L^{-1}, so in order to optimise the buffer concentration in a broad design space, a high buffer concentration of 55 mmol L^{-1} and a lower buffer concentration of 25 mmol L^{-1} was chosen. Figure 2.12 shows a good separation, with resolution values of $\geq$ 2.0 and

tailing values of ≤ 2.0 for all drug substances at mobile phase pH 9.9, so for the DOE, a lower pH value of 9.7 and a higher pH value of 10.1 were chosen to examine the separation.

Table 2.9: Two level fractional factorial DOE for separation of peak pairs (OFL/BMT) and (BUN/DEX).

Run Order	Mobile phase pH	Buffer concentration of mobile phase (mmol L^{-1})	Resolution OFL/BMT	Resolution BUN/DEX
1	9.7	25	3.35	5.24
2	10.1	25	4.88	0.1
3	9.7	55	2.06	6.90
4	10.1	55	3.23	1.25
5 [5]	9.9	40	3.10	3.46

To assess the impact of mobile phase pH and buffer concentration of the mobile phase on the resolution of OFL/BMT and BUN/DEX, a main effects plot[6] and an interaction plot[7] were generated, as shown in Figures 2.16 - 2.19. As shown from the main effects plot for OFL/BMT (Figure 2.16) buffer concentration has slightly bigger main effects on the resolution of OFL/BMT than the mobile phase pH. The opposite is shown for BUN/DEX (Figure 2.17), where mobile phase pH has a much bigger effect on resolution over buffer concentration. An interaction plot shows the impact that changing one factor has on the other factor. Because an interaction of one factor can magnify or diminish main effects, evaluating interactions is extremely important. The significant interaction between pH and buffer concentration shows up as two lines with similar slopes. Parallel lines in an interaction plot indicate no interaction between pH and buffer concentration. The resolution between OFL/BMT at 25 mmol L^{-1} buffer is greater than the resolution between OFL/BMT at 55 mmol L^{-1} buffer at both pH 9.7 pH 10.1 (Figure 2.18). The opposite is shown for BUN/DEX, where the resolution is highest at 55 mmol L^{-1} at both pH 9.7 and pH 10.1 (Figure 2.19). In order to get the

[5] The mobile phase pH 9.9, 40 mmol L^{-1} experiment is the centre point for the DOE.
[6] Used in conjunction with an analysis of variance and design of experiments to examine differences among level means for one or more factors. A main effect is present when different levels of a factor affect the response differently. A main effects plot graphs the response mean for each factor level connected by a line.
[7] When the effect of a one factor depends on the level of the other factor. An interaction plot is used to visualise possible interactions.

highest resolution between OFL/BMT and BUN/DEX, the results for both the main effects plot and the interaction plot suggest a mid-range pH of approximately pH 9.9 and a buffer concentration of approximately 40 mmol L^{-1}.

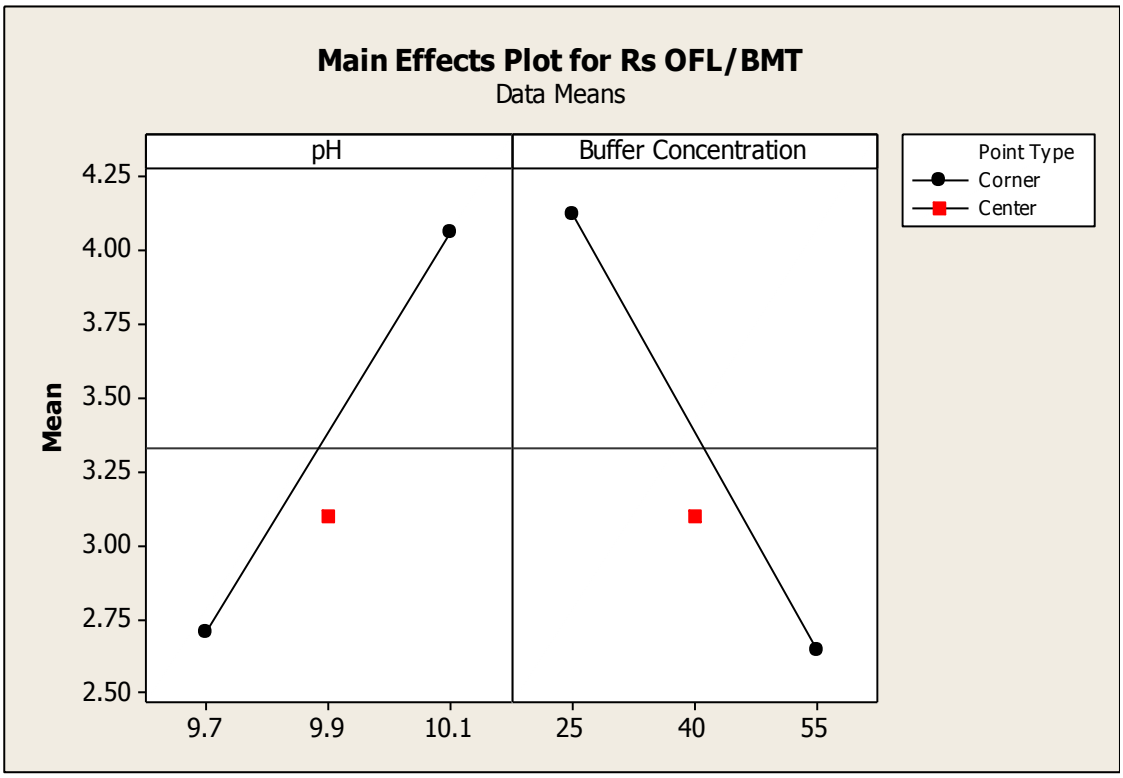


Figure 2.16: Main effects plot for the resolution between OFL/BMT using mobile phase pH and buffer concentration of the mobile phase as the factors.

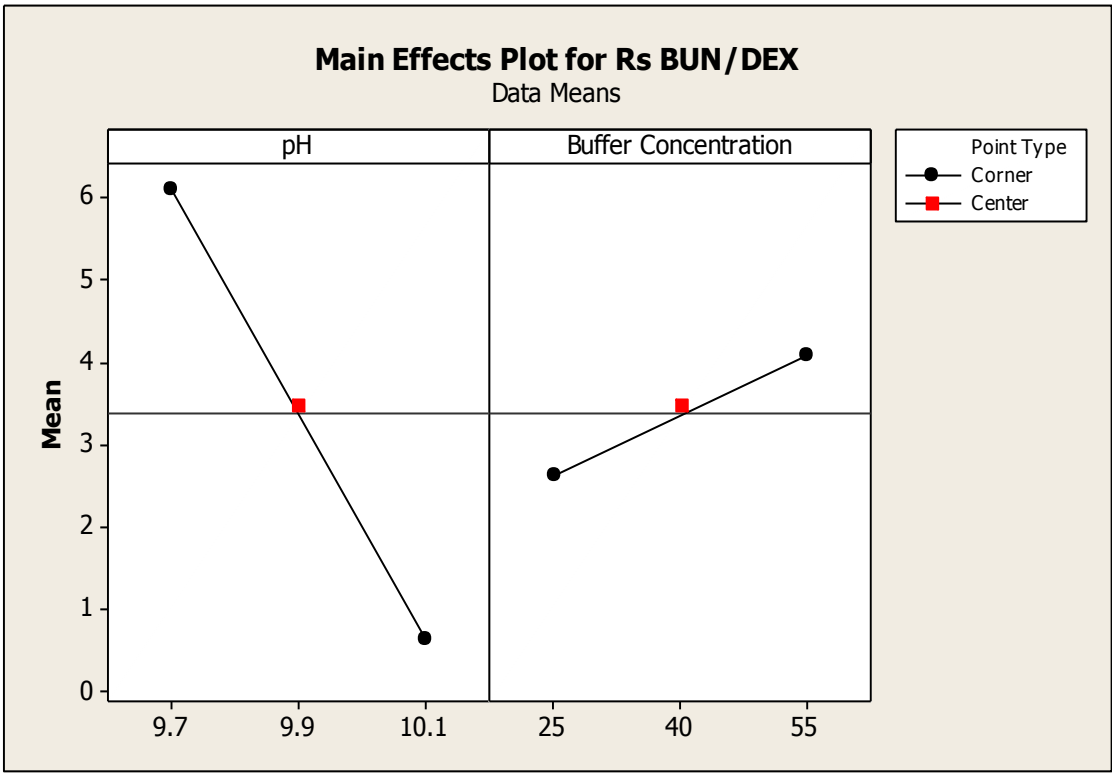


Figure 2.17: Main effects plot for the resolution between BUN/DEX using mobile phase pH and buffer concentration of the mobile phase as the factors.

