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Journal of Chromatography B, 683 (1996) 259–268

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Determination of epimers 22*R* and 22*S* of budesonide in human plasma by high-performance liquid chromatography–atmospheric pressure chemical ionization mass spectrometry

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Received 19 December 1995; revised 27 February 1996; accepted 29 February 1996

Abstract

A highly sensitive and selective method has been developed for the simultaneous quantification of 22*R*- and 22*S*-epimers of budesonide in human plasma. The drug was isolated from human plasma using C_{18} solid-phase extraction cartridges and was acetylated with a mixture of 12.5% acetic anhydride and 12.5% triethylamine in acetonitrile to form the 21-acetyl derivatives. Deuterium-labelled budesonide was synthesized and determined to have an isotopic purity >99%. This was used as the internal standard. Epimers were quantified by automated liquid chromatography–atmospheric pressure chemical ionization mass spectrometry, operating in selected ion mode at m/z 473.2 and m/z 476.2. Linear responses were observed for both epimers over the range 0.25 to 10.0 ng/ml. The average recoveries of 22*R*- and 22*S*-epimers of budesonide from human plasma were 87.4% and 87.0%, respectively. The lower limit of quantification for each epimer was 0.25 ng/ml, corresponding to 50.0 pg of analyte on column. Within- and between-day coefficients of variation were 8.6% and 4.0%, respectively.

Keywords: Budesonide; Glucocorticosteroids

1. Introduction

Budesonide (BUD) is a topically active glucocorticosteroid commonly used in inhalation treatment of asthma and rhinitis [1–5]. The drug is characterized by a therapeutic index of high local anti-inflammatory, but low systemic activity [6–8]. BUD (Fig. 1) contains an asymmetric $16\alpha,17\alpha$-acetal group, resulting in a mixture of two epimers with 22*R* and 22*S* configurations in approximately a 1:1 ratio [9]. A larger volume of distribution and two to three times greater potency of the epimer 22*R* than 22*S* was observed [2,10].

In early studies, the pharmacokinetics of BUD were determined in dogs and in a small number of human volunteers by giving tritiated BUD intravenously and using HPLC methodology with UV detection [11,12]. Since the widespread use of radiolabelled drug in human subjects and patients is neither feasible nor appropriate, a method of radioimmunoassay combined with liquid chromatography (RIA–LC) for sample clean-up was used [13]. Most of the assays used in these studies have involved administration of radiolabelled drug and are

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PII S0378-4347(96)00111-9

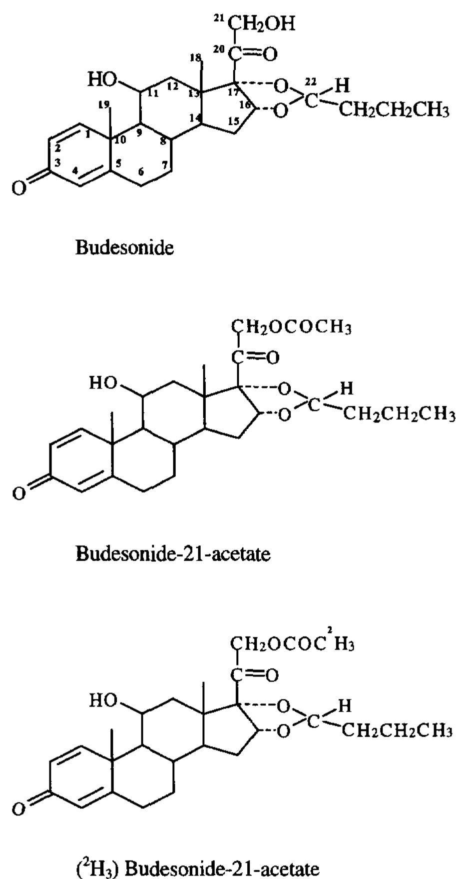

Fig. 1. Structures of budesonide, budesonide-21-acetate and [$^2H_3$]budesonide-21-acetate.

not capable of separating and quantifying each epimer in plasma on a routine basis.

