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An improved method for the determination of fluticasone propionate in human plasma☆

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Abstract

Therapeutic monitoring of the potent, highly lipophilic glucocorticoid, fluticasone propionate (FP), was initially performed by a radioimmunoassay method. However an improved method with a lower limit of quantitation (LLOQ) of at least 25 pg per ml (pg ml^{-1}) was needed to measure the low levels of FP present in human plasma following inhalation administration of doses in the range 50–250 μg twice daily. A sensitive and specific liquid chromatographic, tandem mass spectrometric method (LC-MS/MS) with automated solid phase extraction (SPE) was developed and validated. Fluticasone propionate was extracted from plasma using Bond Elut C18 cartridges and analysed using reverse-phase chromatography with atmospheric pressure chemical ionisation followed by selective reaction monitoring. The method used a ^{13}C-labelled internal standard and was validated over a concentration range of 25–500 pg ml^{-1}. The method was shown to be specific, sensitive and reliable in the analysis of clinical samples. The main advantages of this method over the radioimmunoassay method previously used were improved sensitivity, specificity, ease of sample preparation and shortened analysis time.

Keywords: Fluticasone propionate; Glucocorticoid; Automated assay; Mass spectrometry

1. Introduction

Fluticasone propionate (FP; Fig. 1) is a highly potent, trifluorinated glucocorticosteroid based on the androstane nucleus [1]. FP has been shown to be effective in the treatment of asthma and allergic rhinitis [2]. FP was specifically designed to be metabolically labile and so have low oral bioavailability ($\sim 1\%$) thus minimising any adverse systemic effects [3]. The pharmacodynamics and pharmacokinetics of FP have been reviewed [2–5].

A radioimmunoassay with solid phase extraction method (RIA-SPE) was used initially to determine the concentrations of FP in plasma in the

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early phases of the clinical trials [6]. This RIA-SPE method was capable of detecting FP down to a concentration level of 25 pg ml^{-1} but the precision and accuracy of measurements at this concentration were found to be unacceptable in routine use. The acceptable lower limit of quantification in practice was 50 pg ml^{-1}.

As the bioavailability of FP following inhalation dosage of 1000 μg to healthy volunteers was low (between 11.9 and 16.6%; [7]), an improved assay was needed for the extended clinical trials in patients receiving the lower doses of the compound (50 or 100 μg twice daily) recommended for use in the treatment of asthma. Ideally the new method would enable concentrations in the range of 10–50 pg ml^{-1} to be accurately and reliably measured. An LC-MS method for the determination of FP down to 200 pg ml^{-1} has been described [8]. We wish to present details of an improved analytical method involving automated solid phase extraction (ASPEC) followed by liquid chromatographic, tandem mass spectrometric (LC-MS/MS) quantification with selective reaction monitoring over the concentration range 25–500 pg ml^{-1}. A carbon-13 labelled analogue of FP was used as an internal standard in the procedure. This new method has been shown to be reliable in the analysis of samples from clinical studies.

2. Materials and methods

2.1. Reagents and chemicals

Fluticasone propionate (CCI 18781, batch R6384/028B), isotopically labelled fluticasone propionate (13-C3 CCI18781, batch C2420/265/5; Fig. 1) and the carboxylic acid metabolite of fluticasone (GR36264X, batch RAB/110/2) were supplied to Covance Laboratories by GlaxoWellcome as characterised products. Methanol, ammonium acetate, tricine, sodium azide, formic acid, sodium hydroxide and sodium chloride were purchased from recognized UK suppliers and were of HPLC grade. De-ionised water was further purified via a Milli-Q system (Millipore, Bedford, MA, USA).

Drug-free human plasma was obtained from volunteers at Covance Laboratories with the blood samples being drawn into tubes containing heparin anti-coagulant. The HPLC mobile phase was prepared by adding ammonium acetate solution (5 mM) to HPLC grade methanol (1:4 v/v) with thorough mixing and adding 0.1% (v/v) formic acid, followed by sonication for 10 min. A solvent for the reconstitution of plasma extracts (Section 2.4) was prepared by mixing ammonium acetate (5 mM) with methanol (1:1 v/v) and 0.1% (v/v) formic acid.

