Journal of Chromatography B, 877 (2009) 2290–2300

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Using total error concept for the validation of a liquid chromatography–tandem mass spectrometry method for the determination of budesonide epimers in human plasma☆

B. Streel*, B. Cahay, R. Klinkenberg

Galephar MF, 39, rue du Parc Industriel, B-6900 Marche-en-Famenne, Belgium

ARTICLE INFO

Article history:
Received 26 September 2008
Accepted 3 February 2009
Available online 11 February 2009

Keywords:
Budesonide
LC–MS/MS
Pharmacokinetics
Solid-phase extraction
Validation strategy

ABSTRACT

A robust, sensitive and selective method to quantify budesonide epimers in human plasma using solid-phase extraction and liquid chromatography–tandem mass spectrometry (LC–MS/MS) was developed and fully validated. The drug was first isolated from the biological matrix by automated solid-phase extraction (SPE) on disposable extraction cartridges (C-2). The methanolic eluate was then collected and evaporated to dryness. The residue was dissolved in mobile phase and an aliquot was injected onto a Phenomenex Luna octadecylsilica (C-18) column (50 mm × 4.6 mm i.d., 3 μm). The mobile phase is composed of water containing 10 mM ammonium acetate adjusted to pH 3.2 with glacial acetic acid and acetonitrile (65:35, v/v). The flow-rate was 1.00 ml/min. Hydrocortisone acetate was used as internal standard (IS). Detection of the analytes was achieved using negative atmospheric pressure chemical ionization (APCI) tandem mass spectrometry in selected reaction monitoring (SRM) mode. The MS/MS ion transitions monitored were m/z 489.3 → 357.3 and 463.3 → 403.2 for budesonide epimers and hydrocortisone, respectively. The method was validated using SFSTP (2003) proposal based on total measurement error and accuracy profiles as a decision tool. The most appropriate regression model for the response function as well as the limit of quantitation was first selected during the prevalidation step. These latter criteria were then assessed during the formal validation step. The limit of quantitation (LOQ) was around 50 pg/ml for budesonide epimers. The method was validated with respect to stability, recovery, linearity, precision, trueness and accuracy. Risk and uncertainty were also evaluated. The validated method was finally applied successfully to investigate the plasma concentration of budesonide epimers in a pharmacokinetic study.

1. Introduction

Budesonide (an epimeric mixture of the α- and β-propyl forms of 16α,17α-butylidenedioxy-11β, 21-dihydropregna-1,4-diene-3,20-dione) (Fig. 1) is a non-halogenated glucocorticoid with high topical activity and reduced systemic side effects. Because given a pronounced first-pass metabolism in the liver resulting in low oral bioavailaibility and high hepatic clearance [1], budesonide is very successfully used in the topical treatment of asthma via inhalation therapy [2].

Budesonide is rapidly and almost completely absorbed following oral administration. The major metabolites, 6-β-hydroxybudesonide and 16-α-hydroxyprednisolone have less than 1% of the glucocorticoid activity of unchanged budesonide. Budesonide is reported to have a terminal half time of about 4 h [3].

The fraction of inhaled drug deposited in the lungs is generally to be approximately 20% of the doses. This dose delivered in the lungs is small and thus the amount of drug available for the systemic absorption is very low resulting in low plasma concentrations and a robust, sensitive and selective method was then required for the plasma determination of systemic exposure of the drugs following inhalation [4].

The analysis of budesonide in bulk drugs and pharmaceutical products is well described in the literature [5–9] and it is mentioned either in the European [10] or the United States Pharmacopoeias [11]. The determination of budesonide in biological fluids for pharmacokinetics studies has also been reported. The first sensitive and selective method for the quantitation of budesonide epimers in human plasma published was a method divided into three steps, off-line solid-phase extraction, acetylation, and finally LC–APCI–MS [12]. This first method was not robust enough and endogenous substances appeared in the chromatograms. Then Yuan et al. [4] developed a more selective method. Kronkvist et al. [13] reported

☆ This paper is part of a special issue entitled "Method Validation, Comparison and Transfer", guest edited by Serge Rudaz and Philippe Hubert.

* Corresponding author. Tel.: +32 84 32 04 52; fax: +32 84 32 04 53.
E-mail address: bstre@galephar.be (B. Streel).

1570-0232/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2009.02.004

an automated sample preparation for the determination of budesonide in plasma sample by LC–MS/MS. More recently, Deventer et al. [14] have reported the development and validation of analysis for budesonide in human urine using LC–MS. But Kronkvist et al. [13] and Deventer et al. [14] methods lack the ability to determine the epimers of budesonide individually.

This study describes a new validated method combining automated SPE and liquid chromatography coupled to tandem mass spectrometry to determine budesonide epimers in human plasma. The SPE procedure has been optimized in order to obtain sufficiently high recoveries for budesonide epimers, regarding particularly the selection of the extraction sorbent. The MS/MS ionization (negative) and transitions were optimized in order to avoid endogenous contamination of the chromatogram. The LC conditions were also investigated in order to achieve very low concentrations of the budesonide epimers. The method has been fully validated according to the new strategy proposed by the Commission of the Société Française des Sciences et Techniques Pharmaceutiques (SFSTP) for the validation of quantitative analytical procedure [15]. The validation strategy consists in two steps. The first step, the so-called prevalidation step, has mainly permitted the selection of the most appropriate regression model using the accuracy profile as decision tool [15–21]. The second step, representing the validation itself, consists in testing the method selectivity towards endogenous components and the assessment of method precision, trueness and accuracy [15,22] at different concentration levels over the range investigated as well as the confirmation of the limit of quantitation (LOQ) and the method linearity [15–20]. The limit of quantitation of the method was found to be 41.2 pg/ml for budesonide epimer A and 37.6 for budesonide epimer B. Risk and uncertainty were also assessed in order to evaluate method performance.

Finally the method reported was successfully used to perform the quantitative determination of budesonide in real human plasma samples and was found to be applicable for the quantification of the compound in pharmacokinetics studies, which requires high sensitivity, selectivity and robustness.

2. Experimental

2.1. Chemicals

Budesonide was obtained from Industriale Chimica (Saronno, Italy) and the internal standard (hydrocortisone acetate) was supplied by European Pharmacopoeia (Strasbourg, France). Ammonium acetate and glacial acetic acid were of analytical grade from Merck (Darmstadt, Germany). Acetonitrile and water were of HPLC grade from Biosolve (Valkenswaard, Netherlands). Nitrogen was produced by an on-site nitrogen generator from Air Liquide (Milmort, Belgium).

Isolute DECs (1 ml capacity) filled with 50 mg ethylsilica endcapped ($C2^{EC}$) were obtained from IST (International Sorbent Technology, Mid-Glamorgan, UK). Other Isolute DECs filled with 50 mg of other sorbents such as ethyl (C2), octyl (C8), endcapped octyl ($C8^{EC}$), octadecyl (C18), endcapped octadecyl ($C18^{EC}$), monofunctional octadecyl ($C18^{MF}$), phenyl (PH^{EC}), endcapped phenyl (PH^{EC}) from IST and dualzone octadecyl from Diazem (Midland, MI, USA) were also tested.

The analytical column Luna C18(2) (50 mm × 4.6 mm i.d.) was prepacked with octadecylsilica (particle size 3 μm) from Phenomenex (Utrecht, Netherlands). The analytical column was preceded by a LiChroCart guard column (4 mm × 4 mm i.d.) prepacked with Purospher RP-18e column (5 μm) from Merck.

2.2. Apparatus

The automated sample preparation with extraction cartridges (ASPEC) system from Gilson (Villiers-le-Bel, France) consisted of an automatic sampling injector module equipped with four needles, four model 401 dilutor pipettors and a set of racks and accessories for handling DECs, plasma samples and solvents.

The LC system consisted in a Model 1100 Series liquid chromatograph equipped with a binary pump, a vacuum degasser, a thermostatted column compartment and a thermostatted autosampler, all from Agilent Technologies (Palo-Alto, CA, USA).

