Recombinant Technology

Production of a recombinant anti-morphine-3-glucuronide single-chain variable fragment (scFv) antibody for the development of a "real-time" biosensor-based immunoassay

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Abstract

A recombinant single-chain variable fragment (scFv) antibody to morphine-3-glucuronide (M3G) was produced using genetic material obtained from the spleen cells of mice immunised with a morphine-3-glucuronide-bovine serum albumin (M3G-BSA) conjugate. Immunoglobulin light (V_L) and heavy (V_H) chain genes were amplified and cloned into pAK vectors for generation of recombinant antibody fragments in *Escherichia coli*. A competition ELISA assay was developed in PBS to characterise the ability of the antibody fragments to recognise free drug and the detection limits were found to be as low as 3 ng ml^{-1}. Surface plasmon resonance-based inhibition immunoassays were developed. The recombinant antibody was pre-incubated with various concentrations of free drug followed by injection over a morphine-3-glucuronide-thyroglobulin (M3G-THY) immobilised surface. The response of antibody binding to the surface of the chip was inversely proportional to the amount of free drug in solution. Regeneration conditions for antibody binding to the surface were optimised resulting in a binding-regeneration capacity of at least 30 cycles. The inhibition assay for M3G was tested with assay ranges between 3 and 195 ng ml^{-1} and 3 and 97 ng ml^{-1} in PBS and urine, respectively.

Keywords: Recombinant antibody; Morphine-3-glucuronide; ELISA; Surface plasmon resonance; Biosensor; Inhibition assay

Abbreviations: scFv, single chain variable fragment; M3G-BSA, morphine-3-glucuronide-bovine serum albumin; V_L, immunoglobulin light chain variable genes; V_H, immunoglobulin heavy chain variable genes; PBS, phosphate-buffered saline; M3G-THY, morphine-3-glucuronide-thyroglobulin; ELISA, enzyme-linked immunosorbent assay; M6G, morphine-6-glucuronide; TLC, thin-layer chromatography; GC, gas chromatography; HPLC, high performance liquid chromatography; CE, capillary electrophoresis; PCR, polymerase chain reaction; SOE, splice by overlap extension; SPR, surface plasmon resonance; CM5, carboxy-methylated dextran 5; EDC, *N*-ethyl-*N*(dimethyl-aminopropyl) carbodiimide hydrochloride; NHS, *N*-hydroxysuccinimide; OVA, ovalbumin; NE, non-expression medium; CFU, colony forming units.

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1. Introduction

Heroin is rapidly deacetylated to 6-monoacetyl morphine after ingestion. This is converted to morphine. Glucuronidation of morphine in the liver and intestines via the phenolic hydroxyl group, results in the production of morphine-3-glucuronide (M3G), the main metabolite (55%), followed by glucuronidation via the secondary alcoholic hydroxyl group producing morphine-6-glucuronide (M6G), the minor metabolite (10%), which possesses analgesic properties (Zheng et al., 1998). As M3G is the main metabolite formed in the body for both morphine and heroin (Moffat, 1986; Coyle, 1987; Gough, 1991) it is used as an indicator of drug abuse. Hence, a need exists for sensitive methods of detection, identification and confirmation of opiates in laboratory settings.

Various detection methods for opiates have been developed including thin-layer chromatography (TLC), gas chromatography (GC), high performance liquid chromatography (HPLC) and capillary electrophoresis (CE) (Braithwaite et al., 1995). However, these analytical methods are very time-consuming, require many clean up steps and are expensive. Antibody-based methods have been developed as favourable alternatives for the identification or quantification of opiates as these methods are easy to perform, cost-effective and robust. Previous immunoassays for these compounds (Gough, 1991; Usagawa et al., 1993) have primarily been based on the use of either polyclonal or monoclonal antibodies. However, more recently, recombinant DNA techniques have been used for the production of antibodies in *Escherichia coli* (Hoogenboom et al., 1998; Strachan et al., 2002). This technology facilitates the production of a range of novel antibodies and antibody derivatives and offers several advantages over both polyclonal and monoclonal antibody production. These advantages include the speed of antibody generation, the ability to produce numbers of different antibodies from a library and the possibility of altering the antibody affinity and specificity to suit the requirements of the application. These advantages enable the isolation of specific antibodies tailored for the particular application of interest to the researcher.

The main requirement of recombinant antibody technology, using hybridomas or immune repertoires as the source of DNA, is the reliable cloning of functional light and heavy chain genes. For this purpose, Krebber et al. (1997) developed a standard phage display system optimised for robustness, vector stability, tight control of scFv-Δgene III expression, primer usage for PCR amplification of variable region genes, scFv assembly strategy and subsequent directional cloning using a single rare cutting restriction enzyme. The Krebber system allows the rapid harvesting of antigen binding scFv's derived from spleen cell repertoires of mice immunised with M3G conjugates.