2.2. Equipment

The LC-MS/MS analyses were performed with a Perkin Elmer Sciex API III + mass spectrometer (Perkin Elmer, Thornton, Canada) fitted with an electrospray interface and Windows based software. The mass spectrometer was coupled to a series 410 pump (Perkin Elmer, Beaconsfield, UK), a Gilson 231 autosampler (Anachem, Luton, UK) and a Hypersil BDS C8 column (3 μm; 5 cm × 4.6 mm i.d.). Sample preparation was carried out by an automatic procedure using an ASPEC XL system (Gilson) with a 231 autosampler and Bond Elut C 18 cartridges (200 mg/3 ml; Varian, Surrey, UK).

A refrigerated centrifuge (model 7R or 8R, Damon/IEC, Needham Heights, MA, USA) was used to spin down the thawed plasma prior to sample analysis. Disposable polypropylene transfer pipette tips were used to sample the plasma and the ASPEC eluates which were collected into polypropylene tubes (12 × 75 mm). Screw top conical vials (model 4Sv310) were purchased from

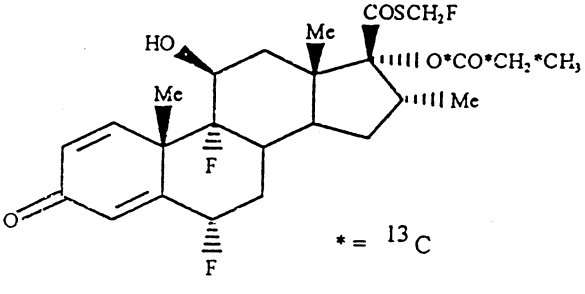

Fig. 1. The structure of fluticasone and its internal standard.

Chromacol (Welwyn Garden City, UK). A sample concentrator system for use at controlled temperatures with nitrogen evaporation (Techne model SC-3, Jencons, Hemel Hempstead, UK) was used to concentrate the samples.

2.3. Preparation of stock solutions, calibration, validation and QC samples

Two separate stock solutions of FP were prepared, one solution (stock solution A) to be used for calibration standards and the other solution (stock solution B) to be used for the preparation of quality control (QC) and validation samples. In each case an accurately weighed sample of FP was dissolved in HPLC grade methanol (10 ml) to give known concentrations of FP ($\sim$ 1000 μg ml^{-1}). Portions of the stock solution (A) were accurately diluted with methanol to provide ten working standard solutions in the concentration range 1–10 000 ng ml^{-1}. Aliquots of these solutions were then used to spike pre-screened human plasma to provide sets of seven calibration standards in the concentration range from 10 to 500 pg ml^{-1}. Using a similar dilution procedure portions of the quality control stock solution (B) were diluted to provide sets of seven validation samples in human plasma in the concentration range of 10–500 pg ml^{-1} and sets of three quality control samples in human plasma (low: 30 pg ml^{-1}; medium: 180 pg ml^{-1}; high: 400 pg ml^{-1}).

A stock solution of the internal standard (stock solution C) was prepared and used to provide working standard solutions in methanol at 50 or 1000 ng ml^{-1}. Portions of these were spiked into human plasma to provide matrix blanks. Portions of the pre-screened human plasma from six different individuals were used as the matrix for these blanks. A solution of the carboxylic acid metabolite of fluticasone in methanol was also prepared.

All standard solutions in methanol were stored refrigerated ($\sim$4°C) when not in use. All the sets of the calibration, validation and QC samples in plasma were stored frozen (about −20°C).

2.4. Sample preparation procedure

The plasma samples were thawed at room temperature, briefly mixed and then centrifuged at 4°C to remove any solid debris. A portion (20 μl) of the internal standard working solution (50 000 pg ml^{-1}) was pipetted into the ASPEC tubes with the exception of those tubes used for the control samples. A portion of plasma (1 ml) was added to the tubes, followed by tricine buffered saline (TBS, 2 ml, pH 7) and the contents of each tube were thoroughly mixed.

