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A sensitive LC–MS/MS method for the quantification of fluticasone propionate in human plasma

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Abstract

A sensitive and selective LC–(APCI) MS/MS method capable of quantifying fluticasone propionate (FP) at levels down to 10 pg ml^{-1} in human plasma is reported. The method was validated over a linear range from 10 to 1000 pg ml^{-1} using a previously published solid-phase extraction procedure with a $^{13}C_3$-labeled internal standard. The inter and intra batch precision (coefficient of variation) and accuracy (% bias) of the quality controls samples (20, 25, 50, 100, 200, 500 and 1000 pg ml^{-1}) were less than 15 and 11%, respectively. The method is robust, rapid (analysis time of 2 min), selective and hence is ideally suited for pharmacokinetic investigations involving inhalation of therapeutic doses of FP. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: APCI; Fluticasone propionate; LC–MS/MS; MRM

1. Introduction

Fluticasone propionate (FP) (Fig. 1) is a new synthetic glucocorticoid with potent anti-inflammatory activity that has been effectively used in the treatment of chronic asthma [1]. Over the years, analytical techniques such as HPLC–UV and radioimmunoassays (RIA) have been used for the determination of glucocorticoids in plasma [2]. However, they either lack the sensitivity needed for detecting levels that are seen with FP after therapeutic doses or selectivity due to interfering endogenous steroids. Alternative methods like HPLC/RIA are extremely time consuming. GC–MS techniques, have been widely used for steroids but they require derivatization [3,4].

Recently, liquid chromatography–mass spectrometric assays (LC–MS) have been developed for the analysis of corticosteroids [5–9]. Over the past few years, atmospheric pressure chemical ionization (APCI) and thermospray (TS) ionization techniques have been used for the quantitation of corticosteroids in various biological fluids [5–9]. However, TS ionization is increasingly less

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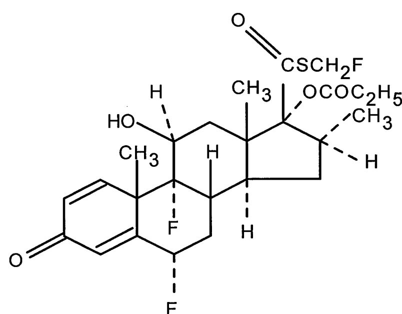

Fig. 1. Structure of FP.

commonly employed due to the lack of reproducibility [7].

Li and coworkers have demonstrated the use of LC–APCI–MS for quantifying FP in plasma with improved sensitivity [9]. The single ion monitoring or recording (SIM or SIR) technique was employed in this assay [9]. Recently, the use of selected or multiple reaction monitoring (SRM or MRM) for quantitation has gained immense popularity because of the additional selectivity it provides by ion monitoring both parent and daughter ions. The increase in specificity consequently enhances the signal-to-noise ratio when only trace quantities of analyte are introduced. This allows the development of assays with minimal sample preparation and also permits some compromises in the chromatography step(s) [10]. This paper describes a rapid and sensitive LC–APCI–MS–MS method using MRM analysis to accurately quantify FP in plasma after inhalation of therapeutically relevant doses.

2. Experimental

2.1. Chemicals

FP and the internal standard (I.S.), $^{13}C_3$-FP, were kindly provided by GlaxoWellcome R&D,

