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Determination of (22*R*,*S*)Budesonide in Human Plasma by Automated Liquid Chromatography/Thermospray Mass Spectrometry

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(22*R*,*S*)Budesonide was isolated from human plasma by solid-phase extraction. Switching from reversed-phase conditions during sample application and washing to normal-phase conditions during elution afforded a very clean extract. Budesonide was derivatized with acetic anhydride to form the 21-acetyl derivative before analysis by reversed-phase liquid chromatography combined with thermospray mass spectrometry. Deuterium-labelled budesonide was used as internal standard. Standard samples prepared in human albumin solution were used for the calibration curve. An automated liquid chromatography/mass spectrometry system, allowing unattended overnight operation, was used for routine analysis. The recovery of budesonide from plasma was 88.9 ± 5.9% (mean ± SD) and the method was linear over the range 0.30–30 pmol (amount analysed), corresponding to plasma concentrations of 0.10–10 nmol l^{-1}. Budesonide could be measured down to 0.10 nmol l^{-1} with a within-day variation of 10–18% (CV). The error was less than ±15% at 0.10 nmol l^{-1} and less than ±7% at concentrations of 0.20 nmol l^{-1} or higher. The total imprecision between days was 9% (CV) at a concentration of 0.30 nmol l^{-1}.

INTRODUCTION

Budesonide (Pulmicort®, Rhinocort®) (Fig. 1) is a topical glucocorticosteroid clinically used in the treatment of asthma and rhinitis.[1] The drug, which is a 1:1 mixture of two epimers with 22*R* and 22*S* configuration, is rapidly and extensively metabolized in the liver.[2,3] Budesonide is pharmacokinetically characterized by low oral bioavailability, a large volume of distribution and high systemic clearance,[4–7] qualities leading to low plasma concentrations of the drug after inhalation of therapeutic doses. Human pharmacokinetic studies of budesonide require a selective and sensitive bioanalytical method, capable of measuring the drug in plasma down to about 0.1 nmol l^{-1}. Early investigations relied on radioactivity measurement after administration of tritiated budesonide.[5] To avoid the administration of radiolabelled drug a method based on radioimmunoassay (RIA)[8] combined with off-line sample clean-up by liquid chromatography (LC) was used.[9] The LC/RIA method, however, was laborious and tedious and not well suited for the analysis of a large number of samples on a routine basis.

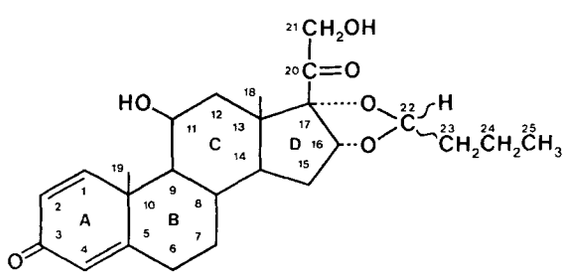

Figure 1. Chemical structure of (22*R*,*S*)budesonide.

We have previously used LC combined with mass spectrometry (MS) with a moving belt interface for the identification of budesonide metabolites.[10] With the commercial introduction of the thermospray interface a more sensitive and robust LC/MS technique became available, permitting quantitative drug analysis on a routine basis. Thermospray LC/MS has been successfully used for the analysis of various corticosteroids.[11–13] The present paper describes a method for routine determination of budesonide in human plasma by use of a previously described[14] automated thermospray LC/MS system. By using a simple and rapid derivatization reaction[15] sufficient sensitivity for clinical samples was achieved.

EXPERIMENTAL

Chemicals

(22*R*,*S*)Budesonide and (22*R*,*S*)($^2H_8$)budesonide [(22*R*,*S*)(22,23,23,24,24,25,25,25-$^2H_8$)-16α,17α-butylidenedioxy-11β,21-dihydroxypregna-1,4-diene-3,20-dione] were obtained from Astra Draco AB (Lund, Sweden). The isotopic purity of deuterated budesonide was determined by LC/MS and selected ion monitoring (SIM) of the MH^+ ion of its 21-acetate ester. The peak area ratio *m/z* 473/481 was 0.004. Tritium-labelled budesonide [(22*R*,*S*)(1,2-^{3}H)budesonide] with a specific activity of 96.5 Ci g^{-1} was obtained from the Radiochemical Centre (Amersham, UK). Acetic acid, ammonium acetate, triethylamine (all of Gold Marke quality) and acetic anhydride (98–99%) were purchased from Aldrich Chemie (Steinheim, Germany). Acetonitrile, ethyl

