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ORIGINAL RESEARCH

Simultaneous quantification of budesonide and its two metabolites, 6β-hydroxybudesonide and 16α-hydroxyprednisolone, in human plasma by liquid chromatography negative electrospray ionization tandem mass spectrometry

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ABSTRACT: A sensitive, rapid and selective liquid chromatography negative electrospray ionization tandem mass spectrometry [LC-(−)ESI-MS-MS] method has been developed and validated for the simultaneous quantification of budesonide (BUD) and its major metabolites, 6β-hydroxybudesonide (OH-BUD) and 16α-hydroxyprednisolone (OH-PRED) in human plasma. The method was validated over a linear range from 0.1 to 10 ng/mL for all three analytes using a solid-phase extraction procedure with 9-fluorohydrocortisone as the internal standard. The between-day and within-day coefficients of variation for all compounds were $\leq$20% at the concentrations of lower limit of quantification and $\leq$15% at other quality control concentrations. The utility of this assay was demonstrated by monitoring BUD, OH-BUD and OH-PRED plasma concentrations in one healthy subject for 24 h following a 3 mg oral dose of budesonide, administered as a pH modified release capsule (Budenofalk®) to healthy volunteers. Copyright © 2003 John Wiley & Sons, Ltd.

KEYWORDS: budesonide; 6β-hydroxybudesonide; 16α-hydroxyprednisolone; LC/MS/MS; ESI; MRM

INTRODUCTION

Budesonide [22(*RS*)-16α-17α-butylenedioxy-11β-21-dihydroxypregna-1,4-diene-3,20-dione], is a non-halogenated glucocorticoid that has been designed to express high topical activity with reduced systemic side effects. This was achieved by incorporating features into the drug molecule that allow a pronounced hepatic metabolism resulting in low oral bioavailability and high hepatic clearance (Spencer and McTavish, 1995). Because of these design features, budesonide has been very successful in the topical treatment of asthma via inhalation therapy (Hvizdos and Jarvis, 2000).

The use of budesonide has also been extended to the local therapy of other diseases. It is currently used or is evaluated in the treatment of chronic inflammatory bowel disease in the form of enteric-coated pellets insoluble in gastric juice, rectal foams and enema (Hamedani *et al.*, 1997). There is evidence suggesting that budesonide can also be successfully applied in certain liver diseases. As an example, a recent open pilot study generated promising results for budesonide in the treatment of autoimmune hepatitis (AIH; Danielsson and Prytz, 1994). Within this patient population it was of interest to evaluate metabolic fate of budesonide in detail, as it is mainly cleared by hepatic metabolism. One of the relevant parameters to be assessed was to determine the metabolic activity and metabolic pathways in this patient population, as differences in the intrinsic clearance, enzyme distribution and oral bioavailability of the drug might be reflected in changes in the budesonide to metabolite ratios. This report describes the analytical procedure suitable to measure budesonide and its main metabolites 6-OH-budesonide budesonide and 16-OH-prednisolone in human plasma by liquid chromatography electrospray ionization tandem mass spectrometry.

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Abbreviations used: BUD, budesonide; FH, 9-flora-hydrocortisone; OH-BUD, 6β-hydroxybudesonide; OH-PRED, 16α-hydroxyprednisolone.

EXPERIMENTAL

Materials

Reference standards of budesonide (BUD) and its two metabolites, 6β-OH budesonide (OH-BUD) and 16α-OH-prednisolone (OH-

