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Journal of Chromatography B, 761 (2001) 177–185

JOURNAL OF CHROMATOGRAPHY B

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Quantification of epimeric budesonide and fluticasone propionate in human plasma by liquid chromatography–atmospheric pressure chemical ionization tandem mass spectrometry

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Received 20 March 2001; received in revised form 3 July 2001; accepted 10 July 2001

Abstract

A highly sensitive and selective liquid chromatography–atmospheric pressure chemical ionization tandem mass spectrometry assay was developed and validated for simultaneous determination of epimeric budesonide (BUD) and fluticasone propionate (FP) in plasma. The drugs were isolated from human plasma using C_{18} solid-phase extraction cartridges, and epimeric BUD was acetylated with a mixture of 12.5% acetic anhydride and 12.5% triethylamine in acetonitrile to form the 21-acetyl derivatives following the solid-phase extraction. Deuterium-labelled BUD acetate with an isotopic purity >99% was synthesized and used as the internal standard. The assay was linear over the ranges 0.05–10.0 ng/ml for epimeric BUD, and 0.02–4.0 ng/ml for FP. The inter- and intra-day relative standard deviations were <14.3% in the assay concentration range.

Keywords: Budesonide; Fluticasone propionate

1. Introduction

Budesonide (BUD) is a potent glucocorticosteroid with high local anti-inflammatory, but low systemic glucocorticoid activity [1]. The drug, a mixture of two epimers with 22*R* and 22*S* configuration at approximately 1:1 ratio, is rapidly and extensively metabolized in the liver [2]. Its pharmacokinetic characterizations of low oral bioavailability, large volume of distribution, and high systemic clearance [3,4] lead to low plasma concentrations following inhalation of therapeutic doses. Fluticasone propionate (FP) is another potent glucocorticoid, which, via pressurized metered dose inhaler (pMDI) [5], appears as effective as BUD [6] and beclomethasone dipropionate (BDP) at half the equivalent micrograms dosage, in patients with moderate to severe asthma [7]. The drug is cleared by hepatic metabolism in experimental animals and in man [2], with a total blood clearance equivalent to hepatic blood flow. Both BUD and FP (Fig. 1) have been reported not to be biotransformed locally in the lung [8]. The fraction of inhaled drugs deposited in the lung is generally accepted to be approximately 20% of the

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0378-4347/01/$ – see front matter
PII: S0378-4347(01)00329-2