The increasing demand for faster and simpler methods of detection increase the need for biosensors. The availability of rapid and simple biosensor procedures could significantly improve the efficiency of routine drug analysis. The BIAcore biosensor is based on the phenomenon of surface plasmon resonance (SPR). This allows the detection of biomolecular interactions in 'real-time'. The BIAcore biosensor continuously monitors changes in the refractive index which are a direct result of changes in the mass or concentration on the surface of the chip and this characteristic of SPR has been used to monitor biological interactions.

BIAcore has been used in many different assay type formats including the two most common types of interactions which are antigen–antibody interactions (Gaudin et al., 2001; Gillis et al., 2002) and receptor–ligand interactions (Rock et al., 1996). Promising novel applications are also becoming available such as mass spectroscopy coupled to BIAcore, the use of SPR for thermodynamic studies and also the use of SPR to monitor purification of ligands (Fivash et al., 1998).

This paper describes the production of anti-M3G recombinant scFv antibodies in *E. coli* using the method developed by Krebber et al. (1997). Characterisation of the recombinant antibody was carried out using ELISA. This was followed by the development of inhibition assays to M3G in urine using sensor-based regenerable assay formats.

2. Materials and methods

All reagents and chemicals were supplied by Sigma (Poole, Dorset, England), unless otherwise stated. Carboxy-methylated dextran was obtained from Fluka Chemicals (Gillingham, Dorset, England). BIAcore 3000™ and CM5 sensor chips were both supplied by BIAcore AB (Uppsala, Sweden).

2.1. Production of protein conjugates to morphine-3-glucuronide

A 50 mg ml^{-1} solution of morphine-3-glucuronide (M3G) was prepared in 50 mM HCl and made up to a final volume of 5 ml with 0.2 M borate buffer, pH 8.5. Concentrations of EDC (*N*-ethyl-*N*-(dimethyl-aminopropyl) carbodiimide hydrochloride) and NHS (*N*-hydroxysuccinimide) were added to give a final molarity of 0.4 and 0.1 M, respectively, and incubated at room temperature without agitation for 10 min. Protein (BSA, OVA, THY) was prepared at a molar ratio of 1:100 to M3G in 5 ml of 0.2 M borate buffer, pH 8.5, and added dropwise to the M3G solution. This solution was then incubated at room temperature for 2 h with stirring. The solution was dialysed against 50 volumes of phosphate-buffered saline solution (PBS-pH 7.3, 0.15 M NaCl) overnight at 4 °C.

2.2. Production of murine scFv antibody libraries to morphine-3-glucuronide

A 1 mg ml^{-1} solution of M3G-BSA was prepared in PBS and added to an equal volume of Freund's complete adjuvant. Five- to ten-week-old BALB/c mice were immunised subcutaneously. Further intraperitoneal immunisations were carried out after 21 days using Freund's incomplete adjuvant. The mice were bled 7 days later and the antibody titre against the specific drug determined. The reboosting and bleeding procedure was repeated until an adequate titre of no less than 1/1000 was obtained. The animals were then immunised intravenously through the tail vein with 1 mg ml^{-1} M3G-BSA in PBS 3–4 days prior to sacrifice. This was followed by sacrifice by cervical dislocation and removal of the spleen. Total RNA was extracted from spleen cells using Trizol Reagent. About 7–10 μg of total RNA was reverse transcribed using random hexamer primers (Promega, Madison, WI 53711-5399, USA) to produce cDNA. ScFv's were constructed by assembling the amplified V_H and V_L cDNA by splice by overlap extension (SOE) PCR using a 20 amino acid linker, as described by Krebber et al. (1997), with minor modifications.

PCR purification of both the V_H and V_L genes was performed using the Wizard PCR prep DNA purification kit (Promega) followed by assembly into the scFv format by SOE-PCR. The SOE-PCR products were again gel purified, SfiI digested and ligated, following the manufacturers instructions (Promega), into the pAK100 vector (kindly donated by Dr. A. Plückthun, Universität Zürich, Switzerland). This was followed by electroporation into *E. coli* XL1-Blue supercompetent cells (Stratagene, La Jolla, CA, USA).

Expression of recombinant antibodies was carried out as described by Krebber et al. (1997) and used for subsequent affinity selection of scFv antibodies by panning to M3G.

2.3. Affinity selection of scFv antibodies by panning

Immunotubes (NUNC) were coated with 100 μg ml^{-1} of conjugate and incubated overnight at 4 °C. The tube was washed five times with PBS containing 0.05% (v/v) Tween (PBST) followed by five washes with PBS. Some 5% (w/v in PBS) marvel milk powder (blocking solution) was added to the immunotube and allowed to incubate for 1 h at 37 °C. After washing the immunotube (as before), 4 ml of PBS containing 2% (w/v) marvel and 10^{11} of phagemid particles was added and incubated for 2 h at room temperature with gentle shaking. This was followed by washing the immunotube to remove any non-specifically bound phage. Specifically bound phage particles were eluted from the immunotube by addition of 800 μl of 0.1 M glycine/HCl, pH 2.2, for 10 min with shaking. Eluted phage particles were then neutralised using 48 μl of 2 M Tris. Phage were reinfected into *E. coli* and grown on agar plates overnight.