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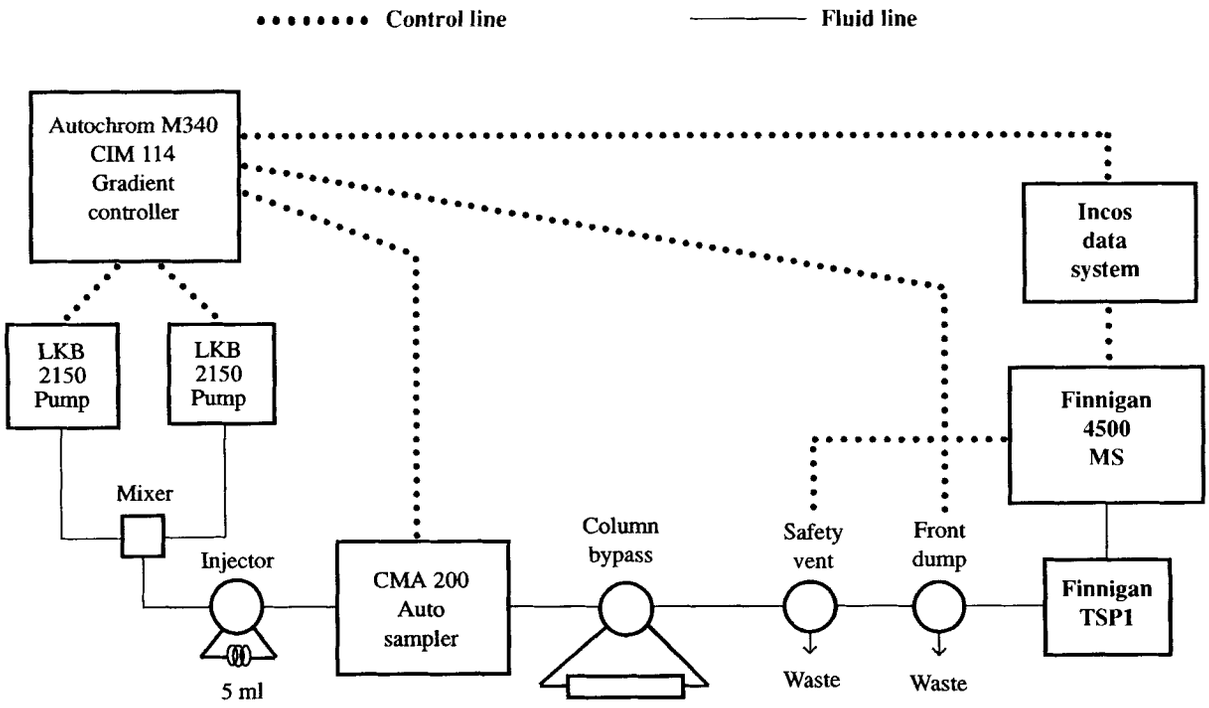

Figure 2. Schematic view of the automated thermospray LC/MS system.

acetate, n-heptane and methanol were all of HPLC grade and obtained from Fison (FSA Laboratory Supplies, Loughborough, UK). Ethanol (99.5%; spectroscopic grade) was obtained from Kemetyl (Stockholm, Sweden) and human albumin solution (50 mg ml^{-1} in glass bottle) from Immuno AG (Vienna, Austria). Water was purified in a MilliQ system (Millipore, Molsheim, France).

LC/MS instrumentation

The automated thermospray LC/MS system (Fig. 2) has been described in detail elsewhere.[14] Gradient LC was performed with two LKB 2150 pumps (Pharmacia LKB, Uppsala, Sweden) controlled by Autochrom M340 software via a CIM 114 interface (Autochrom, Milford, Massachusetts, USA). The chromatographic column used was a 33 × 4.6 mm Supelcosil LC-8-DB (3 μm particles) fitted with a 10 × 3 mm Chromguard R (Chrompack) precolumn. The mobile phases for gradient LC, prepared as described previously,[15] were 30% methanol in 0.1 M ammonium acetate buffer, pH 5, and 90% methanol in 0.1 M ammonium acetate buffer, pH 5. The mobile phases were filtered through a 0.22 μm Durapore filter (Millipore) before use and continuously degassed with helium during LC/MS operation. During analysis the flow rate was kept at 1.40 ml min^{-1} and the mobile-phase composition controlled by a gradient program shown in Table 1. A CMA200 autosampler (CMA Microdialysis, Stockholm, Sweden) was used for automatic sample injection. A Finnigan 4500 mass spectrometer equipped with an Incos data system and a Finnigan thermospray interface (TSP1) was used. The thermospray vaporizer was operated at 95–110 °C. The jet temperature was kept at 180–190 °C, the repeller potential at 35–60 V, the electron multiplier at 1450 V, and the preamplifier at 10^{-8} A V^{-1}. During SIM operation the instrument was set to record the MH^+ ions of the 21-acetate esters of budesonide (*m/z* 473) and ($^2H_8$)budesonide (*m/z* 481) with a scan time of 400 ms over a 0.5 u window for each ion. Tuning of the instrument parameters, to achieve maximum sensitivity and signal stability, was performed by injecting 5 ml of a budesonide solution (2.5 μmol l^{-1} in 66% methanol in 0.1 M ammonium acetate buffer, pH 5) from the manual injector (see Fig. 2) with the analytical column switched to the bypass mode.