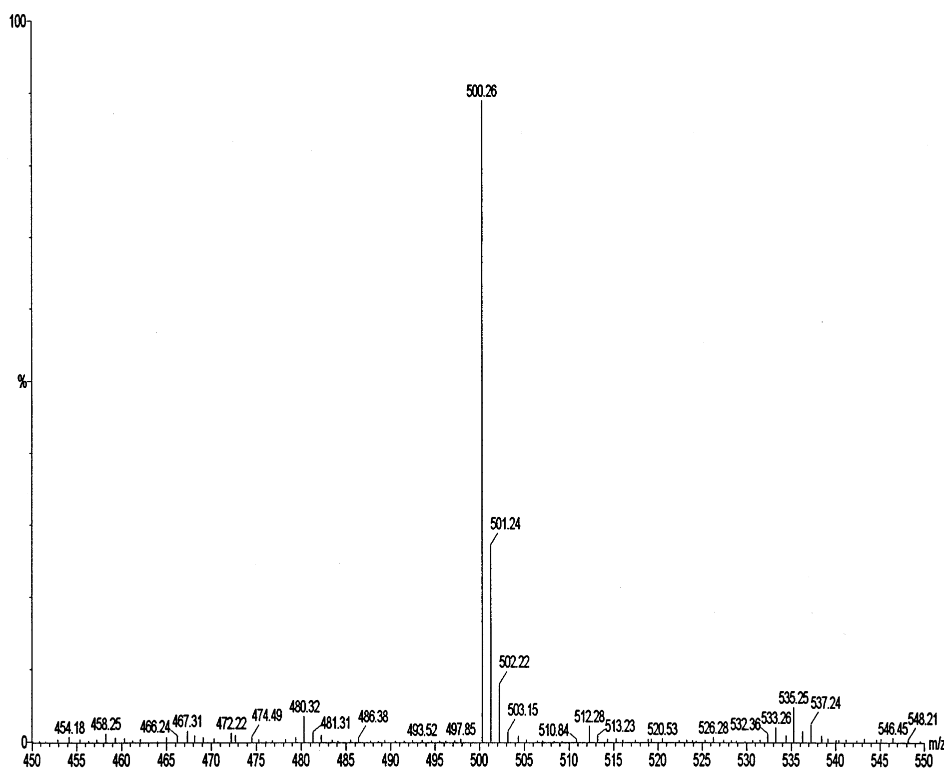

Fig. 2. Full scan mass spectra of FP (m/z 500.2).

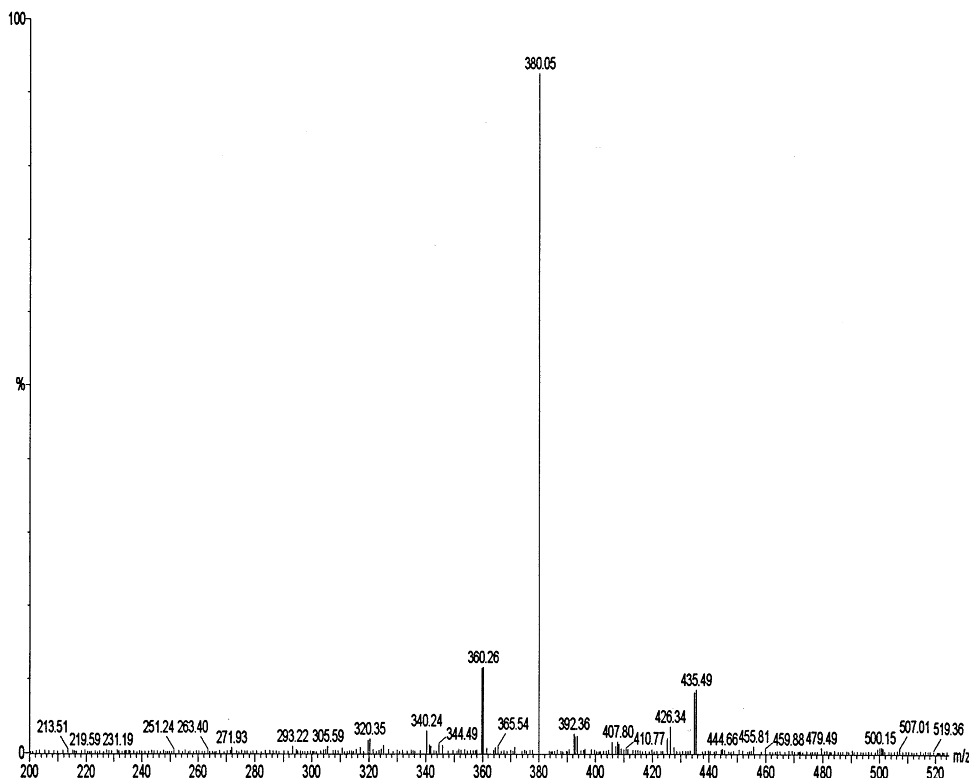

Fig. 3. Daughter scan mass spectra of FP (base peak at *m*/*z* 380).

