Journal of Pharmaceutical and Biomedical Analysis 90 (2014) 198–206

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Validated assay for the simultaneous determination of cortisol and budesonide in human plasma using ultra high performance liquid chromatography–tandem mass spectrometry

András Szeitz[a,*], Jamil Manji[b], K. Wayne Riggs[a], Andrew Thamboo[b], Amin R. Javer[b]

[a] Faculty of Pharmaceutical Sciences, The University of British Columbia, 2405 Wesbrook Mall, Vancouver, British Columbia, V6T 1Z3, Canada
[b] St. Paul's Sinus Centre, St. Paul's Hospital, 1081 Burrard Street, Vancouver, British Columbia, V6Z 1Y6, Canada

ARTICLE INFO

Article history:
Received 14 August 2013
Received in revised form 2 December 2013
Accepted 5 December 2013
Available online 16 December 2013

Keywords:
Cortisol
Budesonide
Liquid chromatography–mass spectrometry
Method validation
Plasma

ABSTRACT

An ultra high performance liquid chromatography–tandem mass spectrometry (UHPLC/MS/MS) method was developed and validated for the quantification of cortisol and budesonide in human plasma. Charcoal stripped human plasma was used as the blank matrix during validation. Cortisol, budesonide, and dexamethasone (internal standard) were extracted from human plasma with methyl-tert-butyl ether, and the chromatographic separation of the peaks was achieved using a Waters Acquity UPLC BEH C_{18}, 1.7 μm, 2.1 mm × 50 mm column with a run time of 4.0 min. Cortisol, budesonide, and dexamethasone were monitored at the total ion current of their respective multiple reaction monitoring transition signals. The UHPLC/MS/MS system consisted of an Agilent 1290 Infinity ultra high performance liquid chromatograph coupled with an AB Sciex Qtrap® 5500 hybrid linear ion-trap triple quadrupole mass spectrometer. The method was validated for accuracy, precision, linearity, range, selectivity, lower limit of quantification (LLOQ), recovery, matrix effect, dilution integrity, and evaluation of carry-over. All validation parameters met the acceptance criteria according to regulatory guidelines. The LLOQ was 1.0 ng/mL for both compounds requiring 100 μL of sample. To our knowledge, this is the first validated LC/MS/MS method for the simultaneous quantitative analysis of cortisol and budesonide in human plasma. The method was applied successfully in a clinical investigation of the impact of nasally administered Pulmicort (budesonide) on the hypothalamic-pituitary-adrenal axis of patients with chronic rhinosinusitis.

1. Introduction

Hypothalamic-pituitary-adrenal (HPA) axis suppression is an indicator of adrenal dysfunction [1]. Primary adrenal insufficiency is associated with an autoimmune disease or an adenoma of the adrenal gland, whereas secondary adrenal insufficiency is generally linked to the long-term use of exogenous corticosteroids, such as budesonide (BUD) [2,3]. The severity of the suppression will depend on several variables, including the pharmacokinetics of the particular drug and the mode of delivery [4]. Symptoms may develop gradually and include hypoglycemia, hypotension, cardiovascular collapse, nausea, and diarrhea. Patients with a chronic inflammatory disease of the nose and paranasal sinuses known as chronic rhinosinusitis (CRS) have been successfully treated with nasally administered BUD. BUD has been demonstrated to have a better side-effect profile compared to other steroids; however, there is still a concern that if administered in sufficient amounts it could cause HPA-axis suppression [5].

Diagnosis of HPA axis suppression involves the administration of the adrenocorticotropic hormone (ACTH) adrenal stimulation test with the subsequent quantification and interpretation of plasma cortisol (CORT) levels [1]. Plasma CORT levels that are within or above the normal range of this test indicate that BUD does not impact the HPA axis. However, CORT levels that are consistently below the normal range indicate that sufficient BUD is being absorbed into the systemic circulation to cause HPA axis suppression [3].

