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A sensitive method for the quantification of fluticasone propionate in human plasma by high-performance liquid chromatography/atmospheric pressure chemical ionisation mass spectrometry

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Abstract

A highly sensitive and selective method has been developed for the quantification of fluticasone propionate (FP) in human plasma. The drug was isolated from human plasma using C_{18} solid-phase extraction cartridges. The analysis was based on high-performance liquid chromatography/atmospheric pressure chemical ionisation mass spectrometry (HPLC/APCI/MS), using the 22R epimer of budesonide (BUD) acetate, synthesised using acetic anhydride, as internal standard. The mass spectrometer was operated in APCI mode with selected ions at tune masses of 473.2 and 501.2 m/z, corresponding to the MH^+ of acetylated (22R)BUD and FP, respectively. The mobile phase used was a mixture of 50% ethanol in water with a flow rate of 0.45 ml min^{-1}. The system was optimised by tuning the capillary and tube lens with a concentrated solution of FP. The recovery of FP from human plasma was 86.3%. Linearity of response was obtained over the concentration range 0.2–4.0 ng ml^{-1}. The intra-assay and inter-assay variability were 6.3 and 2.9%, respectively. The lower limit of quantification was 0.2 ng ml^{-1} when a solid-phase extraction preceded the HPLC/APCI/MS. © 1997 Elsevier Science B.V.

Keywords: Fluticasone propionate; Reverse-phase liquid chromatography; Atmospheric pressure chemical ionisation mass spectrometry

1. Introduction

Fluticasone propionate (FP) is a novel androstane glucocorticosteroid having potent pulmonary anti-inflammatory activity (Fig. 1) [1]. It has been shown to exhibit improved therapeutic efficacy in the treatment of asthma and rhinitis [2] compared with other available inhaled corticosteroids. Clinical research suggests that the high therapeutic index is achieved without an accompanying increase in systemic activity [3].

It is generally accepted that following inhalation administration more than 80% of the dose of

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FP is swallowed and enters the gastrointestinal tract, the remainder enters the airways [4]. The drug is thought to undergo extensive first-pass hepatic metabolism after absorption from the gut. Bioavailability of FP has been calculated to be 1% [5]. The dose available for absorption across the lung vasculature is very low and will thus give rise to very low drug concentrations in plasma. Hence, the development of an analytical methodology with appropriate sensitivity and specificity is challenging.

The effects of systemically acquired FP have been previously compared with other corticoids in healthy subjects by measurement of the relative suppression of hypothalamic–pituitary–adrenal (HPA) function following inhalation of single and multiple doses of the drugs [6–8]. Conventional radioimmunoassay (RIA) [5] and RIA combined with solid-phase extraction [9,10] have been used to measure plasma and urinary cortisol as indicators of glucocorticoid effect on the HPA axis.

Up to the present, the determination of blood and plasma concentrations of FP following its administration by inhalation has involved radioimmunoassay incorporating solid-phase extraction (RIA) [9,10]. The sensitivity of the RIA proved to be very good for the measurement of FP in plasma. However, the method has comparatively poor precision (up to 25% CV). It is also subject to nonspecific interference and varying cross-reactivity with drug metabolites and other unrelated compounds although the antibodies used in the RIA were claimed to be highly specific [10]. Therefore, this paper describes a highly sensitive and selective method using high performance liquid chromatography/atmospheric pressure chemical ionisation mass spectrometry (HPLC/APCI/MS) for quantification of FP in human plasma following administration of therapeutic doses.

2. Experimental

2.1. Chemicals

FP and (22R)budesonide (BUD) were kindly donated by Glaxo Group Research (UK) and Astra Draco (Lund, Sweden), respectively. The 21-acetate ester of the 22R epimer of BUD was synthesised [11] and used as the internal standard (IS). Ethanol, ethyl acetate, *n*-heptane were all of HPLC grade and purchased from Sigma Aldrich (Sydney, Australia). Human blank plasma was obtained from the Red Cross Blood Bank (Sydney, Australia). Water was purified in a MilliQ system (Millipore, Sydney, Australia).