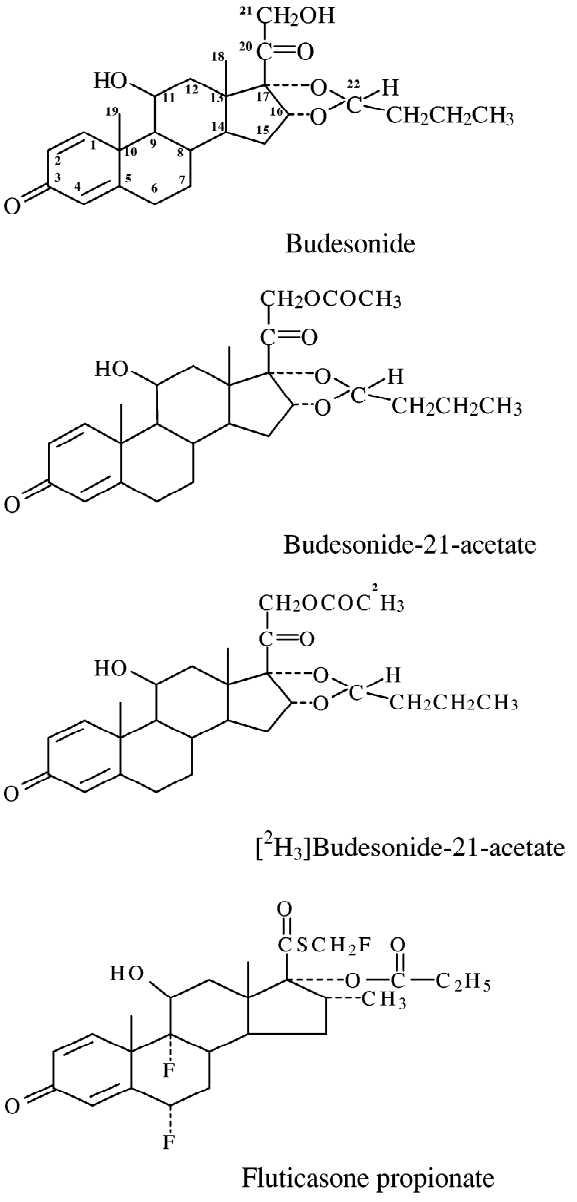


Fig. 1. Structures of budesonide, budesonide-21-acetate, [$^2H_3$]budesonide-21-acetate, fluticasone propionate.

dose and contributes substantially to the systemic availability. The dose delivered to the lung is small, so the amount of drug available for the systemic absorption is very small, resulting in low plasma drug concentrations. A highly sensitive and selective bioanalytical method was required for estimating systemic exposure of the drugs following inhalation.

The pharmacokinetic profile of BUD was previously determined in dogs and a small number of human subjects by giving radio-labelled BUD intravenously using high-performance liquid chromatography (HPLC) methods [3,9]. Radioimmunoassays combined with liquid chromatography (RIA–LC) have been used for determinations of BUD and FP in human plasma [10,11]. Published methods for quantification of BUD in human plasma involved an automated liquid chromatography thermospray mass spectrometry (LC–TSP-MS) [12] and a liquid chromatography atmospheric pressure chemical ionization mass spectrometry (LC–APCI-MS) method for BUD [13] and FP [14] and more recently liquid chromatography atmospheric pressure chemical ionization tandem mass spectrometry (LC–APCI-MS–MS) [15,16]. The LC–TSP-MS method had widely variable thermospray responses from one compound to another and the LC–APCI-MS assay showed occasionally interferences from endogenous substances (Fig. 2). In our previous analysis of authentic clinical samples [13] the ion chromatogram revealed an interfering peak as a shoulder on the back of the 22*R* epimer. More recently, Callejas et al. [17], Laugher et al. [18] and Krishnawami et al. [19] have reported the development and validation of methods for FP in human plasma using LC–MS–MS following automated solid-phase extraction. Other reported methods of analysis for BUD, Kaiser et al. [15] and Kronkvist et al. [16] lack the ability to determine both the 22*R* and 22*S* epimers of BUD individually.

In the present work, we describe a highly sensitive and selective method for the simultaneous quantification of BUD 22*R* and 22*S* epimers and FP in human plasma. The method was employed to determine the plasma concentration profiles of these drugs following inhalation by normal volunteers participating in a cross-over comparative clinical trial of these therapeutic agents.

MS–MS was used for the determination of the BUD and FP as we found that with a number of clinical samples that endogenous material appeared in the chromatograms when using LC–APCI-MS (Fig. 2) for these samples, making quantification impossible. When we developed the LC–APCI-MS–MS method there were no problems with any of the clinical samples and quantifiable data were obtained for all samples. The ability of this assay, which we