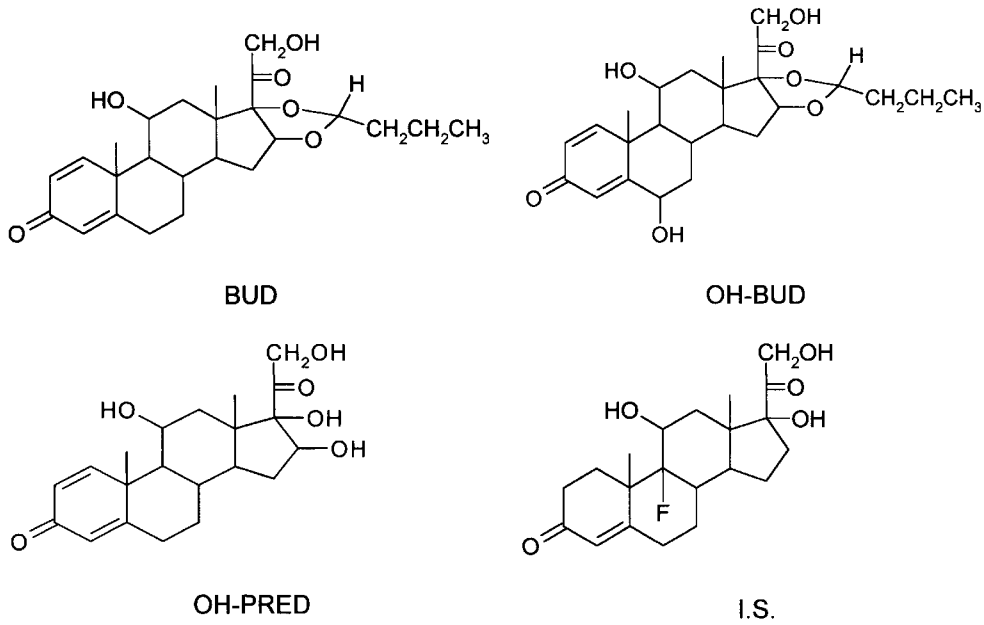

Figure 1. Structures of BUD, OH-BUD, OH-PRED and I.S.

PRED; Fig. 1), were obtained from Sicor, Milano, Italy. 9-fluorohydrocortisone (FH; Fig. 1) was used as internal standard (I.S.) and was obtained from Merck (Darmstadt, Germany). Human blank plasma was obtained from the Civitan regional blood system (Gainesville, FL, USA). Methanol, ethanol, formic acid, ammonium acetate and acetic acid were of HPLC grade and purchased from Fisher Scientific (Springfield, NJ, USA). Double-distilled deionized water was filtered through a 0.2 μm filter. The solid phase LC_{18} (6 mL) cartridges for sample extraction were obtained from Supelco (Bellefonte, PA, USA).

HPLC/MS/MS conditions

The high-performance liquid chromatography was performed isocratically at ambient temperature using a Waters C_{18} 3.5 μm column (Symmetry, 2.1 × 50 mm i.d., Milford, MA, USA) preceded by a Whatman 3.5 μm ODS C_{18} guard column cartridge (Clifton, NJ, USA). The mobile phase consisted of 10 mM ammonium acetate buffer (pH 3.5)-methanol (30:70, v/v). The flow-rate was 0.3 mL/min and the HPLC pump was a LDC Analytical constaMetric®3500 solvent delivery system. The mobile phase was degassed using helium for approximately 10 min before use. The mass spectrometer was a Micromass Quattro-LC-Z (Beverly, MA, USA) triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) ion source. Negative ESI was chosen after tuning with three analytes. The source temperature was set to 120°C, and the desolvation temperature was set to 450°C. Capillary and cone voltages were set to 3.0 kV and 30 V, respectively. 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, Masslynx 3.1. Data analysis was performed using Masslynx software. The calibration curves were plotted as the peak area ratio (analyte/I.S.) vs analyte concentration with a weighting factor of the reciprocal of the analyte nominal concentration.

Preparation of calibration standards and quality control samples

Primary stock solutions were prepared by dissolving the compounds or I.S. in methanol. Appropriate dilutions of the stock solutions with drug-free human plasma were made subsequently in order to prepare the working solutions for all three compounds. Two different series of stock solutions were prepared separately for calibration standards (CCs) and quality control samples (QCs). The CCs included 0.1, 0.15, 0.2, 0.3, 0.5, 1.0, 5.0 and 10.0 ng/mL and QCs included 0.2, 0.75 and 3 ng/mL for all three analytes. Aliquots of 1 mL sample were transferred into thick-walled glass tubes, capped and stored at approximately −69°C. The internal standard stock solution was diluted with methanol to produce a working solution of 20 ng/mL.