Mass spectrometric detection was carried out using an Applied Biosystems API 5000 Triple Quadrupole instrument (Thornhill, Toronto, Canada) equipped with an APCI interface. A PC Dell Precision 390 (Round Rock, TX, USA) equipped with a Analyst 1.4.2 version software from Applied Biosystems was used to control the LC–MS/MS system and to collect and treat the data. The e-Noval® software (Arlenda, Belgium) was used to determine the accuracy profiles as well as all the validation results. The program Kinetica® version 4.4.1 (Innaphase) was applied for the calculations of pharmacokinetics parameters.

2.3. Chromatographic technique

All chromatographic experiments were carried out in the isocratic mode. The Luna C18 (2) analytical column (50 mm × 4.6 mm i.d.) and the precolumn were thermostatted at 25 °C. The mobile phase consisted of a mixture of acetonitrile and 10 mM ammonium acetate adjusted to pH 3.2 (35:65, v/v). The flow-rate was 1.0 ml/min and the volume injected was 100 μl. The thermostatted autosampler was set to 15 °C.

2.4. Mass spectrometric detection

Budesonide and hydrocortisone acetate were detected by tandem mass spectrometric detection with APCI interface in negative mode. Data were acquired using Sciex API5000 triple-quadrupole mass spectrometer in multiple reaction monitoring (MRM) mode. The MS conditions were as follows: *budesonide*: transition $[M+CH_3COO^-] \rightarrow$ product ion mass: 489.3 → 357.3, 250 ms dwell time, declustering potential (DP): −75 eV, collision cell exit potential (CXP): −25 eV, collision energy (CE): −20 eV. *Hydrocortisone acetate*: 463.3 → 403.2, 250 ms dwell time, DP: −85 eV, CXP: −13 eV, CE: −15 eV. *Other parameters*: curtain gas: 20 (arbitrary unit, a.u.), desolvatation gas 1: 85 a.u., Needle current: −5 μA, desolvatation temperature: 550 °C, collision gas: 6 a.u., nitrogen, entrance potential: −10 eV.

2.5. Standard solutions

2.5.1. Stock solutions

A stock solution of budesonide was prepared by dissolving the appropriate amount in methanol in order to obtain a final concentration of 1.0 mg/ml. This solution was then diluted with methanol in order to achieve a final concentration of 2400 ng/ml.

2.5.2. Diluted solutions used for method validation and routine analysis

Seven solutions of budesonide were prepared by diluting the stock solution with the mobile phase to reach concentrations ranging from 1.8 to 120.0 ng/ml. These solutions were then used to spike plasma samples either for calibration curves ranging from 0.09 to 6.00 ng/ml ($m = 7$) or for quality control during the pharmacokinetic study. In chromatographic conditions, both epimers of budesonide were separated. In the standard used, the mass proportion of each epimer is about 1:1, so each epimer of budesonide was

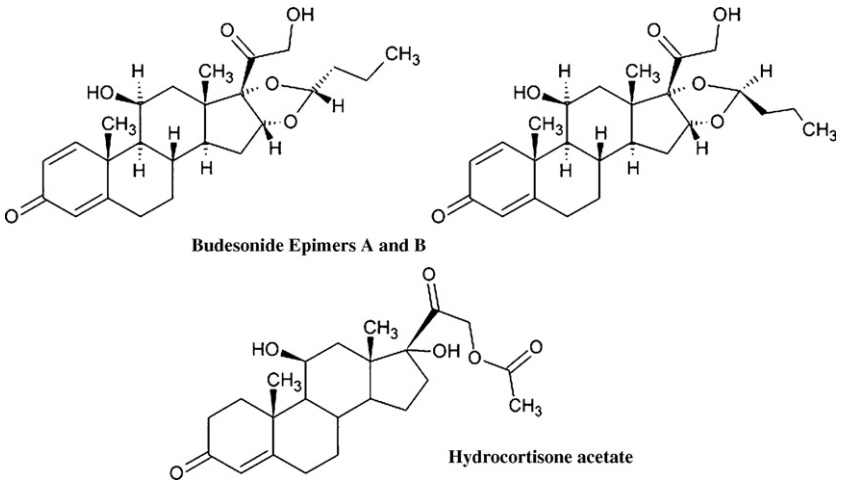

Fig. 1. Structures of budesonide epimers A and B, and hydrocortisone acetate.

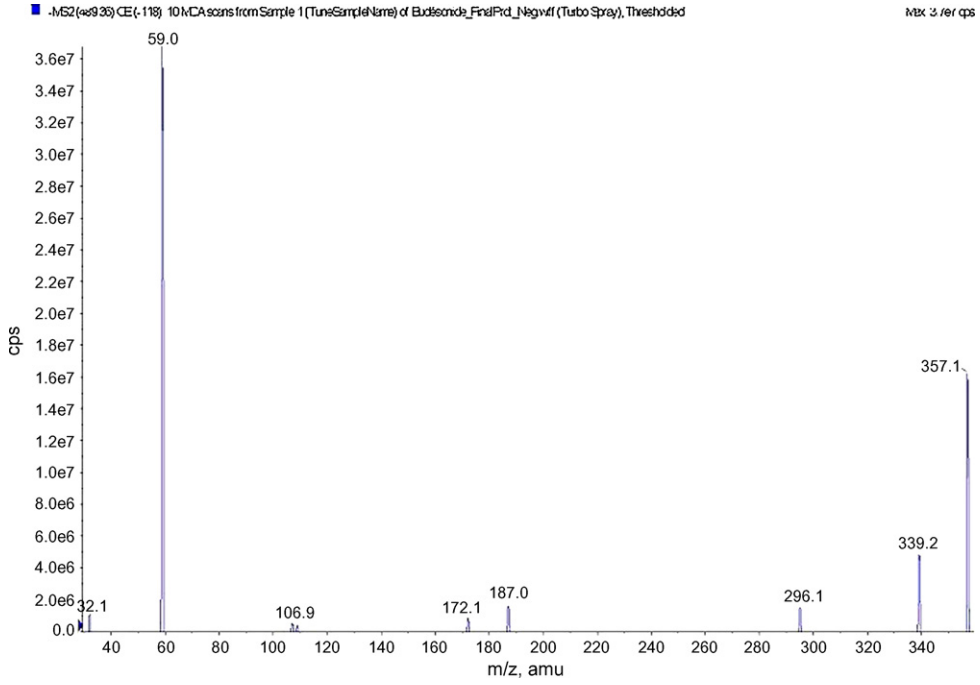

Fig. 2. Product ion mass spectrum of budesonide illustrating the ion m/z 357.

ranging from about 45 to 3000 pg/ml. A stock solution of hydrocortisone acetate (IS) was prepared in methanol. This solution was then diluted with the mobile phase to obtain a final concentration of 6.25 ng/ml.

During the prevalidation and validation phases, three calibration curves ($k = 3$) were performed, each one by using new diluted solutions. Each calibration standard was injected in duplicate ($n = 2$). The independent validation standards were prepared at final concentrations of 45, 90, 750, and 3000 pg/ml ($n = 4$). The same calibration scheme was used in routine analyses.

During the analytical run in the pharmacokinetic study phase, three concentration levels of quality control samples were used: 90, 750 and 3000 pg/ml, repeated three times.

2.6. Sample preparation

After thawing and centrifugation at 3000 × g for 10 min, 1.0-ml of plasma samples was transferred manually to a sample vial, a 0.4-

Table 1A
Selection of the sorbent of the disposable extraction cartridge.

Type of sorbent	Analyte recovery (mean ± S.D., %, $n = 3$)	
	Budesonide epimer B	Budesonide epimer A
Isolute C2	75.7 ± 31.1	73.9 ± 31.3
Isolute C2^{EC}	97.9 ± 13.2	118.8 ± 12.9
Isolute C8	105.5 ± 4.6	127.1 ± 6.2
Isolute C8^{EC}	93.2 ± 10.7	121.0 ± 10.6
Isolute C18	93.9 ± 7.5	99.3 ± 9.4
Isolute C18^{EC}	99.1 ± 5.7	103.1 ± 7.9
Isolute C18^{MF}	103.4 ± 9.3	108.5 ± 9.9
Isolute Phenyl	79.6 ± 8.3	82.2 ± 10.8
Isolute Phenyl^{EC}	75.5 ± 2.2	76.4 ± 1.0
Dualzone C8	72.4 ± 8.9	76.4 ± 11.7

DECs: isolute (50 mg); conditioning: methanol and water (1.0 ml of each); washing: 1.0 ml of water twice; eluting: 1.0 ml of acetonitrile; evaporation; recuperation in 250 μl mobile phase; sample: spiked plasma solution of budesonide and IS.