Two recently published assay methods describe the quantitative analysis of BUD in biological specimens [14,15]. One of the assays provided sufficient sensitivity and selectivity for the routine determination of BUD in human plasma, using automated liquid chromatography–thermospray–mass spectrometry (LC–TSP-MS) [14]. However, thermospray response varies widely from one compound to another. Hence, the magnitude and stability of the LC–TSP-MS response for glucocorticosteroids would need to be improved to make this method reliable. A more recent HPLC method which determines BUD in bronchoalveolar lavage with UV detection has insufficient sensitivity to quantify the drug in plasma and tissue following inhalation of therapeutic doses [15]. Moreover, to our knowledge, no study has reported simultaneous quantification of 22*R*- and 22*S*-epimers of BUD in human plasma at low concentrations without the administration of tritiated drug. The present paper describes a sensitive and specific method for simultaneous determination of the epimers in human plasma by automated liquid chromatography–atmospheric pressure chemical ionization mass spectrometry (LC–APCI-MS). The method makes it possible to evaluate reliably pharmacokinetic parameters of each epimer of BUD, following inhalation.

2. Experimental

2.1. Chemicals and reagents

Budesonide (BUD), $16\alpha,17\alpha$-(22*S*)- and $16\alpha,17\alpha$-(22*R*)-propylmethylenedioxypregna-1,4-diene-11β,21-diol-3,20-dione, and the epimers of 22*R* and 22*S* were kindly provided by Astra Draco (Lund, Sweden). Acetic anhydride-d_6 (99+ atom-% D), acetic anhydride (99%), ethyl acetate (99.8%, HPLC grade), ethyl alcohol (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 Rhône-Poulenc Laboratory Products (Clayton, South Victoria, 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 instrumentation

The analysis of the epimers was performed on a Finnigan/Mat TSQ 7000 LC–MS–MS system (San Jose, CA, USA), operating in the APCI mode. The system was linked to a Hewlett-Packard HP 1090 liquid chromatograph controlled by the software of the TSQ 7000. Separation of the epimers 22*R* and 22*S* was achieved using a 5-μm ODS Hypersil (100×2.1 mm I.D.) narrow-bore column (Hewlett-Packard, Blackburn, Vic., Australia), equipped with a