Sample preparation

Plasma samples were thawed at room temperature. After addition of 50 μL of I.S. working solution to 1 mL plasma, 50 μL of 4% formic acid in water (v/v) was added to acidify the plasma. They were then vortexed, and 1 mL of 30% ethanol (v/v) was added into the plasma sample. After 15 min of incubation at 4°C, the mixture was centrifuged at 4000 rpm for 15 min to remove the protein precipitate. The supernatant was then transferred onto the SPE extraction column, preconditioned using ethanol and water. The column was drained by applying the necessary vacuum to ensure that the samples aspirate at a drop-wise flow rate. The column was then washed with one column volume of 20% methanol solution.

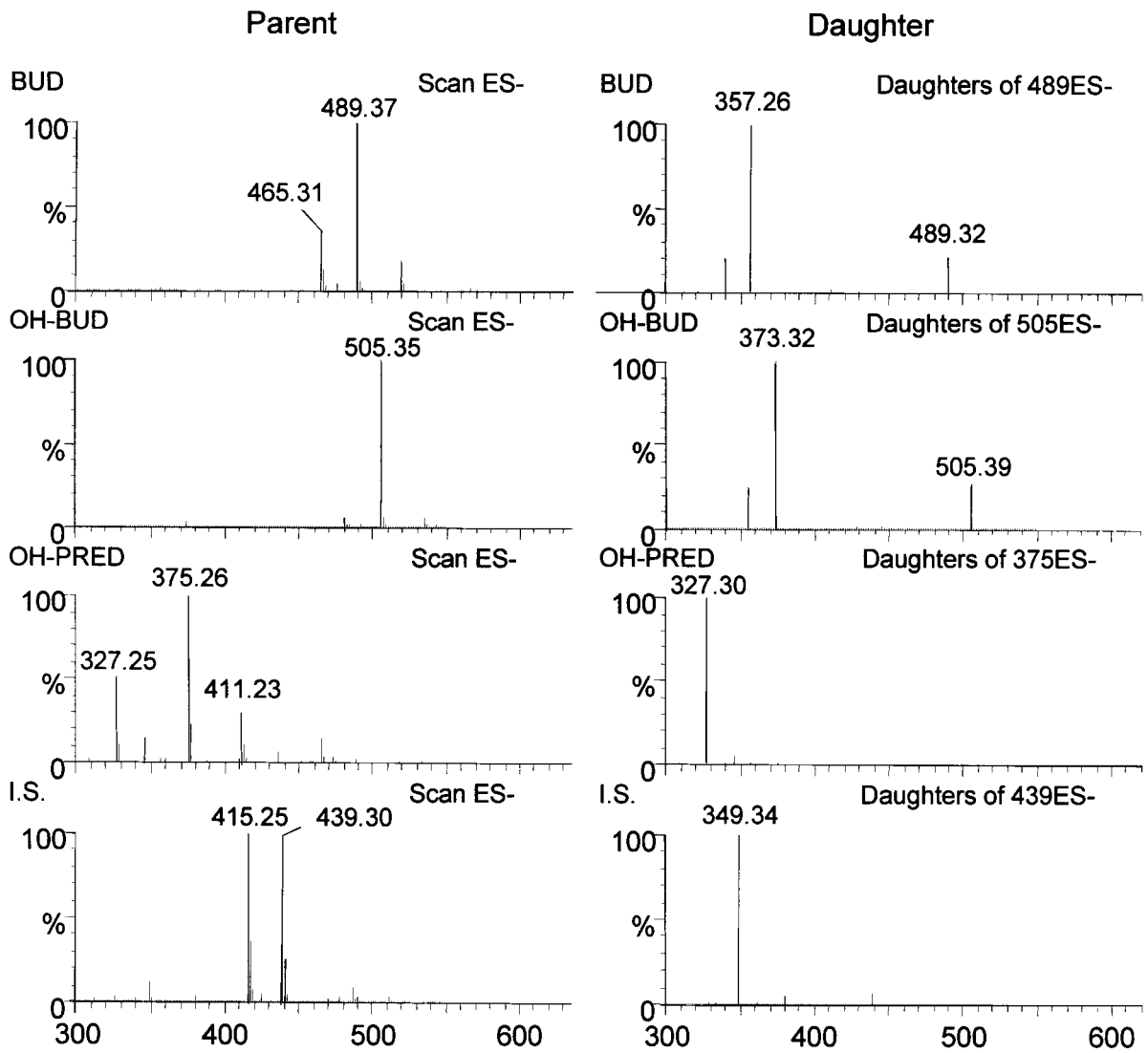

Figure 2. Full scan and daughter scan spectra of BUD, OH-BUD, OH-PRED and I.S.

Finally, the sample was eluted with 2 mL of 80% methanol solution, followed by another 1 mL of 80% methanol solution, at a drop-wise flow rate, evaporated in a vacuum centrifuge, and reconstituted in 100 μL of a 1:1 (v/v) mixture of mobile phase and water. A total sample volume of 40 μL was injected into the HPLC/MS/MS system. Plasma samples were obtained from a pharmacokinetic study after administration of 3 mg budesonide given as pH-modified release capsules (Budenofalk®). Plasma samples were obtained over a 24 h period and stored at −69°C. The study was approved by the local IRB office.

Validation

Selectivity. Drug-free plasma samples from six different individuals were extracted and analyzed to assess the potential endogenous interferences from human plasma. Any apparent response at the retention times of BUD, OH-BUD, OH-PRED and I.S. was compared with the response at the lower limit of quantification for BUD, OH-BUD, OH-PRED and with the response at the working concentration for I.S.

Recovery. The recovery of BUD, OH-BUD and OH-PRED was evaluated in triplicate at three concentrations (0.2, 0.75 and 3 ng/mL) by comparing the peak areas of BUD, OH-BUD and OH-PRED with the peak areas of corresponding compounds in samples prepared by spiking extracted drug-free plasma samples with the same amount of compounds at the step immediately prior to injection. The same method was used to assess the recovery of I.S. at the working concentration.