Bond-Elut cartridges were conditioned with methanol (2 ml) and TBS (2 ml) and allowed to remain wet before the diluted plasma sample was slowly applied to each cartridge. The cartridges were washed with TBS (1 ml) followed by de-ionised water (1 ml). FP was eluted from the cartridges with methanol (2 ml) and the eluates evaporated to dryness under nitrogen at 60°C. Each eluate was re-constituted in the special solvent mixture (200 μl) and the sample centrifuged at 4°C for 10 min. The liquid phase was quantitatively transferred into labelled microvials.

2.5. Chromatographic and mass spectrometric conditions

The reconstituted extracts (100 μl) were injected on to a Hypersil BDS C8 column using an isocratic mobile phase of ammonium acetate:methanol with formic acid at 1 ml min^{-1}.

The mass spectrometer was operated in the multiple-reaction monitoring (MRM) mode using a heated nebulizer (450°C) and heated interface (60°C). Ions were collisionally activated with argon (300×10^{13} molecules cm^2) and the mass spectrometer set to monitor the ions of m/z 501 and 293 amu for FP and of 504 and 313 for the internal standard, with dwell times of 100 ms per transition. Multiple-reaction monitoring data were captured using RAD 2.6 and processed using the MacQuan version 1.3 software packages (Perkin Elmer).

2.6. Sample analysis and assay validation

The agreement between the two primary stock solutions (A,B) was determined by a comparison of the peak areas of replicate injections ($n = 3$) of the two solutions. The intra-batch and inter-batch precision and accuracy of sets of the validation samples at seven concentrations between 10 and 500 pg ml^{-1} were determined. The mean concentration, standard deviation (S.D.), coefficient of variation (CV%) and accuracy (%) were calculated from the replicate analyses ($n = 6$, intra-batch; $n = 7$, inter-batch). The inter-batch precision and accuracy of sets of the seven calibration standards and the inter-batch precision and accuracy of sets of the three QC samples were also determined ($n = 7$). The acceptance criteria were set as $\pm$ 20%. As part of the validation exercise the following items were also investigated: the overall response of the detector to FP, the overall extraction efficiency, the specificity and selectivity, the room temperature stability of FP in human plasma, the freeze-thaw stability of FP in plasma and the stability of FP in sample extracts kept at room temperature.

A set of seven calibration standards, a blank and six QC samples (two of each of the low, medium and high samples) was analysed with each batch of clinical samples. The calibration standards, validation samples, QC samples, and clinical samples were all analysed via selected reaction monitoring of the reactions m/z 501–293 for FP and of m/z 504–313 for the isotopically labelled internal standard of FP (Figs. 1 and 2).

The peak area for each calibration sample was determined and a linear regression describing each calibration line was then calculated using the reciprocal of the drug concentration as weight.

3. Results and discussion

3.1. Mass spectrometry

Under the MS conditions used, FP (Fig. 2) and the internal standard showed protonated ions [M + H] + at m/z 501 and 504 amu, respectively. Fragmentation of these ions using collision activated dissociation (CAD) resulted in strong product ions for FP (293 amu) and its internal standard (313 amu).

The region in the chromatograms corresponding to the retention time of FP ($\sim$ 1 min 4 s) and the internal standard were examined and it was shown that there were no major interfering components present in extracts of matrix blanks (Fig. 3) nor in extracts of reagent blanks (not shown). A total of six different samples of control human plasma as blank samples or spiked with fluticasone at 200 pg ml^{-1} gave observed concentrations below the proposed lower limit (LLOQ) for the blanks or a mean of 197.9 ± 13.4 pg ml^{-1} (CV% = 6.8) for the spiked samples. The presence of the carboxylic acid metabolite was shown not to interfere with the measurement of FP. It was confirmed that the response of the detector to FP was linear over the concentration range studied. Ion chromatograms of one of the calibration standards at 10 pg ml^{-1} and one low level QC sample (30 pg ml^{-1}) are presented in Fig. 4 and show only one peak per channel.