Ware, Herts, UK. Methanol and water were of OPTIMA grade and purchased from Fisher Scientific (Springfield, NJ). Ethanol, ethyl acetate and heptane were of HPLC grade and purchased from Sigma, Aldrich (St. Louis, MO). Drug-free human plasma was obtained from the Civitan regional blood system (Gainesville, FL). The solid phase LC_{18} (6 ml) cartridges for sample extraction were obtained from Supelco (Bellefonte, PA).

2.2. Preparation of standard solutions and plasma samples

Primary stock solutions of FP were prepared by dissolving 10 mg of FP in 50 ml of methanol and these were then stored at -20°C. The working solutions used for the preparation of plasma calibration standards and quality control samples were 10 and 1 ng ml^{-1} in a mixture of methanol–water (80:20, v/v). From one of the stock solutions, a large batch of quality control samples (10, 20, 25, 50, 100, 200, 500, 1000 pg ml^{-1}) was prepared in plasma to be used against independently prepared plasma calibration standards. The calibration curve ranged from 10 to 1000 pg ml^{-1}. The I.S. stock solution was prepared by dissolving 200 µg in 1 ml of methanol and stored at -20°C. A working solution of 20 ng ml^{-1} was prepared by diluting the stock solution with methanol.

2.3. Sample extraction

Plasma samples were thawed at room temperature. After addition of 50 µl of I.S. working solution to 1 ml plasma corresponding to approximately 1000 pg ml^{-1} of $^{13}C_3$-FP, the compounds were extracted using a procedure published before [9]. Briefly, 1 ml of 30% ethanol was added to 1 ml of plasma sample and centrifuged to remove

the protein precipitate. Two milliliters of the supernatant was then extracted using a 3 ml end-capped C_{18} cartridge. The analytes were eluted with 3 ml of a mixture of ethyl acetate–heptane (35:65, v/v). The residue was evaporated under vacuum and reconstituted in 100 μl of a mixture of methanol–water (80:20, v/v). A total sample volume of 80 μl was injected into the HPLC–APCI–MS–MS system.

2.4. LC–MS/MS conditions

The analysis of FP was performed using a Micromass Quattro-LC-Z (Beverly, MA) triple quadrupole mass spectrometer equipped with an APCI ion source. The source temperature was set to 120°C and the APCI probe temperature was set to 500°C. Corona and cone voltages were set to 2.5 kV and 20 V respectively. The mass resolution was set to unit mass. A dwell time of 1 s was used for scanning FP and 0.1 s for the I.S. The MS–MS signals were optimized by injecting a 1 μg ml^{-1} solution of FP in methanol at a flow-rate of 100 μl min^{-1} using a Kd-Scientific® infusion pump. Argon was used as the collision gas. The mass spectrometer was linked to a Perkin Elmer ISS 200 autosampler via contact closure and the operation was controlled by computer software,