Calibration and sample quantification. Duplicate nine point standard curves at 0.1, 0.15, 0.2, 0.3, 0.5, 1.0, 5.0 and 10.0 ng/mL of BUD, OH-BUD and OH-PRED were run on three separate days. Calibration curves ($y = ax + b$), represented by the plots of the peak-area ratios (y) of the analyte to I.S. vs the nominal concentration (x) of the calibration standards, were generated using linear least-square regression with a weighting factor of $1/x$.

Actual, quality control and stability samples were calculated from the resulting area ratio of the analyte to I.S. and the regression equation of the calibration curve.

Accuracy and precision. Within-day accuracy and precision were evaluated by analysis of quality controls (QC) at concentrations of 0.1, 0.2, 0.75 and 3 ng/mL ($n = 6$ at each concentration) on the same day. The selection of the QC concentrations was based on the preliminary results of the analytes concentrations in clinical samples. The same experiment was repeated on three different days to assess the between-day accuracy and precision. Accuracy was calculated as the percentage ratio of measured concentration to nominal concentration. Precision was expressed as the coefficient of variation. The lower limit of quantification (LLOQ) of the assay was also determined during this process. LLOQs were defined as the lowest drug concentration, which can be determined with an accuracy of 80–120% and a precision $\leq$20% on a day-to-day basis (FDA, 2001).

Stability. Freeze and thaw stability was determined by analyzing triplicate quality control samples at the concentrations of 0.2 and 3 ng/mL following three cycles of freezing at −69°C and thawing. Short-term temperature stability of analytes in plasma samples was evaluated at the same concentrations after the samples were thawed and kept at room temperature for 6 h. The bench top stability of extracted and reconstituted samples at the same concentrations was evaluated over 7 h. Long-term stability was also tested after storage at −69°C for one year. Stability was expressed as the percentage ratio of measured concentration to the nominal concentration.