Table 1B
Selection of eluant of the extraction cycle.

	Elution: methanol + 1% formic acid		Elution: acetonitrile + 1% formic acid	
	$C2^{EC}$	$C18^{EC}$	$C2^{EC}$	$C18^{EC}$
Analytical recovery (mean ± S.D., %, $n = 3$)				
Budesonide epimer B	90.0 ± 1.4	116.5 ± 10.8	99.9 ± 21.6	76.9 ± 13.6
Budesonide epimer A	107.7 ± 6.1	103.3 ± 7.1	88.1 ± 19.7	66.2 ± 14.7
Hydrocortisone acetate	78.6 ± 5.5	36.6 ± 18.3	32.4 ± 16.4	21.6 ± 13.8

DECs: isolute (50 mg); conditioning: methanol and water (1.0 ml of each); washing: 1.0 ml of water/methanol (95:5, v/v) twice; evaporation; recuperation in 250 μl mobile phase; sample: spiked plasma solution of budesonide and IS.

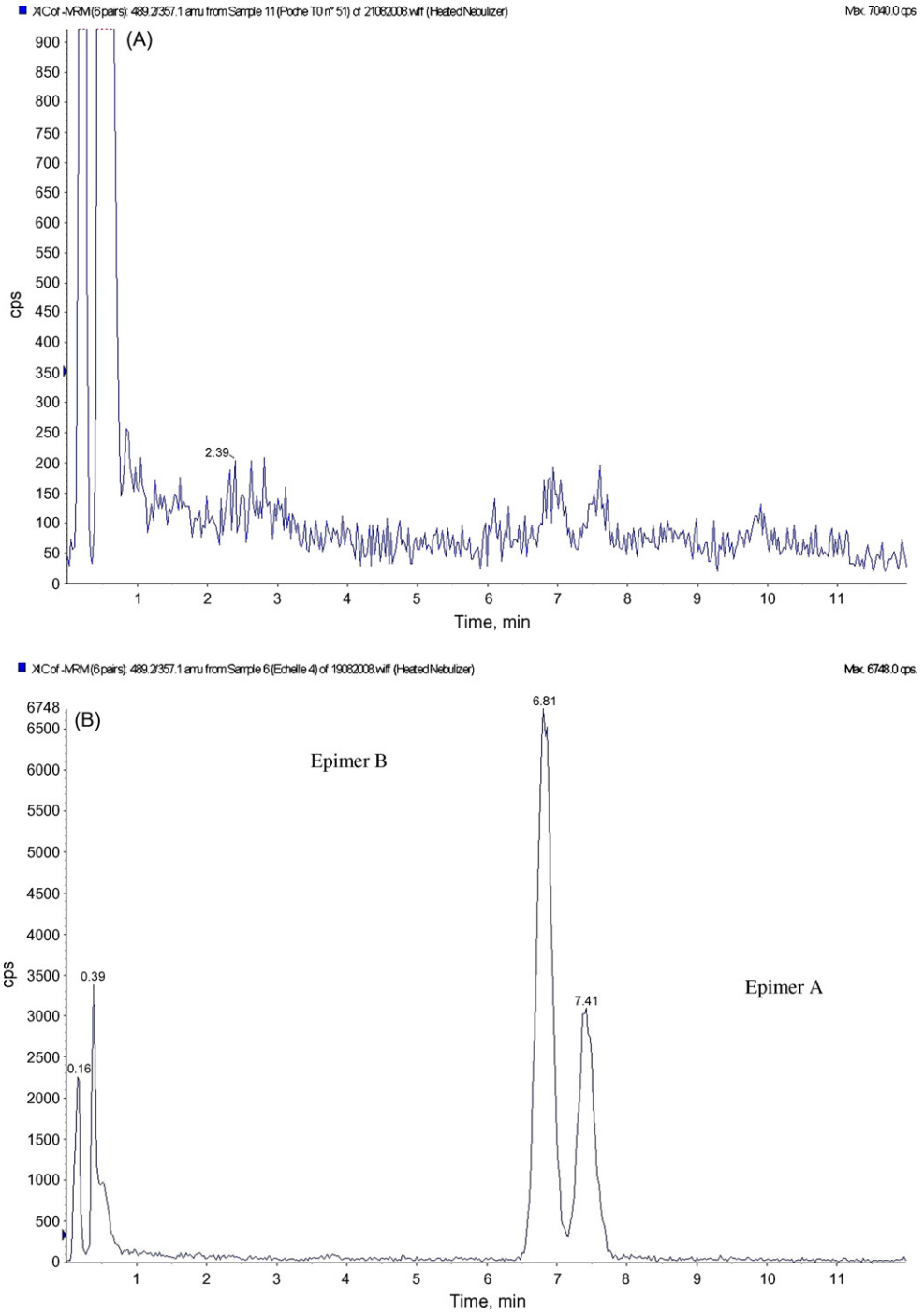

Fig. 3. Determination of method selectivity. (A) Chromatogram of blank plasma with budesonide quantitation parameters. (B) Chromatogram of plasma spiked with budesonide at 375 pg/ml. (C) Chromatogram of blank plasma with IS quantitation parameters. (D) Chromatogram of plasma spiked with internal standard at 5000 pg/ml.

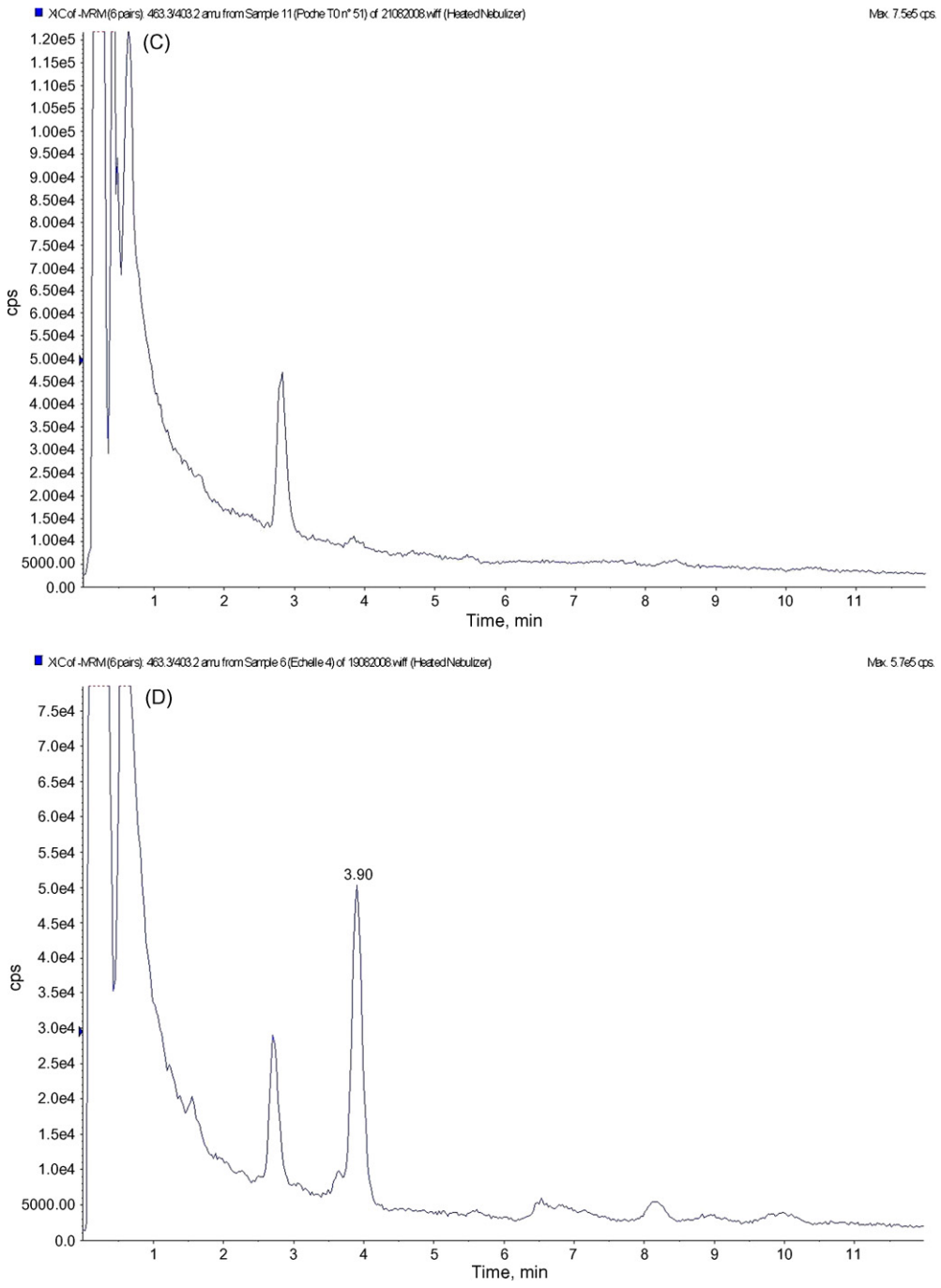

Fig. 3. (*Continued*).