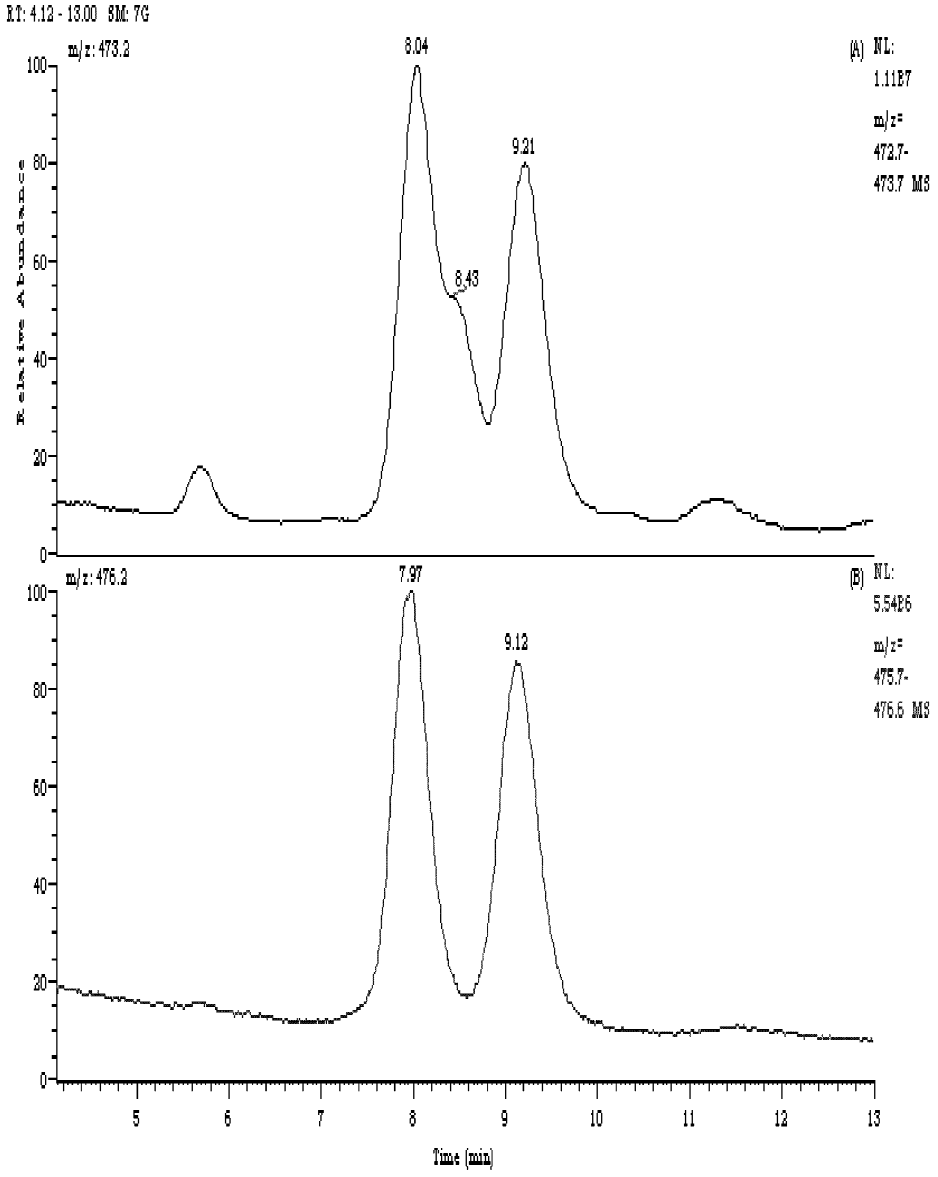


Fig. 2. Representative interfering LC–APCI-MS chromatograms of (A) epimeric budesonide (2.0 ng/ml); (B) blank plasma; (C) internal standard (5.0 ng/ml).

describe, to resolve the epimers of BUD may be an important clinical consideration.

2. Experimental

2.1. Chemicals

BUD, 16α,17α-(22*S*)- and 16α,17α-(22*R*)-propylmethyl enedioxy pregna-1,4-diene-11β,21-diol-3,20-dione, the epimers of 22*R*, 22*S*, respectively, and FP, were kindly provided by AstraZeneca Lund (Sweden), and GlaxoSmithKline (UK), respectively. Acetic anhydride-d_6 (99+ atom-% D), acetic anhydride (99%), ethyl acetate (99.8%, HPLC grade), ethanol (reagent, denatured, HPLC grade), heptane (99+%, HPLC grade), and triethylamine (minimum

99%) were purchased from Sigma–Aldrich (Sydney, Australia). Acetonitrile (ChromAR HPLC grade) was obtained from Rhone-Poulenc Laboratory Products (Clayton, Australia).

Solid-phase extraction C_{18} cartridges (Extract Clean; 500 mg, 6 ml) were purchased from Alltech (Sydney, Australia). Drug-free human plasma used in this study was supplied by the Red-Cross Blood Bank (Sydney, Australia) and stored at −20°C. Aqueous solutions were prepared using doubly distilled water.