Single colonies were selected from these plates and used to inoculate individual wells of a 96 well cell culture plate containing 200 μl 2 × TY containing 1% (v/v) glucose, 25 μg ml^{-1} chloramphenicol and 30 μg ml^{-1} tetracycline. Phage particle production was induced and 75 μl of the supernatant produced from each clone was used for analysis of phage by direct ELISA using an anti-M13 rabbit antibody to detect phage.

Positive individual clones were then inoculated into 5 ml of non-expression (NE) medium followed by inducing phage particle production for analysis by competitive ELISA to characterise the clones specific for free drug. ScFv's, with the ability to bind to free drug, were characterised by competitive ELISA.

2.4. Ligation of light and heavy chain genes from positive clones into pAK400 for soluble expression of scFv antibodies

Plasmids possessing the specific antibody genes were purified and digested with SfiI followed by gel purification of the scFv fragment. This was followed by ligation into previously digested pAK400 vector. JM83 calcium-chloride competent cells were transformed with pAK400 vector harbouring the plasmid containing the heavy and light chain genes for the scFv to M3G.

Soluble expressed of the scFv was carried out as described by Krebber et al. (1997) and purified using a 5 ml ProBond™ Resin column. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis were used to assess the purity of the antibody solution.

2.5. Development of BIAcore-based immunoassay

2.5.1. Coupling reaction of morphine-3-glucuronide-THY to CM-dextran gel

The carboxymethylated dextran (CM-dextran) matrix was activated by mixing equal volumes of 100 mM NHS (*N*-hydroxysuccinimide) and 400 mM EDC (*N*-ethyl-*N*-(dimethyl-aminopropyl) carbodiimide hydrochloride) prepared in ultra-pure water and injecting the mixture over the sensor chip surface for 7 min at a flowrate of 5 μl min^{-1}. The M3G-THY conjugate to be immobilised (concentration, 50–200 μg ml^{-1}) was dissolved in 10 mM acetate buffer, pH 4.3, and injected over the surface for 20 min at a flowrate of 2 μl min^{-1}. The unreacted sites on the sensor chip surface were then capped by injection of 1 M ethanolamine prepared in ultra-pure water, pH 8.5, for 7 min.

2.5.2. Sample preparation for sensor analysis and regeneration conditions required

The antibody was diluted in phosphate-buffered saline solution (PBS, pH 7.3, 0.15 M NaCl) at a concentration of 488 μg ml^{-1}. The antibody displayed negligible non-specific interactions for the immobilised protein THY and the CM-dextran surface negating the need for any pre-incubation steps with either THY or CM-dextran. All buffers and solutions used were made up using ultrapure water, de-gassed and sterile filtered.

Regeneration conditions for the removal of the scFv antibody from the surface of the chip were optimised and found to require a 1-min pulse of 10 mM NaOH.

2.5.3. Morphine-3-glucuronide preparation for use in inhibitive assay

A 10 mg ml^{-1} solution of free M3G was prepared in 50 mM HCl to reduce insoluble complexes forming. Standards of M3G were prepared in PBS containing 0.5 mM HCl ranging from 1.5 to 3125 ng ml^{-1} for the E3 scFv antibody. The same standard concentrations were prepared in urine when performing drug analysis in urine. Each sample was incubated separately with an equal volume of neat (976 μg ml^{-1}) E3 scFv antibody (for urine analysis the E3 scFv was prepared in 2 × PBS) and allowed to equilibrate for 1 h, and then passed over the surface of the chip. This was carried out three times for each concentration of drug for assay variability studies.

3. Results and discussion

3.1. Production of murine scFv antibody libraries to morphine-3-glucuronide

BALB/c mice were immunised with a carbodiimide-coupled bovine serum albumin conjugate to M3G (M3G-BSA). Serum from mice gave a very high response to M3G-OVA and the antibodies titred out at about 1/300,000. These serum titrations indicated a sufficient response from the host to justify isolating splenic mRNA. The concentrations of isolated mRNA taken from mice were approximately 10 μg ml^{-1}.

This splenic mRNA was used in the production of a recombinant antibody library. cDNA was initially produced followed by the amplification of the antibody light and heavy chain genes. Heavy and light chain genes were amplified by using primers which amplified a single band of approximately 400 bp for both (Krebber et al., 1997).

In many cases, primer sets have been too restricted to amplify either particular light or heavy chain genes. However, the set of primers developed by Krebber et al. (1997) and used in this paper have been extended and optimised. They incorporate all mouse V_H, V_λ and V_κ sequences collected by the Kabat data base (Kabat et