3.2. Analytical performance of the method

The peak areas of replicate injections of the calibration and QC stock solutions (A,B) were found to be within 1.53% of each other. The inter-batch precision and accuracy data (not shown) for the seven calibration standards showed good accuracy for the back-calculated data from 25 to 500 pg ml^{-1} (98.6–106.8%) but poorer accuracy (86%) for the 10-pg ml^{-1} standard. This value was within the acceptable limit of $\pm$ 20%. The calibration lines were linear over the range from the LLOQ to the highest concentration evaluated (500 pg ml^{-1}). All coefficients of determination of the calibration lines during the validation exercise were considered acceptable, varying between 0.992681 and 0.999712.

The method showed good inter- and intra-batch precision and accuracy for the validation samples over the concentration range 25–500 pg ml^{-1} (Table 1). The intra-batch precision values were less than or equal to 11.5% (CV) and the inter-batch precision values were less than or equal to 18.5% (CV). However the method was

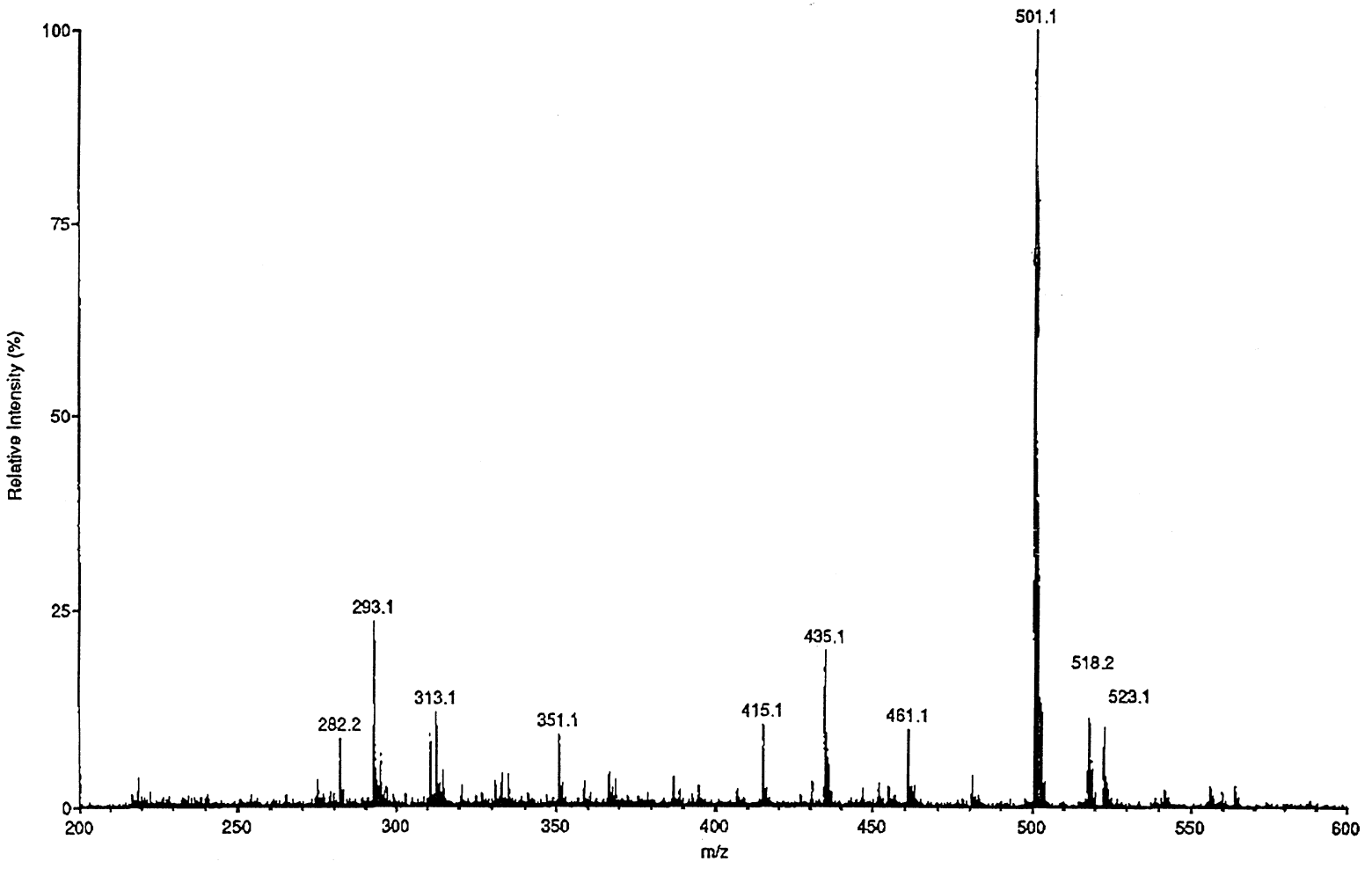

Fig. 2. The fragmentation pattern of fluticasone.