ml volume of internal standard solution (6.25 ng/ml) was added and mixed. The vial is transferred on the appropriate rack of the ASPEC system. The conditioning of the sorbent was achieved by passing first 1.0-ml of methanol and then 1.0-ml of ammonium acetate 10 mM through the DEC. The mixed sample (1.3-ml) was then aspirated by the autosampler needle from the corresponding vial and applied onto the DEC. The washing step was then performed by dispensing twice 1-ml of water/methanol (95:5, v/v). A 1-ml volume of methanol/formic acid (99:1, v/v) was then dispensed on the DEC and the eluate was collected in the tube positioned under the DEC. The collected eluate was evaporated under reduced pressure at 60 °C for 180 min, 250-μl of mobile phase was added to the residue, agitated and transferred into a chromatographic vial. Vials were transferred manually to the LC autosampler rack for analysis.

2.7. Pharmacokinetic study

The developed LC–MS/MS procedure was used to investigate the plasma profiles of budesonide after a multiple inhaled administration to 24 healthy volunteers. Each volunteer received a dose of 300 μg of budesonide twice a day. The study lasted 8 days. Seventy blood samples were withdrawn at different times from day 5 to day 8 after the administration of the medication.

3. Results and discussion

3.1. Optimization of MS conditions

The LC–MS/MS method for the determination of budesonide was first investigated. For the optimization of MS conditions, each compound was directly introduced in the MS detector using APCI ionization and parameters such as corona discharge, orifice voltage, ring voltage, flow of nebulizer and auxiliary gas (N_2) and temperature of auxiliary gas (N_2) were investigated in order to obtain the deprotonated pseudomolecular ions of budesonide and hydrocortisone acetate (IS) (Fig. 1).

The pseudomolecular ions [M−CH_3COO^-] observed on the full scan mass spectra of budesonide were *m*/*z* 489.3. The collision energy in Q2 produced five significant fragment ions with *m*/*z* ranging from 59 to 357 (Fig. 2). MS–MS fragments gave higher abundance were *m*/*z* 59 and *m*/*z* 357. *m*/*z* 59 fragment comes from the deacetylation of the budesonide acetate adduct and thus will not be stable and robust. Thus the MS/MS transition *m*/*z* 489/357 was selected.

3.2. LC optimization

Even if the MS detection coupled to the LC separation can be considered as a very selective method and allows to quantify with a high level of precision some coeluted peaks, it is generally admitted that it is a better way to dispose of a complete chromatographic separation of the compounds. The complete separation of budesonide epimers and hydrocortisone acetate was obtained on an octadecyl silica stationary phase using a mobile phase consisting of a pH 3.2 ammonium acetate buffer–acetonitrile (65:35, v/v) mixture.

3.3. Selection of SPE sorbent

Different kinds of DECs containing bonded silicas with various polarities were tested. Spiked plasma solutions were used as samples and the corresponding recoveries of budesonide were determined (Table 1A). The recoveries were calculated by comparing the peak areas obtained from freshly prepared samples extracts with those found by direct injection of aqueous solutions at the same concentration into the LC–MS/MS system, using the same autosampler. As can be seen in Table 1A, best results were obtained with $C2^{EC}$, C8, $C8^{EC}$, C18, $C18^{EC}$ and $C18^{MF}$. Two types of sorbent were selected to perform the optimization of the extraction cycle, $C2^{EC}$ and $C18^{EC}$.

Parameters such as washing solvent and elution solvent were then optimized. Table 1B shows the comparison between $C2^{EC}$ and $C18^{EC}$ with elution solvent methanol/formic acid (99:1, v/v) and acetonitrile:formic acid (99:1, v/v). Best results were obtained with $C2^{EC}$ with methanol:formic acid (99:1, v/v).

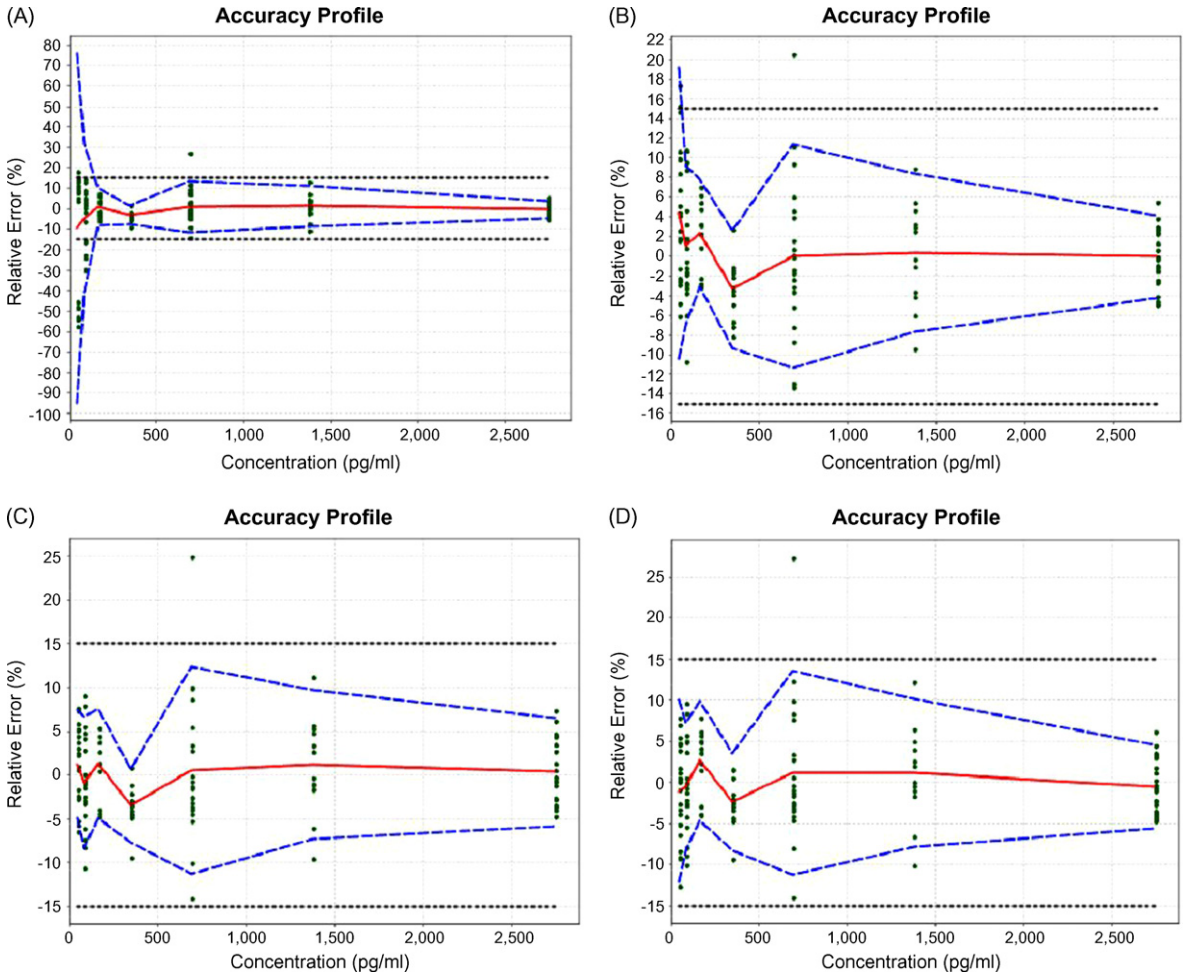

Fig. 4. Accuracy profile, at 17.5%, of budesonide epimer A in human plasma using (A) a linear regression model, (B) a quadratic regression model, (C) a linear regression model after logarithmic transformation and (D) a weighted 1/*X* linear regression model. Relative bias (—), acceptance limits (- - -), β-expectation tolerance limits (– – –) and relative back-calculated concentrations (■).