2.2. LC–APCI-MS–MS instrumentation

The analysis of BUD epimers and FP was performed on a Finnigan/Mat TSQ 7000 LC–MS–MS system (San Jose, CA, USA). A Hewlett-Packard HP 1090 liquid chromatograph controlled by the software of the TSQ 7000 was coupled with the system. An APCI interface was used in the positive ionization mode. The temperatures of heated capillary and vaporizer were 180°C, and 475°C respectively. Selected reaction monitoring (SRM) was used for the analytical assay, monitoring 473 to 455 for BUD-21-acetate, 476 to 455 for [$^2H_3$]BUD-21-acetate and 501 to 313 for FP. Argon was used as the collision gas at 2.0 mTorr and the corona current 5 μA (1 Torr=133.322 Pa). Separation of BUD epimers and FP was achieved using a 5 μm ODS Hypersil (100× 2.1 mm I.D.) narrow-bore column (Hewlett-Packard, Blackburn, Australia) equipped with a 5 μm ODS Hypersil C_{18} (20×2.1 mm I.D.) guard column cartridge (Hewlett-Packard). The mobile phase used was a mixture of ethanol–water (43:57, v/v) with a flow-rate of 500 μl/min, and filtered through a 0.45-μm HVLP filter (Millipore, Sydney, Australia) before use.

2.3. Preparation of [$^2H_3$]BUD-21-acetate and standard solutions

The I.S., deuterium-labelled BUD-21-acetate was synthesized by dissolving 20.0 mg of BUD in 5 ml of a solution comprising of 12.5% acetic anhydride-d_3 and 12.5% triethylamine in acetonitrile. The procedures of synthesis and purification were described previously [13]. The working solution of the I.S. was prepared in ethanol at a concentration of 0.2 μg/ml.

Stock solutions of BUD and FP were prepared in ethanol at concentrations of 5.0 and 2.0 mg/ml, respectively and stored at −20°C for maximum 8 weeks. Serial dilution of the stock solution was carried out to prepare working solutions at concentrations of 0.5 and 0.2 μg/ml for BUD and FP, respectively. Six 100-ml standard plasma samples including blank human plasma were prepared in three replicates through the serial dilutions of the working solutions spiked into blank human plasma in the concentration range of 0.05–10.0 ng/ml for BUD, and 0.02–4.0 ng/ml for FP. All working solutions of BUD, FP and I.S. were kept at −20°C and were allowed to warm to room temperature with protection of light before use.

2.4. Analytical procedure

To a 50-μl volume of the working solution of the I.S., corresponding to 10.0 ng of [$^2H_3$]BUD-21-acetate, plus 1 ml of 30% ethanol in water was added to 1 ml of the standard plasma samples in the concentration range 0.05–10.0 ng/ml for BUD, and 0.02–4.0 ng/ml for FP, as prepared as per Section 2.3. Samples were carefully vortex-mixed and allowed to stand for 15 min before centrifugation at 1200 *g* for 20 min to remove protein precipitates, resulting from the addition of 30% ethanol. The supernatants were transferred to the solid-phase extraction C_{18} cartridges pre-conditioned by rinsing them twice with 3 ml of ethanol and twice with 3 ml of water. A 24-port manifold (Supelco, Bellefonte, PA, USA) 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 at a drop wise flow-rate. The cartridges were then washed consecutively with 3.0 ml of 25% ethanol, 3.0 ml of water, and 2.0 ml of 2% ethyl acetate in heptane. The analytes were eluted with 2.0 ml of 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 to elute analytes possibly retained on the cartridge, which was performed for every 20 extracts on a standard plasma sample of BUD and FP at 5.0 and 2.0 ng/ml, respectively. The solvent was evaporated to dryness under a stream of N_2 at 35°C, and the residue was treated with 100 μl of derivatizing reagent containing 12.5% acetic anhydride and

12.5% triethylamine in acetonitrile for 15 min at room temperature.

After evaporating the derivatizing reagent to dryness under a stream of N_2 without heating, the samples were 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 25 μl was injected into the LC–APCI-MS–MS system.

3. Results and discussion

3.1. Solid-phase extraction

The recoveries of 22*R*,*S*-BUD-21-acetate and FP are shown in Table 1. The extraction procedure for epimeric BUD [13] and FP [14] reported previously were employed. Mean recoveries were 88.5±4.13% for BUD, and 88.2±7.34% for FP.