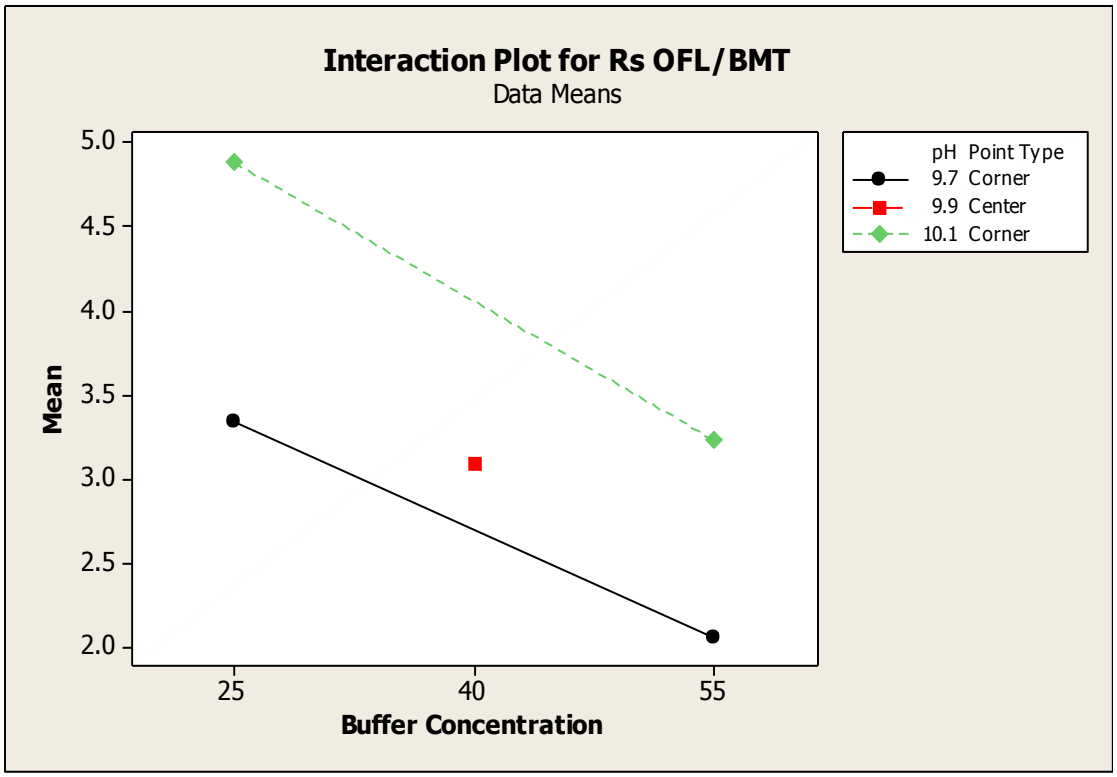


Figure 2.18: Interaction plot for the resolution between OFL/BMT using mobile phase pH and buffer concentration of the mobile phase as the factors.

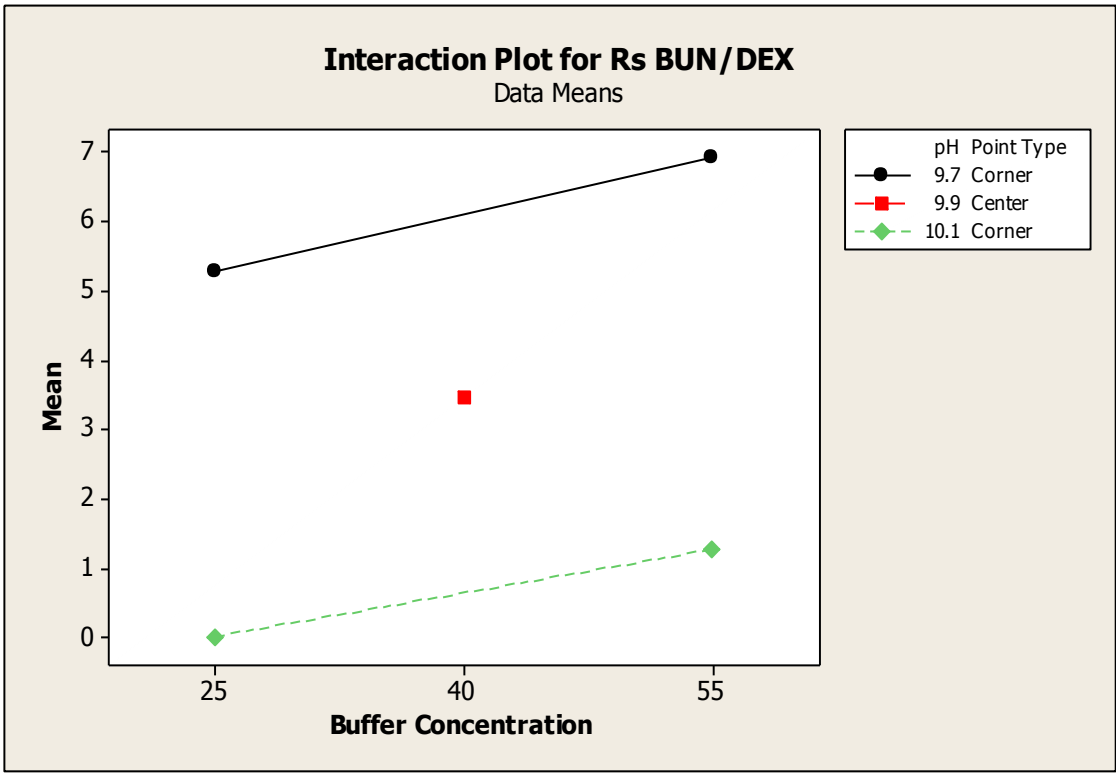


Figure 2.19: Interaction plot for the resolution between BUN/DEX using mobile phase pH and buffer concentration of the mobile phase as the factors.

To enable an assessment of the method parameters simultaneously and allow for optimisation of the analytical method, an optimisation plot was utilised as shown in Figure 2.20. Using the data generated from the DOE, this tool allows the user to modify each parameter and assess its impact on the separation. Each point is modified so that it operates on a plateau of composite desirability[8] ensuring that the method will operate within the centre of a design space while still maintaining optimal resolution. An R_S of 1.5, 2.0 and 3.0 were input as the lower, target and upper values, respectively for both OFL/BMT and BUN/DEX. The optimisation plot indicates that to achieve resolution of approximately 3.1 between OFL/BMT and 3.4 between BUN/DEX, a mobile phase of approximately pH 9.9 and a buffer concentration of 40 mmol L^{-1} is required. The final parameters decided were a mobile phase pH of 9.85 and a buffer

[8] Assesses how well a combination of input variables satisfies the defined goals for the responses. Individual desirability (d) evaluates how the settings optimise a single response; composite desirability (D) evaluates how the settings optimise a set of responses overall. Desirability has a range of zero to one. One represents the ideal case; zero indicates that one or more responses are outside their acceptable limits.

concentration of 40 mmol L^{-1}. As shown previously at pH 10.1 (Table 2.9), BUN/DEX co-elute, so to ensure method robustness, a slightly lower mobile phase pH of 9.85 was chosen.

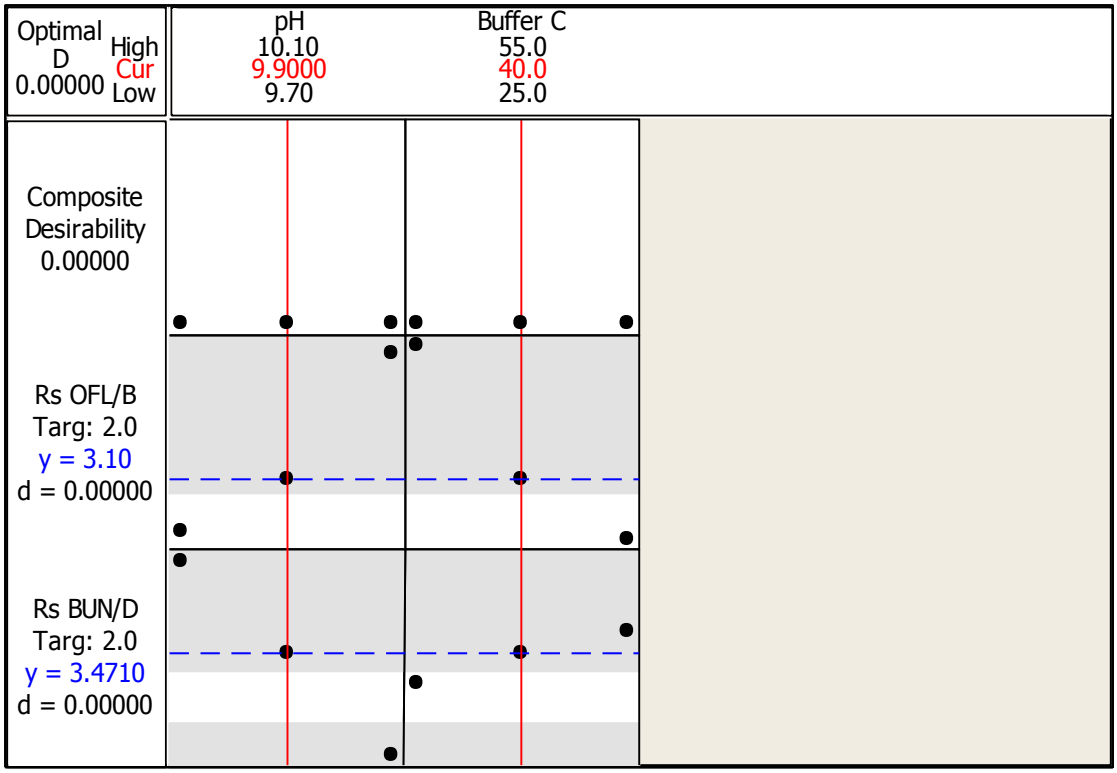


Figure 2.20: Optimisation plot for the resolution between OFL/BMT and BUN/DEX using mobile phase pH and buffer concentration of the mobile phase as the factors.

