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Short communication

Modification of the ultrafiltration technique to overcome solubility and non-specific binding challenges associated with the measurement of plasma protein binding of corticosteroids

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Abstract

Plasma protein binding (PPB) methodology suitable for application in the lead optimisation of a corticosteroid series known to demonstrate non-specific binding (NSB) and poor solubility has been established. The method involved a modification to standard ultrafiltration (UF) techniques. In parallel with each experimental plasma sample, a control plasma sample was also processed by ultrafiltration. The retentate from experimental and control plasma samples were mixed back into the filtrate of the partner sample. The resulting regenerated plasma samples, one representing the experimental filtrate and one representing the experimental retentate, were then analysed by LC/MS/MS. Varying degrees of NSB were demonstrated with a number of corticosteroids, and this effect was eliminated using the modified method. Validation using a panel of established corticosteroids showed good agreement with published PPB figures. The published PPB figure for fluticasone propionate (FP) was, however, found to be an underestimate, and this was subsequently confirmed, at clinically relevant plasma concentrations, to be 99.3%. The modified method was particularly suited to lead optimisation because it provided samples in a consistent matrix compatible with standard high throughput LC/MS/MS analysis.

Keywords: Plasma protein binding; Non-specific binding; Ultrafiltration; Corticosteroids; Fluticasone propionate

1. Introduction

It is well established that plasma protein binding (PPB) can have a significant impact on the pharmacokinetics and pharmacodynamics of a drug. Only the unbound fraction of a drug in plasma is available for many pharmacokinetic and pharmacodynamic processes, such as membrane permeation and receptor binding [1–3]. In the case of inhaled corticosteroids, it is widely believed that high protein binding is desirable in order to minimise systemic side effects and hence maximise the therapeutic index [4–6]. For this reason it was necessary to have an appropriate screen for plasma protein binding in place during the lead optimisation stage of a glucocorticoid receptor agonist research programme.

The two methods most commonly used to determine plasma protein binding are equilibrium dialysis (ED) and ultrafiltration (UF). In a lead optimisation setting, ultrafiltration generally has the advantage over equilibrium dialysis being a less time consuming process, and therefore, likely to have a higher throughput, although 96-well equilibrium dialysis methodologies with improved throughput have been reported [7,8]. The disadvantage of both equilibrium dialysis and conventional ultrafiltration is that they can be susceptible to non-specific binding (NSB) of test compounds to the polymer-constructed components of these devices. Whilst using a higher throughput method was desirable, it was known that the more lipophilic corticosteroids suffered from NSB to glass and plastic in the absence of organic solvents or protein. Lee et al. [9] describe a modification to the UF technique where pre-treatment of the filter membranes can significantly reduce NSB, however, with the corticosteroids, it was determined that there was NSB to the filtrate collection tubes. A simple modification to the UF technique is described

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that was devised in order to overcome the NSB problems associated with corticosteroids, but this method could have wider applicability.

2. Materials and methods

2.1. Materials

Ultrafiltration units (Microcon YM-10; MWCO 10 K) and filtrate collection tubes were obtained from Millipore (Bedford, MA). A pool of control human plasma was obtained by centrifugation of blood obtained from five healthy volunteers and was stored frozen prior to use. Fluticasone propionate (FP), dexamethasone (Dex), triamcinolone acetonide (TAA), budesonide (Bud), ciclesonide active principle (Cic AP), prednisolone (Pred), methylprednisolone (M Pred) and mometasone furoate (MF) were synthesised by GlaxoSmithKline. Phosphate buffer (10 mM; pH 7.4) was supplied by Sigma Chemical Co. (St. Louis, MO). All other materials and reagents were obtained from Fisher Scientific (Fair Lawn, NJ) or Sigma Chemical Co. (St. Louis, MO) and were used as received.

Log *P* was calculated using software supplied by ACD/Labs, Toronto, Canada.