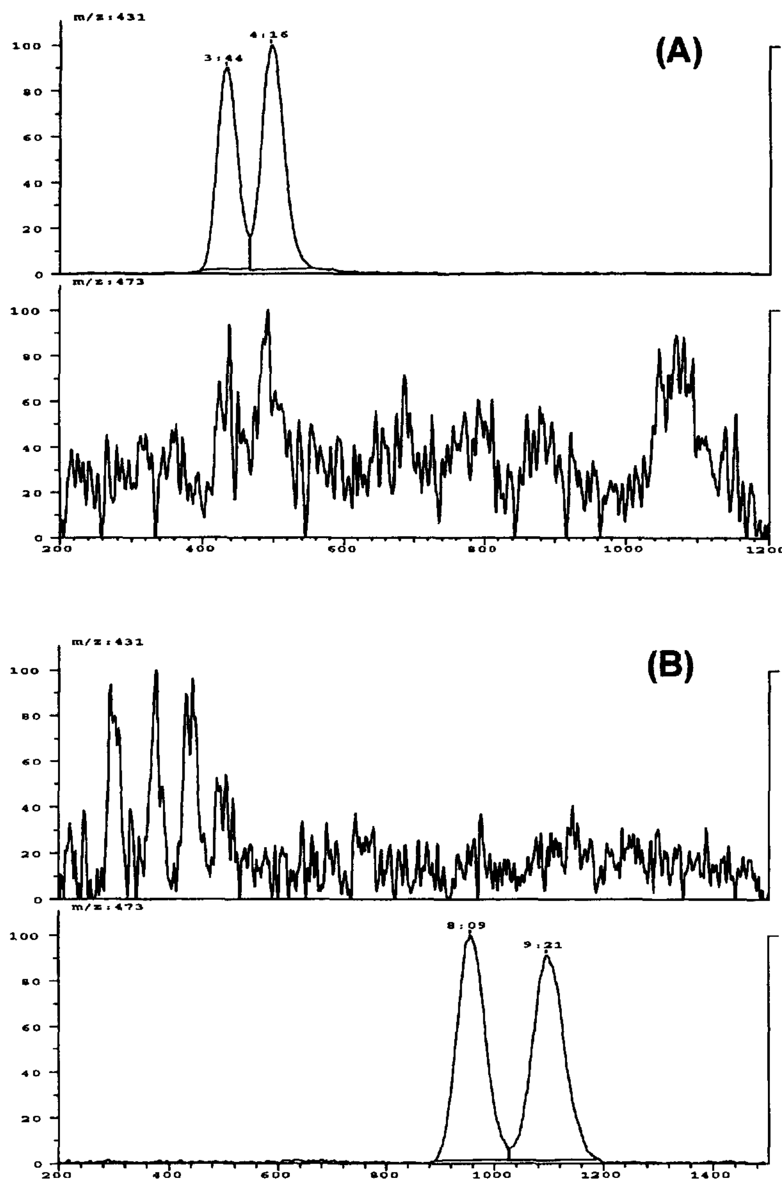

Fig. 2. Mass chromatograms of budesonide epimers (A) without and (B) with derivatization. The chromatograms were obtained from two identical samples at a concentration of 5.0 ng/ml for each epimer.

5-μm ODS Hypersil C_{18} (20×2.1 mm I.D.) guard column cartridge (Hewlett-Packard). The mobile-phase was ethanol–water (43:57, v/v) with a flow-rate of 500 μl/min, and was 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 *m/z* and 476.2 *m/z*, corresponding to the MH^+ of acetylated BUD and deuterium-labelled BUD-21-acetate, respectively. The system was tuned first 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 the 21-acetyl derivative of BUD. Optimum sensitivity was achieved by using an open resolution of approximately 1.5.