Bond Elut sample preparation system

The Bond Elut columns (Bond Elut® C18 LRC, 200 mg, 10 ml reservoir) from Analytichem International (Harbor City, California, USA) were used with a home-made stainless steel vacuum box holding up to 60

Table 1. Gradient program for the LC/MS analysis of budesonide in plasma

Time (min)	Solvent composition (% methanol)	Control signals[a]
0	64.2	Flow to waste
		Autosampler inject
1.5	64.2	Flow to mass spectrometer
5.3	69.0	
5.7	|	Flow to waste
7.0	84.0	
8.0	84.0	
8.5	64.2	
9.5	64.2	Flow to mass spectrometer

[a] The control of the automated LC/MS system is shown schematically in Fig. 2.

columns. The top of the box was covered with a transparent macrolon plate with conical holes, adapted for disposable pipette tips in which the columns were mounted. The columns were conditioned by rinsing them twice with 3 ml of ethanol and twice with 3 ml of water. After conditioning the columns were ready for sample application.

Estimation of recovery

The extraction recovery of budesonide was determined with tritium-labelled budesonide added to blank plasma at a concentration of 2.0 nmol l^{-1} and taking the samples through the Bond Elut extraction procedure. The radioactivity in various fractions collected (sample application, washes and eluate) was determined by liquid scintillation counting.

Blood sampling, storage and transportation

Blood was collected into sodium-heparinized Venoject® vacuum tubes (two 10 ml tubes on each sampling occasion). The tubes were inverted four times and then immediately centrifuged at approximately 1500 × *g* for 10 min to separate the plasma. The plasma fractions were pooled, carefully mixed, and transferred to two polystyrene tubes and frozen. Samples were transported in boxes containing dry ice and stored frozen at −20 °C.

Preparation of standard solutions

A stock solution of budesonide was prepared in ethanol at a concentration of 2.00 μmol l^{-1}. The stock solution was diluted with human albumin solution to give a budesonide concentration of 10 nmol l^{-1}. From this solution, six standard solutions in the concentration range 0.20–6.40 nmol l^{-1} were prepared in human albumin solution. The standard solutions were divided into 3.4 ml aliquots and stored frozen in polystyrene tubes. A stock solution of the internal standard [($^2H_8$)budesonide] was prepared in ethanol at a concentration of 150 μmol l^{-1}, from which a working solution was prepared in 30% ethanol in water at a concentration of 15 nmol l^{-1}. The stock solutions of budesonide and ($^2H_8$)budesonide were kept at −20 °C for a maximum of six months and the internal standard working solution in a refrigerator for a maximum of one month. The working solution was allowed to warm at room temperture in a dark cabinet for about 1 h before use.

Quality-control samples

Spiked quality-control samples were prepared by adding budesonide to a pool of human blank plasma to give a final budesonide concentration of 0.30 nmol l^{-1}. The plasma pool was divided into 3.4 ml aliquots and stored frozen at −20 °C. Authentic quality-control samples were prepared from plasma obtained after administration of budesonide (inhalation) to healthy volunteers. The plasma was pooled and divided into 3.4 ml aliquots and stored frozen at −20 °C.

Analytical procedure

The frozen plasma samples and two quality-control samples (one spiked and one authentic) were thawed together with duplicate samples of the albumin standard solutions and blank albumin solution. After careful mixing and centrifugation 3.00 ml of the samples were pipetted into 10 ml polypropylene tubes. The amounts of budesonide in the standard samples were equivalent to 0.60, 1.20, 2.40, 4.80, 9.60 and 19.2 pmol. After addition of 200 μl of the internal standard working solution [corresponding to 3.00 pmol of ($^2H_8$)budesonide], plus 2.80 ml of 30% ethanol in water, using a MicroLab-M (Hamilton, Bonaduz, Switzerland) programmable dispenser, the samples were carefully mixed and allowed to stand for at least 15 min. Protein precipitation, caused by the addition of 30% ethanol, was removed by centrifugation at approximately 1500 × *g*. The supernatant fractions were transferred to, and aspirated through, conditioned Bond Elut columns. The columns were rinsed with 3.0 ml of 25% ethanol in water, 3.0 ml of water, and 2.0 ml of 2% ethyl acetate in n-heptane. Budesonide and the internal standard were eluted with 2.0 ml of 35% ethyl acetate in n-heptane into 4.5 ml polypropylene tubes (the eluting solvent was allowed to wet the columns for 5 min before applying vacuum). The solvent was evaporated to dryness at 40 °C in a Savant Speedvac vacuum centrifuge (Savant Instruments, Farmingdale, New York, USA), the tube being left in the vacuum centrifuge for a maximum of 2 h. The residue was treated with 200 μl of a mixture containing 12.5% acetic anhydride and 12.5% triethylamine in acetonitrile at room temperature for 15 min. The derivatization reagent was evaporated to dryness (no heating) in a Savant Speedvac vacuum centrifuge. After addition of 200 μl of 50% methanol in 0.1 M ammonium acetate buffer, pH 5, the tube was allowed to stand at room temperature for 30 min to make sample dissolution complete. The solution was transferred to autosampler vials and the total sample volume (190 μl) was injected into the thermospray LC/MS system. Samples were injected in the following order: budesonide-21-acetate reference solution (1.9 pmol), blank albumin solution, standard samples (low to high concentration), authentic quality-control sample, plasma samples with unknown budesonide concentration, spiked quality-control sample, blank albumin solution, standard samples (low to high concentration), reference solution (1.9 pmol).