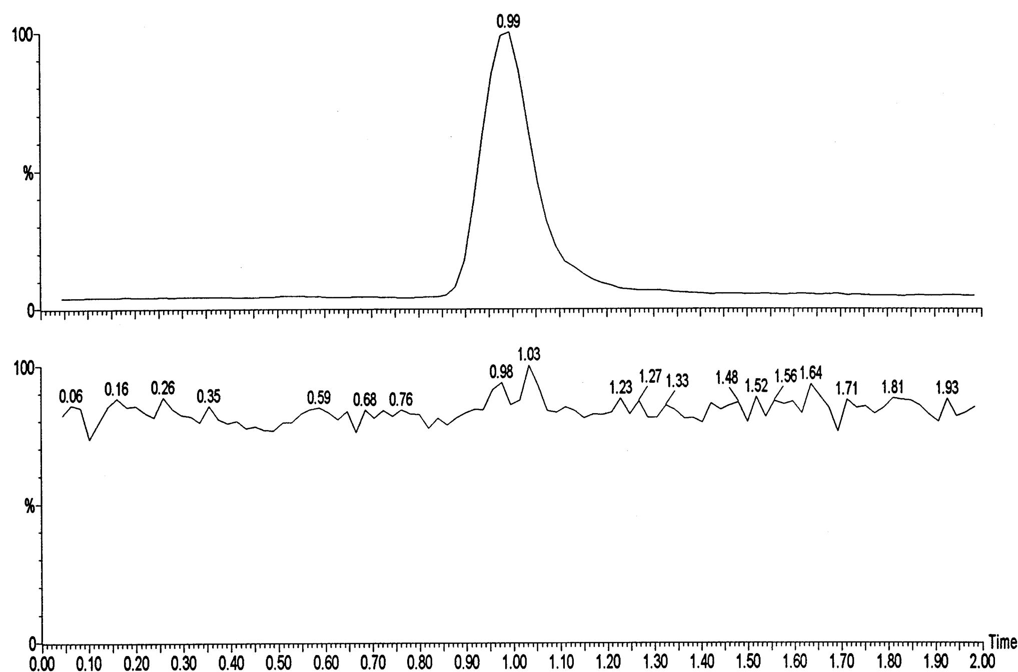

Fig. 4. TIC chromatograms of blank plasma (bottom) and plasma spiked with I.S. (top).

Table 1
Inter-day precision and accuracy for FP standard concentrations

FP (pg ml^{-1})[a]							
Theoretical concentration	20	25	50	100	200	500	1000
Mean ± S.D.	20.1 ± 0.8	27.6 ± 1.8	56.0 ± 1.3	89.0 ± 2.3	188.6 ± 2	517.0 ± 3.4	995.1 ± 5.5
% Bias	0.28	10.4	11.9	−11.2	−5.7	3.4	−0.5
CV (%)	4.1	6.6	2.3	2.6	1.1	1.9	0.5

[a] $n = 18$.

Table 2
Inter and intrabatch precision and accuracy for FP quality control samples

FP (pg ml^{-1})	10	20	25	50	100	200	500	1000
Batch 1 (n = 6)								
Mean ± S.D.	9.8 ± 1.3	20.6 ± 1.9	25.2 ± 3.0	53.4 ± 2.3	111.8 ± 4.9	216.2 ± 7.7	526.2 ± 24	992.8 ± 44
% Bias	−2.0	2.9	0.8	6.4	10.5	7.5	5.0	0.7
CV (%)	13.3	9.2	11.9	4.4	4.5	3.6	4.6	4.5
Batch 2 (n = 3)								
Mean ± S.D.	9.25 ± 1.2	19.0 ± 2.7	26 ± 1.4	52.5 ± 2	103 ± 8.1	198.5 ± 5	503.7 ± 39	938 ± 11.7
% Bias	−7.5	−5	4	5	3	−0.7	0.7	−6.2
CV (%)	12.9	14.2	5.4	4	7.9	2.6	7.9	1.2
Batch 3 (n = 3)								
Mean ± S.D.	9.16 ± 1.6	19.6 ± 1.5	24 ± 0.5	45.7 ± 2.1	97.6 ± 16.8	185.3 ± 7.4	453.7 ± 21	914.7 ± 65
% Bias	−10	−2.5	−5	−6	−7.7	−8.6	−7.3	−7.6
CV (%)	17.5	7.6	2.1	6.8	11.1	4.3	5.8	6.1