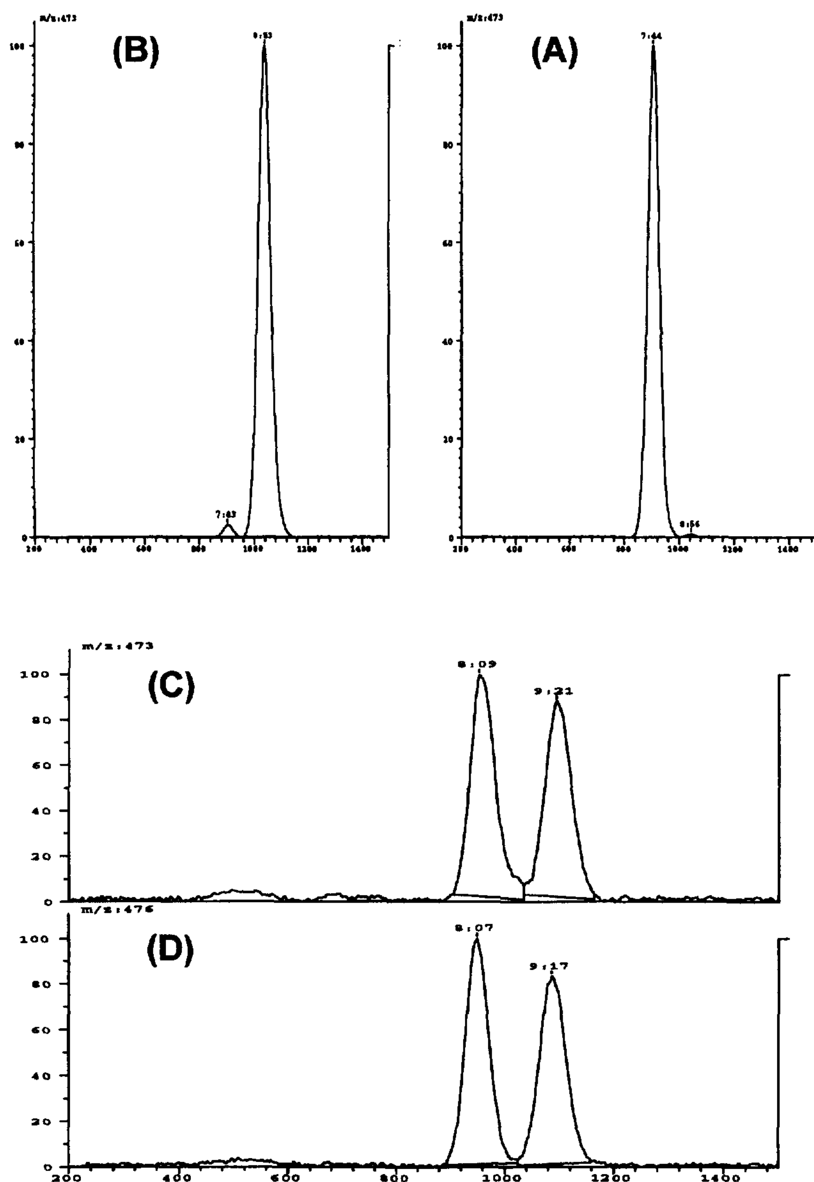

Fig. 3. Representative mass chromatograms of 21-acetyl derivatives and internal standard (I.S.): (A) pure standard epimer 22*R* acetate, (B) pure standard epimer 22*S* acetate, (C) budesonide-21-acetate, (D) [$^{2}H_{3}$]budesonide-21-acetate (I.S.).

2.3. Preparation of standard solutions

A stock solution of BUD was prepared in ethanol at a concentration of 2.0 mg/ml and stored frozen at −20°C for eight weeks. The stock solution was diluted with ethanol to give a BUD working solution of 0.2 μg/ml. The working solutions were prepared every two weeks and stored at −20°C. Six standard solutions (including blank human plasma) were prepared in three replicates from the working solution spiked to blank human plasma in the concentration range 0.5–20.0 ng/ml, giving an epimer concentration of 0.25 to 10.0 ng/ml, based on a 1:1 ratio of 22*R*/22*S*.