The clinical investigation associated with the present analytical study sought to determine the impact of a novel intranasal steroid delivery device, the mucosal atomization device (MAD), (LMA North America, Inc., formerly Wolfe-Tory Inc.) on the systemic absorption of BUD. To achieve this goal, a sensitive and validated assay was required for the simultaneous quantification of CORT and BUD in human plasma.

There are several analytical methods available for the separate determination of CORT or BUD. CORT was determined in a variety of matrices using radioimmunoassay [6], liquid chromatography–mass spectrometry cubed [7], and liquid chromatography–tandem mass spectrometry (LC/MS/MS) [8–10]. Similarly, BUD was quantitated in a range of biological matrices

* Corresponding author. Tel.: +1 604 8273347; fax: +1 604 8223035.
E-mail address: szeitz@mail.ubc.ca (A. Szeitz).

0731-7085/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jpba.2013.12.006

using LC/MS/MS [11–13]. None of the above methods attempted the simultaneous analysis of CORT and BUD in the same sample.

Although, there have been several attempts in the literature to quantify both CORT and BUD in the same sample, those studies had their limitations. They have either used different methodologies or laboratories for these two analytes [14–16], were done in matrices other than human plasma [17,18] or have not been validated for both CORT and BUD [19].

In the present study, an ultra high performance liquid chromatography–tandem mass spectrometry (UHPLC/MS/MS) method is presented for the simultaneous determination of CORT and BUD in human plasma using charcoal stripped human plasma as the blank matrix. The assay was validated according to regulatory guidelines [20] and applied successfully to measure the CORT and BUD plasma levels of human volunteers who received ACTH stimulation followed by BUD treatment, 1 mg, twice a day for 60 days. To our knowledge, this is the first validated LC/MS/MS assay for the simultaneous quantitation of cortisol and budesonide in human plasma.

2. Experimental

2.1. Chemicals and standards

Hydrocortisone (≥98%), budesonide (≥99%), dexamethasone (≥98%), and ammonium formate (99.995 + % metals basis) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Methyl-tert-butyl ether, acetonitrile, and methanol (HPLC grade) were purchased from Fisher Scientific (Fair Lawn, NY, USA), formic acid (puriss. p.a. for mass spectroscopy) from Fluka (Steinheim, Germany), hydrochloric acid (1.0 M) from VWR (West Chester, PA, USA). Human plasma (2× charcoal stripped, K_2 EDTA-treated) was purchased from Bioreclamation, LLC (Westbury NY, USA). Ultra pure water was prepared in our laboratory using a Milli-Q Synthesis system (Millipore, Billerica, MA, USA). Cortrosyn® (cosyntropin) for injection, sterile lyophilized powder vials (containing 0.25 mg cosyntropin, 10 mg mannitol) were manufactured by Amphastar Pharmaceuticals, Inc., (Rancho Cucamonga, CA, USA), and the saline solution was manufactured by Hospira Canada (Montreal, Quebec, Canada). Pulmicort (budesonide) nebuamp capsules (0.5 mg budesonide in 2 mL saline) were manufactured by Astrazeneca (Wilmington, DE, USA).

2.2. BUD administration to human volunteers

Human volunteers with CRS were recruited from the St. Paul's Hospital Sinus Center in Vancouver, British Columbia, Canada, for a clinical safety and efficacy trial of BUD delivered via MAD. Ethics approval was obtained from the University of British Columbia Providence Health Care Research Ethics Board prior to the start of the study. Patient consent was obtained two months prior to starting the trial in order to ensure that patients currently on BUD treatment could undergo a four week 'washout period' consistent with previously published data on the pharmacokinetics and pharmacodynamics of BUD [16,21]. Patients were provided with a bailout dose of BUD in the event they required immediate therapy for an acute sinusitis flare-up during the washout period. Patients were seen three times over the 60-day trial. Prior to the start of BUD therapy, patients' plasma CORT was measured at the first visit in order to establish a baseline plasma CORT level. The ACTH stimulation test was then administered to rule out pre-existing adrenocortical insufficiency in the patient. The ACTH stimulation test is the standard to test for suspicion of adrenocortical insufficiency [1]. An intramuscular injection of cosyntropin 0.25 mg (synthetic ACTH) reconstituted in 1 mL of saline was administered followed by a post-stimulation blood sampling at 60 min [22]. A post-stimulation level of plasma CORT, which was above a threshold of 18 mg/dL or twice the baseline amount, was expected in healthy patients [23]. At the end of the first visit, patients were randomly divided into two equal groups, i.e., the experimental group (BUD via MAD) and control group (BUD via impregnated nasal saline irrigation). For the duration of the study, patients were administered 1 mg of BUD twice daily (2 mg/day) for 60 days. The ACTH stimulation test was administered at days 30 and 60. Blood samples were collected in a consistent manner at each visit, immediately processed for plasma, and stored at −80 °C until the analysis by UHPLC/MS/MS.