Figure 2.21 (a) shows the separation of the nine drug substances under the optimised conditions. It was concluded that a mobile phase of approximately pH 9.85 and a high buffer concentration of 40 mmol L^{-1} gave the optimal separation in terms of retention, resolution, and tailing. As it can be seen, the nine drug substances are well separated from each other and all drug substances have tailing of ≤ 2.0 and resolution of ≥ 2.0. The resolution of OFL/BMT is 4.3 and resolution for BUN/DEX is 8.8. Figure 2.21 (b) shows the same chromatogram ran at 295 nm, the wavelength which was used for quantitation of OFL, KET and TIM which are shown in the figure enclosed in parentheses.

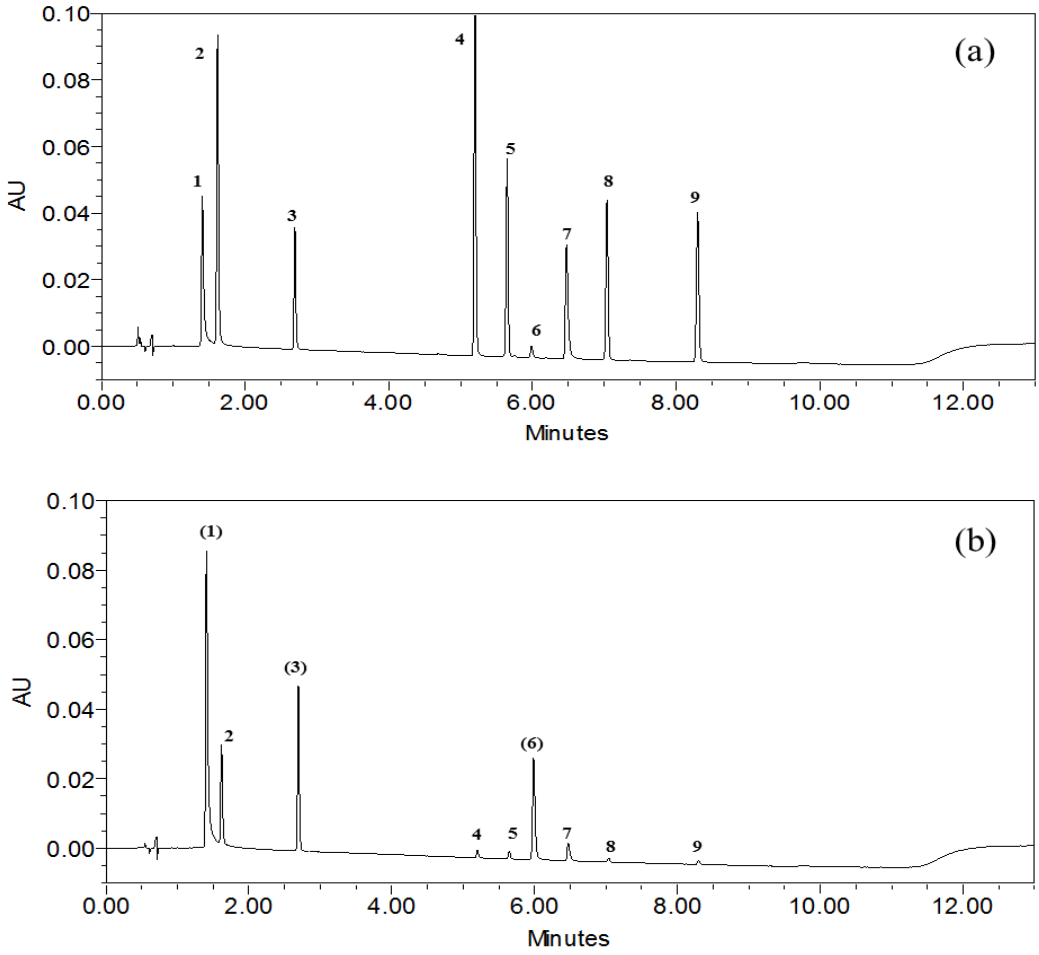


Figure 2.21: Final optimised chromatographic conditions for the separation of 9 selected drug substances at (a) 254 nm and (b) 295 nm. *Column B*: Waters Acquity Charged Surface Hybrid (CSH) C_{18} 1.7 µm, 2.1 mm x 100 mm column. Mobile phase A: 40 mmol L^{-1} ammonium acetate buffer (pH 9.85), mobile phase B: 40 mmol L^{-1} ammonium acetate buffer (pH 9.85)/ACN (50/50, v/v) Gradient program: 0-10 minutes (15-50% B), with a 3 minute equilibration time. Flow rate: 0.4 mL min^{-1}. Column temperature: 30 °C. Injection volume: 5 µL. Peaks: (**1**) OFL, (**2**) BMT, (**3**) KET, (**4**) FBP, (**5**) POH, (**6**) TIM, (**7**) BUN, (**8**) DEX, and (**9**) FML.

2.3.1.7. Optimisation of injection volume

During development of the chromatographic conditions, an injection volume of 5 µL was used. However, in order to meet the limits of quantitation that were required for the cleaning method, an injection volume of 30 µL was considered suitable. All method validation criteria were met as shown in Section 2.3.2. In addition, an injection volume of 30 µL was used for all swab sampling studies in Chapter 3.

2.3.2. Method validation

Note: *The PAC standard (containing the impurities P17-AC and HAC) was injected separately for the method validation due to co-elution issues as discussed in Section 2.3.1.3. Furthermore, PAC contains POH, so for quantitation purposes, the PAC was run on separate injections.*

The validation data for the analytical method is shown in Table 2.10 and the results are discussed below from Section 2.3.2.1 to Section 2.3.2.4.

2.3.2.1. Linearity

The linear range of the method was examined using standard solutions of the ten drug substances. Each drug substance was prepared at seven different concentrations (n=7) and injected in duplicate. The method provided linear standard curves ($r^2 > 0.999$) for each drug substance of interest over a wide concentration range (Table 2.10).

2.3.2.2. Precision

A standard solution of the ten drug substances was injected six times for testing peak area, retention time and concentration precision, resulting in % RSD values of < 2.0% for all drug substances. The same standard solution was prepared and tested by a single analyst on different days, to establish Day-to-Day precision (equivalency)[9]. The ratio of drug substance concentration Day 2 versus drug substance concentration Day 1 was measured and the % RSD values were < 2.0% for all drug substances as listed in Table 2.10.

2.3.2.3. Limit of Quantitation and Recoveries

The limit of quantitation limit (LOQ) of each drug substance was determined by recovery data and by the R.S.D of recovery data using peaks areas of three (n=3) separate injections of standard solutions. An R.S.D of ≤ 10% for the LOQ concentration and a recovery of 80-120% were achieved for all drug substances. OFL is linear from 0.10 to 10.0 µg/mL; however at 0.10 µg/mL the recovery of the OFL

[9] Ratio of drug substance concentration Day 2 versus drug substance concentration Day 1

solution was only 30%. The lowest OFL concentration to achieve a recovery of 80-120% was 1.0 µg/mL. However, this quantitation limit is higher than the residue limit for an OFL swab sample as defined by Allergan Pharmaceuticals. For commercial reasons, this residue limit is proprietary information.

2.3.2.4. Robustness

To evaluate the robustness of the analytical method, experimental conditions were deliberately altered to assess the effect of column temperature, flow rate, mobile phase buffer concentration, and mobile phase pH on the separation achieved. The column temperature was set to 30 ± 2 °C, the mobile phase buffer concentration was varied from 40 ± 5 mmol L^{-1} ammonium acetate buffer, the pH of the mobile phase was varied from pH 9.85 ± 0.1, and a flow rate of 0.40 ± 0.04 mL min^{-1} was applied. For each condition investigated, three injections of the standard solution were injected to determine system suitability. For all parameters altered, a resolution of ≥ 2.0 was maintained between all drug substances in the standard injections. All drug substances for each robustness test investigated were compared to the nominal conditions and the concentration results were found to be equivalent as shown in Table 2.10. There was no significant difference from nominal conditions for all the drug substances under the various robustness challenges indicating that the method is capable of withstanding small changes in the parameters whilst still retaining the robustness necessary for the QC laboratory.

Table 2.10: Analytical performance criteria for the determination of ten drug substances.