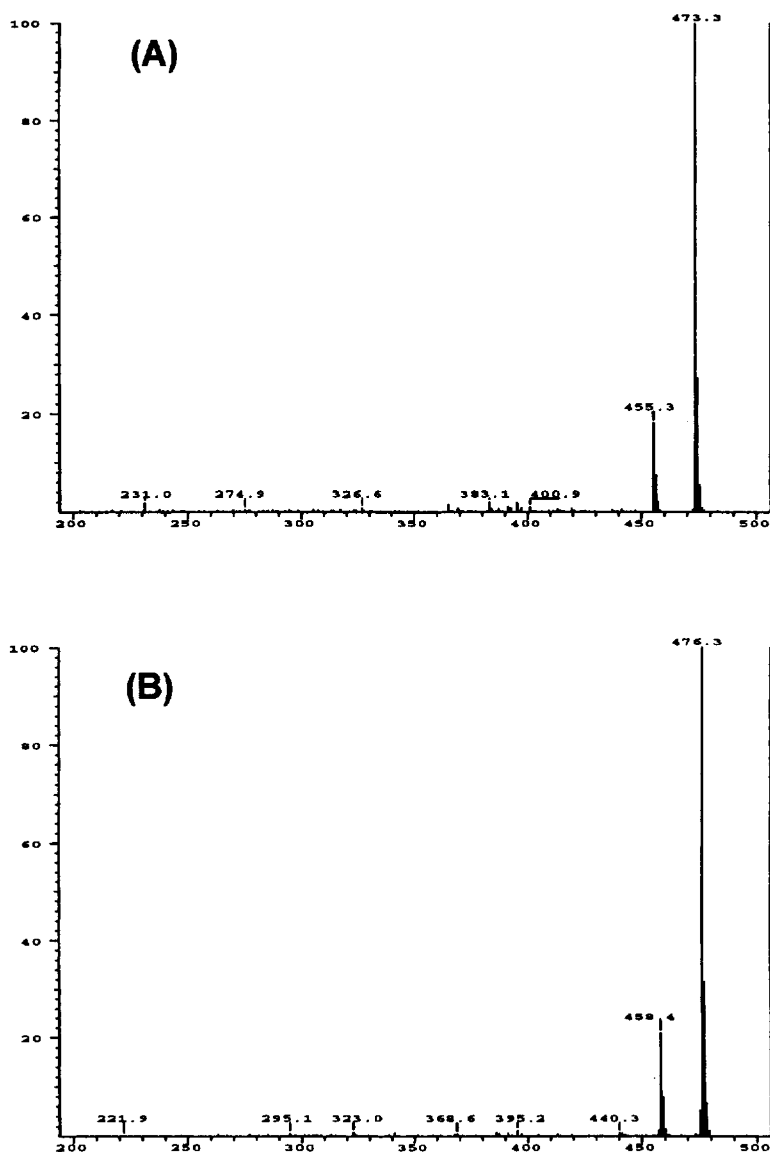

Fig. 4. Full scan APCI mass spectra of the 21-acetyl derivatives of budesonide: (A) budesonide-21-acetate, (B) [$^{2}H_{3}$]budesonide-21-acetate (I.S.).

2.4. Preparation of [$^{2}H_{3}$]BUD-21-acetates

[$^{2}H_{3}$]BUD-21-acetate was prepared by dissolving 20.0 mg of BUD in 5 ml of a solution consisting of 12.5% acetic anhydride-d_6 and 12.5% triethylamine in acetonitrile and was used as internal standard (I.S.). The mixture was allowed to stand for 15 min at room temperature after vortex-mixing, then evaporated to dryness under a stream of N_2 at 40°C. The crude product was purified with water and methanol. A stock solution of the I.S. was prepared in ethanol to obtain a concentration of 20.0 μg/ml and was stored frozen at −20°C for a maximum of three months. A working solution was prepared from the stock in ethanol at a concentration of 0.2 μg/ml and kept at −20°C. Before use, the working solutions

Table 1
Recoveries of the epimers of budesonide and trideuterated internal standard from solid-phase extraction (n=4)

Concentration (ng/ml)	Recovery (% of total)			
	Mean		Range	
	22*R*	22*S*	22*R*	22*S*
0.5	80.8	86.8	79.1–82.7	84.3–88.9
2.0	79.4	72.1	75.4–81.9	72.4–75.2
5.0	104.4	105.8	80.3–88.3	82.1–90.2
5.0 (I.S.)	82.0	80.1	73.4–90.2	73.0–86.2
10.0	90.3	90.3	88.2–92.2	88.3–91.6

were allowed to warm to room temperature for about 1 h, with protection from light.

2.5. Analytical procedure

A 50-μl volume of the working solution of the I.S., corresponding to 10.0 ng of [$^2H_3$]BUD-21-acetate (5.0 ng for each epimer), plus 1 ml of 30% ethanol in water were added to aliquots of the standard solutions. 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 that had been 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 was operated at approximately $5.1 \cdot 10^3$ Pa. Supernatant fractions were aspirated through the cartridges at a dropwise 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 of 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 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 being transferred to the autosampler vial. A total sample volume of 20 μl was injected onto the LC–APCI-MS system.

2.6. Estimation of recovery

The recovery for each epimer of BUD was assessed at concentrations of 0.5, 2.0, 5.0 and 10.0 ng/ml in replicates. The working solutions of BUD and the I.S. (10.0 ng/ml in total) were added to 1 ml of blank human plasma and 2 ml of 35% ethyl acetate in heptane simultaneously to give the above concentrations.

The plasma samples were extracted with an equal volume of 35% ethyl acetate solution as described in Section 2.5. Recovery was calculated as the percentage recovered from plasma relative to the amount determined in the ethyl acetate–heptane matrix. The recovery of the I.S. was estimated at an epimeric concentration of 5.0 ng/ml by repeating the above procedures.