2.3. Instrumentation and experimental conditions

The UHPLC/MS/MS system consisted of an Agilent 1290 Infinity Binary Pump, a 1290 Infinity Sampler, a 1290 Infinity Thermostat, and a 1290 Infinity Thermostatted Column Compartment (Agilent, Mississauga, Ontario, Canada) connected to an AB Sciex QTrap® 5500 hybrid linear ion-trap triple quadrupole mass spectrometer equipped with a Turbo Spray source (AB Sciex, Concord, Ontario, Canada). The mass spectrometer was operated in positive ionization mode and data were acquired using the Analyst 1.5.2. software on a Microsoft Windows XP Professional operating platform.

Chromatographic separation was achieved using a Waters Acquity UPLC BEH C_{18}, 1.7 μm, 2.1 mm × 50 mm column maintained at 30 °C, and the autosampler tray temperature was maintained at 10 °C. Solvent A was water with ammonium formate (AF) (2.5 mM), solvent B was methanol with AF (2.5 mM). The mobile phase initial conditions were solvent A (40%) and solvent B (60%), which was ramped to solvent A (5%) by 2.0 min, held until 3.0 min and followed by an equilibration with solvent A (40%) and solvent B (60%) for 1 min. The flow rate was 0.2 mL/min, injection volume was 15 μL with a total run time of 4.0 min.

Mass spectrometric conditions were as follows: curtain gas 30 units, collision gas (CAD) high, ionspray 5500 V, temperature 450 °C, ion source gas 1, 40 units, ion source gas 2, 60 units. Nitrogen gas was used for curtain gas, collision gas, ion source gas 2 (vaporizing gas), and zero air was used for ion source gas 1 (nebulizing gas). Entrance potential 10 units, resolution Q1 low, resolution Q3 unit, and dwell time was 100 ms for all the compounds.

CORT and BUD were quantitated using the total ion current (TIC) of the multiple reaction monitoring (MRM) transitions as follows. For CORT (declustering potential DP, 80, collision cell exit potential CXP, 12), m/z 363.0 → 327.3 (collision energy CE, 23), m/z 363.0 → 267.0 (CE, 26), m/z 363.0 → 121.2 (CE, 34), for BUD (DP, 70, CXP, 18), m/z 431.0 → 323.2 (CE, 19), m/z 431.0 → 173.1 (CE, 38), m/z 431.0 → 147.1 (CE, 47). Dexamethasone (DEX) was monitored (DP, 70, CXP, 14) using the TIC of MRMs of m/z 393.0 → 373.4 (CE, 13), m/z 393.0 → 355.1 (CE, 17), and m/z 393.0 → 337.3 (CE, 19). To protect the mass spectrometer from contamination from the samples and to reduce the solvent load in the source, the mobile phase flow was diverted to the waste before 1.2 min and after 3.4 min during the chromatographic run.