Table 1. The transition channels for three analytes and the internal standard

Compound	Parent ion	Daughter ion
Budesonide	489.30	357.30
6β-Hydroxy-budesonide	505.20	373.10
16α-Hydroxy-prednisolone	375.30	327.30
9-Fluoro-hydrocortisone (I.S.)	439.00	349.00

RESULTS AND DISCUSSION

Mass spectrometry/chromatography

Both atmosphere pressure chemical ionization (APCI) and electrospray ionization (ESI) modes were tuned for all compounds. ESI was chosen for the better sensitivity. In positive electrospray ionization (ESI+), the base peaks observed for all compounds are dominated by sodium and potassium adducts ($[M + Na]^+$ and $[M + K]^+$). In

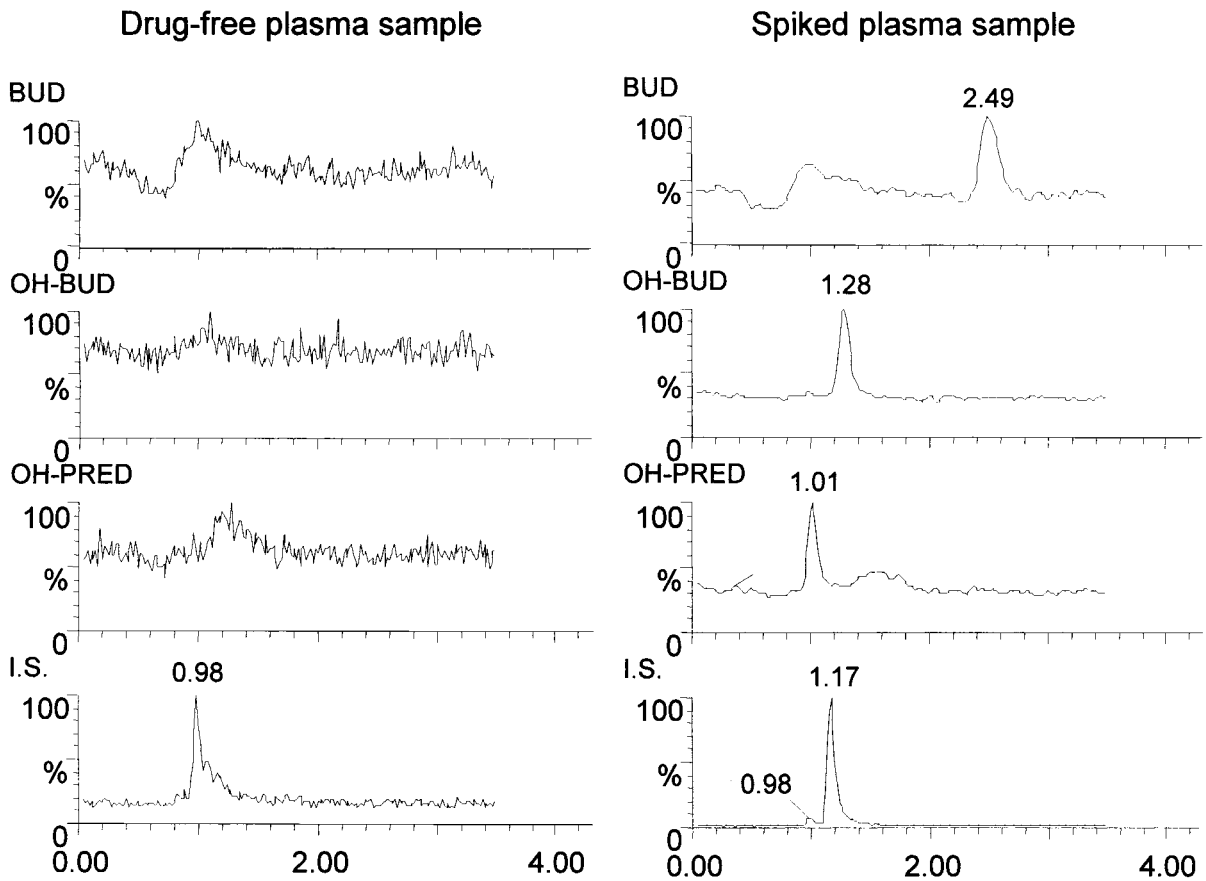

Figure 3. Chromatograms of blank plasma and plasma spiked with 0.1 ng/mL analytes and 1 ng/mL I.S.