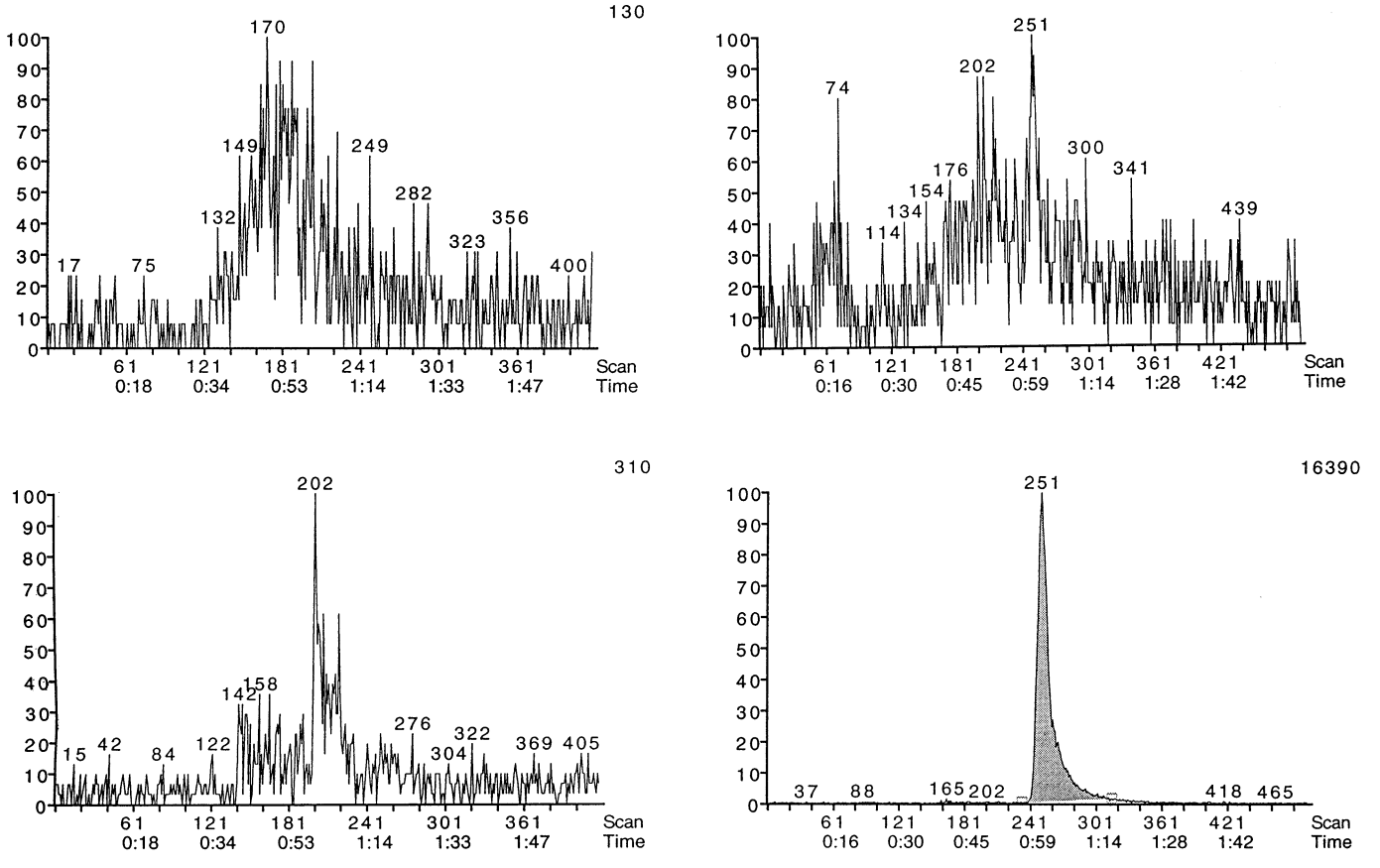

Fig. 3. LC-MS/MS MRM ion current chromatograms of (a) an extract of control human plasma, and (b) an extract of control human plasma spiked with internal standard obtained by multiple-reaction monitoring of m/z 501–293 for FP and 504–313 for the internal standard.