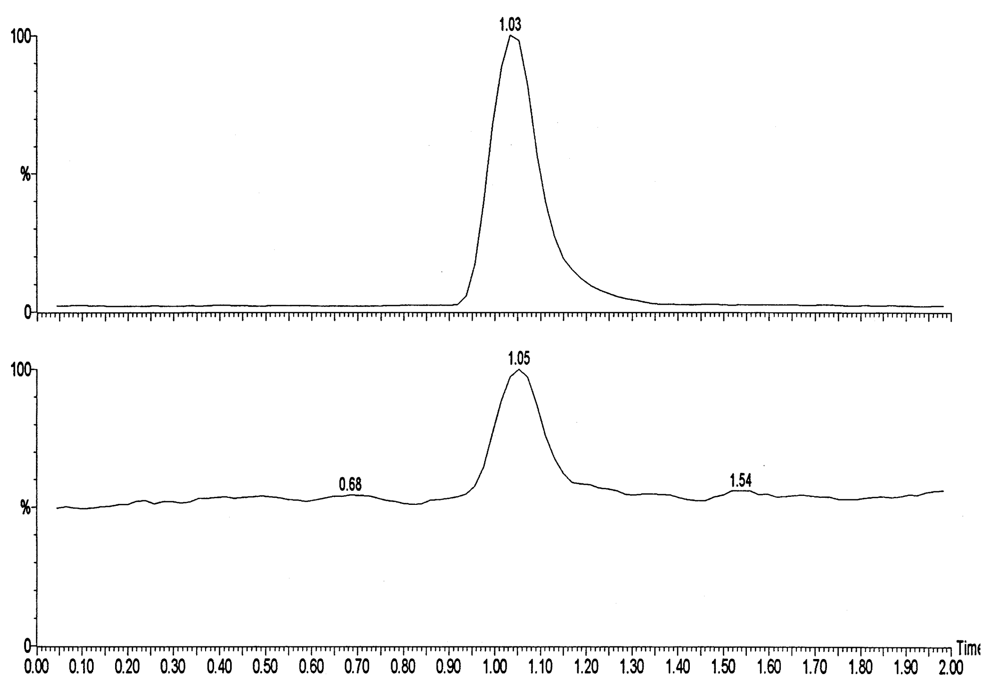

Fig. 5. TIC chromatograms of FP 10 pg ml^{-1} (bottom) and I.S (top) in plasma.

MASSLYNX 3.1. The mobile phase was a mixture of methanol–water (80:20, v/v) delivered at a flow-rate of 1.2 ml min^{-1} by a LDC/Milton Roy CM4000 multiple solvent delivery system. Chromatographic separations were achieved using a Waters 5-μm ODS2 (4.6 × 50 mm i.d.) column (Milford, MA) preceded by a Whatman 5-μm ODS C_{18} guard column cartridge (Clifton, NJ). Data analysis was performed using MASSLYNX software. The calibration curves were plotted as the peak area ratios of FP to internal standard against FP concentration using

a weighted (1/x) linear regression model with nine concentration points (including blank plasma) ranging from 10 to 1000 pg ml^{-1}.

3. Results and discussion

Preliminary experiments were carried out by tuning for FP on both positive and negative ion APCI modes. In the positive ion mode, the MH^+ ion of FP at m/z 501.3 gave two major product ion peaks at m/z 293 and 313. The negative ion mode was chosen because of enhanced signal-to-noise ratio for the product ion peak. Fig. 2 shows the full scan MS1 mass spectrum of FP in the negative ion mode where the molecular ion ($M^{-\bullet}$) is m/z 500.2 The I.S. had a molecular ion at m/z 503.3. The selected precursor ions (m/z 500.2 and 503.3) were introduced into the collision cell to obtain the product spectrum. Both FP and $^{13}C_3$-FP had a similar fragmentation pattern with the base peak of the product ion spectrum at m/z 380 (Fig. 3). The maximum abundance of the daughter ion was obtained at a collision energy of 10 eV. The transitions selected for monitoring FP and $^{13}C_3$-FP were m/z 500.2–380 and 503.3–380, respectively. The purity of the I.S. reference material was tested by monitoring the FP transition upon injection of a concentrated solution (1 μg ml^{-1}) of I.S. prepared in methanol–water (80:20, v/v) mixture. No peaks were observed in the FP transition, which indicated that there was no interference from the internal standard reference material.

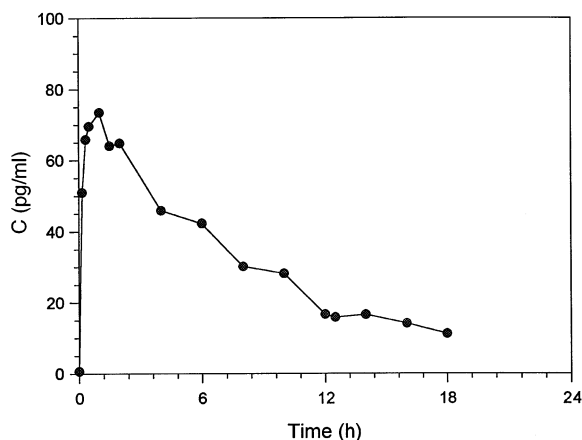

Fig. 6. Pharmacokinetic profile of a subject dosed with 500 μg of FP via inhalation.