2.2. Determination of plasma protein binding using a modified ultrafiltration method

Stock solutions of drugs in acetonitrile were added to human plasma (1%, v/v) to provide plasma samples at a nominal concentration of 1 μg/mL. This concentration was selected in order that analytical sensitivity would allow the detection of at least 0.5% free fraction in plasma. Aliquots of plasma were immediately removed for determination of the initial drug concentration. The remaining plasma samples were placed in a rolling incubator at 37 °C for 30 min to ensure equilibrium was established. Following incubation, an aliquot of plasma was removed for determination of the drug concentration post incubation. Triplicate 200 μL aliquots of the plasma samples were added to the sample reservoir of the UF unit having pre-weighed the filtrate collection tubes. For each unit loaded with a plasma sample, a partner UF unit was loaded with control plasma. All ultrafiltration units were centrifuged at 10,000 × *g* for 10 min at ambient temperature using a Heraeus Picofuge (Herts, UK). The sample reservoirs containing the plasma retentate were removed and retained whilst the filtrate collection tubes were re-weighed to determine the volume of ultrafiltrate produced. The sample reservoirs containing plasma retentate were then inverted and placed on the filtrate collection tubes of the partner ultrafiltration unit. The ultrafiltration units were centrifuged a second time at 700 × *g* for 3 min at ambient temperature using a Heraeus Picofuge such that the retentate was mixed with the filtrate of the partner sample. The result is that two reconstituted plasma samples are produced, one representing drug in the filtrate and one representing drug in the retentate. This methodology is described diagrammatically in Fig. 1. Analysis of the samples enables determination of both free and bound drug. In addition, the analysis of both filtrate and retentate enables the calculation of the recovery of drug from the device.

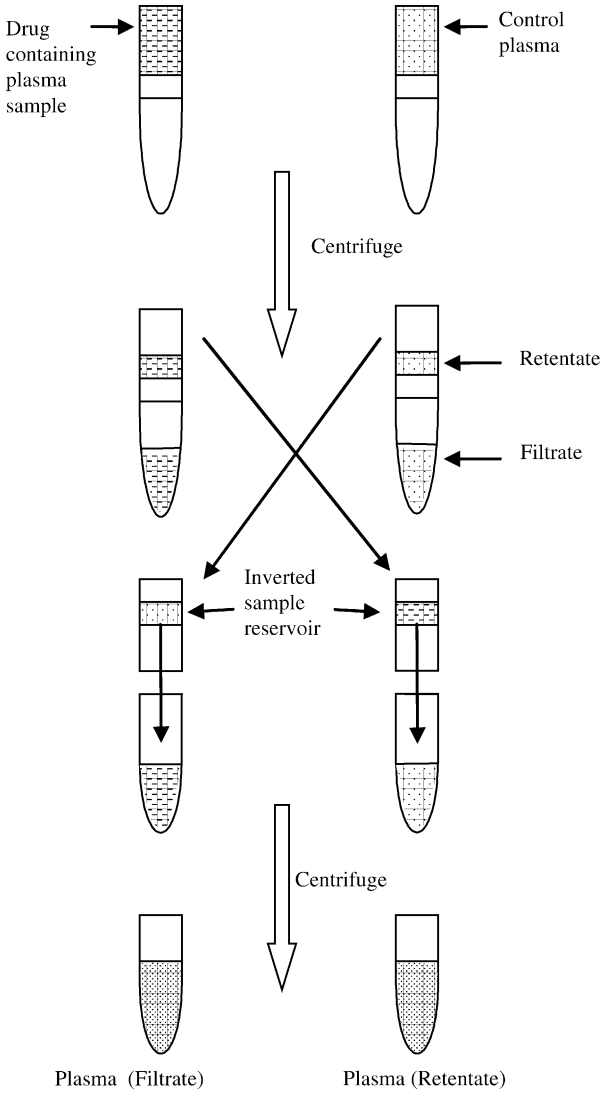

Fig. 1. Schematic diagram of the modified ultrafiltration (UF) technique for plasma protein binding determination.

2.3. Determination of the extent of non-specific binding

These experiments were conducted to demonstrate the NSB problem that exists with lipophilic corticosteroids and that it can be overcome by the use of the modified methodology described. These experiments were conducted at a nominal concentration of 10 ng/mL. This is a concentration which can be detected by LC/MS/MS and is within the range of likely free concentrations of drug in the ultrafiltrate.

2.4. Experiment 1 (uncorrected for NSB)

Stock solutions of each compound in acetonitrile were added to phosphate buffered saline (pH 7.4) (1%, v/v) in filtrate collection tubes to provide solutions of total volume 200 μL at a nominal concentration of 10 ng/mL (Sample A). The solu-

tions were incubated for 30 min at room temperature. A 100 μL aliquot of each sample was removed and placed in a separate filtrate collection tube (Sample B). A 100 μL of control plasma was added to all the tubes, and the tubes mixed.

2.5. Experiment 2 (correction for NSB)

Stock solutions of each compound in acetonitrile were added to either phosphate buffered saline (pH 7.4) (Sample C) or control human plasma (Sample D) (1%, v/v) in filtrate collection tubes to provide solutions of total volume 200 μL at a nominal concentration of 10 ng/mL. The samples were incubated for 30 min at room temperature. Control human plasma (200 μL) was added to the drug containing buffer samples and control phosphate buffered saline (pH 7.4) (200 μL) was added to the drug containing plasma samples and the tubes mixed.