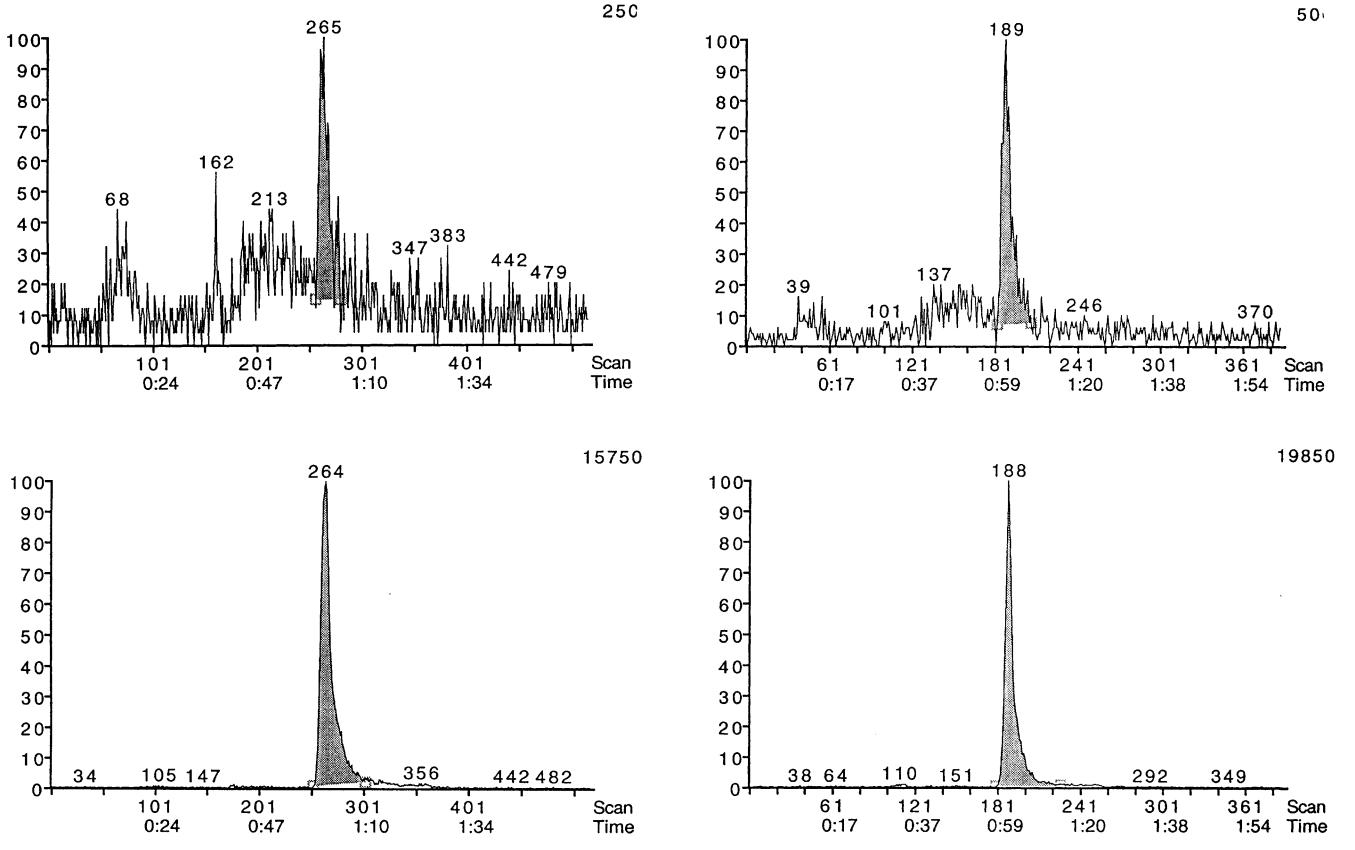

Fig. 4. LC-MS/MS MRM ion chromatogram ion current chromatograms of (a) an extract of a calibration sample (10 pg ml^{-1}), and (b) an extract of a QC sample (30 pg ml^{-1}) obtained by multiple-reaction monitoring of m/z 501–293 for FP and 504–313 for the internal standard.

Table 1
The intra-batch and inter-batch precision and accuracy data[a]

	Nominal concentration of validation sample (pg ml^{-1})						
	10	25	50	100	200	350	500
Intra-batch data Replicate	(Observed concentration)						
1	7.9	25.1	47.3	103.6	221.8	337.3	486.5
2	8.0	24.6	47.1	109.3	219.5	349.5	508.6
3	10.4	20.4	50.8	101.2	218.7	357.3	502.1
4	9.4	22.8	46.1	96.0	209.7	370.0	498.4
5	8.6	20.4	50.7	101.4	212.8	364.9	503.6
6	8.0	26.2	50.5	102.8	212.2	363.8	478.5
Mean	8.7	23.3	48.8	102.4	216.1	357.1	496.3
S.D. (n^{-1})	1.0	2.47	2.14	4.30	4.61	12.02	11.45
CV (%)	11.5	10.6	4.4	4.2	2.1	3.4	2.3
Accuracy (%)	87.0	93.2	97.6	102.4	108.1	102.0	99.3