Linearity	**DEX**		**FML**		**POH**		**PAC**		**KET**		**FBP**		**OFL**		**TIM**		**BUN**		**BMT**	
Linearity r^2 (n=7)	> 0.999		> 0.999		> 0.999		> 0.999		> 0.999		> 0.999		> 0.999		> 0.999		> 0.999		> 0.999	
Linear Range (µg/mL)	0.01-10.0		0.01-10.0		0.01-10.0		0.01-10.0		0.02-10.0		0.01-10.0		0.1-10.0		0.03-10.0		0.03-10.0		0.02-10.0	
Equation of the line	y = 56439x - 236.8		y = 130841x - 534.5		y = 163863x - 1007.7		y = 148651x + 536.8		y = 94119x + 66.9		y = 265272 + 24.6		y= 85075x - 15161		y = 92771x - 2744.9		y = 119839x - 895.0		y = 212523x - 436.3	
Limits of Quantitation[a]	**DEX**		**FML**		**POH**		**PAC**		**KET**		**FBP**		**OFL**		**TIM**		**BUN**		**BMT**	
LOQ µg/mL	0.01		0.01		0.01		0.01		0.02		0.01		1.0		0.03		0.03		0.02	
% Recovery (at LOQ)	105		108		107		108		103		101		81		95		94		85	
% Recovery RSD	0.5		1.8		0.0		1.3		1.3		1.5		1.7		4.1		3.5		5.2	
Precision[b] (%RSD)	Day-1	Day-2	Day-1	Day-2	Day-1	Day-2	Day-1	Day-2	Day-1	Day-2	Day-1	Day-2	Day-1	Day-2	Day-1	Day-2	Day-1	Day-2	Day-1	Day-2
Retention time	0.21	0.02	0.19	0.01	0.24	0.02	0.03	0.00	0.43	0.02	0.25	0.03	0.07	0.23	0.22	0.02	0.21	0.02	0.40	0.02
Area	0.67	0.06	0.93	0.13	0.47	0.09	0.07	0.03	0.30	0.11	0.42	0.14	1.12	0.98	0.29	0.14	0.25	0.24	0.14	0.14
Concentration	0.19	0.09	0.29	0.09	0.17	0.06	0.13	0.06	0.17	0.08	0.28	0.08	1.05	0.65	0.38	0.32	0.63	0.28	1.08	0.38
Equivalency[c]	1.01		1.01		1.00		1.00		1.02		0.99		0.98		1.01		1.01		1.03	
Robustness[d]	**DEX**		**FML**		**POH**		**PAC**		**KET**		**FBP**		**OFL**		**TIM**		**BUN**		**BMT**	
Temperature: 28 °C	1.00		1.00		1.00		0.99		0.99		0.99		0.99		0.99		1.00		0.99	
Temperature: 32 °C	0.98		1.00		1.00		0.99		0.99		0.99		0.99		1.00		1.00		0.99	

Table 2.10: (continued)

Robustness[d]	DEX	FML	POH	PAC	KET	FBP	OFL	TIM	BUN	BMT
Flow: 0.36 mL min^{-1}	1.00	0.98	1.00	0.99	0.99	0.99	0.99	0.99	1.00	0.99
Flow: 0.44 mL min^{-1}	1.00	1.00	0.99	0.99	0.99	0.99	0.99	1.00	1.00	0.99
Buffer Conc: 35mmol L^{-1}	0.99	1.00	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99
Buffer Conc: 45mmol L^{-1}	1.00	1.00	1.00	0.99	0.99	0.99	0.99	1.00	1.00	0.99
Mobile phase pH: 9.75	1.00	1.00	1.00	0.99	0.99	0.99	0.99	0.99	1.00	0.99
Mobile phase pH: 9.95	0.99	1.00	0.99	0.99	0.99	0.99	0.99	0.99	1.00	0.99

[a] n=3

[b] n=6

[c] Ratio of drug substance concentration Day 2 versus drug substance concentration Day 1

[d] Ratio of drug substance concentration versus drug substance concentration under optimum (nominal) conditions

2.4. Conclusion

A relatively rapid, robust UPLC method was developed for the determination and quantitation of up to ten drug substances (dexamethasone, fluorometholone, prednisolone acetate prednisolone, ketorolac tromethamine, flurbiprofen, timolol maleate, levobunolol hydrochloride, brimonidine tartrate and ofloxacin) simultaneously which can be used to verify that production equipment surfaces have been cleaned to an extent that meets existing regulatory requirements. Prednisolone acetate was validated on a separate injection due to co-elution issues. Standard analytical performance criteria were used for evaluation purposes, and the method was found to be both linear and precise, with LOQs meeting the requirements for analysis of DEX, FML, POH, PAC, KET, FBP, TIM, BUN and BMT trace residues. This work demonstrates for the first time a single UPLC method directly applicable for analysis of a range of APIs simultaneously and provides the pharmaceutical industry with a robust analytical cleaning method with broad applicability.

2.5. References

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Chapter 3

Development of Swab Sampling Methodology in Support of Cleaning Validation

3.1. Introduction

The aim of the developed swab sampling procedure described in this Chapter was to demonstrate that the selected drug substance residues could be adequately removed and quantitated from various product-contacting surfaces that included stainless steel, glass, teflon, and silicone. The analytical cleaning method that was developed and validated in Chapter 2 was used in combination with the developed swab sampling methodology described in this Chapter to verify that adequate removal of the drug substance residues from the various product contacting surfaces was achievable.

There are many developed analytical methods and related swab sampling methodologies in the literature that describe the use of various product contacting surfaces, swabs and solvents. Polyester swabs are generally the most widely used swabs in pharmaceutical industries [1-3], however cotton swabs [4-6] are also used depending on the drug substance and recovery values obtained from the swab sampling analysis.

This Chapter describes a swab sampling procedure that enables the removal and quantitation of up to 9 drug substances from various product-contacting manufacturing equipment surfaces, which covers a broad range of ophthalmic solutions. Therefore, the swab sampling methodology has a broad applicability. Removal of the drug substances from the selected surfaces were evaluated using a variety of swabbing materials (foam, cotton, polyester), and different solvents etc. A Design of Experiments (DOE) approach utilising Minitab software was adopted to determine the most appropriate swab and solvent for the swab sampling methodology.

Because the product-contacting surfaces on the manufacturing equipment trains consist mainly of stainless steel, stainless steel was the surface primarily used in optimizing the swab sampling conditions. Different swabs, extracting solvents and sampling techniques were investigated in this research to evaluate for recoveries of the APIs on the various surfaces whilst ensuring minimal interferences from the swabbing materials and the product contacting surfaces.

3.2. Experimental

3.2.1. Reagents and materials

Reference materials, dexamethasone (DEX, 99.7%), prednisolone acetate (PAC, 99.4%), and fluoromethalone (FML, 99.7%) were purchased from Sanofi Aventis (Paris, France), levobunolol hydrochloride (BUN, 99.7%) and brimonidine tartrate (BMT, 99.9%) from Piramal Healthcare (Andhra Pradesh, India), timolol maleate (TIM, 99.9%) from PCAS Finland OY (Turka, Finland), ketorolac tromethamine (KET, 98.7%) from Recordati (Milan, Italy), ofloxacin (OFL, 99.7%) from Chemo (Madrid, Spain), prednisolone (POH, 98.5%) from Sigma Aldrich (St. Louis, Missouri, US), and flurbiprofen (FBP, 99.6%) from Aesica Pharmaceuticals Limited (Cramlington, UK).

HPLC grade methanol (MeOH), acetonitrile (ACN), and ethanol (EtOH) were purchased from Labscan (Tallaght, Dublin, Ireland). All chemicals were used as received, without any further purification. Deionised water was obtained from a Millipore Milli-Q water purification unit (Millipore, Bedford, MA, USA).

3.2.2. Instrumentation

The chromatographic separation performed was as described in Section 2.2.2 using a Waters Acquity Charged Surface Hybrid (CSH) C18 (1.7 µm, 2.1 mm x 100 mm) column (Waters, Santry, Dublin, Ireland) (*Column B*). The compounds analyzed in this section were BMT, FBP, PAC, POH, BUN, DEX and FML (254 nm), and KET, OFL, and TIM (295 nm). A Branson sonication bath model 8510 (Branson Ultrasonics Corporation, Danbury, USA) was used for dissolving the standard reference materials and swab samples, and the balance used was a Mettler Toledo model XS205 (Manson Technology, Dublin, Ireland). TX761 swabs (ITW Texipe, NJ, USA) were used to recover the drug substances from the coupons. The coupons used were 316 stainless steel, toughened borosilicate glass, teflon (PTFE, Polytetrafluoroethylene) and platinum cured silicone, FDA approved, all obtained from Globe Pharma, NJ, USA. Minitab software was used to predict the most appropriate swab and solvent for the swab sampling procedure.

3.2.3. Method

The chromatographic analysis was performed on *Column B* using 40 mmol L^{-1} ammonium acetate buffer (pH 9.85, adjusted with ammonium hydroxide solution) as mobile phase A and 40 mmol L^{-1} ammonium acetate buffer (pH 9.85)/ACN (50/50, v/v) as mobile phase B. The gradient programme was a linear gradient from 0-10 minutes (30-100% B), with a 3 minute column re-equilibration time[10]. The flow was set at 0.4 mL min^{-1} and the column temperature was maintained at 30 °C. The injection volume was 30 µL, with detection by UV at 254 nm for BMT, FBP, PAC, POH, BUN, DEX and FML, and 295 nm for OFL, KET and TIM.

3.2.4. Swab standard solution preparation

Stock standard solutions of BMT, KET, FBP, TIM, and BUN were prepared in deionised water; PAC, POH, OFL and DEX in MeOH:water (70:30, v/v); and FML in methanol, each at a concentration of 2500 µg/mL. The standards were stored in a refrigerator at 4 °C, for two weeks with no significant loss of API. Appropriate dilutions were made with MeOH to obtain solutions containing 2 µg/mL, 10 µg/mL and 50 µg/mL.

3.2.5. Swab sample preparation

3.2.5.1. Product contacting surface preparation

The selected surfaces (25 cm^2) of stainless steel, glass, teflon and silicone were cleaned and dried. 100 µL of the swab standard solution was applied onto the coupons as shown in Figure 3.1 at the three concentration levels in triplicate to give theoretical concentrations of 0.2 µg/25 cm^2, 1 µg/25 cm^2 and 5 µg/25 cm^2. The coupons were allowed to dry before swabbing. The swabbing procedure was carried out as shown in Section 3.2.5.2 and Figure 3.2.

[10] 3 minute column equilibration time; The column was washed for 3 minutes at the end of each chromatographic run with the same mobile phase composition as the start of the gradient. This step was employed to re-equilibrate the column back to the original conditions before each injection.