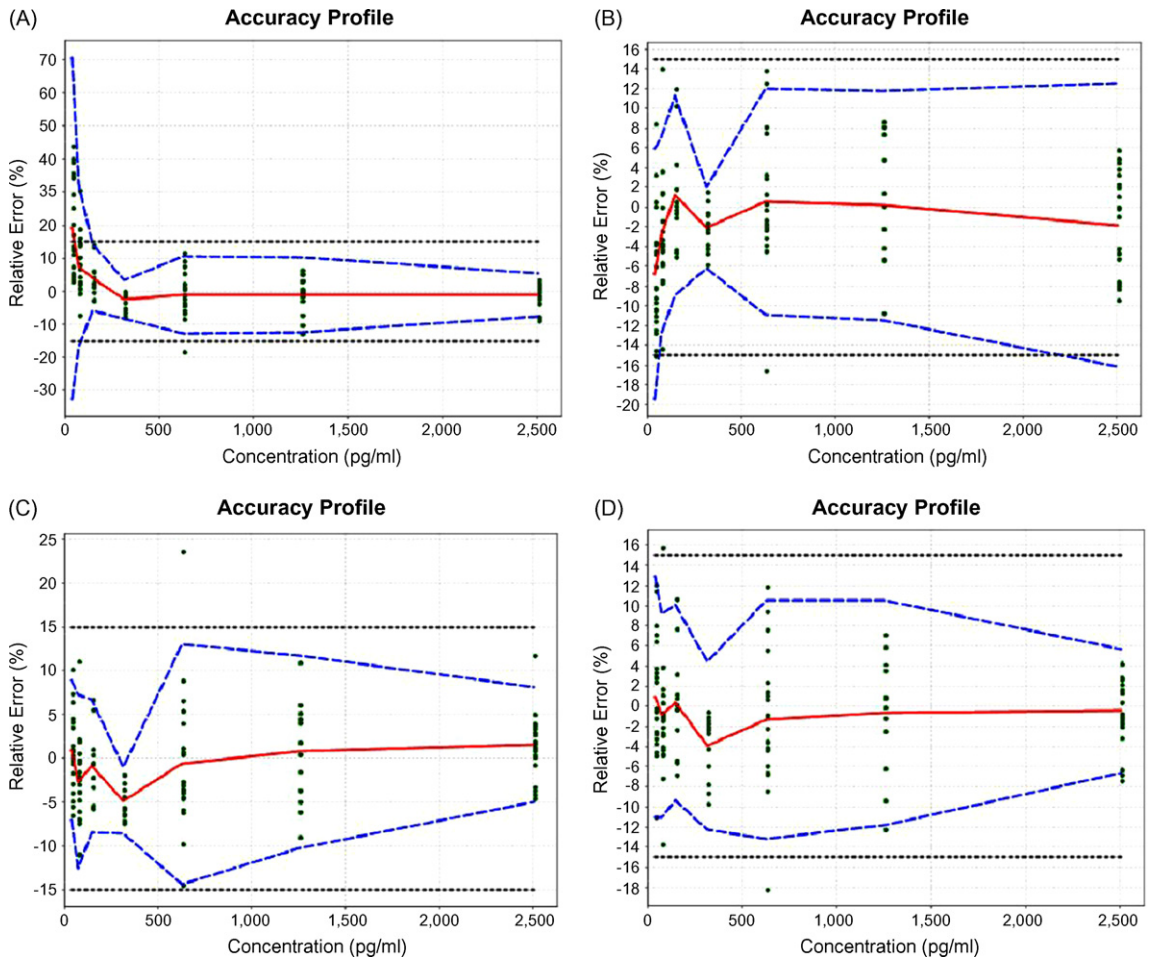

Fig. 5. Accuracy profile, at 10.0%, of budesonide epimer B in human plasma using (A) a linear regression model, (B) a quadratic regression model, (C) a linear regression model after logarithmic transformation and (D) a weighted 1/*X* linear regression model. Relative bias (—), acceptance limits (- - -), β-expectation tolerance limits (– – –) and relative back-calculated concentrations (■).

3.4. Validation step

3.4.1. Selection of the calibration model

An important step in the validation phase of an analytical method consists in the assessment of the relationship between the response and concentration in order to avoid serious difficulties in the estimation of other validation criteria. In order to select the most appropriate response function, the SFSTP approach based on β expectation tolerance interval (or accuracy profile) for total measurement error – including both bias and precision – of calibration samples has been used [15–19]. This approach reflects more directly the performance of individual assays and will result in fewer rejected in-study runs than the current procedure that compares point estimates of observed bias and precision with the target acceptance criteria, i.e. 15% according to the Washington conference [23] or FDA document [24]. Once the calibration experiments have been performed, the response function can be determined by applying different regression models and, from both analytical responses and regression line obtained, selecting the most suitable accuracy profile for the intended use of the analytical method [15,17–19]. Regarding the obtained accuracy profiles for both budesonide epimers (Fig. 4 for epimer A and Fig. 5 for epimer B), regression analysis could be performed in the present study using either the linear regression after log transformation or the weighted 1/X linear regression (Figs. 4(C) and (D) and 5(C) and (D)). Nevertheless, the accuracy profiles obtained with the linear regression after log transformation reach the acceptance limits at one concentration for budesonide epimer B determination. The weighted 1/*X* linear regression model was finally selected.

The obtained equations are presented in Tables 3 and 4.

Accuracy profiles were build using a 82.5% (Risk $\alpha = 17.5\%$) tolerance interval for budesoinde epimer A and a 90.0% ($\alpha = 10.0\%$) tolerance interval for budesonide epimer B. The tolerance intervals for both epimers were selected according to the maximum risk (α) to have future measurements during routine analysis outside the acceptance limits of the ±15% β-expectation tolerance interval (see Section 3.4.10). The determination coefficients (r^2) obtained for both regression lines demonstrate the good relationship between peak area ratio and epimer concentration.

Furthermore, by use of the weighted linear regression model, the procedure was able to quantify over the whole range under investigation. This was of particular interest in the present study since very low concentrations of budesonide should be measured.

3.4.2. Stability

The stability of the whole procedure was studied by considering the different steps of the method.

For the evaluation of stability, the concentration levels of 90 and 3000 pg/ml were used and the number of replicate was 3. The stability of stock solutions (9 days at 5 °C), autosampler eluate (48 h at 20 °C), plasma sample (48 h at 20 °C), plasma storage (7 months at −80 °C) and after three freeze and thaw cycles was investigated. The determination of budesonide and IS were performed at the beginning and at the end of each storage period. The results obtained

Table 2
Matrix effect.

Budesonide epimer A		Budesonide epimer B	
90 pg/ml	3000 pg/ml	90 pg/ml	3000 pg/ml
Analytical recovery (mean ± S.D., %, $n = 3$)			
96.3 ± 8.5	95.6 ± 1.9	93.7 ± 2.8	91.2 ± 3.3

were all included between 85% and 115% of the initial value. No significant degradation of budesonide and internal standard was observed.

3.4.3. Selectivity and matrix effect

Potential interfering substances in a biological matrix include endogenous matrix components, related substances, metabolites and concomitant medication drugs such as OTC drugs (aspirin, acetaminophen, caffeine and ibuprofen). The selectivity was studied by injecting 6 different sources of plasma spiked with these compounds in the chromatographic system after DECs and by analyzing 18 different sources of plasma. No endogenous source of interference was observed at the retention times of the analytes. Typical chromatograms obtained with a blank plasma and a plasma containing 120.2 pg/ml of budesonide epimer A and 90.5 pg/ml budesonide epimer B are presented in Fig. 3(A) and (B). Chromatograms obtained with a blank plasma and a plasma containing 5000 pg/ml of internal standard are presented in Fig. 3(C) and (D).