2.7. Yield of derivatives

Plasma samples (n=8) spiked with BUD (5.0 ng/ml for each epimer) and the I.S. (10.0 ng/ml in total) were subjected to the analytical procedure outlined in Section 2.5. Four of the extracts (Set I) were acetylated as described in Section 2.4, while the remaining four (Set II) were evaporated to dryness. The residues were reconstituted with 100 μl of the mobile phase and 20 μl of each sample was injected into the LC–APCI-MS system. The amounts of the underivatized epimers present in the two sets of samples were measured by peak-area ratio of m/z 431 (BUD epimers) to m/z 476 (I.S.). The differences in the amounts of the underivatized epimers between Sets II and I provide an estimate of the yield of each derivatized epimer.

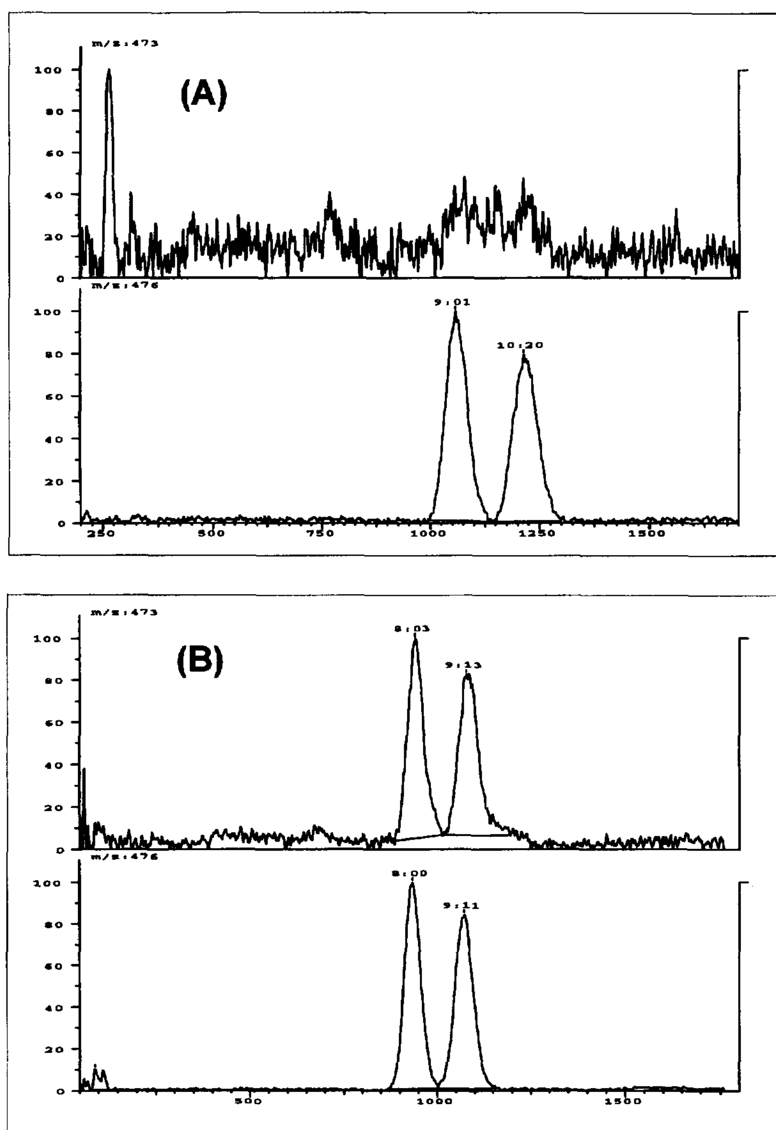

Fig. 5. Mass chromatograms of human plasma samples: (A) blank human plasma spiked with I.S. (5.0 ng/ml for each epimer), (B) blank human plasma spiked with budesonide (0.5 ng/ml for each epimer) and I.S. (10.0 ng/ml in total).