2.2. Preparation of standard solutions and plasma samples

Stock solutions (3.5 mg ml^{-1}) of FP were prepared in ethanol at two monthly intervals and stored at $-20°C$. Working solutions of FP (0.05 g ml^{-1}) were prepared fortnightly by dilution of the stock standard solution with ethanol. The IS working solution (0.5 μg ml^{-1}) was also prepared in ethanol. Replicate ($n = 3$) 1 ml plasma samples (including blank plasma) were spiked with known quantities of FP to cover the FP concentration range 0.2–4.0 ng ml^{-1}. These concentrations were used to conduct standard curve.

2.3. HPLC/APCI/MS

The analysis of FP was performed on a 5-μm ODS Hypersil (100 × 2.1 mm i.d.) narrow-bore column (Hewlett Packard, Blackburn, Victoria, Australia) equipped with a 5-μm ODS Hypersil C_{18} (20 mm × 2.1 mm i.d.) guard column cartridge (Hewlett Packard) using a Finnigan/Mat TSQ 7000 LC–MS–MS system (San Jose, CA)

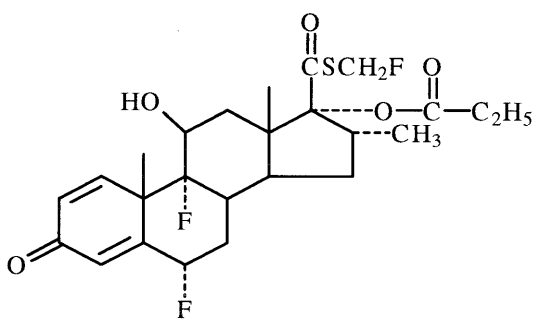

Fig. 1. Structure of fluticasone propionate.

operating in APCI mode. The system was linked to a Hewlett Packard HP 1090 liquid chromatograph controlled by the software of the TSQ 7000. The mobile phase was a mixture of ethanol–water (50:50, v/v) with a flow-rate of 0.45 ml min^{-1}, and filtered through a 0.45-μm HVLP filter (Millipore, Sydney, Australia) before use. The mass spectrometer was operated in selected-ion mode at the tune masses of 473.2 and 501.2 m/z, corresponding to the MH^+ of acetylated (22R)BUD and FP, respectively. The system was tuned previously in electrospray (ESI) mode then when in APCI mode the system was optimised by tuning the capillary and tube lens with a concentrated solution of FP.

2.4. Analytical procedure

Plasma samples were thawed at room temperature. After addition of 10 μl of IS working solution, corresponding to 5.0 ng of (22R)BUD acetate, plus 1 ml of 30% ethanol in water, samples were vortexed and allowed to stand for 15 min. Samples were centrifuged for 15 min at $1800 \times g$ to remove protein precipitates caused by addition of 30% ethanol. The supernatants were transferred to the solid-phase extraction C_{18} cartridges pre-conditioned by rinsing twice with 3 ml ethanol and twice with 3 ml water. A 24-port manifold (Supelco, Bellefonte, PA) equipped with an oil vacuum pump was used to accommodate the cartridges and operated at approximately $5.1 \cdot 10^3$ Pa. Supernatant fractions were aspirated through the cartridges dropwise. The cartridges were then washed consecutively with 3.0 ml 25% ethanol, 3.0 ml water, and twice with 2.0 ml 2% ethyl acetate in heptane. The cartridges were dried under vacuum for 15 min before analytes were eluted with 2.0 ml 35% ethyl acetate in heptane into 5 ml borosilicate tubes. To check the recovery a final wash with 2.0 ml 100% ethanol was used occasionally to elute analytes possibly retained on the cartridge. The solvent was evaporated to dryness under a stream of N_2 at 35°C, and the residue was reconstituted with 100 μl of the mobile phase and allowed to stand for at least 15 min at room temperature before transferring to the autosampler vial. A total sample volume of 20 μl was injected into the HPLC/APCI/MS system.

Table 1
Recoveries of FP and IS from solid-phase extraction

Concentration (ng ml^{-1})	Recovery (% of total)
0.5	81.6 ± 3.6
1.0	86.4 ± 6.5
4.0	90.8 ± 7.6
5.0 (IS)	85.3 ± 5.4

$n = 5$.