2.4. Preparation of stock solutions

Two separate master stock solutions of CORT (100 μg/mL), and BUD (100 μg/mL) were prepared in methanol. The CORT and BUD master stock solutions were combined in equal parts into a mixed working stock solution of CORT and BUD (50 μg/mL, each). The mixed working stock solution was further diluted with methanol to yield a series of diluted working stock solutions. The series of diluted working stock solutions were used to prepare the calibration standards as described in Section 2.5. A 100 μg/mL DEX internal standard (IS) solution was also prepared in methanol and

diluted with methanol to yield the 50 ng/mL DEX working stock solution. The solutions were stored at −20 °C until analysis.

2.5. Preparation of calibration standards

During the current validation procedure, charcoal stripped human plasma was used as the validation blank matrix. Further on, charcoal stripped human plasma is referred to as human plasma. The series of diluted working stock solutions of CORT and BUD were used to prepare the calibration standards in human plasma. The calibration standards were prepared by pipetting 90 μL of blank human plasma into tubes, adding 200 μL of water and spiking 10 μL aliquots of appropriately diluted working stock solutions into the mixture. A volume of 50 μL of the 50 ng/mL IS solution was added and the samples were further processed as described in Section 2.6. Calibration curves were prepared freshly on the day of a batch analysis in the concentrations of 1.0, 2.5, 5.0, 10, 20, 50, 100, 500 ng/mL of CORT and BUD in human plasma. A blank and a zero sample (matrix blank containing IS) were also prepared.

2.6. Sample preparation

Plasma samples obtained from human volunteers were stored at −80 °C until analysis. On the day of sample analysis, the samples were thawed at room temperature (ca. 20 °C) and processed as follows. A volume of 100 μL of sample was pipetted into disposable borosilicate glass tubes and 200 μL of water was added. A volume of 50 μL of the 50 ng/mL IS solution was added to the mixture followed by the addition of 100 μL of 1 M HCl solution. The samples were vortex-mixed for at least 15 s, and 2.0 mL of methyl-tert-butyl ether was added. The samples were vortex-mixed for at least 45 s followed by placing them at −80 °C for at least 10 min. The tubes were removed from the −80 °C freezer and the top layers were transferred to a clean set of tubes. The organic layer was brought to dryness in a Zymark TurboVap LV sample evaporator (Zymark Corporation, Hopkinton, Mass, USA), under nitrogen, at ca. 35 °C, and the dried residues were reconstituted with 100 μL of water:methanol, 2:3 (v/v) mixture containing 2.5 mM AF. The samples were analyzed with UHPLC/MS/MS.

2.7. Preparation of quality control samples

Quality control (QC) samples were prepared as QC-Low (3.0 ng/mL), QC-Mid (80 ng/mL), and QC-High (400 ng/mL) samples in human plasma. A volume of 5 mL of QC samples at each concentration level was prepared by spiking the appropriately diluted mixed working stock solutions of CORT and BUD, prepared in Section 2.4, into blank human plasma. The QC samples were dispensed in equal aliquots (approx. 130 μL) into vials and stored at −80 °C until use. For each batch analysis, fresh aliquots of QC-Low, QC-Mid, and QC-High samples were thawed, 100 μL analyzed, and the rest discarded.

The method was validated for accuracy, precision, linearity, range, selectivity, lower limit of quantification (LLOQ), recovery, matrix effect, dilution integrity, and evaluation of carry-over in human plasma using 100 μL of samples.

2.8. Method validation

2.8.1. Accuracy

Six replicate spiked samples of QC-Low, QC-Mid, and QC-High were prepared in human plasma, and analyzed. Accuracy was expressed as the percentage deviation of the measured CORT and BUD concentration against the added concentration, according to the following formula: %Deviation = [(measured amount/added amount) × 100] − 100 with negative %Deviation representing under-estimation, and positive %Deviation representing over-estimation of the true value. The acceptance criterion for accuracy was %Deviation ± 15% for the QC-Low, QC-Mid, and QC-High samples. For intra-day accuracy, six replicates of QC-Low, QC-Mid, and QC-High samples were prepared and analyzed on the same day. Inter-day accuracy was determined by repeating intra-day accuracy experiments for three separate days, and using the combined results of the three intra-day accuracy experiments, the inter-day accuracy was calculated.