Blank plasma from 16 humans was screened during method validation and no interference was observed. Figs. 4 and 5 show the TIC chromatograms for blank plasma and FP (10 pg ml^{-1}), respectively, spiked with the I.S. The analysis time was 2 min with both FP and I.S. eluted at 1 min. The resulting calibration curves were linear with coefficient of determination (r^2) > 0.995 with slopes of 1.004 ± 0.11 ($n = 21$).

3.1. Recovery

The recovery of FP was 81.8% ± 4.7 for a 100 pg ml^{-1} solution in plasma ($n = 6$) which was comparable to that published earlier [9].

3.2. Validation

In the first part of the study, as a pre-validation evaluation of the method and instrument performance, six calibration curves from one batch (range: 20–1000 pg ml^{-1}) were run each day on 3 separate days. Consistently good correlation ($r^2 >$ 0.995) was obtained throughout the process. Table 1 shows the inter-day precision and accu-racy data for each standard concentration. Based on the results, it was decided to add a lower concentration of 10 pg ml^{-1} for the standard curve and the quality controls.

In the second part of the validation study, calibration curves were used to calculate the concentrations of independently prepared QCs over 3 days. The calibration curves used on those three occasions were also prepared independently by separately weighing out FP. Table 2 displays the inter and intrabatch precision and accuracy data for eight quality control levels. The data shows that this LC–MS–MS method is consistent and reliable with good accuracy (< 11%) and precision (17% at the lower limit of quantitation and < 15% at other concentrations).

This MRM method, with a lower limit of quantitation (LLOQ) of 10 pg ml^{-1} and signal to noise ratio of > 5, is substantially more sensitive than the SIR method described by Li et al., which had an LLOQ of 200 pg ml^{-1} [9]. MRM analysis, therefore, has not only provided additional selec-

tivity by monitoring the fragment ion specific to FP, but has also provided with increased sensitivity and turnover as shown in this report.

The presented method is slightly more sensitive than the one reported recently by Pleasance et al. employing turboionspray positive ionization for quantifying FP to levels down to 20 pg ml^{-1} [11]. Using a different LC–MS–MS instrument than the one used by Pleasance et al., initial attempts to ionize FP using electrospray in the positive ion mode resulted in the formation of major sodium adduct ions that underwent little fragmentation and as a result yielded inadequate sensitivity. The presented method using atmospheric pressure chemical ionization in the negative ion mode provides an alternative for quantifying FP using instruments that are susceptible to sodium adduct interference.

3.3. Stability

The freeze thaw stability of a 500 pg ml^{-1} solution in plasma of FP was evaluated. Fluticasone propionate (FP) was stable in plasma even after four freeze thaw cycles (ANOVA, $P = 0.63$). The bench top stability of a 100 pg ml^{-1} plasma FP solution after extraction and reconstitution was evaluated over 24 h. FP showed good stability over the time period studied (ANOVA, $P = 0.42$).

3.4. Clinical application

This LC–MS–MS method was used to provide pharmacokinetic data for FP in human plasma following inhalation. Volunteers were given single and multiple doses of 200 and 500 μg of FP and subsequently sampled at specific times for the determination of FP in plasma. A plot of FP concentrations versus time after treatment from a healthy volunteer is shown in Fig. 6. Approximately 1100 clinical samples from 12 subjects were analyzed in a short period of time.

4. Conclusions

A simple, sensitive and selective LC–MS–MS method was developed using a solid phase extraction procedure for quantifying FP in human plasma. Validation results have shown that the method is robust and meets the requirements of the pharmacokinetic investigation after inhalation of therapeutic doses. Finally, the reported method is sensitive (LOQ 10 pg ml^{-1}) and rapid (analysis time of 2 min) and hence ideally suited for pharmacokinetic investigations involving large number of samples.

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