3. Results and discussion

3.1. Solid-phase extraction

Preliminary experiments were carried out to determine the most effective procedure for extracting FP from human plasma. A range of organic solvents was investigated for solid-phase extraction such as methanol and methylene chloride. Use of ethanol and ethyl acetate in heptane gave the best compromise between extraction efficiency and cleanliness. However, it became clear early on in our investigation that it is critically important to carefully maintain a dropwise flow rate, during the elution of the analyte in solid-phase extraction, in order to obtain clean extracts, even with the lengthy clean-up procedure. The recoveries of FP and 22R epimer of BUD at various concentrations are given in Table 1. The mean recoveries of FP and (22R)BUD acetate were 86.3 and 85.3%, respectively.

3.2. HPLC/APCI/MS

Initial attempts to validate the procedure employed deuterated cortisol acetate, the synthesised compound, as IS. However, this gave unacceptably low precision, perhaps because of a relatively significant difference in retention time. As tritium-labeled FP was not available, the 22R epimer of BUD acetate was used as IS. The inclusion of this IS gave much more consistent recovery than when [$^2H_3$]cortisol acetate was used. Typical mass chromatograms are shown in Fig. 2C and A, where FP and the 22R epimer of BUD elute with retention times of 3.09 and 3.35 min, respectively.

In the positive ion APCI mode utilising corona discharge, the instrument was used in single scanning mode, the second and third quadruples acting in transmission mode only. Only the first quadrupole was used because the sensitivity in selected ion monitoring (SIM) was found to be sufficient for the analysis. A triple quadrupole has greater inherent sensitivity in quadrupole 1 (Q1) mode compared to a single quadrupole instrument. In order to obtain maximum response for the analyte the retention time was minimised to give sharper peaks. This was achieved firstly by using a short HPLC column and secondly by a high concentration (50%) of ethanol in the mobile phase.

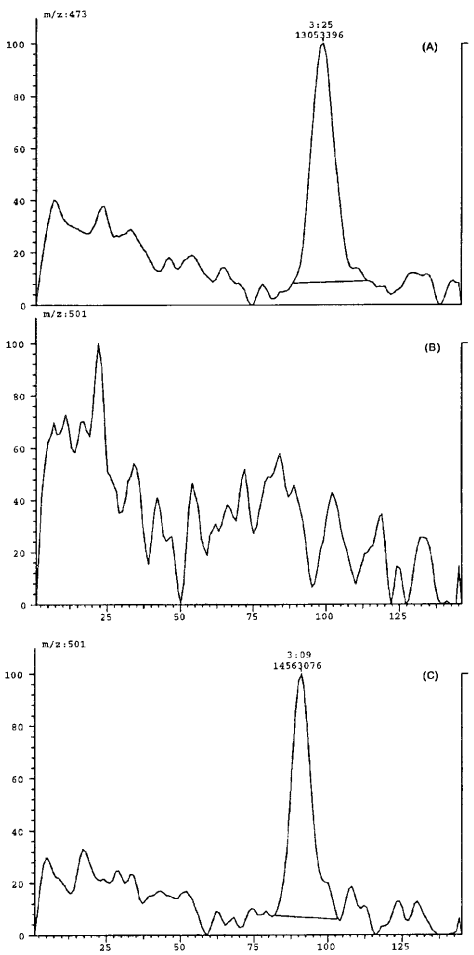

Fig. 2. Mass chromatograms of human plasma sample: A, spiked with IS 22R budesonide acetate (5.0 ng ml^{-1}); B, blank human plasma; C, spiked with FP (0.2 ng ml^{-1}).

3.3. Selectivity

RIA methods have reportedly been used for the quantitative determination of FP in human plasma following oral [5], intravenous and inhalation [9] administration [10], respectively. Concentrations of FP lower than 50 pg per tube could often be detected in samples using these methods but potential interference by endogenous materials make those methods of questionable reliability. The cross-reactivity of antibodies with endogenous steroids, potential FP metabolites and potentially coadministered steroids have been determined in these studies. Three compounds were found to show a significant degree of cross-reactivity, although these compounds differ from FP at the 11- or 6-positions [9]. The non-specific binding was low ($< 5\%$), but it would make a considerable contribution to unacceptable error in quantification of FP at the ng level of concentration. This meant a more specific method was required. As might be expected, thermospray HPLC/MS has been employed and offered a significant specificity for the quantification of corticosteroids such as cortisol [12] and BUD [13] in human plasma, but the technique has been shown to be compound dependent in terms of ionisation responses [14]. Hence, HPLC/APCI/MS was selected as the most appropriate method because it was highly specific and sensitive to FP. The method has also been successfully applied to the quantification of epimeric BUD in human plasma [11].