2.8.2. Precision

A single spiked sample for QC-Low, QC-Mid, and QC-High (5 mL each) was prepared in human plasma. Six aliquots (100 μL each) were removed from each of the QC-Low, QC-Mid, and QC-High samples and analyzed. The relative standard deviation (%RSD) of the CORT and BUD concentrations measured in each QC sample were calculated. The acceptance criterion for precision was %RSD ≤ 15% for the QC-Low, QC-Mid, and QC-High samples. For intra-day precision, six aliquots were removed from each of the QC-Low, QC-Mid, and QC-High spiked samples and analyzed on the same day. Inter-day precision was determined by repeating intra-day precision experiments for three separate days, and using the combined results of the three intra-day precision experiments, the inter-day precision was calculated.

2.8.3. Linearity and range

Calibration curves were prepared for each batch analysis in the following concentrations: 1.0, 2.5, 5.0, 10, 20, 50, 100, 500 ng/mL in human plasma. Calibration curves were constructed by plotting the concentrations of CORT and BUD on the x-axis, vs. the chromatographic peak area ratio of CORT and BUD to IS on the y-axis. Linear regression analyses were performed using the calibration curve data. Using the $y = mx + b$ equation, the y-intercept (b), slope (m) and correlation coefficient (r) were calculated. The calibration curves were weighted using the weighting factor of $1/x^2$. The acceptance criterion for linearity was the coefficient of determination $r^2 \geq 0.99$ for CORT and BUD after weighting with $1/x^2$. CORT and BUD concentrations were calculated by the Analyst 1.5.2. software using the following formula: $x = (y - b)/m$, where y = CORT and BUD to IS peak area ratio, b = weighted y-intercept, m = weighted slope. The range of the assay was established as the section of the calibration curve where the curve was linear, i.e., $r^2 \geq 0.99$, the calibration levels were accurate (%Deviation ± 15%) and precise (%RSD ≤ 15%).

2.8.4. Selectivity

Selectivity was determined in human plasma samples spiked at the LLOQ level. Triplicate samples were prepared. Blank human plasma samples without CORT, BUD and DEX were also prepared. The blank samples were visually compared to the LLOQ samples for any significant interference at the retention times of CORT, BUD and DEX. The acceptance criteria for selectivity were that the mean response, i.e., signal-to-noise ratio (S/N) of CORT and BUD in the LLOQ samples were at least 5-times the response compared to the blank samples, and there was no significant matrix interference at the retention times of CORT, BUD and DEX in the blank samples compared with the LLOQ samples.

2.8.5. LLOQ

To determine LLOQ, six replicates of the 1.0 ng/mL calibration standard were prepared in human plasma and analyzed. The mean response (S/N), accuracy and precision were determined from the samples. The LLOQ was determined as the lowest concentration level of the calibration curve which met the following acceptance criteria. The mean response of CORT and BUD peaks in the samples were at least 5-times the response compared to the blank sample (S/N was calculated using Analyst software). The CORT and

BUD peaks were identifiable, discrete, and reproducible, with an accuracy (%Deviation) of ±20% and precision (%RSD) ≤20%.

2.8.6. Recovery

Samples of QC-Low, QC-Mid, and QC-High were prepared in human plasma and analyzed. The CORT and BUD concentrations of the extracted samples were compared to the CORT and BUD concentrations of the unextracted standards of the same concentration. Four determinations per concentration were performed and recovery was calculated according to the following formula:

$$\%\text{Recovery} = \left(\frac{\text{extracted QC plasma concentrations}}{\text{unextracted standard concentrations}}\right) \times 100.$$

2.8.7. Matrix effect

Matrix effect, which may cause ionization suppression or enhancement of the analytes, was determined in four replicate samples. Samples of QC-Low, QC-Mid, and QC-High were prepared in human plasma and in water and analyzed. The CORT and BUD concentrations of the plasma samples were compared to the concentrations of samples prepared in water. Matrix effect was calculated according to the following formula: %Matrix effect = [(QC plasma concentration − QC concentration in water)/QC concentration in water] × 100. Matrix effect was considered acceptable if no more than 15% difference in the concentrations of CORT and BUD was observed in the human plasma samples compared to the samples prepared in water. Negative %Matrix effect represented ionization suppression, and positive %Matrix effect represented ionization enhancement.