Table 2. Within and between-day precision and accuracy for quality control samples

	0.1	0.2	0.75	3
BUD (ng/mL)				
Within-day day 1 ($n = 6$)				
Accuracy (%)	96.9	98.3	106.2	100.4
Precision (%)	9.1	6.1	9.9	6.6
Within-day day 2 ($n = 6$)				
Accuracy (%)	93.1	97.5	103.3	108.9
Precision (%)	16.7	13.3	9.1	2.2
Within-day day 3 ($n = 6$)				
Accuracy (%)	84.3	97.4	98.1	101.4
Precision (%)	17.8	14.7	9.5	6.5
Between-day ($n = 3$)				
Accuracy (%)	91.4	97.7	102.5	103.6
Precision (%)	15.1	11.2	9.5	6.3
OH-BUD (ng/mL)				
Within-day day 1 ($n = 6$)				
Accuracy (%)	96.5	93.8	107.9	106.0
Precision (%)	10.4	7.7	5.1	6.2
Within-day day 2 ($n = 6$)				
Accuracy (%)	97.7	97.5	106.7	106.9
Precision (%)	7.2	9.5	4.3	4.3
Within-day day 3 ($n = 6$)				
Accuracy (%)	94.9	102.7	103.8	98.3
Precision (%)	10.4	5.3	6.3	9.2
Between-day ($n = 3$)				
Accuracy (%)	96.4	98.0	106.2	103.8
Precision (%)	8.9	8.1	9.9	7.4
OH-PRED (ng/mL)				
Within-day day 1 ($n = 6$)				
Accuracy (%)	91.1	90.7	93.7	96.6
Precision (%)	7.6	7.3	3.3	8.4
Within-day day 2 ($n = 6$)				
Accuracy (%)	98.1	97.1	93.2	94.7
Precision (%)	17.6	10.6	9.7	3.8
Within-day day 3 ($n = 6$)				
Accuracy (%)	92.5	100.6	111.1	107.4
Precision (%)	14.0	12.7	8.5	3.2
Between-day ($n = 3$)				
Accuracy (%)	93.9	96.1	99.3	99.6
Precision (%)	13.5	10.9	11.3	7.8

Accuracy: 100% measured concentration/nominal concentration.
Precision: coefficient of variation (100% standard deviation/mean).

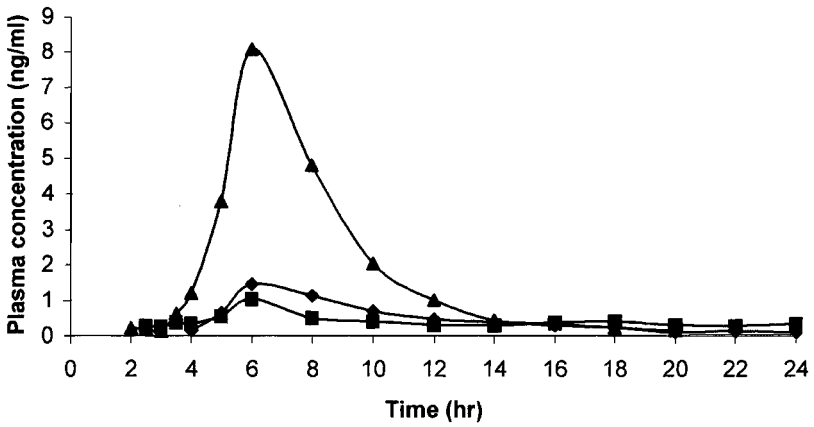

Figure 4. Concentration–time profiles of BUD (■), OH-BUD (◆) and OH-PRED (▲) after a single oral dose of 3 mg BUD as a pH-modified release capsule (Budenofalk®).