Typical mass chromatograms obtained from extracted FP samples are shown in Fig. 2A and B. where the mass chromatogram of blank human plasma indicates no co-extracted endogenous compound from the FP sample.

3.4. Precision and accuracy

The intra-day precision was determined by replicate analyses of human blank plasma to which known amounts of FP had been spiked.

Table 2
Intra-day precision and accuracy of the FP HPLC/APCI/MS investigated on three different occasions

Concentration prepared (ng ml^{-1})	Occasion No.	Concentration measured (ng ml^{-1})	R.S.D. (%)	Bias (%)
0.20	1	0.18 ± 0.014	7.8	−10.0
	2	0.17 ± 0.016	9.4	−15.0
	3	0.19 ± 0.010	5.2	−5.0
0.50	1	0.44 ± 0.048	10.9	−12.0
	2	0.45 ± 0.058	12.9	−10.0
	3	0.47 ± 0.038	8.1	−6.0
1.0	1	0.91 ± 0.097	10.7	−9.0
	2	0.94 ± 0.024	2.6	−6.0
	3	0.93 ± 0.029	3.1	−7.0
2.0	1	1.77 ± 0.036	2.0	−11.5
	2	1.74 ± 0.020	1.1	−13.0
	3	1.93 ± 0.029	1.5	−3.5
4.0	1	4.10 ± 0.119	2.9	2.5
	2	4.03 ± 0.180	4.5	0.8
	3	3.86 ± 0.460	11.9	−3.5

$n = 5$.

The intra-day accuracy was also estimated from these samples by the percent difference of the mean concentration determined from the nominal concentration: ((measured concentration − nominal concentration) ÷ nominal concentration × 100%). The investigation was performed on three different occasions (Table 2). The R.S.D. was 7.5% at 0.2 ng ml^{-1}, 10.6% at 0.5 ng ml^{-1}, and better than 4.5% at higher levels. The error was less than ± 12.0%, except for one occasion at 0.2 ng ml^{-1} (− 15%).

The inter-day variation was evaluated by measuring replicates of the same samples at 3 different concentrations over a period of 2 weeks. The results are given in Table 3.

3.5. Linearity and sensitivity

The calibration curves of the peak area ratio of FP to IS versus drug concentration were linear over the range 0.2–4.0 ng ml^{-1}. Previous studies indicate that the peak plasma concentration following inhalation of 1.0 mg FP approximates 2.5 ng ml^{-1}. The drug plasma concentration was determined in the range 0.2–4.0 ng ml^{-1} using RIA combined with solid-phase extraction in healthy volunteers as previously reported [9]. It is believed that this upper limit will rarely be exceeded in clinical samples. The mean linear regression equation was $y = 0.0854x + 0.0023$, in which y represents the peak area ratio of FP to IS and x

Table 3
Inter-day precision and accuracy of the FP HPLC/APCI/MS

Concentration prepared (ng ml^{-1})	Concentration measured (ng ml^{-1})	R.S.D. (%)	Bias (%)
0.2	0.21 ± 0.005	2.4	5.0
0.5	0.48 ± 0.015	3.1	−4.0
1.0	0.98 ± 0.046	4.7	−2.0
4.0	3.95 ± 0.050	1.3	−1.3

$n = 6$.

corresponds to the drug concentration in ng ml^{-1}. The correlation coefficients (r) were better than 0.998.

The lower limit of quantification was 0.2 ng ml^{-1} at a signal-to-noise ratio of 3:1. Fig. 2C shows mass chromatograms of 1 ml of plasma sample spiked with 0.2 ng of FP.

4. Conclusion

In this study we have demonstrated a sensitive and selective assay for quantification of FP in human plasma. The precision and bias of this assay were acceptable over the concentration range 0.2–4.0 ng ml^{-1}. The methodology reported herein will provide a sound basis for monitoring plasma concentrations of FP in man.

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