Aliquots of each sample were extracted with acetonitrile and analysed by LC/MS/MS as described below. A peak area ratio of the test compounds: internal standard was obtained by integration of the test compound and internal standard compound peak areas. No calibration was required for the non-specific binding experiments. The difference in peak area ratio between samples A and B and between samples C and D represent the non-specific binding properties of the compound to the filtrate collection tube. Experiment 1 (A and B) represents the NSB associated with the conventional ultrafiltration technique, whereas Experiment 2 (C and D) represents the NSB associated with the modified ultrafiltration technique.

2.6. Sample preparation and analysis

A calibration line over an appropriate analytical range was prepared for each compound in control human plasma. Aliquots of the calibration standards and reconstituted plasma samples representative of both ultrafiltrate and retentate were extracted by protein precipitation on a 96 well filter plate using three times the volume of acetonitrile containing an analogue compound to act as an internal standard. The sample extracts were evaporated to dryness under a stream of heated nitrogen gas at 40 °C and reconstituted in 100 μL of 50% acetonitrile (aq) containing 0.1% formic acid (v/v). Aliquots of the sample extracts (10–20 μL) were analysed by high performance liquid chromatography with mass spectrometry detection (HPLC/MS/MS) to assess the concentration of drug in the samples. The HPLC system consisted of a HP1100 binary pump (Agilent Technologies, Foster City, CA), HP1100 vacuum degasser (Agilent Technologies, Foster City, CA), HTS PAL autosampler (Leap Technologies, Inc., Carrboro, NC) and column switching valve (Valco Instruments, Inc., Houston, TX). Samples were analysed using a Luna C18 5 cm × 2.1 mm column, 5 μm particle size, (Phenomenex, Macclesfield, UK) operating at 40 °C and were eluted at 800 μL/min using a gradient mobile phase consisting of 0.1% formic acid (v/v) in water and 0.1% formic acid (v/v) in acetonitrile. A Sciex API4000 mass spectrometer using Turbo IonSpray (Applied BioSystems, Foster City, CA) operating in positive ion mode with a source temperature of 550 °C was used for the detection of all compounds. Multiple reaction monitoring was performed using nitrogen as the collision gas with a dwell time of 150 ms. Total analysis time was 5 min per sample. Data collection and processing was performed using Analyst 1.3 (Applied BioSystems, Foster City, CA). Experimental procedures were performed with a minimum of three replicates. Plasma protein binding and recovery are reported as mean ± % coefficient of variation (%CV).

2.7. Protein binding calculation

The volume of filtrate produced was calculated from the weights of the collection vials measured both pre-and post filtrate generation assuming 1 mg was equivalent to 1 μL. The concentration of drug in the filtrate was then determined by correction for dilution of the filtrate with control retentate, i.e., to a total volume of 200 μL.

Protein binding was calculated using Eq. (1):

$$\text{Protein binding (\%)} : \left[\frac{C_t - C_u}{C_t}\right] \times 100 \quad (1)$$

where C_t is the total drug concentration in the plasma and C_u is the corrected concentration of unbound drug in the filtrate.

2.8. Calculation of non-specific binding

Non-specific binding was calculated using Eq. (2):

$$\text{Uncorrected NSB (\%)} : \left[\frac{\text{PAR}_A}{\text{PAR}_{(A+B)}}\right] - \left[\frac{\text{PAR}_B}{\text{PAR}_{(A+B)}}\right] \times 100 \quad (2)$$

where PAR is peak area ratio.

Note: For corrected NSB, substitute A and B for C and D, respectively.

3. Results and discussion

The extent of non-specific binding of a selection of corticosteroids using the conventional UF technique (uncorrected values) and the modified UF technique (corrected values), are presented in Table 1. Using the conventional UF technique all the corticosteroids exhibited extensive NSB, which in general seemed to be more pronounced at higher lipophilicity. NSB is effectively eliminated using the modified UF technique, where

Table 1
The % non-specific binding (NSB) of test compounds to filtrate collection tubes in the presence (corrected) and absence (uncorrected) of human plasma

Compound	*c* Log *P*	% NSB (uncorrected)	% NSB (corrected)
Dexamethasone	2.06	23.2	2.5
Methylprednisolone	2.18	7.5	6.3
Triamcinolone acetonide	2.25	11.0	−2.6
Budesonide	2.89	36.0	0.2
Ciclesonide AP	3.89	48.2	−2.6
Fluticasone propionate	3.89	59.6	−2.0
Mometasone furoate	4.73	63.9	−2.2

Table 2
The plasma protein binding and recovery of test compounds from the ultrafiltration device