Table 3. Stability of BUD, OH-BUD and OH-PRED under various conditions[a]

	Nominal concentration (ng/mL)					
	BuD		OH-BuD		OH-PRED	
	0.2	3	0.2	3	0.2	3
Freeze–thaw (three cycles)	93.9 ± 2.0%	106.6 ± 5.6%	103.7 ± 5.4%	107.8 ± 5.5%	87.7 ± 2.9%	98.6 ± 1.0%
Short-term (6 h)	90.1 ± 17.1%	101.9 ± 9.5%	93.87 ± 3.6%	104.0 ± 4.1%	107.1 ± 8.6%	105.2 ± 7.9%
Bench-top (7 h)	98.5 ± 10.1%	101.1 ± 10.3%	102.9 ± 15.4%	101.2 ± 4.9%	94.3 ± 7.7%	94.5 ± 5.7%
Long-term (1 year)	103.4 ± 13.8%	99.7 ± 9.9%	99.8 ± 3.1%	94.7 ± 9.9%	103.3 ± 13.8%	99.7 ± 9.9%

[a] Stability was expressed as the percentage ratio of measured concentration to the nominal concentration ($n = 3$).

negative electrospray ionization (ESI−), the base peaks for BUD, OH-BUD and I.S. were acetate adducts $[M + AcO]^-$ and the base peak for OH-PRED corresponded to its pseudo-molecular ion $[M - H]^-$. All these parent ions generated specific strong daughter ions under multiple reaction monitoring (MRM) mode. As a result, ESI− was chosen for analysis. The mass spectra and product ion mass spectra of three analytes and of the I.S. are shown in Fig. 2. The transitions selected for monitoring all compounds are listed in Table 1. The instrument was tuned to give maximum abundance of the daughter ion for each compound. Figure 3 shows the typical chromatograms for a plasma sample spiked with 0.1 ng/mL BUD, OH-PRED and OH-BUD and 0.4 ng/mL I.S. The analysis time was 3.5 min with OH-PRED eluted at 1.01 min, OH-BUD at 1.27 min, I.S. at 1.16 min and BUD at 2.49 min. The resulting calibration curves were linear with correlation coefficients >0.996.

Selectivity

Drug-free plasma samples from six individuals were screened during method validation and Fig. 3 compares the chromatograms for extracted drug-free plasma and a plasma sample spiked with 0.1 ng/mL BUD, OH-PRED and OH-BUD and with 0.4 ng/mL I.S. No significant interferences were observed at the retention times for BUD, OH-BUD and OH-PRED. Even though a minor peak was observed near the retention time of I.S., the peak area of the interfering peak is less than 2% of the peak area of I.S.

Recovery

The recoveries from plasma samples for BUD, OH-BUD and OH-PRED were 85.0 ± 7.0, 70.1 ± 10.5 and 66.2 ± 10.2%, respectively, at 0.2 ng/mL ($n = 3$), 74.1 ± 3.1, 68.1 ± 2.2 and 60.0 ± 7.2%, respectively, at 0.75 ng/mL ($n = 3$) and 74.2 ± 2.6, 64.2 ± 2.3 and 66.9 ± 10.5% respectively at 3 ng/mL ($n = 3$). I.S. recovery was also determined to be 70% at the working concentration of 1 ng/mL. Preliminary experiment data indicated that the relatively low recoveries were mainly due to the loss of analytes during solid-phase extraction and reconstitution with low volume of reconstituting solvent. Despite the relatively low values, the recoveries were fairly reproducible, as shown by the data.

Precision and accuracy

Table 2 lists the within and between-day precision and accuracy data for four quality control levels. Accuracy was calculated as the percentage ratio of measured concentration to nominal concentration. Precision was expressed as the coefficient of variation. The results showed that this assay is consistent and reliable with good accuracy (84.3–98.1% at LLOQ and 90.7–111.1% at other concentrations) and precision (<18% at LLOQ and <15% at other concentrations). LLOQs were set at 0.1 ng/mL for all three analytes with an accuracy of 80–120% and a precision ≤20% on a day-to-day basis (FDA, 2001). The high QC, 3 ng/mL, was selected according to the preliminary results of the analytes concentrations in clinical samples. As a result, a linear range of 0.1 to 3 ng/mL was fully justified. However, the linearity of standard curve between 0.1 and 10 ng/mL suggests that a further validation with a higher QC concentration near 10 ng/mL should expand the linear range of this assay up to 10 ng/mL.