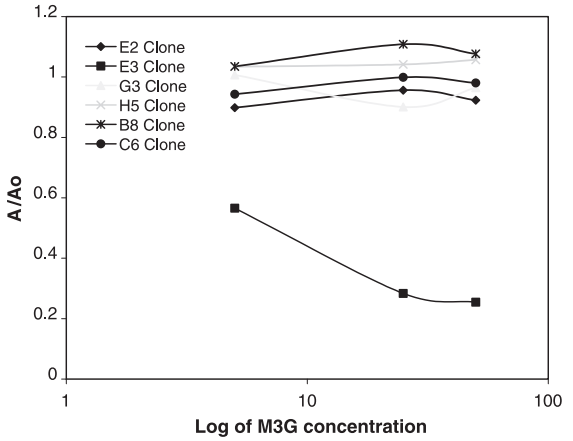

Fig. 1. Competition enzyme immunoassay carried out for free M3G at drug concentrations of 5, 25 and 50 μg ml^{-1} on supernatants from six scFv antibody clones picked from the first round of panning. B8, C6, E2, G3 and H5 scFv antibody clones showed no competition for free M3G. However, the E3 clone clearly shows competition.

al., 1991) and combine extended primer sets described by Kettleborough et al. (1993), Ørum et al. (1993) and Zhou et al. (1994). In all cases, the first PCR amplification yielded sufficient amounts of products for cloning, with a sharp band produced at the predicted band of 375–402 bp for V_L or 386–440 bp for V_H.

Purification of amplified light and heavy chain genes was performed followed by assembling both together by splice by overlap extension (SOE) PCR (Horton et al., 1989). To avoid the occurrence of incorrect overlaps during assembly PCR, the four (Gly_4Ser) repeats in the single chain linker region were encoded by different codons (Krebber et al., 1997). ScFv fragments may have a tendency to dimerize or aggregate. In order to reduce the occurrence of this, the linker between the V_L and V_H regions is 20 amino acids in length rather than the 15 amino acids long variant which is frequently used (Krebber et al., 1997). The resulting SOE-PCR product was a fragment with a combined molecular weight of approximately 800 bp. The SOE-PCR products and pAK100 vector were then digested with SfiI restriction enzyme followed by ligation of SOE-PCR with the pAK100 vector at a molar ratio of vector to insert of 1.5:1. These ligation products were then transformed into supercompetent *E. coli* XL1-Blue cells, with a transformation efficiency of 1.0×10^9 cfu/μg of DNA and used for the production of a recombinant antibody library to M3G with approximately 5×10^3 transformants. The failure to produce high numbers of transformants may be due to the inefficiency of the SOE-PCR and pAK100 to digest or to ligate together properly. Inadequate overhangs can reduce the efficiency of restriction digestion. However, due to the pre-immunised nature of the splenomic cells, which contain high levels of mRNA encoding antibodies specific for M3G, low levels of efficiency may in fact produce specific scFv antibodies.

3.2. Production of single chain Fv antibodies to morphine-3-glucuronide

After transformation of the ligated reaction into *E. coli* strain XL1-Blue, phage were produced (approx-

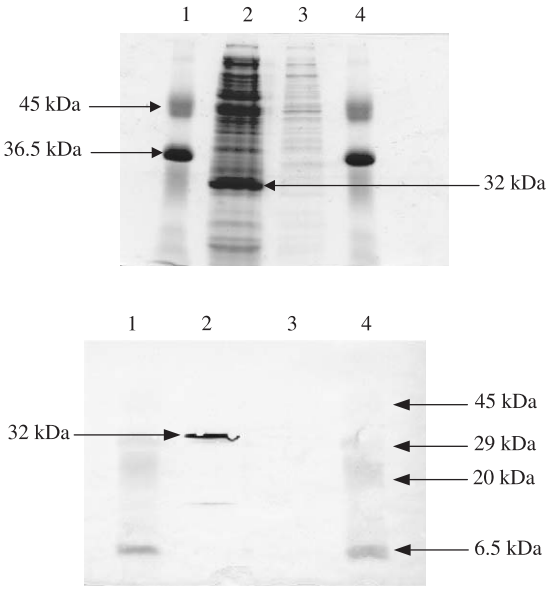

Fig. 2. SDS-PAGE gel analysis of purified E3 scFv antibody. Lanes 1 and 4 contain prestained molecular weight markers ranging from 26 to 116 kDa. Lane 2 represents concentrated purified E3 scFv antibody showing a strong molecular weight band of 32 kDa relative to the 36.5 kDa marker (Lactic Dehydrogenase). Lane 3 represents supernatant from E3 clone. (a) Western blot analysis of purified E3 soluble scFv antibody. Lanes 1 and 4 contain prestained molecular weight markers ranging from 6.5 to 45 kDa. Column 2 represents concentrated purified E3 scFv antibody showing a specific molecular weight of 32 kDa. Lane 3 represents supernatant from E3 clone. However, the supernatant was dilute and showed no band at 32 kDa.

imately 4×10^{10} phage particles) and panned initially against M3G-OVA as described in Section 2.3. Ninety-five individual clones from the M3G recombinant antibody library, following the first round of affinity selection, were screened on a plate coated with M3G-thyroglobulin (THY) for antigen binding in a typical phage ELISA. Results from the phage ELISA showed that all clones analysed possessed affinity to the conjugate producing absorbance readings of 1 or higher. Six positive clones, B8, C6, E2, E3, G3 and H5 were selected on the bases of the strength of their absorbance readings for characterisation with respect to their affinity for free drug by competition phage ELISA. Results showed that the E3 clone exhibited affinity for free M3G (Fig. 1).