If, for practical reasons, it is necessary to make an overnight break before LC/MS analysis, the analytical procedure can be interrupted after derivatization. The samples can be left in the Savant Speedvac after evaporation of the derivatization reagent, or in the autosampler vials (kept dark at room temperature) after the final dissolution in 50% methanol in ammonium acetate buffer.

Calculations

The peak areas of budesonide and ($^2H_8$)budesonide were calculated by the Incos data system without any smoothing of the chromatograms. The peak area was defined as 21 scans centred around the scan of maximum intensity, thereby disregarding the tailing end of the chromatographic peak. The calibration curve was constructed by plotting the peak area ratios of budesonide over ($^2H_8$)budesonide versus the amount of budesonide. A straight line was fitted to the data by unweighted least-squares regression analysis. The amounts of budesonide in unknown samples were calculated from calibration curves defined by the three closest standard concentrations (six data points).

RESULTS AND DISCUSSION

Solid-phase extraction

The recovery of tritium-labelled budesonide in various fractions collected during Bond Elut extraction is shown in Table 2. The investigation was performed on three different batches of Bond Elut columns. Plasma samples had to be diluted with aqueous ethanol to avoid losses of budesonide during sample application, possibly due to strong plasma protein binding of the drug. Virtually complete extraction of budesonide from the plasma was achieved at an ethanol concentration of 15%. After sample application the column was rinsed with 25% ethanol in water. At 30% ethanol appreciable leakage of budesonide from the column was observed. The column was further rinsed with water and 2% ethyl acetate in n-heptane, the last step resulting in a change to normal phase conditions. Budesonide was eluted with 35% ethyl acetate in n-heptane, giving a very clean extract and a recovery of 88.9% ± 5.9% (mean ± SD, $n = 34$) (cf. Table 2). The total recovery of budesonide, calculated from the mean of all fractions, was only 92.7%. The incomplete recovery may be due to losses of budesonide during the protein precipitation process, achieved by the addition of 30% ethanol to the plasma sample. New batches of Bond Elut columns were tested with respect to recovery before use.

Table 2. Recovery of tritium-labelled budesonide in various fractions collected during solid-phase extraction

Fraction	Radioactivity (% of total) Mean	Range	n
Sample application	1.9	0.6–2.4	46
Washes			
25% ethanol	0.6	0.3–1.2	24
water	<0.02		8
2% ethyl acetate in heptane	0.7	0.3–1.2	10
Elution			
35% ethyl acetyl in heptane	88.9	81.6–100.2	34
100% ethanol[a]	0.6	0.2–1.4	34

[a] 100% ethanol was used to achieve complete elution of budesonide from the column.

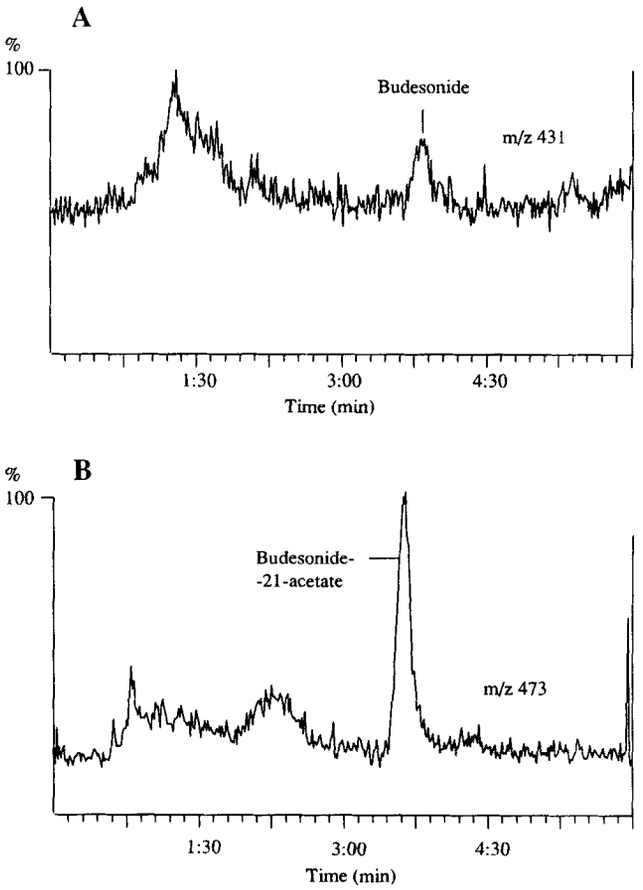

Figure 3. Mass chromatograms of two identical plasma samples, (a) without derivatization and (b) with derivatization. The sample containing underivatized budesonide was chromatographed with a mobile phase containing 60% methanol.

Derivatization

We have previously shown that derivatization with acetic anhydride increases the thermospray response for cortisol.[15] The reaction can be applied to other corticosteroids as well and in this case acetylation of the 21-hydroxyl group of budesonide increased sensitivity by a factor of about 10. The reaction required the presence of triethylamine and was complete after 5–10 min at room temperature. The 11-hydroxyl group is sterically hindered and formation of the 11,21-diacetyl derivative was not observed. Figure 3 shows mass chromatograms of equimolar amounts of underivatized and derivatized budesonide for comparison.