The matrix effect was evaluated by injected six samples of different plasma spiked with budesonide solution after DECs and compared with pure solution of the same concentration.

Results presented in Table 2 show that matrix effect is negligeable.

3.4.4. Response function

The response function of an analytical procedure is, within the range selected, the existing relationship between the response (signal) and the concentration (quantity) of the analyte in the sample system [15–19,21,22]. The validation results of the response function are presented in Table 3 (epimer A) and Table 4 (epimer B). The response function was evaluated on the validation solutions to confirm that the selected weighted $1/X$ linear regression model obtained with the calibration standards is valid and may be applied on real routine samples.

3.4.5. Trueness

Trueness refers to the closeness of agreement between the mean value obtained from a series of measurements and an accepted reference value [15,22]. As can be seen from the results in Table 3 (epimer A) and Table 4 (epimer B), trueness was expressed in terms of absolute bias (in pg/ml) or relative bias (%). It was assessed by means of validation standards in the matrix at four concentration levels ranging from 41.2 to 2745 pg/ml ($k = 3$, $n = 6$) for budesonide epimer A and ranging from 37.6 to 2506 pg/ml ($k = 3$, $n = 6$) for budesonide epimer B. Compared to the regulatory requirements for

Table 3
Validation of the method of determination of budesonide epimer A in human plasma.

Validation criterion for budesonide epimer A			
Response function	Series 1	Series 2	Series 3
Calibration model	Weigthed $1/X$ linear regression; calibration range ($m = 7$); 41.2–2745 pg/ml		
Slope	4.37×10^{-4}	3.32×10^{-4}	3.04×10^{-4}
Intercept	1.73×10^{-3}	-7.63×10^{-4}	-4.00×10^{-6}
r^2	0.9974	0.9980	0.9977

Trueness ($k = 3$; $n = 6$) (pg/ml)	Absolute bias (pg/ml)	Relative bias (%)
41.2	1.3	3.0
82.4	0.4	0.5
686	−7.9	−1.2
2745	−84.0	−3.0

Precision ($k = 3$; $n = 6$) (pg/ml)	Repeatability (R.S.D., %)	Intermediate precision (R.S.D., %)
41.2	4.1	5.8
82.4	5.1	6.6
686	8.6	8.9
2745	2.8	3.8

Accuracy ($k = 3$; $n = 6$) (pg/ml)	β-Expectation tolerance limit (pg/ml)	Relative β-expectation tolerance limit (%)
41.2	[38.4; 46.5]	[−6,8; 12,9]
82.4	[73.8; 91.7]	[−10.4; 11.4]
686	[588; 769]	[−14.4; 12.1]
2745	[2486; 2836]	[−9.5; 3.3]

Linearity ($k = 3$; $n = 6$)	
Range (pg/ml)	41.2–2745
Slope	0.9679
Intercept	5.942
r^2	0.9972
LOD (pg/ml)	4.7
LOQ (pg/ml)	41.2

k, number of series; n, number of replicates per series; m, number of concentration levels for calibration standards.

Table 4
Validation of the method of determination of budesonide epimer B in human plasma.

Validation criterion for budesonide epimer B			
Response function	Series 1	Series 2	Series 3
Calibration model	Weigthed $1/X$ linear regression; calibration range ($m = 7$); 37.6–2506 pg/ml		
Slope	7.58×10^{-4}	5.79×10^{-4}	1.71×10^{-3}
Intercept	2.72×10^{-3}	-1.89×10^{-4}	4.98×10^{-3}
r^2	0.9991	0.9981	0.9978

Trueness ($k = 3$; $n = 6$) (pg/ml)	Absolute bias (pg/ml)	Relative bias (%)
37.6	0.5	1.4
75.2	−0.7	−0.9
626	−13.9	−2.2
2506	−20.1	−0.8

Precision ($k = 3$; $n = 6$) (pg/ml)	Repeatability (R.S.D., %)	Intermediate precision (R.S.D., %)
37.6	4.3	5.6
75.2	5.0	6.0
626	6.3	6.8
2506	3.3	3.5

Accuracy ($k = 3$; $n = 6$) (pg/ml)	β-Expectation tolerance limit (pg/ml)	Relative β-expectation tolerance limit (%)
37.6	[33.8, 42.5]	[−10.1, 12.9]
75.2	[65.7, 83.3]	[−12.6, 10.8]
626	[534.3, 690.9]	[−14.7, 10.3]
2506	[2324.0, 2648.0]	[−6.6, 5.7]

Linearity ($k = 3$; $n = 6$)	
Range (pg/ml)	37.6–2506.0
Slope	0.9922
Intercept	−2.241
r^2	0.9978
LOD (pg/ml)	3.2
LOQ (pg/ml)	37.6

k, number of series; n, number of replicates per series; m, number of concentration levels for calibration standards.

bioanalytical method validation [23,24], the trueness of the proposed method was demonstrated since the relative bias did not exceed the values of 15%, including the lower limit of quantitation.

3.4.6. Precision

The precision of the bioanalytical method was evaluated at two levels: repeatability and intermediate precision, at the same concentration levels as those mentioned above for each budesonide epimer. The variance of repeatability and time dependent intermediate precision as well as the corresponding relative standard deviation (R.S.D.) were calculated from the estimated concentrations [16,17] and are presented in Tables 3 and 4. R.S.D. values for repeatability and intermediate precision were between 2.8% and 8.9% for budesonide epimer A and between 3.3% and 6.8% for budesonide epimer B, illustrating the good precision of the proposed method.

3.4.7. Accuracy

The accuracy takes into account the total error, i.e. systematic and random errors, related to the test result [16,22]. The upper and lower β-expectation tolerance limits expressed in absolute concentration (pg/ml) and in relative value (%) are presented in Tables 3 and 4 as a function of the introduced concentrations. As can be seen from the results, the method was considered as accurate, since the tolerance intervals are included in the ±15% acceptance limits [23,24] for all the concentration levels tested including the lowest one (41.2 pg/ml for epimer A and 37.6 pg/ml for epimer B).

3.4.8. Linearity

The linearity of an analytical method is its ability within a definite range to obtain results directly proportional to the concentrations (quantities) of the analyte in the sample [15–17]. Therefore, for all the series, a regression line was fitted on the estimated concentrations as a function of the introduced concentrations by applying the linear regression model based on the least squares method. The regression equation is presented for each budesonide epimer in Tables 3 and 4. Moreover, in order to demonstrate method linearity, the approach based on the absolute β-expectation tolerance limits as illustrated in Fig. 6 (epimer A and epimer B) can be applied [21]. The linearity of the present method was demonstrated for both epimers since the absolute β-expectation tolerance limits were within the absolute acceptance limits.

3.4.9. Detection and quantitation limits

The limit of detection (LOD) was estimated using the mean intercept of the calibration model and the residual variance of the regression [25]. By applying this computation method, the LOD of the developed method was 4.7 pg/ml for budesonide epimer A and 3.2 pg/ml for budesonide epimer B. The lower limit of quantitation of an analytical procedure is the smallest amount of the targeted substance in the sample that can be assayed under the experimental conditions prescribed with a well defined accuracy [16], i.e. taking into account the systematic and random errors [23,24].