For the production and purification of soluble scFv antibodies to M3G, the E3 clone antibody light and heavy chain genes were digested with SfiI restriction

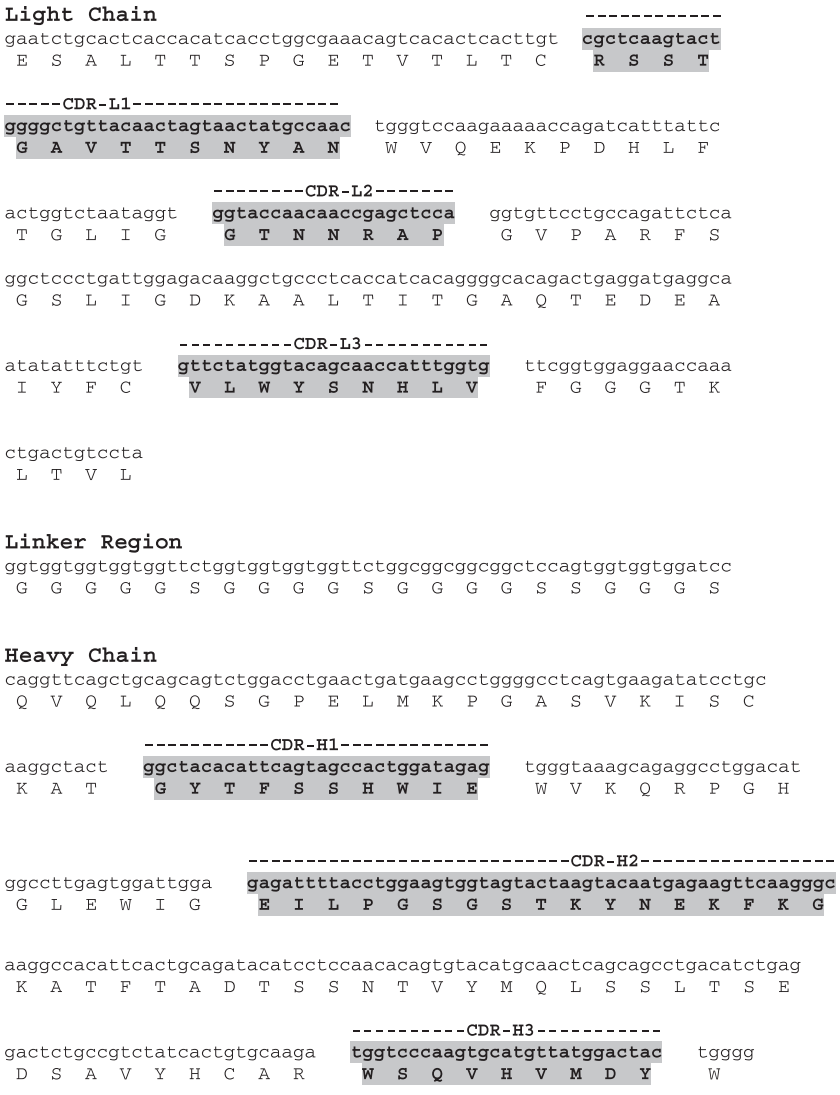

Fig. 3. Amino acid sequence of the V_H and V_L domains of the anti-morphine-3-glucuronide antibody. The CDR L1-3 and CDR H1-3 domains are clearly present. The glycine-serine sequence linker peptide can also clearly be seen. Numbering of amino acid residues and CDR designation is according to Kabat et al. (1991).

enzyme and ligated into previously digested pAK400 vector. These ligation reactions were transformed into calcium chloride JM83 competent cells resulting in the production of E3 clones capable of producing soluble scFv antibodies. Expression medium devoid of glucose was used to increase expression levels of recombinant protein (De Bellis and Schwartz, 1990). The pAK400 vector also contains a much stronger Shine-Dalgarno sequence (Krebber et al., 1997), resulting in a further significant enhancement of protein expression. However, the expected increase in expression levels did not occur for the E3 clone as a titration of purified and concentrated scFv was carried out in which the scFv titred out at a concentration of 30.5 μg ml^{-1} (1/32 dilution). However, adequate concentrations of antibody was produced to carry out further analysis.

A ProBond™ resin column was used in the purification of scFv antibodies. ScFv fragments produced in this library contain a six histidine tag allowing purification by IMAC (Lindner et al., 1992). These soluble E3 scFv antibodies were characterised by using SDS gel (Fig. 2) and by Western blot analysis (Fig. 2a). However, this strategy was not successful as can be seen in Fig. 2, as the concentrated sample of scFv contains a number of non-specific bands. This problem has been encountered before and may be due to the protein of interest being present in small fractions only. This results in several contaminating proteins binding to the column and co-eluting with the specific protein (Müller et al., 1998). Examples of such contaminating proteins produced by *E. coli* including superoxidase dismutase, chloramphenicol acetyltransferase, cAMP receptor protein, heat-shock protein and many more as described by Wülfing et al. (1994). The scFv was purified and concentrated to a total protein concentration of 976 μg ml^{-1} including contaminating proteins. The scFv sequence was also determined as shown in Fig. 3 illustrating the three CDR light and heavy regions and the linker region.