3.2. Derivatization

We have previously shown that derivatization with acetic anhydride increases about fivefold of the APCI-MS response for the epimeric BUD [13]. SRM of the daughter ions was performed in the present work and showed the same magnitude of sensitivity increase as may be expected. There was no evidence of FP reacting with the derivatization agent, verified by running full scan LC–MS on both derivatized and underivatized FP to show that no modification to FP had occurred the mass m/z and relative sensitivity for both were identical.

3.3. Separations of the epimers 22R and 22S, and FP

The epimeric derivatives of BUD were well separated by the present isocratic chromatographic method. Baseline resolutions of each epimer and FP were achieved, free from interfering endogenous substances in plasma in this study. Typical LC–APCI-MS–MS chromatograms of the epimer-21-acetyl derivatives and FP (Fig. 3) indicate the relatively short retention times (approximately 8.1 min for 22*R*, 9.3 min for 22*S*, and 7.4 min for FP). There was no baseline shift observed in the LC–MS–MS chromatograms at the lowest concentrations, enabling accurate determination of the 22*R* and 22*S* epimers of BUD.

3.4. Mass spectrometry

Full scan positive ion spectra for epimeric BUD-21-acetate, FP and I.S. gave ions of m/z 473.1, m/z 501.2, and m/z 476.1, respectively. MS–MS fragmentation of these ions were monitored by using instrument settings that gave maximum intensity to one major product ion for each component (Table 2). Fig. 4 shows full scan MS spectra of the analytes.

The isotopic purity of trideuterated BUD-21-acetate (>99%) was determined from the peak area ratio of BUD-21-acetate (m/z 473) to [$^2H_3$]BUD-21-acetate (m/z 476) by LC–APCI-MS [13].

3.5. Selectivity

In our previous analysis of clinical samples using LC–APCI-MS, interfering substance were occasionally observed (Fig. 2). The present LC–APCI-MS–MS method improved the selectively and the typical LC–APCI-MS–MS chromatograms are free from interfering substance as shown in Fig. 3. Plasma blanks (n=5) were extracted under the same conditions as described in Section 2.4 with and without I.S. to determine if any endogenous substances may interfere with the analysis. The blanks did not

Table 1
Recovery (n=3) of epimeric budesonide and fluticasone propionate from solid-phase extraction

Concentration (ng/ml)	Recovery (% of total)			
	22*R*	22*S*	Concentration (ng/ml)	FP
1.0	90.9±4.44	90.5±5.28	1.0	79.7±1.61
4.0	80.2±2.58	90.4±2.84	2.0	92.0±0.87
10.0	89.0±1.78	90.2±6.98	4.0	92.8±1.70