Liquid chromatography

The 22*R*- and 22*S*-epimers of budesonide can be completely separated on a C18 column.[16] The epimers of budesonide-21-acetate were more or less separated even on a short C18 column but co-eluted on a short (33 mm) C8 column, packed with 3 μm particles. The latter

type of column was chosen because co-determination of the budesonide epimers made it possible to measure budesonide at lower concentrations. The gradient program for the analysis of budesonide (Table 1) started with isocratic conditions at 64.2% methanol, the eluent initially being directed to waste. After 1.5 min the flow was switched into the mass spectrometer and a weak gradient up to 69.0% methanol during 3.8 min was started. After elution of budesonide (retention time about 3.2 min) the flow was again directed to waste and the column washed with 84% methanol during 1 min. The column was equilibrated at initial conditions for 4.5 min before the next injection, giving a total cycle time of 13 min.

Mass spectrometry

Figure 4 shows the thermospray mass spectra of the 21-acetyl derivatives of budesonide and ($^2H_8$)budesonide. The MH^+ ions at *m/z* 473 and *m/z* 481 were chosen for selected ion monitoring. There was no difference in mass spectrometric response between the two budesonide epimers (tested as the 21-acetyl derivatives). The mass spectrum of the internal standard showed that it contained 76% of ($^2H_8$)budesonide, the rest being made up of ($^2H_7$)budesonide and species with a lower degree of deuterium substitution. Trace amounts of unlabelled budesonide [0.4% relative to ($^2H_8$)budesonide] did not cause any problems during quantification of low levels of budesonide.

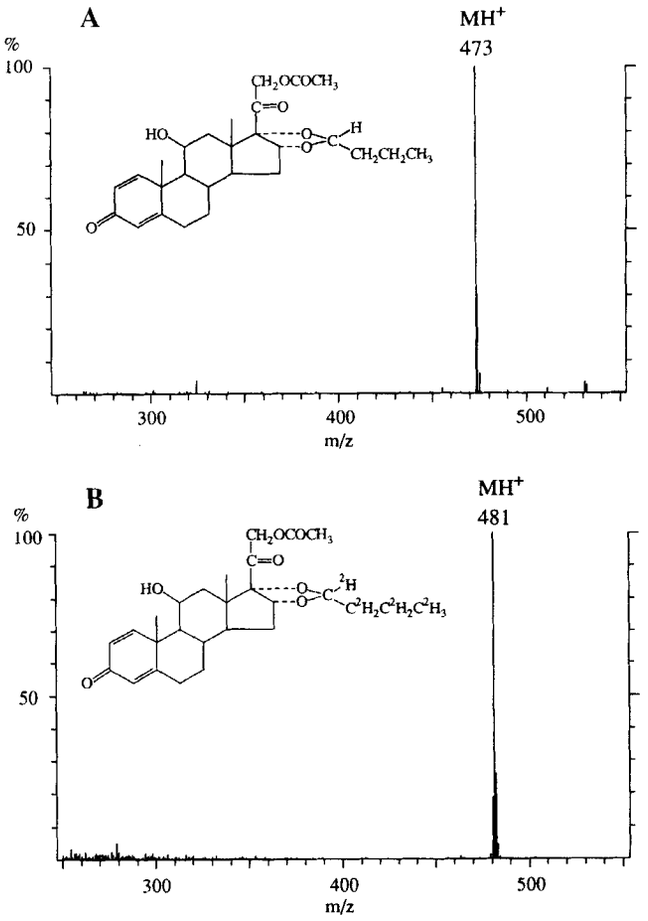

Figure 4. Thermospray mass spectra of the 21-acetyl derivatives of (a) budesonide and (b) ($^2H_8$)budesonide (internal standard).

Selectivity

A chromatogram of human blank plasma showed no peaks interfering with budesonide (Fig. 5). Most metabolites of budesonide, with the exception of Δ^6-budesonide and 1,2-dihydrobudesonide, are well separated from the parent drug on a reversed-phase system.[2] The major budesonide metabolites, 16α-hydroxyprednisolone and 6β-hydroxybudesonide, were tested (after derivatization with acetic anhydride) and found not to interfere. 24-Hydroxybudesonide was also