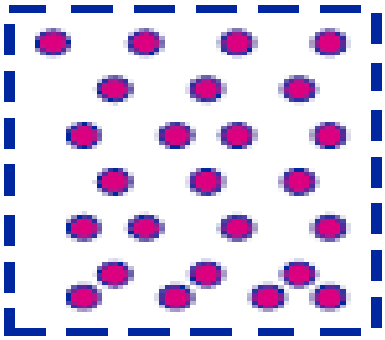

Figure 3.1: Application of the swab standard solution onto the coupons.

3.2.5.2. Swabbing procedure

- The region to be swabbed was defined.
- The swab head was dampened with the selected extraction solvent.
- As shown from Figure 3.1, Step 1 and Step 2, the product contact surface was swabbed with an overlapping pattern. The swab was flipped over and the step was repeated, passing the swab in a perpendicular direction. The product contact surface was swabbed with the entire swab head flat against the surface.
- The above swabbing process was repeated with a second swab at a 45° angle as shown in Step 3, Figure 3.2. The swab was flipped over and the step was repeated, passing the swab in a perpendicular direction (step 4).
- The swab heads was cut, placed into a sample vial and sonicated for 5 minutes to extract the residue.
- The extracted sample was transferred to a HPLC vial for analysis.

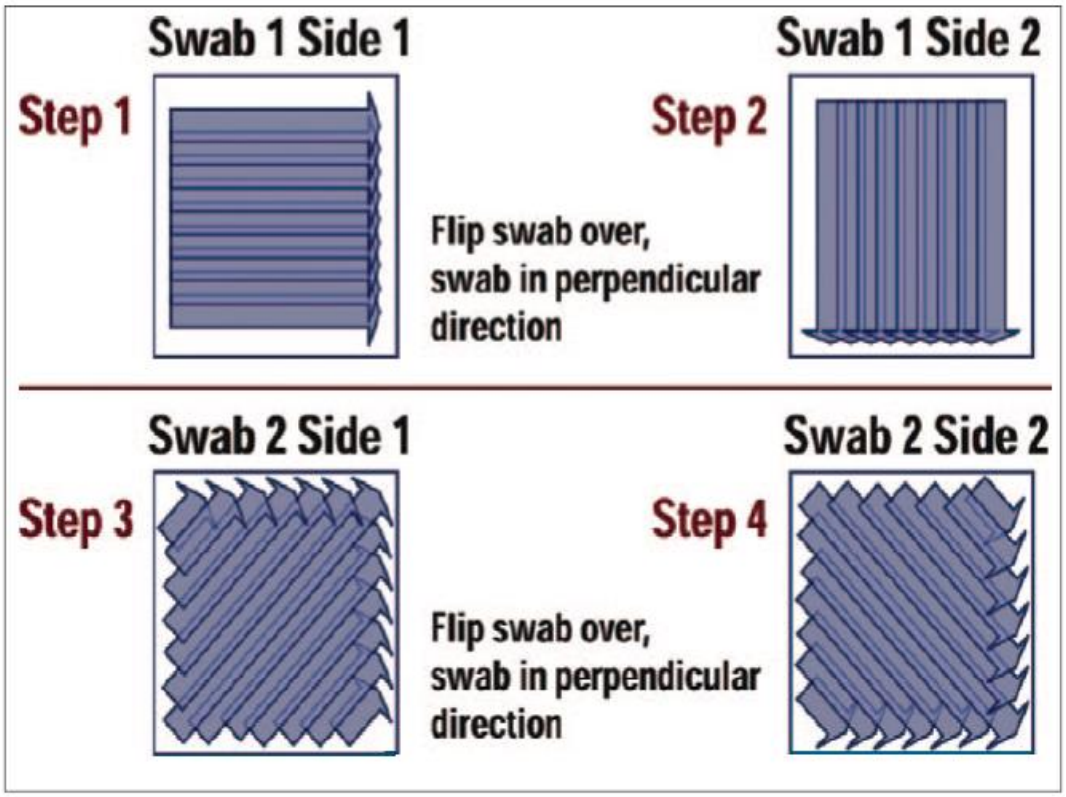

Figure 3.2: Swabbing procedure, taken from [10].

3.3. Results and discussion

3.3.1. Optimisation of swab sampling methodology

The surface of a manufacturing equipment line consists of mainly (>95%) stainless steel, but there are critical surfaces, which are made of teflon (PTFE, Polytetrafluoroethylene), glass, and silicone. The desired criteria were minimal interferences from the swab, stainless steel, glass, teflon and silicone surfaces. Sampling recoveries of ≥ 70% with an RSD value of ≤ 15% were the desired criteria, but swab sampling recoveries of ≥ 50% with an RSD value of ≤ 15% were deemed acceptable for the selected APIs (BMT, OFL, TIM, BUN, POH, DEX, KET, PAC, FML and FBP), as a correction factor will be applied to correct for the true amount of residue present on the manufacturing equipment surfaces as discussed in Chapter 1, Section 1.4.4.2.

3.3.1.1. Swab interferences

One of the most widely used types of swab material for cleaning validation is a polyester swab. Polyester swabs generally offer high absorbency of residues, ultra- low particulates, and minimal extractable interferences [7-10]. Several different swabs were analysed to determine the level of particulates and extractable interferences as shown in Figure 3.3.

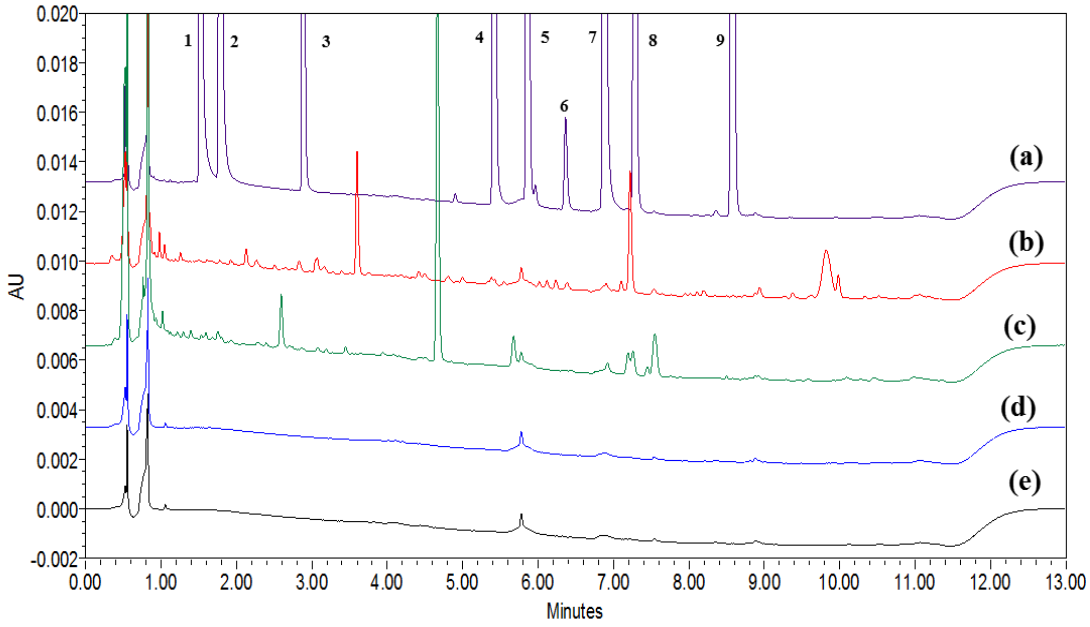

Figure 3.3: The comparison of interferences from different types of swabs overlaid with a standard containing the nine drug substances: (a) (1) OFL, (2) BMT, (3) KET, (4) FBP, (5) POH, (6) TIM, (7) BUN, (8) DEX, and (FML), (b) a foam swab, (c) a cotton swab, (d) a TX761 polyester swab and (e) a constix SP-3 polyester swab.

3.3.1.1.1. Impact of interferences from swab

To determine the impact of particulates and extractable interferences from the swab, several different types of swabs were placed into 5 mL of water, sonicated for 5 minutes and analysed by UPLC. The effect of particulates and extractable interferences from each swab is shown in Figure 3.3. The swabs with the least amount of particulates and extractable interferences were the polyester swabs; TX761 (d), and the constix swab (e). However, as can be seen from Figure 3.3, there is a potential interference from the TX761 (d) and Constix (e) swab at approximately 5.8 minutes.

3.3.1.2. Optimisation of swab sampling methodology using Minitab

As shown previously from Figure 3.3, the polyester swabs; TX761 and the Constix SP-3 swab showed the least amount of particulates and extractable interferences. Therefore, these two swabs were evaluated for recoveries for the ten APIs on stainless steel surfaces using Minitab. As described previously in Chapter 2, a two level fractional factorial Design of Experiments (DOE) was used to investigate several different factors simultaneously instead of changing only one factor at a time. The other factors investigated were the use of 1 swab/2 swabs and solvent type (ACN and MeOH). A standard solution (BMT, OFL, TIM, BUN, POH, DEX, KET, FML, FBP, and a separate solution containing PAC) was applied onto stainless steel coupons to give a theoretical concentration of 0.2 µg/25 cm^2. The coupons were swabbed with one/two Constix/Texwipe swabs with either ACN or MeOH as the swabbing solvent. The swabs were placed in 5 mL of deionised water and sonicated for five minutes. The DOE parameters are outlined in Table 3.1.

Table 3.1: Two level fractional factorial DOE for swab recoveries on stainless steel.