Stability

Table 3 lists the data for the freeze–thaw stability, the short-term stability under room temperature, the bench-top stability and long-term stability at low concentration (0.2 ng/mL) and high concentration (3 ng/mL) in plasma samples. Stability was expressed as the percentage ratio of measured concentration to the nominal concentration. The results show that all three analytes were stable under the conditions investigated in this study since the measured concentrations were all within 85–115% of the nominal concentrations.

Application to clinical sample analysis

Because of the lack of clinical samples available from

liver disease patients during assay validation, the method was applied to a single oral dose pharmacokinetic study. Plasma samples from one volunteer were analyzed and the concentration vs time profiles for BUD, OH-BUD and OH-PRED are shown in Fig. 4. These results seem to suggest that the predominant metabolite is hydroxy-prednisolone, while the lowest concentrations are observed for budesonide.

CONCLUSIONS

A simple, sensitive and selective LC-(ESI−)-MS-MS method was developed using a solid-phase extraction procedure for simultaneously quantifying budesonide and its two major metabolites in human plasma. Even though it is not the optimum method for measuring BUD, since there are other HPLC/MS/MS methods available with LLOQ of 50 pg/mL (Li *et al.*, 2001) or 15 pg/ml (Kronkvist *et al.*, 1998) and some methods with the selectivity to separate *R* and *S* form of BUD (Li *et al.*, 1996, 2001), this assay is the first reported method to simultaneously quantify budesonide and its two major metabolites in human plasma at fairly low concentrations. With oral administration of 3 mg BUD, the reported assay was able to monitor the levels of three analytes over 24 h after dosing, providing enough information for pharmacokinetic analysis. 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 rapid (analysis time of 3.5 min) and therefore ideally suited for pharmacokinetic studies involving a large number of samples.

Acknowledgments

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REFERENCES

Danielsson A and Prytz H. Oral budesonide for treatment of autoimmune chronic active hepatitis. *Alimentary Pharmacological Therapy* 1994; **8**(6): 585–590.

FDA. Guidance for Industry, Bioanalytical Method Validation. US Department of Health and Human Services, Food and Drug Administration. May, 2001.

Hamedani R, Feldman RD, Feagan BG. Drug development in inflammatory bowel disease: budesonide—a model of targeted therapy. *Alimentary Pharmacological Therapy* 1997; **11**(Suppl 3): 98–108.

Hvizdos KM and Jarvis B. Budesonide inhalation suspension: a review of its use in infants, children and adults with inflammatory respiratory disorders. *Drugs* 2000; **60**(5): 1141–78.

Kronkvist K, Gustavsson M, Wendel AK, Jaegfeldt H. Automated sample preparation for the determination of budesonide in plasma samples by liquid chromatography and tandem mass spectrometry. *Journal of Chromatography A* 1998; **823**((1–2)): 401–409.

Li N, Tattam B, Brown KF, Seale JP. Quantification of epimeric budesonide and fluticasone propionate in human plasma by liquid chromatography–atmospheric pressure chemical ionization tandem mass spectrometry. *Journal of Chromatography B: Biomedical Science Applications* 2001; **761**(2): 177–185.

Li YN, Tattam B, Brown KF, Seale JP. Determination of epimers 22R and 22S of budesonide in human plasma by high-performance liquid chromatography–atmospheric pressure chemical ionization mass spectrometry. *Journal of Chromatography B: Biomedical Applications* 1996; **683**(2): 259–268.

Spencer CM and McTavish D. Budesonide. A review of its pharmacological properties and therapeutic efficacy in inflammatory bowel disease. *Drugs* 1995; **50**(5): 854–72.