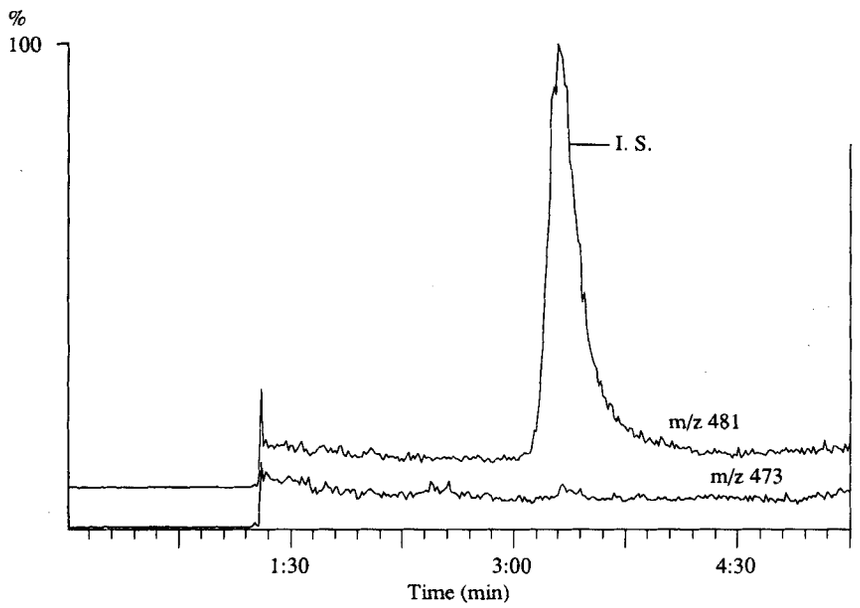

Figure 5. Mass chromatogram of human blank plasma. ($^2H_8$)Budesonide (3.0 pmol) was added as internal standard (I.S.).

tested, as a model compound for the known metabolite 23-hydroxybudesonide,[2] and found not to interfere. Although the 21-acetyl derivatives of Δ^6-budesonide and 1,2-dihydrobudesonide co-elute with budesonide-21-acetate on the C8 column used, they are minor metabolites and the molecular weight of their 21-acetyl derivatives differs from that of budesonide-21-acetate by 2 u (*m/z* 471 and *m/z* 475, respectively), making any serious interference unlikely. The measured peak area ratio of *m/z* 473/471 after injection of acetylated Δ^6-budesonide was 0.07 and the ratio of *m/z* 473/475 after injection of acetylated 1,2-dihydrobudesonide was less than 0.01. Other natural and synthetic corticosteroids like cortisol, cortisone, prednisolone, prednisone, 16β-methylprednisolone, beclomethasone, beclomethasone-17α-propionate, and beclomethasone-17α,21-dipropionate were found not to interfere with budesonide in the present method.

Precision and accuracy

The within-day variation was investigated by replicate analysis of human blank plasma to which known amounts of budesonide had been added (Table 3). The accuracy was also estimated from these samples by calculating the amount of budesonide from a separate calibration curve analysed at the same time. The investigation was performed on three different occasions during a period of eight months. The coefficient of variation was 10–18% at 0.3 pmol, 6–11% at 0.6 pmol, and better than 7% at higher levels. The error was less than $\pm$10%, except for one occasion at the 0.3 pmol level ($-$15%).

The total imprecision between days was calculated from quality-control samples analysed on each occasion. At the time of analysis one spiked and one authentic quality-control sample was thawed, mixed and centrifuged, and 3.00 ml of plasma of each was taken for analysis. The results are given in Table 4. The authentic quality-control samples showed a coefficient of variation of 6.7% over a period of 17 months. Three batches of spiked quality-control samples were analysed, each with a coefficient of variation of about 9% and an error less than 2%.

Table 3. Within-day variation of the method investigated on three different occasions

Amount added (pmol)	Occasion no.	Amount found (pmol; mean ± SD)	CV (%)	Error (%)	*n*
0.30	1	0.29 ± 0.042	15	−4.6	7
	2	0.27 ± 0.026	9.5	−9.6	6
	3	0.25 ± 0.046	18	−15	6
0.60	1	0.58 ± 0.037	6.4	−2.6	7
	2	0.61 ± 0.064	11	1.9	7
	3	0.56 ± 0.038	6.7	−6.5	7
19.2	1	19.5 ± 0.36	1.9	1.6	7
	2	19.4 ± 0.63	3.2	1.1	6
	3	19.5 ± 1.10	5.6	1.6	7
30.0	3	30.3 ± 1.99	6.6	1.0	6

Table 4. Total imprecision between days calculated from quality-control samples

Sample	Amount added (pmol)	Amount found (pmol; mean ± SD)	CV (%)	Error (%)	*n*
Spiked no. 1	0.90	0.92 ± 0.082	9.0	2.0	21
Spiked no. 2	0.90	0.91 ± 0.080	8.8	0.9	23
Spiked no. 3	0.90	0.90 ± 0.082	9.2	0.0	24
Authentic no. 1		6.12 ± 0.413	6.7		60

Comparison between human plasma and human albumin solution

In order to reduce the consumption of human blank plasma during routine analysis this matrix was replaced by human albumin solution in the standard samples prepared for the calibration curve. A comparison of calibration curves obtained from either human blank plasma or human albumin solution showed no significant difference ($0.2 > p > 0.1$) between the slopes of the curves. Quality-control samples prepared in human plasma gave the correct value when measured against calibration curves obtained from albumin standards.

Linearity

The correlation coefficient of the calibration curve, covering the range 0.6–19.2 pmol, was generally better than 0.998 during routine operation. The linearity was further investigated by studying the precision and accuracy at one concentration below (0.30 pmol) and one above (30.0 pmol) the calibration curve (cf. Table 3). It can be concluded that the method is linear at least between 0.3 and 30 pmol. Samples with an estimated amount of budesonide above 30 pmol were renalysed after diluting the sample with human blank plasma.