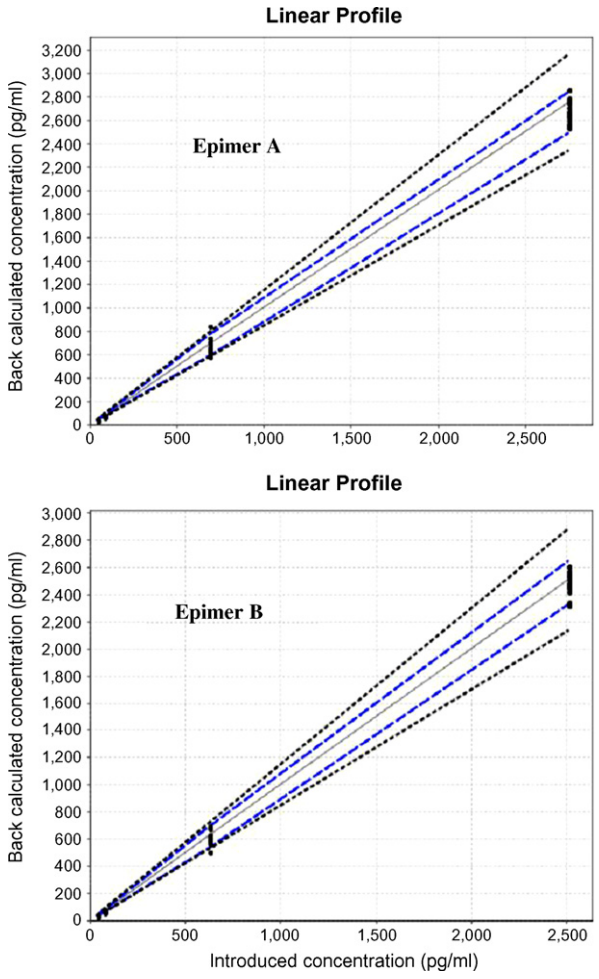

Fig. 6. Linear profile of budesonide. The dashed limits on this graph correspond to the accuracy profile, i.e. the β-expectation confidence limits expressed in absolute values. The dotted curves represent the acceptance limit at 15% expressed in the concentration units.

The lowest concentration levels studied for each epimer were considered to be the LOQ since the accuracy profiles are within the acceptance limits. Precision and trueness were also demonstrated at these concentration levels (see Tables 3 and 4).

3.4.10. Risk and uncertainty assessments

The maximum risk (α; $1-\beta$) to have future measurements during routine analysis outside the acceptance limits of the ±15% β-expectation tolerance interval was set at 17.5% for budesonide epimer A and 10.0% for budesonide epimer B [26]. Accuracy profiles with the selected regression model allow to estimate the probability to obtain such results. Fig. 7 shows the risk profiles associated at each level studied for both epimers. The maximum risk obtained

Table 5
Uncertainty of measurements for each concentration level of the budesoinde epimers A and B validation standards.

Analyte	Concentration (pg/ml)	Uncertainty of the bias (pg/ml)	Uncertainty (pg/ml)	Expended uncertainty (pg/ml)	Relative expanded uncertainty (%)
Budesonide epimer A	41.2	1.1	2.6	5.2	13
	82.4	2.3	5.9	12	14
	686	17	64	127	19
	2745	45	115	229	8.3
Budesonide epimer B	37.6	0.9	2. 3	4.6	12
	75.2	1.7	4.8	9.6	12
	626	12	44	89	14
	2506	26	92	184	7.3

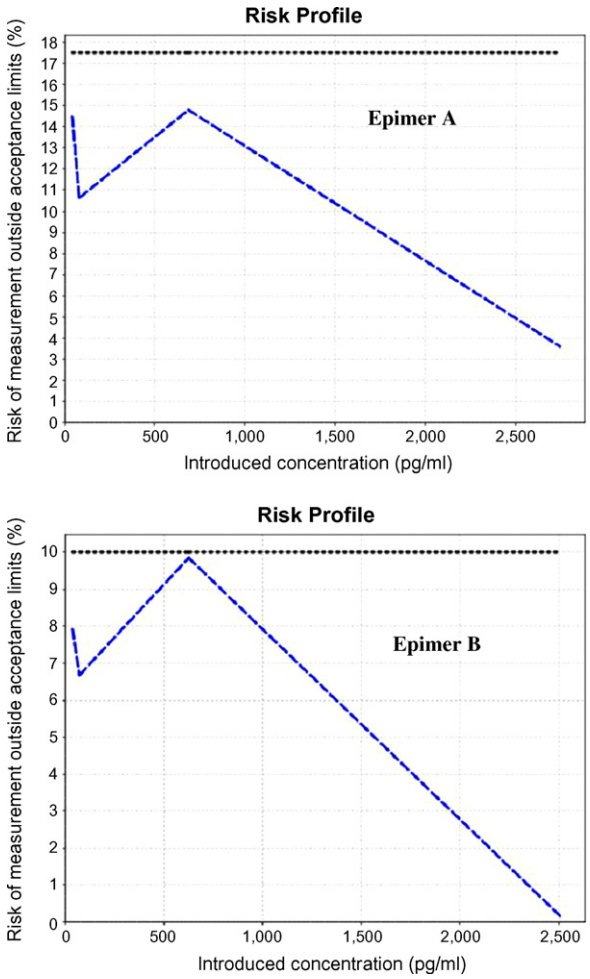

Fig. 7. Risk profile in % of having future measurements falling outside the ±15% acceptance limits in routine analysis for the quantitation of budesonide epimer A and B in human plasma, obtained by considering the weighted (1/*X*) linear regression.

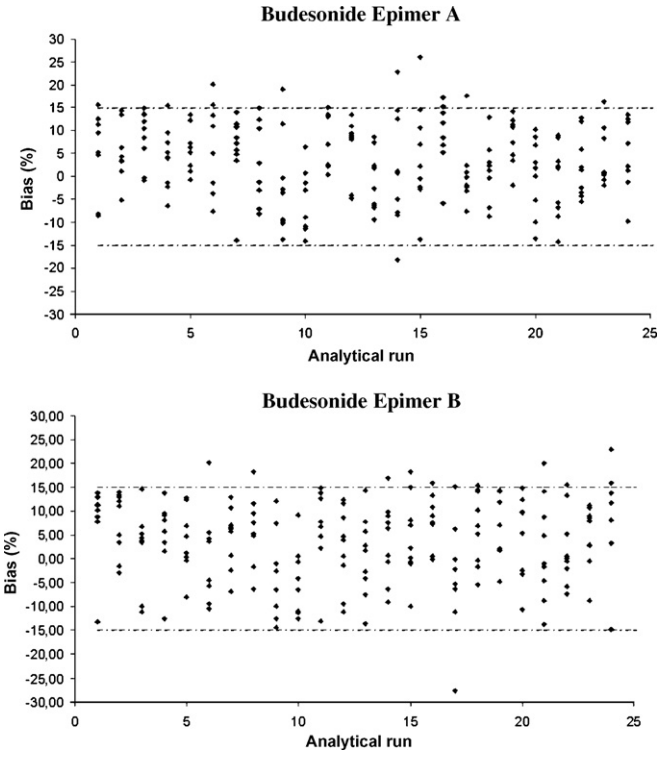

Fig. 8. Chart of quality control during routine analysis (*N* = 207).

is less than 15% for the quantitation of budesonide epimer A and less than 10% for the quantitation of budesonide epimer B. It can be noticed that a risk of 33% is accepted in routine analysis according to the 4-6-20 rule of the FDA guidance [23,24].

Measurements uncertainty was also evaluated during the validation phase. The uncertainty characterizes the dispersion of the values that could reasonably be attributed to the measurand. The expanded uncertainty was computed using a coverage factor of $k = 2$ [27–29], representing an interval around the results were the unknown true value can be found with a confidence level of 95%. The relative expanded uncertainty of budesonide epimer A in human plasma is less than 20% (Table 5) and less than 15% for epimer B. This means that, with a confidence level of 95%, the unknown true value is situated at maximum ±20% for epimer A and ±15% for epimer B around the measured result.

3.5. Routine analysis

3.5.1. Method follow-up during routine analysis

In order to assess the method performances during routine analysis, quality control samples at different concentration levels have to be analysed. The procedure most widely used for the continuous evaluation of assay performance involves the construction of QC charts. In the present study, the acceptance limits have been fixed at ±15% of the observed bias according to the Washington conference [23] and the FDA document [24]. Three concentration levels for each method were monitored. The QC charts presented in Fig. 8 demonstrate that the analytical procedures were undercontrol during routine analysis. Indeed, at least 67% of the QC samples were within 15% of their nominal values and definitely less than 33% of the QC samples without replicates at the same concentration levels were outside the ±15% of the nominal value [23,24]. Moreover, the QC bias (%) and the QC R.S.D. values which are presented in Table 6 illustrates the very good reliability of the described method.

Table 6
QC interday precision and trueness during routine analysis.