3. Results and discussion

3.1. Derivatization of BUD

It has been shown previously that acetylation of the 21-hydroxyl group of corticosteroids increases the TSP response [16]. When applied to BUD it was shown that thermospray sensitivity increased by a factor of about ten [14]. In this study, the acetylation of BUD also increased the response of APCI-MS, but the sensitivity increase was about five fold. The acetylation was effectively completed in less than 15

Table 2
Intra-day precision and accuracy data ($n=5$) of the epimers of budesonide

	Amount added (ng/ml)	Amount found (mean±S.D.)	R.S.D. (%)	Error (%)
22*R*	0.6	0.62±0.030	4.8	3.3
	4.0	3.56±0.460	12.9	−11.0
	6.0	5.68±0.690	12.1	−5.3
	10.0	9.72±0.470	4.8	−2.8
	14.0	14.11±1.620	11.5	0.8
22*S*	0.6	0.64±0.050	7.8	6.7
	4.0	3.64±0.361	9.9	−9.0
	6.0	5.81±0.140	2.4	−3.2
	10.0	9.74±0.750	7.7	−2.6
	14.0	14.43±1.750	12.1	3.1

min at room temperature. The 11,21-diacetyl derivatives were not formed, due to the 11-hydroxyl group being sterically hindered.

Selected ion monitoring of MH^+ ions at *m/z* 431 and *m/z* 476 was performed to estimate the yield of the derivatized epimers. Mass chromatograms of the epimers without and with derivatization (Fig. 2) indicate that the derivatization yield approached 100%. Following the derivatization reaction, underivatized epimers were not detectable.

3.2. Separation of the epimers

The epimeric derivatives of BUD were well separated by the present isocratic chromatographic method. Baseline resolution of each epimer was achieved, free from interfering endogenous substances in plasma. Typical mass chromatograms of the epimer-21-acetyl derivatives (Fig. 3) indicate the relatively short retention times (approximately 8.0 min for 22*R* and 9.0 min for 22*S*) for both epimers.

Table 3
Inter-day data ($n=5$) obtained from the analysis of the same samples over three weeks

	Amount added (ng/ml)	Amount found (mean±S.D.)	R.S.D. (%)	Error (%)
22*R*	2.0	1.92±0.035	1.8	−4.0
	4.0	4.01±0.284	7.1	0.3
	10.0	9.90±0.360	3.6	−1.0
22*S*	2.0	2.00±0.042	2.1	0.0
	4.0	3.87±0.210	5.4	−3.3
	10.0	9.92±0.410	4.1	0.8

There was no baseline shift observed at low concentration. By comparing the derivative of pure standard epimer 22*R* with that of standard epimer 22*S*, it was observed that derivatized epimer 22*R* eluted at a shorter retention time. The epimeric purity of 22*S* was found to be about 99%, based on the peak-area ratio of 22*S*/22*R*. In contrast, higher purity of epimer 22*R* was observed in the analysis by LC–APCI-MS.

3.3. Mass spetrometry of [$^2H_3$]BUD-21-acetate

Fig. 4 shows full-scan APCI mass spectra of the 21-acetyl derivatives of BUD and [$^2H_3$]BUD. The isotopic purity of trideuterated BUD-21-acetate 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. No trace of undeuterated BUD-21-acetate was observed in the APCI mass spectrum of [$^2H_3$]BUD-21-acetate, which indicates that >99% isotopic purity of [$^2H_3$]BUD-21-acetate was obtained.

The [$^2H_3$]BUD-21-acetate was reacetylated after solid-phase extraction with and without the presence of BUD. The MH^+ ions of *m/z* 473 and *m/z* 476 were selected for ion monitoring. Reacetylated [$^2H_3$]BUD-21-acetyl derivative was stable and the relative ion abundance of *m/z* 473 in deuterated samples is not appreciable or measurable, having a poor signal-to-noise ratio (*S/N*) of only 2:1. No trace of unlabelled BUD derivatives was found at the epimeric concentration of 5.0 ng/ml.