Limit of quantification

The lower limit of quantification was generally set at 0.30 pmol, corresponding to a plasma concentration of 0.10 nmol l^{-1} at a sample volume of 3 ml. The coefficient of variation was estimated at 10–18% at this level, with an error of $-$5% to $-$15% (cf. Table 3). On rare occasions the limit was set at 0.60 pmol due to lack of instrumental sensitivity. Figure 6 shows mass chromatograms of samples spiked with 0.30 pmol and 0.60 pmol of budesonide.

Sample storage and budesonide stability

Plasma samples were stored frozen and the stability of budesonide during storage was estimated from the quality-control samples (cf. Table 4) which were analysed repeatedly over an extended period. The authentic quality-control samples were analysed during 17 months and each batch of the spiked samples during five months. Linear regression analysis of the results showed no significant change of the budesonide concentration over the period studied (Fig. 7).

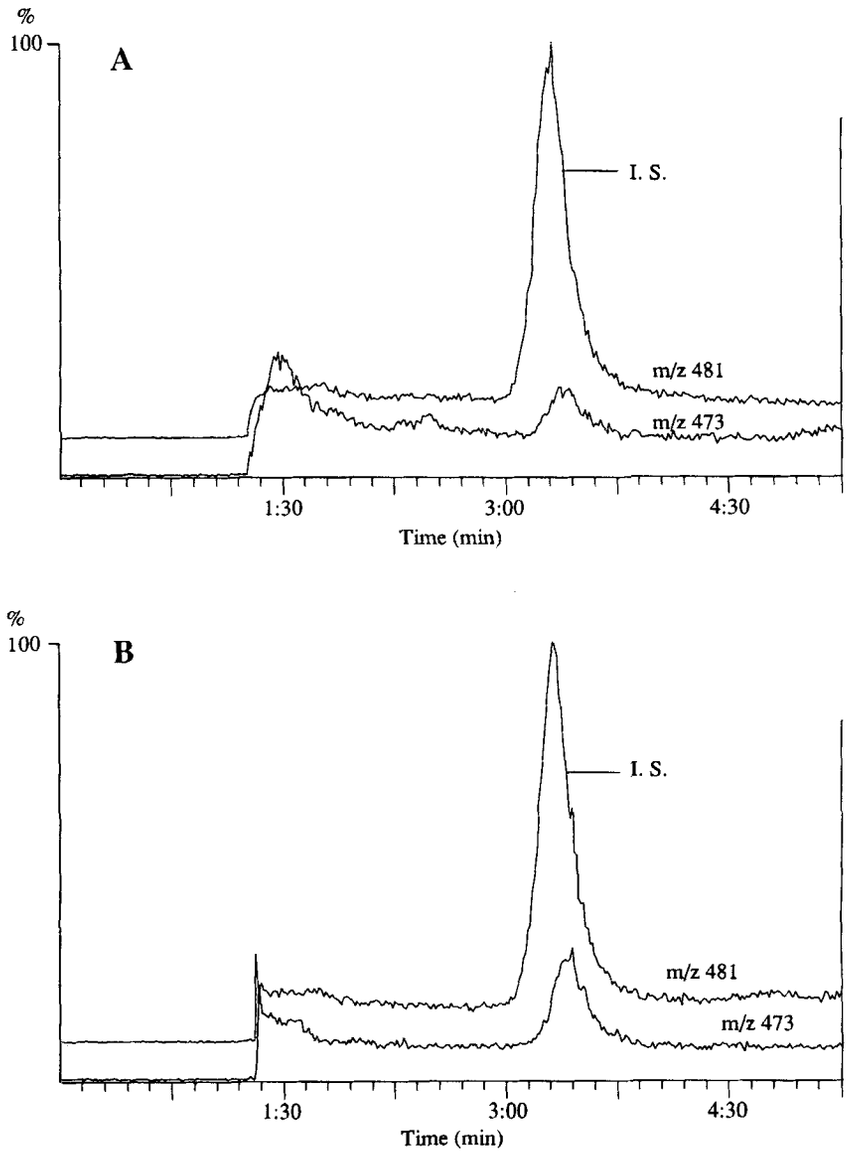

Figure 6. Mass chromatograms of standard samples spiked with (a) 0.30 pmol of budesonide in human blank plasma and (b) 0.60 pmol of budesonide in human albumin solution. ($^2H_8$)Budesonide (3.0 pmol) was added as internal standard (I.S.).

Budesonide is stable in ethanol solution at 5 °C for ten months.[17] Standard solutions of budesonide and ($^2H_8$)budesonide were prepared in ethanol and stored at −20 °C for a maximum of six months. The working solution of the internal standard was freshly prepared each month.

Budesonide was found to be unstable after extraction and evaporation to dryness if kept underivatized overnight. If necessary, the sample work-up procedure could be interrupted after derivatization and subsequent evaporation of the reagent. The extracts were redissolved in methanol/ammonium acetate buffer and transferred to autosampler vials before LC/MS analysis. The dissolved samples were stable for at least seven days when kept dark at room temperature. Any degradation of budesonide that might occur after addition of the internal standard is probably well compensated for by concurrent degradation of ($^2H_8$)budesonide and should not affect the accuracy of the results.