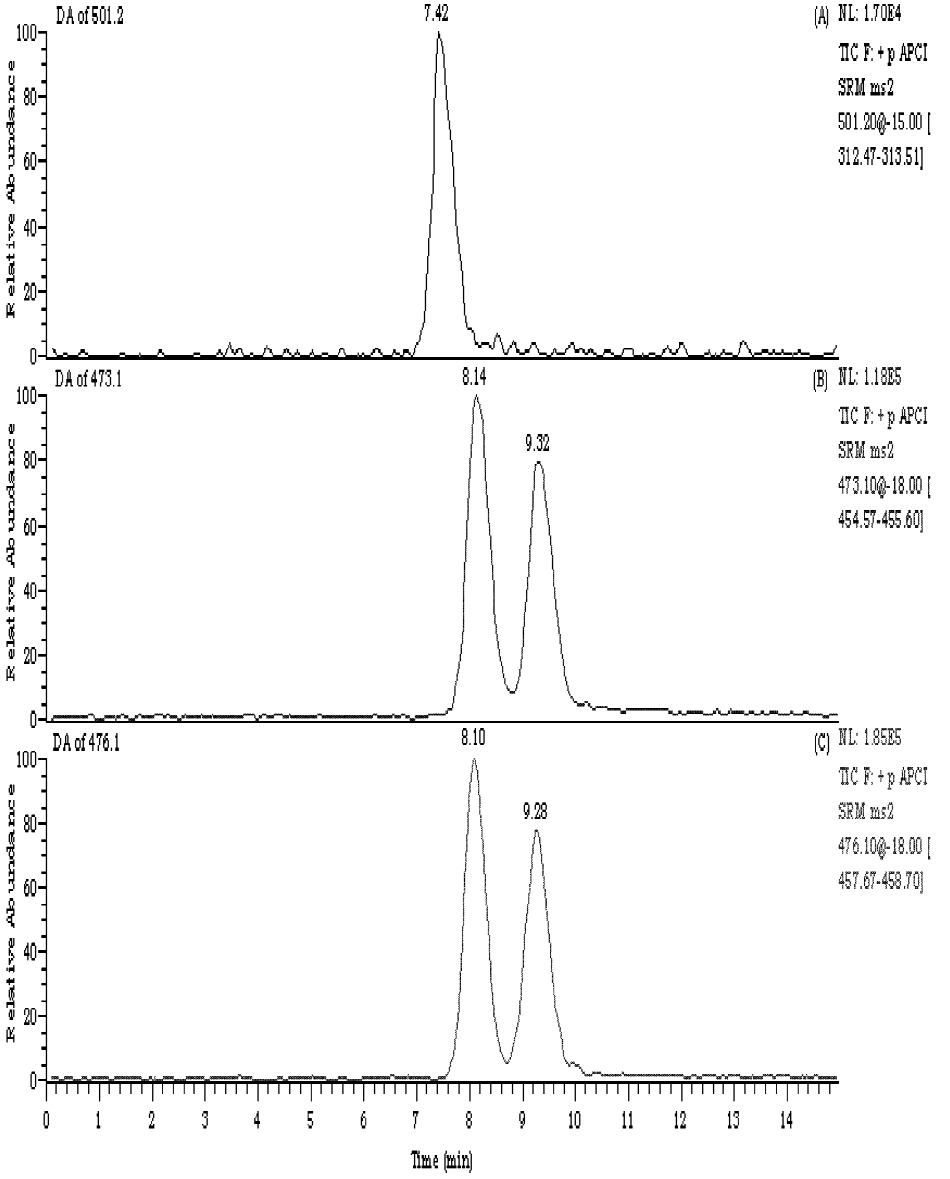


Fig. 3. Typical LC–APCI-MS–MS chromatograms obtained from plasma extracts fluticasone propionate (0.02 ng/ml); (B) epimeric budesonide (0.1 ng/ml); (C) internal standard (5.0 ng/ml).

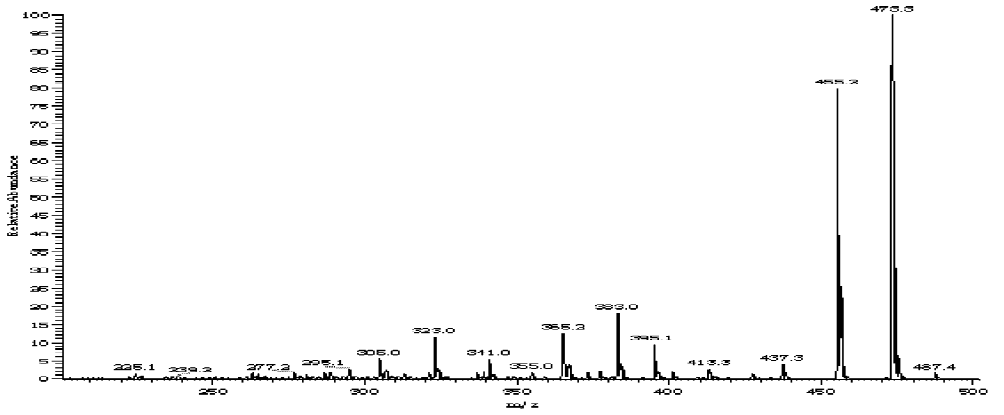
Table 2
Fragmentation of precursor ions

Precursor ion	Product ion	Analyte
473	455	BUD-21-acetate
476	458	[$^{2}H_{3}$]BUD-21-acetate (I.S.)
501	313	FP