3.3. Development of a competitive enzyme-linked immunosorbent assay (ELISA) for morphine-3-glucuronide

The working dilution of these antibodies was determined using a checkerboard ELISA resulting in the optimal conjugate loading density of M3G-THY of 50 μg ml^{-1} and with the optimal antibody concentration determined to be 97.6 μg ml^{-1}.

To carry out intra-day assay variability studies, three sets of standards were prepared for the antibody and assayed on the same day and their means plotted. The intra-day assay variability study for E3 antibody had a linear range of detection between 3.05 and 48.8 ng ml^{-1}. The coefficients of variation (CVs) were determined to assess the precision of the analytical method at each drug concentration, expressing standard deviation as a percent function of the mean. These ranged from 2.64% to 8.03%. The inter-day assay variability study for E3 scFv antibody also had a linear range of detection between 3.05 and 48.8 ng ml^{-1}, with CVs between 2.52% and 12.55%. Overall the assay displayed excellent linearity with low limits of detection. However, the high concentration of antibody required (97.6 μg ml^{-1}) limited the number of assays that could be performed.

3.4. Cross-reactivity studies of E3 scFv antibody

Standard curves for each of the drugs were produced as already described for competitive enzyme-linked immunosorbent assay (ELISA) for detection of morphine-3-glucuronide. The results were normalised and plotted. The slope of the linear range of the standard curve for each drug was expressed as the percentage of the slope of the line for binding to morphine-3-glucuronide.

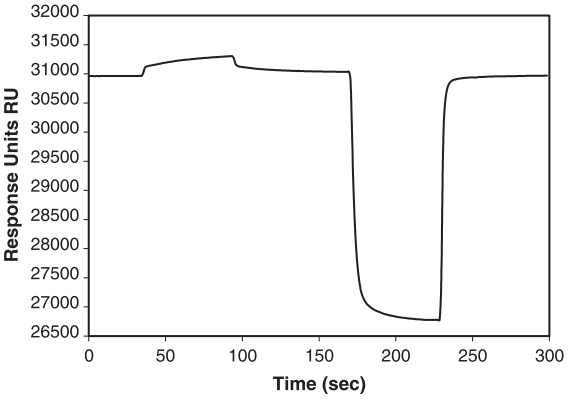

Fig. 4. Sensorgram illustrating a binding regeneration cycle. Antibody was passed over the surface of the chip followed by regeneration of the surface with a 1-min pulse of 10 mM NaOH.

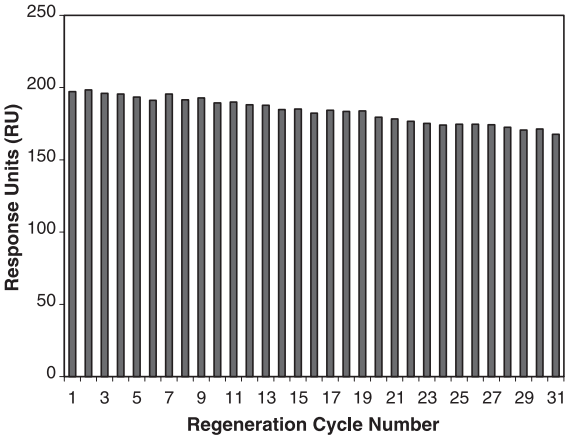

Fig. 5. Typical regeneration profile for approximately 30 cycles of a 2-min binding pulse of purified E3 antibody for the surface of a chip immobilised with M3G-THY. A total of 488 μg ml^{-1} of E3 scFv antibody was used and the surface regenerated with a 1-min pulse of 10 mM NaOH. After 30 cycles of binding and regeneration, the response after the injection of the last binding pulse was 167.7 RU compared with the first binding pulse of 197.2 RU. The ligand binding capacity was shown to decrease by 14.9% over the course of the regeneration cycles.

Results indicate the scFv antibody shows some cross reactivity with a number of structurally related molecules including morphine (23.9%), codeine (28.4%) and 6-monoacetylmorphine (15.7%) with very little cross reactivity to nor-codeine (6.5%). These results demonstrate that the scFv recognises an epitope common to some of the molecules related to M3G. Having shown that the E3 scFv antibody was suitable for use in an ELISA assay of this type, the aim was to develop a similar method of detection of M3G using the BIAcore biosensor.

3.5. Development of a recombinant antibody-based assay on BIAcore

For the development of a model inhibition BIAcore assay to M3G in PBS morphine-3-glucuronide-thyroglobulin (M3G-THY) was immobilised onto the surface of a CM5 sensor chip. The immobilisation chemistry used was EDC-mediated NHS esterification followed by passing over M3G-THY prepared in a solution of 10 mM sodium acetate, pH 4.3. Approximately 15,000 RU of M3G-THY were immobilised on the surface of the chip.