Application to clinical samples

The described method has been in routine use for about two years for the determination of budesonide plasma concentrations in connection with pharmacokinetic studies in patients and healthy volunteers. Figure 8 shows an example of the plasma concentration–time curve of budesonide obtained after administration of a 1 mg dose from a Pulmicort® metered dose inhaler. The sensitivity of the method allowed plasma concentrations to be followed for 8 h.

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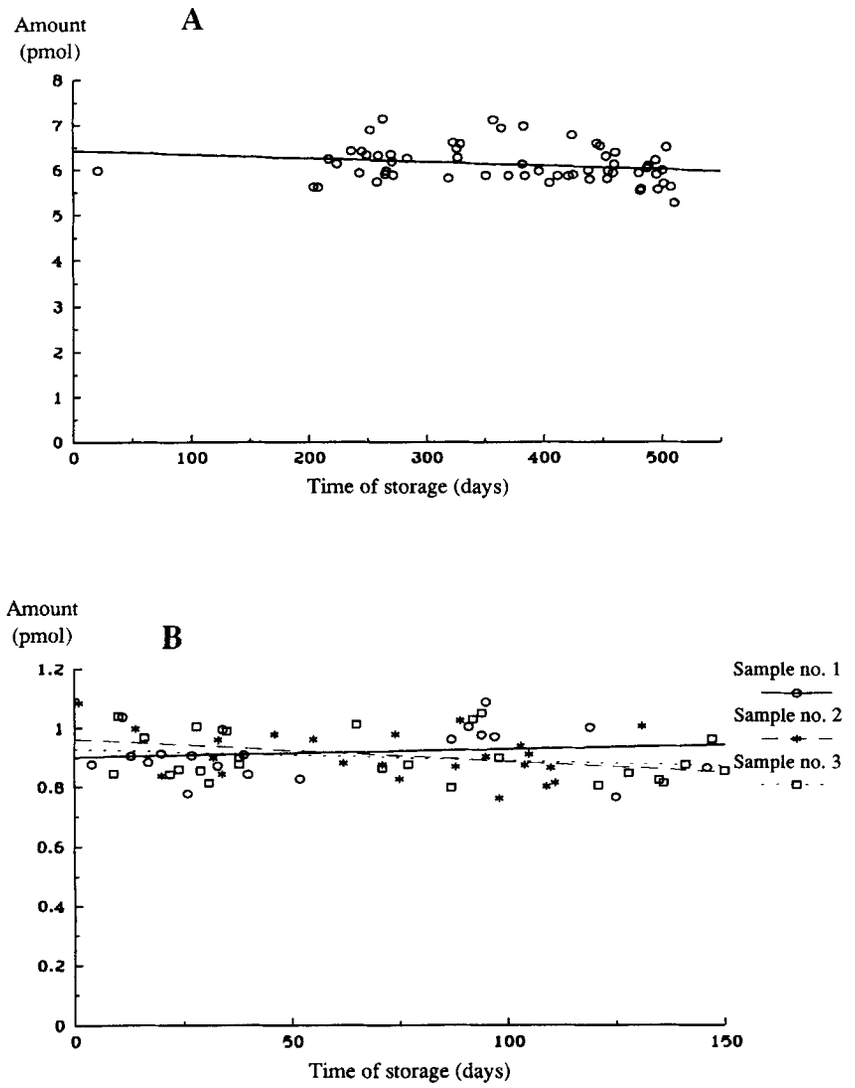

Figure 7. Results from the analysis of (a) authentic quality-control samples and (b) spiked quality-control samples (three batches), showing the stability of budesonide during long-term storage. The slopes of the regression lines did not differ significantly from zero ($p > 0.09$).

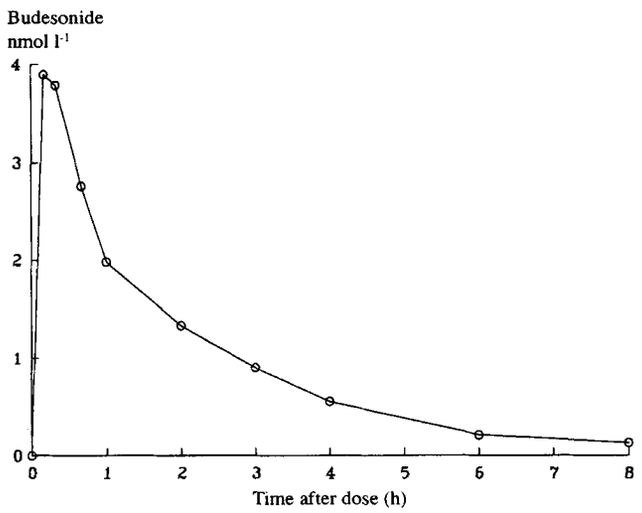

Figure 8. Plasma concentration–time curve of budesonide after inhalation of a 1 mg dose.

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