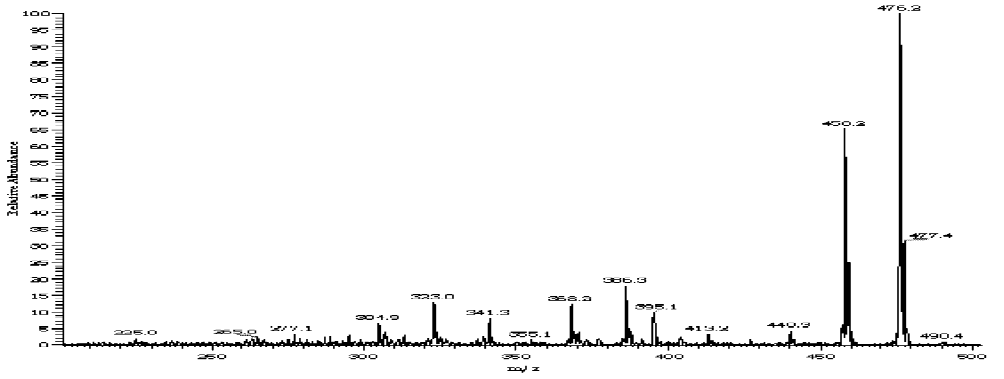
produce any peaks in the SRM experiments that interfered with the quantitation.

3.6. Precision and accuracy

The intra-day variation of the assay method was



(A)



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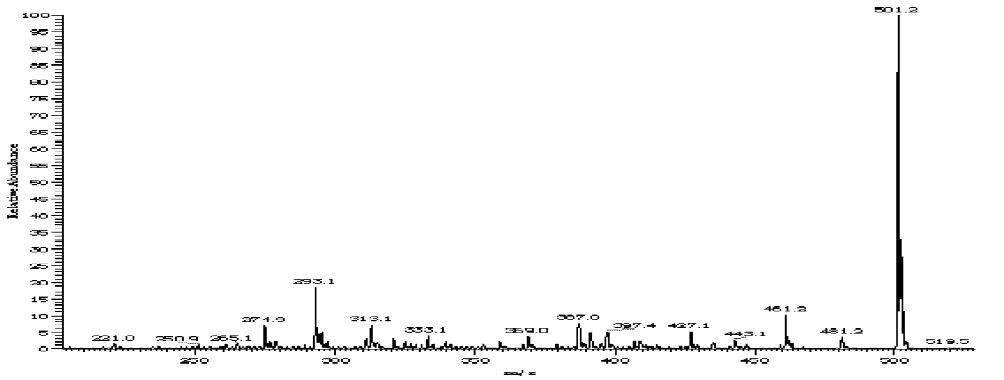

(C)

Fig. 4. MS–MS product ion spectra of (A) budesonide-21-acetate, (B) [$^2H_3$]budesonide-21-acetate (I.S.), (C) fluticasone propionate.

Table 3
Precision and accuracy of budesonide added to human plasma ($n=5$)

Nominal concentration (ng/ml)	Intra-day				Inter-day			
	RSD (%)		Bias (%)		RSD (%)		Bias (%)	
	22*R*	22*S*	22*R*	22*S*	22*R*	22*S*	22*R*	22*S*
0.05	13.8	8.0	6.0	11.2	12.7	13.5	7.3	10.5
1.0	8.1	3.0	4.4	4.2	5.3	8.6	−6.1	8.3
4.0	3.6	4.6	6.4	12.2	3.6	4.4	5.9	4.8
10.0	2.9	4.0	2.7	4.1	3.9	2.7	3.2	1.5

determined by replicate analysis ($n=5$) of blank human plasma spiked with BUD and FP at different concentrations in the range 0.05–10.0 and 0.02–4.0 ng/ml, respectively. The inter-day precision was evaluated by measuring replicates of the same samples at four different concentrations over a period of 2 weeks. The precision was expressed as a percentage by calculating the intra- and inter-day relative standard deviations (RSDs). The accuracy was estimated by the percent difference of the mean concentration determined from the known concentration: [(measured concentration−nominal concentration)÷nominal concentration×100%]. All intra- and inter-day data are summarized in Tables 3 and 4. The peak area ratio of FP to the epimer 22*R* of the I.S. was used for estimation of FP concentrations.