Run Order	No. Swabs	Swab Type	Solvent
1	1	Texwipe TX761	ACN
2	2	Texwipe TX761	ACN
3	1	Constix SP-3	ACN
4	2	Constix SP-3	ACN
5	1	Texwipe TX761	MeOH
6	2	Texwipe TX761	MeOH
7	1	Constix SP-3	MeOH
8	2	Constix SP-3	MeOH

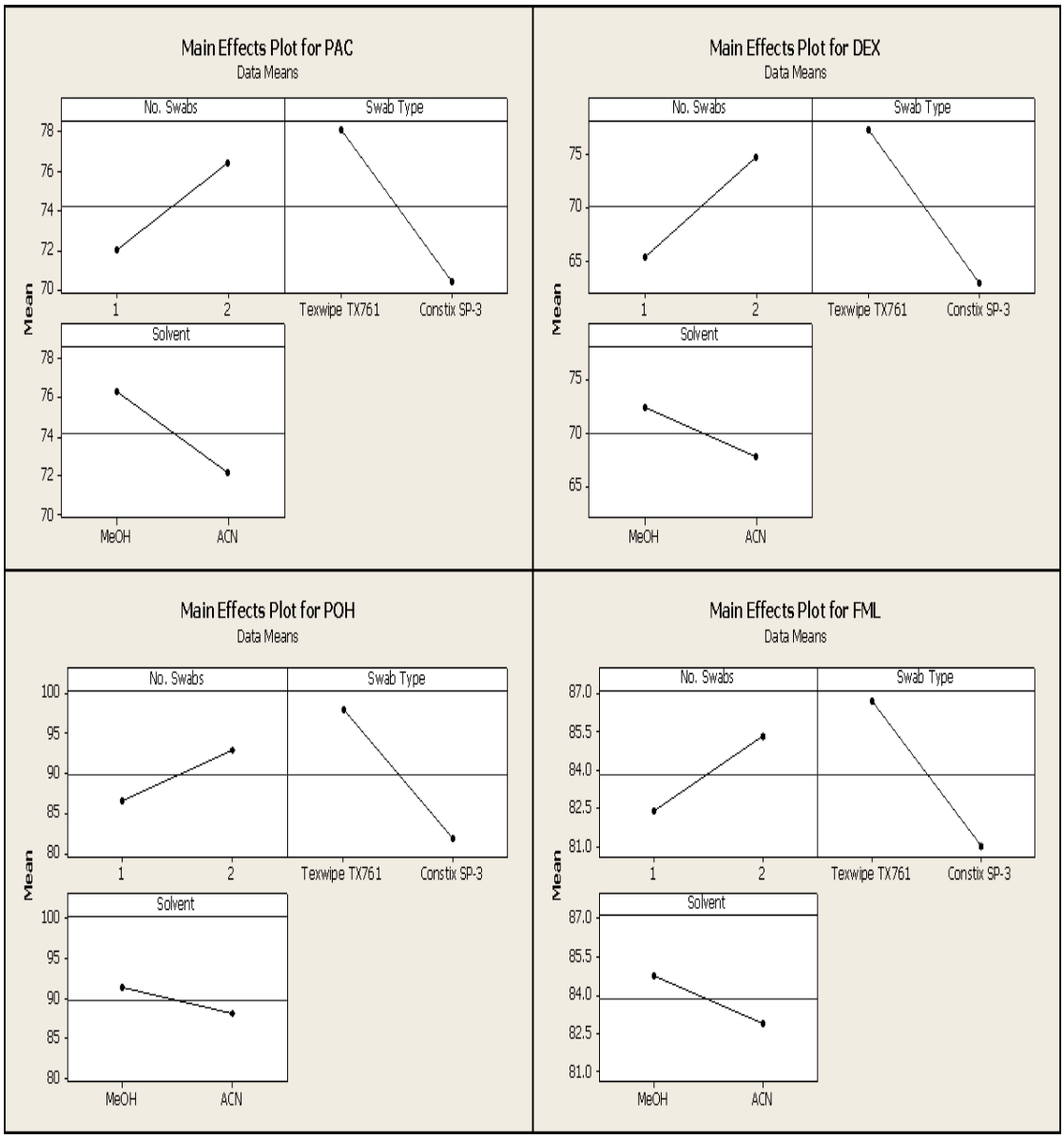

Figure 3.4: Main effects plot for PAC, DEX, POH and FML % recoveries on stainless steel using number of swabs, type of swab and type of solvent as the factors.

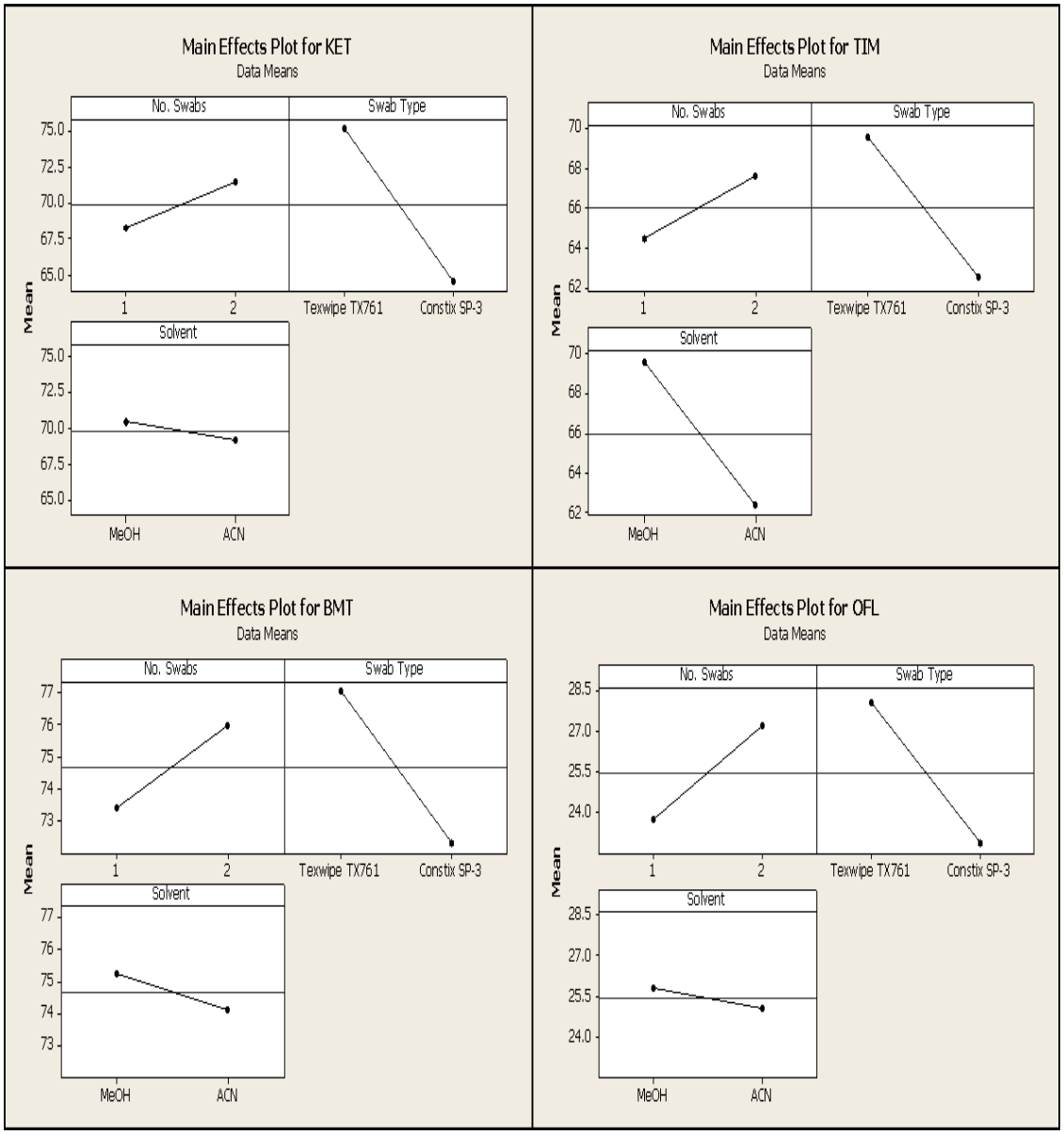

Figure 3.5: Main effects plot for KET, TIM, BMT and OFL % recoveries on stainless steel using number of swabs, type of swab and type of solvent as the factors.