An important consideration when using BIAcore as an immunoassay tool is the ability of the surface of the chip to be reproducibly regenerated. The binding-capacity of antibody to conjugate immobilised surfaces must not decrease by more than 20% (Wong et al., 1997). A 488 μg ml^{-1} concentration of E3 scFv antibody was found to produce a binding response of approximately 200 RU. Non-specific interactions of the E3 scFv antibody for both the carrier protein

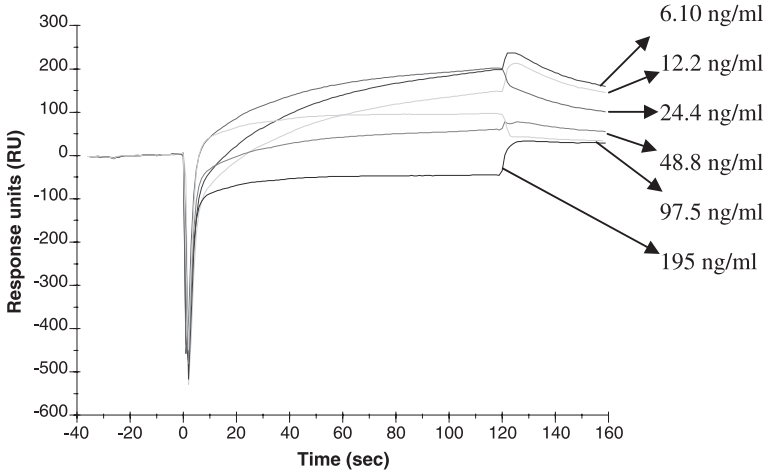

Fig. 6. An overlay plot for a typical set of binding curves in an inhibition assay. M3G standards ranging from 6.10 to 195 ng ml^{-1} of free drug were mixed with E-3 scFv antibody and passed over an M3G-THY-immobilised surface. The measured binding response was used to construct the calibration curves for the intra-day assay variability studies, and used to calculate the normalised binding response in conjunction with two other normalised binding response curves for the inter-day assay variability studies.

used to prepare the conjugate and the dextran matrix on the surface of the sensor chip were analysed and found to be negligible. Regeneration conditions for the E3 scFv antibody were optimised and found to require a 1-min pulse of 10 mM NaOH.

Surface regeneration studies were conducted by a series of antibody binding and regeneration pulses (Fig. 4). These studies demonstrated that it was possible to regenerate the surface up to approximately 30 times (Fig. 5) with a 15% decrease in antibody binding capacity.

This was followed by the development of an inhibition assay. Free drug and antibody were pre-incubated and then passed over the surface of a chip immobilised with M3G-THY. A typical intra-day antibody binding response for each mixture of antibody and drug concentration is shown in Fig. 6 illustrating a decrease in binding as drug concentration in solution increases. For the determination of the range of detection of M3G for a BIAcore inhibition assay, standards of M3G were prepared in PBS ranging from 1.5 to 3125 ng ml^{-1} for the E3 scFv antibody. The range of detection for the E3 scFv antibody was found to be between 3.05 and 195 ng ml^{-1}. Intra- and inter-day (Fig. 7) assay variability studies were carried out and CVs ranged from 0.62% to 1.55% and 0.96% to 6.36% (Table 1), respectively. Overall, these results indicate that the E3 scFv anti-M3G antibody gives accurate, specific and reproducible assays in PBS.

Table 1
Inter-day assay CVs for E3 scFv antibody BIAcore inhibition assay

M3G concentration (ng ml^{-1})	Calculated mean ± S.D. (R/R_0)	Coefficients of variation (CVs) (%)
195	0.186 ± 0.008	4.56
97.5	0.212 ± 0.011	5.51
48.8	0.342 ± 0.016	4.77
24.4	0.600 ± 0.038	6.36
12.2	0.820 ± 0.040	4.94
6.10	0.898 ± 0.051	5.72
3.05	0.945 ± 0.009	0.96

Three sets of seven standards ranging from 3.05 to 195 ng ml^{-1} were analysed over three different days and the CVs calculated.

The quantitative determination of M3G in urine samples was also investigated. Known samples of M3G ranging from 1.5 to 3125 ng ml^{-1} were spiked into control urine and assayed for the presence of free drug using the E3 scFv antibody. A calibration curve was constructed and the range of detection in urine

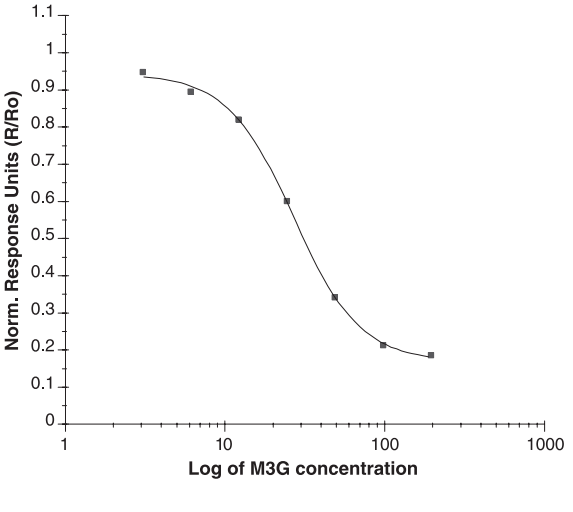

Fig. 7. Inter-day assay curve for M3G using the E3 scFv antibody on an M3G-THY-immobilised chip surface. The results shown are the average of triplicate results and the range of detection of free M3G was found to be between 3.05 and 195 ng ml^{-1}. The binding response at each M3G concentration (R_{AG}) was divided by the antibody binding response determined in the presence of zero M3G concentration (R_0) to give a normalised binding response (R/R_0).