Inter-batch data Batch number	(Observed concentration)						
1	8.0	26.2	50.5	102.8	214.2	363.8	478.5
2	NR	22.9	52.1	103.8	212.4	NR	444.1
3	10.7	22.9	52.4	95.2	203.4	361.2	508.4
4	15.7	35.1	65.1	96.5	197.8	358.3	493.4
5	9.9	21.0	58.9	105.7	231.3	384.5	478.5
6	7.1	25.1	53.4	111.1	194.0	374.0	475.2
7	NR	23.5	48.8	101.6	208.8	296.6	387.2
Mean	10.3	25.2	54.5	102.8	208.8	356.4	466.5
S.D. (n^{-1})	3.35	4.66	5.65	5.42	12.37	30.84	40.07
CV (%)	32.5	18.5	10.4	5.3	5.9	8.7	8.6
Accuracy (%)	103.0	100.8	109.0	102.4	104.4	101.8	93.3

[a] CV, coefficient of variation; NR, no result.

not sufficiently sensitive for the 10-pg ml^{-1} sample to be reproducibly measured within acceptable limits. Whilst the intra-batch data for the 10-pg ml^{-1} validation sample showed a reasonable precision (11.5%), the inter-batch precision was unacceptable (32.5%).

The inter-batch precision values for the three sets of QC samples ranged from 6.1 to 12.5% (CV) and the accuracy was between 94.7 and 101.5% for seven batches.