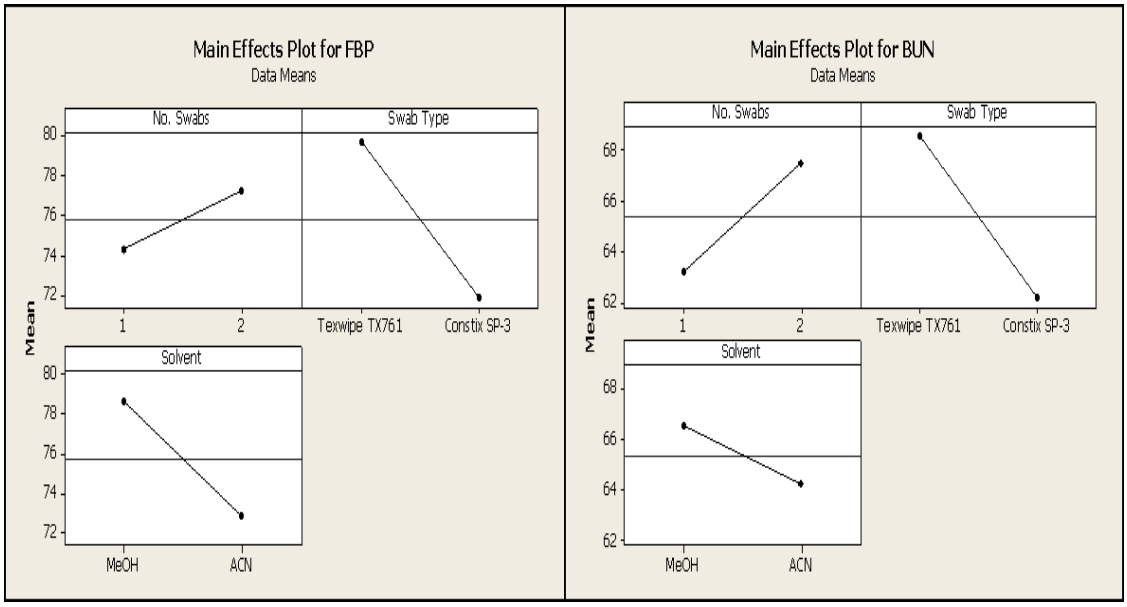

Figure 3.6: Main effects plot for FBP and BUN % recoveries on stainless steel using number of swabs, type of swab and type of solvent as the factors.

The impact of the various factors on the % recoveries of the selected APIs from stainless steel was assessed by a main effects plot as shown in Figures 3.4 - 3.6. As shown from the main effects plots, the use of 2 tex-wipe TX761 swabs using methanol as the swabbing solvent gave the best recoveries for the stainless steel surface. The recoveries of OFL from all the different runs however were only ≤ 30%. However, this was not surprising as 0.2 µg/swab equates to 0.04 µg/mL, after dilution (5 mL) which is below the LOQ of the analytical method for OFL[11].

Removal of the APIs from glass, teflon and silicone were evaluated using the optimised parameters from the Minitab experiment. Recoveries were acceptable for glass and teflon (≥ 50%) at 0.2 µg/25 cm^2 for all drug substances, but only DEX, POH, PAC, FML, BMT, and KET met the target of ≥ 50% for the silicone surface, as shown in Table 3.2. TIM, BUN and FBP did not meet the target acceptance criteria of ≥ 50% as outlined in Table 3.2. Optimisation of the drug substance recoveries from the silicone surface is described in Section 3.3.1.3.

[11] The swab recoveries of OFL were not sufficient at 0.2 µg/25 cm^2, and as mentioned in Chapter 2, the limit of quantitation of the analytical method is higher than the residue limit for an OFL swab as defined by Allergan Pharmaceuticals. Therefore, no further recovery work for OFL was carried out simultaneously with the other selected drug substances.

3.3.1.3. Optimisation of swab recoveries on silicone surfaces

As mentioned above BUN, FBP, and TIM did not the acceptance criteria of ≥ 50% recovery from the silicone product-contacting surfaces. Therefore, ethanol and isopropanol were investigated as alternative solvents for the removal of the selected APIs from the silicone surfaces. The API swab standard solution was applied onto the silicone surface at 0.2 µg/swab and was swabbed as per swabbing procedure in Section 3.2.5.2.

Table 3.2: Swab recoveries from silicone surfaces.

API	**% recovery using methanol**	**% recovery using ethanol**	**% recovery using isopropanol**
BMT	61.8	64.6	63.4
BUN	31.9	61.1	42.9
DEX	71.1	74.7	70.9
FBP	28.0	33.3	28.8
FML	71.6	82.2	80.8
POH	95.4	95.4	95.4
PAC	73.5	76.9	75.6
KET	64.1	71.1	64.2
TIM	23.5	51.3	33.3

3.3.1.3.1. Impact of methanol on recoveries from silicone surfaces

The impact of using methanol to recover the selected APIs from silicone was evaluated and the results show that BMT, DEX, FML, POH, PAC, and KET can be recovered to an acceptable level (≥ 50%). However, the results show that BUN, FBP and TIM obtained recoveries of approximately 20-30% which is not an acceptable amount to recover, as per regulatory standards.

3.3.1.3.2. Impact of isopropanol on recoveries from silicone surfaces

The results from using isopropanol to recover the APIs from the silicone surfaces are quite similar to the methanol results. Again, BUN, FBP and TIM did not achieve the ≥ 50% criterion, however the recoveries for BUN and TIM were approximately 10% higher, but the recovery of FBP remained the same (28%).

3.3.1.3.3. Impact of ethanol on recoveries from silicone surfaces

As shown from Table 3.2, using ethanol to recover the APIs from the silicone surfaces achieved the best results overall. BMT, BUN, DEX, FML, POH, PAC, KET, and TIM all obtained recovery values of ≥ 50% which was deemed as an acceptable result. However, FBP still only obtained recoveries of approximately 30% using ethanol.

3.3.1.3.4 Impact of ethanol, methanol and isopropanol on recoveries of FBP from silicone surfaces

Recoveries of ≥ 50% for FBP were not achievable from the silicone surfaces at 0.2 µg/25 cm^2 using methanol, ethanol or isopropanol. Therefore, an additional study was carried out where a standard solution was prepared containing FBP only. The standard containing only FBP was applied to the silicone surface at 0.2 µg/25 cm^2 and swabbed as before using ethanol. Ethanol was the solvent chosen as it gave the best recoveries overall for the removal of the selected APIs from the silicone surfaces and ethanol also gave slightly higher recoveries for FBP over methanol or isopropanol. The result achieved from this analysis was 51%, which just meets the ≥ 50% criterion.

3.3.1.4. Discussion of silicone surface recovery results

The results from Section 3.3.1.3 show that certain APIs are more difficult to remove from silicone than other product contacting surfaces. As shown, the steroids (PAC, POH, DEX and FML) are more easily recovered from the silicone surfaces than the salt based APIs (BUN, TIM, BMT). It is interesting to note that the TIM, BUN, and BMT are more water soluble than the steroids and most all the drug substances are 'slightly soluble' in alcohol according to USP solubility descriptions.

According to USP solubility descriptions, FBP is 'freely soluble' in dehydrated alcohol (ethanol), but this drug substance showed the lowest recovery values from silicone than the other drug substances. FBP is relatively non polar as it is 'sparingly soluble' in water, therefore it may have been more tightly bound to the nonpolar silicone surface than some of the other drug substances. It can be difficult to predict what APIs will be easily recovered or more difficult to recover from certain surfaces such as silicone as discussed below.

One example of insufficient recoveries obtained from silicone for certain drug substances is from a paper regarding a UPLC method and related swab sampling methodology that was developed for a range of steroids in a hormone pilot plant. The surfaces that were evaluated in this research were stainless steel, plexi-glass, teflon, textile and silicone. Recoveries of ≥ 70% were achieved for the seven steroids for stainless steel, plexi-glass, teflon and textile. However, only three of the steroids achieved recoveries of ≥ 70% from the silicone surface. Three of the steroids did not exceed the value of 70% (50-70%) and one steroid obtained a value of ≤ 50%. Additional studies were performed investigating the use of a variety of solvents that included methanol, acetonitrile and ethanol. However, the use of these solvents did not increase the recoveries of the problematic steroids [11].

Another study was conducted where recovery results were evaluated from a series of tests and showed that different materials of construction could be grouped into five different categories based on recovery performance, taking into account factors such as swab material, material of construction, solvent type, residue solubility, and sampling technique. These grouping however could not be correlated to the product contacting surface properties, e.g. different types of plastic were noted in the five different groupings. One point mentioned in the article is that for materials such as neoprene, EDPM, latex or silicone, the transport of compounds through these materials occurs more rapidly. It is also noted that the recovery of a selected API on a particular product contacting surface cannot be predicted. In order to predict the recovery outcome of the API from the surface, specific measurements of the fundamental transport properties from the solvents, APIs and materials of construction would have to be evaluated [12-13].

3.3.1.5. Interferences from the TX761 swab and selected surfaces

As shown from Figure 3.3, there is a potential interference near the retention time of POH from the TX761 swab. The chromatogram below shows the swab standard solution swabbed from stainless steel, glass, teflon and silicone using the TX761 swab, as well as the standard solution just spiked onto the TX761 swab. The only potentially interfering peak is at approximately 5.6 minutes with a resolution of 1.3 between the peak originating from the TX761 swab and the POH peak. This does not interfere with the quantitation of POH.