3.4. Solid-phase extraction

Liquid-phase extraction did not provide sufficient plasma clean-up for the analysis of the epimers by LC–APCI-MS. Thus, the drug and the I.S. were isolated from human plasma using a solid-phase extraction technique that had been reported previously [14]. The solid-phase extraction was performed on two different batches of Extract Clean C_{18} cartridges. During sample elution, a dropwise flow-rate was controlled to obtain a very clean extract with mean recoveries of 87.4±10.4% (mean±S.D.) for epimer 22*R* and 87.0±12.6% (mean±S.D.) for epimer 22*S* (Table 1). During the column wash with 25% ethanol in water and 2.0% ethyl acetate in heptane,

the fractions were analysed and leakage of the analytes was not detected. After elution of analytes with 35% ethyl acetate in heptane, 100% ethanol was used to completely elute the analytes from the column. The amount of the epimers and I.S. retained on the column after elution was negligible. Incomplete recovery may have resulted from losses of analytes during deproteination.

3.5. Linearity and sensitivity

Insufficient quantities of the pure epimers were available for this study. Thus, calibration curves for each epimer were constructed by separating BUD and quantifying the epimers. Linear responses were obtained for both epimers over the range 0.25–10.0 ng/ml of human plasma. A typical calibration curve of epimer 22*R* was described by the equation $y=0.2363x+0.0127$ ($r=0.9961$). The corresponding equation for the quantification of epimer 22*S* was $y=0.2356x+0.0186$ ($r=0.9978$). In the equations, y represents the peak-area ratio of the analyte to I.S., where x corresponds to plasma concentration in ng/ml. The regression lines were not forced through the origin.

The theoretical limit of detection was 0.7 pg for both epimers of BUD at a *S/N* of 5, from analysis of the standard BUD-21-acetate under the optimum conditions of the LC–APCI-MS system. Compared to the electrospray chemical ionization (ESI), the APCI gave better responses of the 21-acetyl derivatives of BUD. The quantifiable limit of the assay method was 0.25 ng/ml of human plasma for both epimers of BUD at a *S/N* of 7. Fig. 5 presents typical mass chromatograms of standard samples of blank human plasma spiked with I.S. and BUD, respectively. A total volume of 20 μl of the sample was injected onto the LC–APCI-MS system.

3.6. Precision and accuracy

The intra-day variation of the assay method was determined by replicate analysis of blank human plasma spiked with BUD at different concentrations in the range 0.6–14.0 ng/ml for each epimer of BUD. The inter-day precision was evaluated by measuring replicates of the same samples at three different concentrations over a period of three weeks. The precision was expressed as a percentage by calculating the intra- and inter-day relative standard deviations (R.S.D.). The accuracy was estimated by the percentage 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 Table 2 and Table 3.

3.7. Selectivity of the method

As the metabolites of BUD are not commercially available, none of them could be tested in this study. A mass chromatogram of blank human plasma showed no peaks interfering with the epimers of BUD (Fig. 5). In previous studies, most metabolites of BUD were reported to be well separated from the parent drug on a reversed-phase system [17]. Lindberg et al. [14] also reported that natural and synthetic corticosteroids, such as cortisol, beclomethasone dipropionate did not interfere with BUD in their LC–TSP-MS method. Therefore, the metabolites of BUD and other corticosteroids are unlikely to cause any serious interference in the present method.

4. Conclusions

The method described in this study is sensitive, stereospecific, linear, precise and accurate in the concentration range 0.25–10.0 ng/ml for both epimers of BUD. The process followed for injecting and analysing samples provides automated performance from the LC–APCI-MS system on a routine basis. The method should be capable of quantifying each epimer of BUD simultaneously in human plasma following inhalation of therapeutic doses. Thus, it should enable real characterisation of the pharmacokinetic parameters of the epimers of BUD following inhaled dose used in the therapy of asthma.

Acknowledgments

We are grateful to Astra Draco (Lund, Sweden) for the supply of BUD and its 22*R*- and 22*S*-epimers.

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