Compound	*c* Log *P*	Protein binding		
		% Binding (literature values)	% Binding[a] (modified UF values)	Recovery from UF device (%)[b]
Prednisolone	1.69	[c]	57.6	117.6
Dexamethasone	2.06	72	70.9	106.6
Methylprednisolone	2.18	78.5	81.1	120.3
Triamcinolone acetonide	2.25	71	73.2	104.7
Budesonide	2.89	88	91.4	97.5
Ciclesonide AP	3.89	99	98.7	101.4
Fluticasone propionate	3.89	90	98.1[d]	109.8
Mometasone furoate	4.73	98.5	99.5	95.4

[a] Prednisolone, mean $n = 3$, CV 7.9%; dexamethasone, mean $n = 6$, CV 2.2%; methylprednisolone, mean $n = 3$, CV 1.1%; TAA, mean $n = 5$, CV 6.8%; budesonide, mean $n = 15$, CV 1.8%; ciclesonide AP, mean $n = 15$, CV 0.3%; fluticasone propionate, mean $n = 12$, CV 0.3%; mometasone furoate, mean $n = 3$, CV 0.1%.
[b] Prednisolone, mean $n = 3$, CV 5.7%; dexamethasone, mean $n = 6$, CV 2.4%; methylprednisolone, mean $n = 3$, CV 1.4%; TAA, mean $n = 5$, CV 18.7%; budesonide, mean $n = 15$, CV 8.0%; ciclesonide AP, mean $n = 15$, CV 5.5%; fluticasone propionate, mean $n = 12$, CV 8.1%; mometasone furoate, mean $n = 3$, CV 5.2%.
[c] Literature value not quoted as concentration dependent binding suspected (11).
[d] 99.3% when investigated at more clinically relevant concentrations (5, 10 and 100 ng/mL).

human plasma retentate is added to the filtrate collection tube before sampling for analysis.

Table 2 lists the calculated lipophilicity of each corticosteroid, the plasma protein binding values and the associated drug recovery obtained using the modified UF technique. Plasma protein binding was found to increase with increasing lipophilicity with values ranging from 58% to 99.5%. This indicated that the modification to the method has not affected the ability of the technique to discriminate binding over a broad dynamic range. The device recovery was generally within the range 100% ± 10%, with the exception of prednisolone (+17%) and methylprednisolone (+20%). Analytical error is likely to account for a considerable portion of this variability. This was demonstrated when this technique was applied to two novel radiolabelled corticosteroids currently of interest to GlaxoSmithKline (data not shown). The increased precision provided by radiolabel analysis using liquid scintillation counting provided recoveries of 100% ± 2%. This precision demonstrates that recovery using this technique is essentially complete.

The modified method was validated by comparison of the plasma protein binding values with those quoted in the literature [8,10–16]. A comparison of the values is shown in Table 2 and a correlation of $R = 0.98$ indicates that the modified method compares well with the literature data. However, the literature value for fluticasone propionate (90%) [10] does not correlate with the value produced using the modified UF technique. This discrepancy in the plasma protein binding of FP was further investigated using the modified UF technique at more clinically relevant concentrations of 5, 10 and 100 ng/mL in plasma from three different subjects. The mean binding value at these concentrations was calculated to be 99.3%, thus, confirming the high binding observed at 1 μg/mL. Furthermore, the new protein binding for FP corresponds with the observed correlation of protein binding and lipophilicity.

4. Conclusions

Corticosteroids can exhibit poor aqueous solubility and a high degree of non-specific binding which prevent accurate determination of plasma protein binding by standard techniques. This work demonstrates that a modification to the standard ultrafiltration methodology, mixing of control plasma retentate with the filtrate, can eradicate NSB. It has been demonstrated that the non-specific binding of corticosteroids to the filtrate collection tubes can be reversed by the addition of retentate from a control plasma sample. The protein content of the retentate is bringing the corticosteroids into solution either by providing a large number of additional binding sites, possibly of a higher affinity, or by saturating the non-specific binding sites on the wall of the plastic tube. Although the observed NSB appeared to increase with increasing lipophilicity, the modification overcame NSB for even the most lipophilic corticosteroids investigated. This modification provides plasma samples representing the filtrate and the retentate in a consistent matrix compatible with a high throughput mass spectrometric end-point. The validity of the modified method has been demonstrated by the complete recovery of test compounds and by the good correlation with published values for plasma protein binding. The modified method has been successfully used to filter out low binding compounds (<98%) during lead optimisation and has confirmed the high binding of candidate molecules. Although the modifications were specifically introduced to overcome issues with corticosteroids, the method could have wider applicability beyond corticosteroids.

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