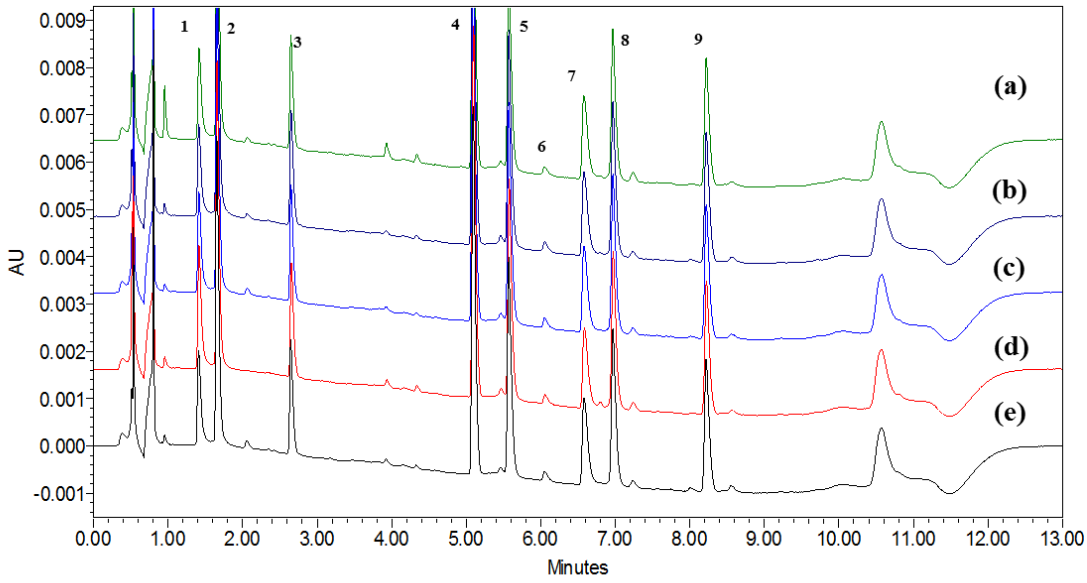

Figure 3.7: The comparison of interferences from the selected surfaces and the TX761 swab; (a) silicone, (b) teflon, (c) glass, (d) stainless steel, (e) TX761, (1) OFL, (2) BMT, (3) KET, (4) FBP, (5) POH, (6) TIM, (7) BUN, (8) DEX, and (FML).

3.3.2. Swab sampling validation

3.3.2.1. Specificity

Specificity was determined by demonstrating that the swabs and stainless steel, glass, teflon and silicone surfaces did not interfere with the quantitation of BMT, BUN, DEX, FBP, FML, POH, PAC (254 nm), and KET, and TIM (295 nm). To show that the determination of the drug substance residues were selective and free from any interferences, blank extractions of the swab material and blank swabs from the stainless steel, glass, teflon and silicone surfaces were injected. There were no interferences at the retention times of BMT, BUN, DEX, FBP, FML, POH, PAC (254 nm), and KET, and TIM (295 nm) that affected the recovery of the APIs from the selected surfaces. Therefore, the method can be considered as a specific method for these ten drug substances.

3.3.2.2. Accuracy (Swab sampling recovery studies)

The nine drug substances were spiked onto and sampled from stainless steel, glass, teflon and silicone at 3 different concentrations (0.2 µg/25 cm^2, 1.0 µg/25 cm^2 and 5.0 µg/25 cm^2) and accuracy was determined by the recovery data. The overall recovery (n=9) for each drug substance was calculated and the results are shown in Table 3.3. Overall recovery values of ≥ 70% were achieved for all drug substances on stainless steel, glass and teflon. For the silicone surface, FBP, TIM and BUN recoveries did not exceed 70%. However, the overall recoveries of these three drug substances were ≥ 50%, which meets regulatory standards.

3.3.2.3. Precision

Precision was established by the % RSD values of the accuracy recovery data (n=9) for each drug substance on each sampling surface. All % RSD values were ≤ 15% for the nine drug substances for stainless steel, glass, teflon and silicone as listed in Table 3.3.

3.3.2.4. Swab sample stability

The stability of the swab sample solutions (spiked and swabbed from stainless steel, glass, teflon and silicone) were studied at a concentration of 1.0 µg/swab for BMT, BUN, DEX, FBP, FML, POH, PAC, KET, and TIM. The sample solutions were stored in a sample compartment and were chromatographed five times within a 20 hour period. For each surface investigated, two injections of the swab sample solution were injected at 0, 5, 10, 15 and 20 hours. All drug substances for each time point investigated were compared to the 0 hour time point and the ratio of mean results were calculated. The sample solutions proved to be stable for each drug substance within a 20 hour period for all surfaces with ≤ 3% difference observed for each time point.

Table 3.3: Swab studies performance criteria for the determination of nine drug substances

Parameters	**DEX**	**FML**	**POH**	**PAC**	**KET**	**FBP**	**TIM**	**BUN**	**BMT**
Specificity[a]	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
Accuracy[b]	**DEX**	**FML**	**POH**	**PAC**	**KET**	**FBP**	**TIM**	**BUN**	**BMT**
Steel	84.7	83.4	103.1	70.7	86.8	85.6	82.2	76.8	86.3
Glass	86.8	83.4	100.2	84.4	80.4	86.2	83.1	78.7	78.2
Teflon	79.0	76.4	98.9	73.4	73.0	84.2	78.7	75.6	73.3
Silicone	81.1	70.8	98.1	78.8	77.9	52.5	67.8	69.5	84.8
Precision[c]	**DEX**	**FML**	**POH**	**PAC**	**KET**	**FBP**	**TIM**	**BUN**	**BMT**
Steel	3.3	5.1	2.5	9.6	2.9	2.3	10.2	13.9	3.9
Glass	4.3	9.1	4.4	13.2	13.2	3.4	9.4	8.3	10.2
Teflon	3.6	10.6	2.0	12.2	12.7	2.2	5.7	10.3	10.5
Silicone	6.8	10.1	6.5	6.9	3.0	12.7	10.8	7.7	8.6
Swab Sample stability[d]	**DEX**	**FML**	**POH**	**PAC**	**KET**	**FBP**	**TIM**	**BUN**	**BMT**
Steel	0.99	0.99	1.00	0.99	0.99	0.99	1.01	0.97	0.99
Glass	1.01	1.00	1.00	0.99	1.00	1.00	1.00	1.00	1.00
Teflon	1.00	1.00	1.00	0.99	1.00	1.00	1.02	0.98	1.00
Silicone	1.00	1.00	1.00	0.99	1.00	1.00	0.99	0.98	1.00

[a] The method is specific as there are no interferences from the stainless steel, glass, teflon and silicone, and TX761 swab that interfere with the quantitation of the drug substances
[b] Calculated n=9 on overall recovery for each drug substance
[c] Relative standard deviation (R.S.D.) of recovery data (n=9)
[d] Ratio of drug substance concentration at 20 hours versus drug substance concentration at 0 hours

3.4. Conclusion

A swab sampling methodology was developed in combination with the analytical UPLC method that was developed in Chapter 2 to show that trace levels of API residues can be effectively removed from the selected surfaces on the manufacturing equipment. The swab sampling methodology was found to be specific, precise and accurate as per guidances and regulations. The swab sampling recovery results show a recovery of ≥ 70% for the selected APIs on stainless steel, glass and teflon. An overall recovery of ≥ 70% was achieved for DEX, FML, PAC, POH, KET and BMT for silicone and a recovery of between 50 and 70% was achieved for BUN, FBP and TIM which meets regulatory standards. However, the recovery values obtained for the removal of FBP from silicone surfaces were approximately 50%. In a situation where the recoveries obtained are ≤ 50%, it is advised to apply these silicone surfaces, i.e. gaskets, as dedicated parts of the manufacturing equipment.

3.5. References

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Chapter 4

Final Conclusions

In recent years, increased emphasis has been placed in pharmaceutical companies for implementing robust validated cleaning procedures. The cleaning procedure is one of the most important tasks to avoid contamination and cross-contamination of APIs in pharmaceutical products manufactured on the same equipment trains. The cleaning procedures must be supported by appropriate analytical methods and related sampling methodologies. The cleaning analytical methods and related sampling methodologies are critical in establishing quality validated cleaning programs, as they quantitatively determine the amount of residual API, if present, on the manufacturing equipment post-cleaning. Therefore, the aim of this project was to develop and validate a robust cleaning analytical method and related sampling methodologies for the determination and quantitation of up to ten APIs in ophthalmic solutions. The challenge was to chromatographically separate up to 10 different drug substances which were a mixture of acidic, basic and neutral drug substances. In addition, the sampling methodologies were a critical aspect of the project. Several different product contacting surfaces were evaluated to determine whether the API residues could be adequately removed from the surfaces whilst ensuring minimal interferences from the swab and manufacturing equipment surfaces.

The results in Chapter 2 show the development of a relatively rapid and robust UPLC method for the determination and quantitation of nine drug substances simultaneously. The APIs were efficiently separated on a C18 charged surface hybrid column (CSH) with sub-2 µm particles. The method was run at a low flow rate, thus using small amounts of mobile phase. Standard analytical performance criteria were used for evaluation purposes, and the method was found to be both linear ($r^2 > 0.999$) and precise (%RSD < 2.0%), with LOQs as low as 0.01 µg/mL.

The results in Chapter 3 show the development of a robust swab sampling procedure for the adequate removal of nine API residues from various product contacting manufacturing surfaces including stainless steel, silicone, glass and teflon. The APIs were sufficiently removed using methanol as the swabbing solvent for stainless steel, glass and teflon and ethanol was chosen for the removal of the various APIs from the silicone surfaces. The swab chosen for this was the TX761 polyester swab which also proved to be the swab with the least amount of interferences and extractables that may interfere with the analysis of the selected APIs. The swab sampling methodology was found to be both accurate and precise with recoveries of ≥ 50% for all surfaces with RSD values of ≤ 15% which meet regulatory standards.

However, it has been shown that flurbiprofen was more difficult to remove from the silicone surfaces than the other APIs. The surfaces of most manufacturing equipment trains consist of mainly stainless steel, but there are surfaces such as gaskets or transfer line pipes that are made from silicone. These critical surfaces that cannot achieve a recovery of $\geq$ 50% during the swab sampling studies should become dedicated pieces of equipment to the production of the formulation containing the problematic API.

Future work could involve an examination of the removal of problematic APIs from critical surfaces on the manufacturing equipment trains such as silicone.