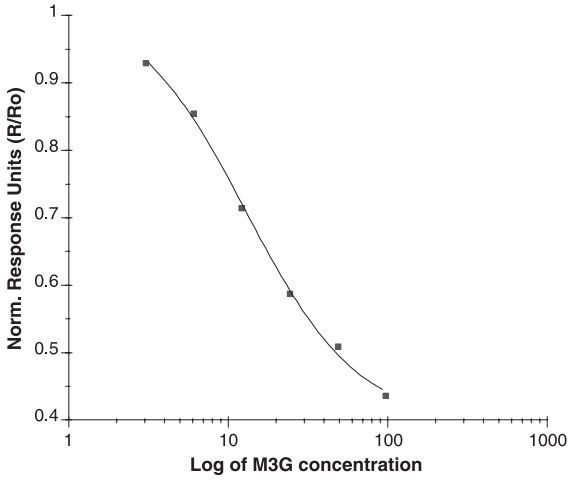

Fig. 8. Inter-day assay curve for M3G in urine using the E3 scFv antibody on an M3G-THY-immobilised chip surface. The results shown are the average of triplicate results and the range of detection of free M3G was found to be between 3.05 and 97.5 ng ml^{-1}. The binding response at each M3G concentration (R_{AG}) was divided by the antibody binding response determined in the presence of zero M3G concentration (R_0) to give a normalised binding response (R/R_0).

Table 2
Inter-day assay CVs for E3 scFv antibody BIAcore inhibition assay in urine

M3G concentration (ng ml^{-1})	Calculated mean ± S.D. (R/R_0)	Coefficients of variation (CVs) (%)
97.5	0.435 ± 0.002	0.59
48.8	0.508 ± 0.093	18.38
24.4	0.587 ± 0.052	8.98
12.2	0.714 ± 0.068	9.58
6.10	0.854 ± 0.004	0.48
3.05	0.929 ± 0.007	0.77

Three sets of six standards ranging from 3.05 to 97.5 ng ml^{-1} were analysed over three different days and the CVs calculated.

was found to be between 3.05 and 97 ng ml^{-1}. The intra-day assay variability study for E3 had a range of detection between 3.05 and 97.5 ng ml^{-1} with CVs between 0.39% and 5.35%. The inter-day assay variability study carried out for the E3 scFv antibody had a range of detection between 3.05 and 97.5 ng ml^{-1} as shown in Fig. 8 and CVs ranged from 0.48% to 18.38% as shown in Table 2. A CV of 18.38% was seen for the 48.8 ng ml^{-1} concentration of M3G in the inter-day variability studies. However, all other CVs were below 10%, indicating that the assay may be used for sample analysis.

The assays developed in our lab to M3G using both the ELISA and BIAcore formats compare favourably to previously developed assays in the literature. Of the analytical techniques available, HPLC assays developed by Low and Taylor (1995), Zheng et al. (1998) and Beike et al. (1999) have detection limits down to low ng ml^{-1} levels compared to a detection limit of 3 ng ml^{-1} for the assays described here. However, the HPLC assay developed by Meng et al. (2000) and the RIA assays developed by Spector (1971) and Steiner and Spratt (1978) all have detection limits in the low pg ml^{-1} levels making these assays much more sensitive than the assays described in this paper. The suitability of our scFv-based assay for sample analysis is possible, as the assays range is suitable for the confirmatory cut-off concentrations for opiate abuse (i.e. morphine and codeine-2000 ng ml^{-1}, 6-monoacetylmorphine (MAM), 10 ng ml^{-1}).

When comparing the assays developed in both the ELISA and BIAcore formats, results show the detection ranges for M3G to be approximately the same for both. However, previous assays developed in our laboratory to M3G using polyclonal antibodies show the ELISA to provide lower detection limits compared to the BIAcore biosensor (Dillon et al., 2003) indicating the advantage of the scFv for analysis on the BIAcore biosensor. Furthermore, when comparing the M3G assay developed in this paper to previous assays developed to molecules such as comarin (Keating et al., 1999) and aflatoxin B_1 (Daly et al., 2000), a comparison of detection limits has proved favourable. Clearly, in both the ELISA and biosensor formats, the scFv produced is very sensitive and has low limits of detection in ELISA while also being suitable for use in biosensor applications. Once isolated large-scale production of antibody is relatively cheap making this approach important for analysis in future assay developments. The availability of such an antibody-based biosensor system may also have a significant impact on routine surveillance and analysis of drug abuse.

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