The overall extraction efficiency of FP from human plasma ranged between 81.6 and 97% over six concentrations. The efficiency was 85.2% at 25 pg ml^{-1} and 97% at 500 pg ml^{-1}, but was not acceptable at 10 pg ml^{-1}. The check on the room temperature stability of FP in human plasma varied between -4.7% and $+3.7\%$ at 4 h and between -4.3% and $+1.5\%$ at 24 h, relative to the zero time analyses. The stability of FP in human plasma following three freeze-thaw cycles was between -6.9% and $+2.3\%$ for the third thaw relative to the zero-time analyses. FP was also shown to have acceptable stability in sample extracts held at ambient temperature for the duration (5.5 h) of a typical analytical run of 100 samples.

The concentration at 25 pg ml^{-1} was taken as the lower limit of quantitation with the upper limit being 500 pg ml^{-1}.

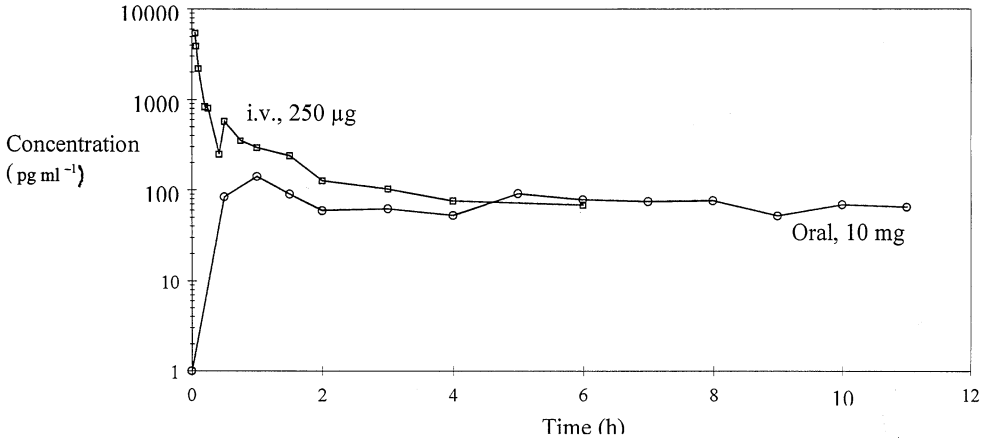

Fig. 5. Concentration versus time profile following administration of fluticasone propionate to a volunteer at either 250 µg by the i.v. route or 10 mg by the oral route. Concentration, pg ml^{-1}; time, h; upper plot: intravenous dose; lower plot: oral dose.

3.3. Application of the method to clinical samples

The validated method was subsequently used to determine the concentrations of FP in human plasma following intravenous, oral or inhalation administration at various dose levels. A plasma concentration versus time profile following intravenous dosage of a single dose of 250 µg of FP is compared to the profile following an oral dose of 10 mg (Fig. 5). The peak plasma concentrations following a single oral dose of 10 mg to five volunteers was low (range 47–179 pg ml^{-1}) confirming that FP had a low bioavailability.

During the analyses of clinical samples from an inhalation study in which FP was administered to 12 subjects as three separate treatments, the inter-batch precision (between 3.9 and 12.7%) and accuracy (between 92.7 and 108.8%) of the calibration samples was found to be acceptable. The coefficient of determination ranged between 0.9803 and 0.9933 for 11 batches of samples. The performance of the QC samples was also shown to be satisfactory (precision: low QC, 14.6%; medium QC, 7.3%; high QC, 7.0%; accuracy: low QC, 110.9%; medium QC, 108.0; high QC, 100.7%). Comparable performance data were obtained during a second inhalation study in which the half-life of elimination was shown to be ~ 6 h and independent of dose [9].

In conclusion it has been shown that LC-MS/MS can be used to determine the low picogram concentrations of FP in human plasma which might be expected following administration of low microgram levels of the compound for the treatment of asthma and rhinitis. The validated method has acceptable precision and accuracy over the concentration range of 25–500 pg ml^{-1} and is more sensitive than the alternative RIA-SPE method used previously. It surpasses that technique in terms of specificity, reliability and throughput and so is better suited to the determination of FP in clinical samples.

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