Epimer A		
Trueness ($k = 24$; $n = 3$) (pg/ml)		Absolute bias: pg/ml (relative bias: %)
84.4		−3.3 (−3.9)
703		−19.3 (−2.7)
2810		−121.7 (−4.3)
Precision ($k = 24$; $n = 3$) (pg/ml)	Repeatability (R.S.D., %)	Intermediate precision (R.S.D., %)
84.4	8.3	9.6
703	5.6	7.2
2810	5.8	8.4
Epimer B		
Trueness ($k = 24$; $n = 3$)		Absolute bias: pg/ml (relative bias: %)
95.6		−4.6 (−4.8)
797		−29.2 (−3.7)
2190		−99.0 (−3.1)
Precision ($k = 24$; $n = 3$) (pg/ml)	Repeatability (R.S.D., %)	Intermediate precision (R.S.D., %)
95.6	9.6	10.1
797	6.7	7.8
2190	6.3	8.4

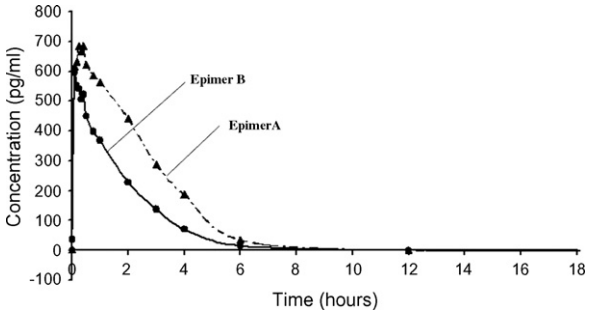

Fig. 9. Plasma concentration–time profile of budesonide (epimer A + epimer B) after a multiple inhaled administration to 24 healthy volunteers.

The proportion of the QC samples outside the limits of ±15% (i.e. the acceptance limit) was 6.8% for budesonide epimers A and B. As these values are less than the risk associated to the predictive model, we can assess the good accuracy of the validated model.

3.5.2. Pharmacokinetics

The LC–MS/MS procedure developed was used to investigate the pharmacokinetics profile and the bioavailability of budesonide after the administration of multiple inhaled doses to 24 healthy volunteers. Plot of the plasma concentration of budesonide (pg/ml) versus post-dose sampling time (h) is presented in Fig. 9. Pharmacokinetics parameters calculated from these data are as follows: budesonide epimer A: AUC_{0-12}: 1746.3 ± 922.3 pg/ml h, C_{max}: 879.5 ± 436.2 pg/ml and T_{max}: 0.5 ± 0.6 h. Budesonide epimer B: AUC_{0-12}: 1429.5 ± 2151.3 pg/ml h, C_{max}: 776.3 ± 428.1 pg/ml and T_{max}: 0.36 ± 0.45 h.

4. Conclusions

A sensitive and accurate procedure based on the solid-phase extraction coupled at-line to a LC–MS/MS determination has been developed for the assay of budesonide. The extraction procedure and the MS/MS conditions were optimized in order to have a sensitive method. The procedure was fully validated to meet the requirements of the pharmacokinetic investigation of this compound. The procedure developed was successfully applied to the determination of budesonide plasma levels for investigating a pharmacokinetic study.

Acknowledgments

Many thanks are due to Ch. Laine, N. Gengler, S. Matagne and C. Zimmer for technical assistance.

References

[1] C.M. Spencer, D. McTavish, Drugs 50 (5) (1995) 854.
[2] K.M. Hvizdoz, B. Jarvis, Drugs 60 (5) (2000) 1141.
[3] Y. Wang, Y. Tang, H. Moellmann, G. Hochhaus, Biomed. Chromatogr. 17 (2003) 158.
[4] Y.N. (Benny) Li, B. Tattam, F. Brown Kenneth, J. Paul Seale, J. Chromatogr. B 761 (2001) 177.
[5] H. William Kelly, Ann. Pharmacother. 32 (1998) 220.
[6] K.H. Assi, W. Tarsin, H. Chrystyn, J. Pharm. Biomed. Anal. 41 (2006) 325.
[7] G. Hochhaus, P. Fröehlich, R. Hochhaus, A. Möllmann, H. Derendorf, H.W. Möllmann, J. Pharm. Biomed. Anal. 17 (1998) 1235.
[8] S. Hou, M. Hindle, P.R. Byron, J. Pharm. Biomed. Anal. 39 (2005) 196.
[9] M. Gupta, H.N. Bhargava, J. Pharm. Biomed. Anal. 40 (2006) 423.
[10] European Pharmacopoeia, 6th ed., Directorate for the Quality of Medicines of the Council of Europe, Strasbourg, France, Monograph 01/2008, p. 1075.
[11] United States Pharmacopoeia, 307th ed., suppl. 1, Rockville, MD, USA, 2007, p. 3756.
[12] Y.N. (Benny) Li, B. Tattam, K.F. Brown, J.P. Seale, J. Chromatogr. B 683 (1996) 259.
[13] K. Kronkvist, M. Gustavsson, A.K. Wendel, H. Jaegfeldt, J. Chromatogr. A 823 (1998) 01.
[14] K. Deventer, P. Mikuleikova, H. Van Hoecke, P. Van Eenoo, F.T. Delbeke, J. Pharm. Biomed. Anal. 42 (2006) 474.
[15] Ph. Hubert, J.-J. Nguyen-Huu, B. Boulanger, E. Chapuzet, P. Chiap, N. Cohen, P.-A. Compagnon, W. Dewe, M. Feinberg, M. Lallier, M. Laurentie, N. Mercier, G. Muzard, C. Nivet, L. Valat, STP Pharma Pratiques 13 (3) (2003) 101.
[16] E. Chapuzet, N. Mercier, S. Bervoas-Martin, B. Boulanger, P. Chevalier, P. Chiap, D. Grandjean, Ph. Hubert, P. Lagorce, M. Lallier, M.C. Laparra, M. Laurentie, J.C. Nivet, STP Pharma Pratiques 7 (1997) 169.
[17] Ph. Hubert, P. Chiap, J. Crommen, B. Boulanger, E. Chapuzet, N. Mercier, S. Bervoas-Martin, P. Chevalier, D. Grandjean, P. Lagorce, M. Lallier, M.C. Laparra, M. Laurentie, J.C. Nivet, Anal. Chim. Acta 391 (1999) 135.
[18] Ph. Hubert, J.-J. Nguyen-Huu, B. Boulanger, E. Chapuzet, P. Chiap, N. Cohen, P.-A. Compagnon, W. Dewé, M. Feinberg, M. Lallier, M. Laurentie, N. Mercier, G. Muzard, C. Nivet, L. Valat, E. Rozet, J. Pharm. Biomed. Anal. 45 (2007) 70.
[19] Ph. Hubert, J.-J. Nguyen-Huu, B. Boulanger, E. Chapuzet, N. Cohen, P.-A. Compagnon, W. Dewé, M. Feinberg, M. Lallier, M. Laurentie, N. Mercier, G. Muzard, L. Valat, E. Rozet, J. Pharm. Biomed. Anal. 45 (2007) 82.
[20] B. Boulanger, W. Dewe, P. Chiap, J. Crommen, Ph. Hubert, J. Pharm. Biomed. Anal. 32 (2003) 753.
[21] B. Boulanger, W. Dewe, M. Feinberg, M. Laurentie, J. Crommen, Ph. Hubert, J. Pharm. Biomed. Anal. 36 (2004) 579.
[22] ISO 5725-1, Application of the Statistics—Accuracy (Trueness and Precision) of the Results and Methods of Measurement. Part 1. General Principles and Definitions, International Organization for Standardization (ISO), Geneva, Switzerland.
[23] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittman, S. Spector, Pharm. Res. 9 (1992) 588.
[24] Guidance for Industry: Bioanalytical Method Validation, U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER), May 2001.
[25] J.C. Miller, J.N. Miller, Statistics for Analytical Chemistry, 5th ed., Ellis Horwood, New York, 2000, p. 96.
[26] B. Boulanger, W. Dewe, A. Gilbert, B. Govaerts, M. Maumy-Bertrand, Chemometr. Intell. Lab. Syst. 86 (2007) 198.
[27] Analytical Methods Committee, Analyst 120 (1995) 2303.
[28] Eurachem/Citac Guide, Quantifying the Uncertainty in Analytical Measurement, 2nd ed., 2000.
[29] EA-4/16, EA Guidelines on the Expression of Uncertainty in Quantitative Testing, 2004, http://www.european-accreditation.org.