3.7. Linear response and limit of quantification

Linear responses were obtained for BUD epimers and FP over the concentration ranges 0.05–10.0 and 0.02–4.0 ng/ml, respectively. The limit of detection (LOD) was 0.01 ng/ml for FP, and 0.025 for both epimers of BUD at a signal-to-noise ratio (S/N) of 6 under the optimum LC–APCI-MS–MS conditions, these limits do not represent a quantifiable amount, i.e., it was not reproducible to a RSD<20%. The limit of quantification (LOQ) was determined to be 0.05 ng/ml for BUD and 0.02 ng/ml for FP. An improvement in sensitivity over that previously reported in our work involving LC–APCI-MS [13] for the epimers of BUD is increased by a factor of 10 and we have improved on the sensitivity reported for FP in Ref. [14] to the same level.

3.8. Application to clinical samples

The LC–APCI-MS–MS method has been used to support an open-label, randomized, placebo-controlled, seven-period crossover study for comparing the short-term effects of different doses of BUD (400, 800, 1600 μg b.i.d.) and FP (375, 750, 1000 μg b.i.d.) via pMDI. On the last day (day 5) of each high-dose treatment (BUD 1600 μg b.i.d. and FP 1000 μg b.i.d.), 6 ml venous blood samples were collected in a random subgroup of nine individuals at 10:00 (immediately prior to the scheduled dose), then at 15, 30 min, and 1, 2, 4, 6 and 8 h post-dose for direct assay of BUD and FP concentrations in plasma. Fig. 5 shows a representative plasma concentration profile of the epimers 22*R*, 22*S* of BUD

Table 4
Precision and accuracy of fluticasone propionate added to plasma ($n=5$)

Nominal concentration (ng/ml)	Intra-day		Inter-day	
	RSD (%)	Bias (%)	RSD (%)	Bias (%)
0.02	14.3	−4.0	13.5	7.3
1.0	11.0	9.1	13.4	6.2
2.0	5.3	2.2	4.2	−1.4
4.0	1.2	1.8	2.6	3.3

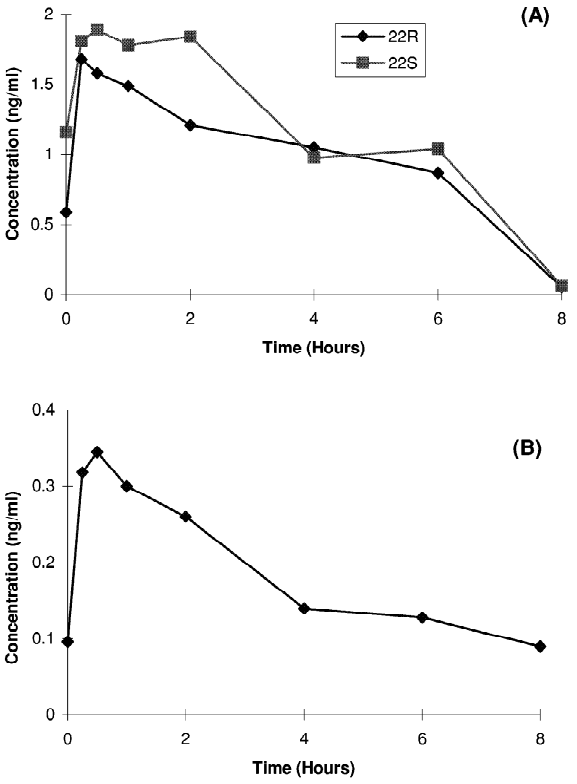

Fig. 5. Representative plasma concentration profiles of (A) epimeric budesonide (1600 μg b.i.d.), and (B) fluticasone propionate (1000 μg b.i.d.) in human plasma following inhalation.

and FP obtained by LC–APCI-MS–MS. The sensitivity of the method allowed plasma concentrations to be followed for 8 h after inhalation of therapeutic doses.

Acknowledgements

The authors would like to thank AstraZeneca Lund (Sweden) for its sponsorship of the clinical study and the supply of BUD and epimers. We are also grateful to GlaxoSmithKline (UK) for the supply of FP.

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