2.8.8. Dilution-integrity

Six aliquots of QC-High samples were diluted 10-fold with blank human plasma and analyzed. The acceptance criteria for CORT and BUD were the accuracy (%Deviation) ± 15% from the actual value (40 ng/mL), and precision (%RSD) ≤ 15% from the six determinations.

2.8.9. Evaluation of carry-over

An aliquot of a QC-High sample was prepared and three injections were made, immediately followed by three injections of a blank human plasma sample. Carry-over was expressed as the percentage difference between the mean CORT, BUD or DEX peak area counts in blank human plasma samples and the mean CORT and BUD or DEX peak area count in the QC-High sample. Carry-over was considered acceptable if the mean peak area counts of CORT, BUD and DEX were not more than 20% for CORT and BUD, and 5% for DEX, compared to the area counts in the LLOQ sample [24].

3. Results and discussion

The objective of this study was to develop and validate a sensitive and selective UHPLC/MS/MS method for the simultaneous quantification of CORT and BUD in human plasma. This method was needed to measure the CORT and BUD plasma levels of patients with CRS who were placed on BUD treatment and were receiving the ACTH stimulation test.

3.1. Mass spectrometry/liquid chromatography

To monitor CORT, BUD, and DEX with mass spectrometry, the molecular ions (i.e., precursor ions) and their fragments (i.e., product ions) were determined. Literature shows that the molecular ions of these compounds depend on the additive used in the mobile phase [17]. In the present study, using AF (2.5 mM) in the mobile phase and Turbo Spray, positive ionization mode, an intense signal of the positively charged molecular ions of CORT, BUD, and DEX were observed. The molecular ions were determined by the direct infusion of about 10 μg/mL solutions in water:methanol, 1:1 (v/v) containing AF (2.5 mM) into the mass spectrometer. The molecular ions were for CORT m/z 363.0, for BUD m/z 431.0, and for DEX m/z 393.0. The product ions were determined by fragmenting the precursor ions, and the origin of the product ions, which were selected for quantification, was confirmed by performing precursor ion scans. Representative product ion mass spectra with the proposed fragmentation pattern of CORT, BUD, and DEX are presented in Fig. 1. The mass spectrometric parameters were optimized to achieve the most abundant MRM response for each compound, and CORT, BUD, and DEX were monitored using the TIC of their corresponding MRM signals. According to literature data, similar MRM transitions were used for the detection of CORT [9,10,15], BUD [12,13,15], and DEX [13,15]. The Waters Acquity UPLC BEH C_{18}, 1.7 μm, 2.1 mm × 50 mm column, the mobile phase composition and gradient programming used in this study provided a fast analysis with a run time of 4.0 min. Baseline separation between CORT and BUD, and near baseline separation between CORT and DEX were achieved. The selective detection characteristics of MRM eliminated any possible interference between the channels of CORT and DEX during sample analysis. Using the current experimental conditions the retention times were for CORT 1.73 min, for BUD 2.64 min, and for DEX 1.98 min. A representative chromatogram of a QC-Mid sample prepared in charcoal stripped human plasma is presented in Fig. 2.

3.2. Sample preparation

Charcoal stripped human plasma was used as the validation matrix to avoid any potential interference from the endogenous CORT content of the neat blank human plasma. Charcoal stripped human plasma has been previously used as the blank matrix to quantitate CORT, and other corticosteroids, in human plasma, using HPLC/UV [25], and LC/MS/MS techniques [9,26], and these methods were validated [25] according to FDA guidelines [9,26].

3.3. Method validation

3.3.1. Accuracy and precision

The results for accuracy and precision are presented in Table 1. Intra-day accuracy for CORT ranged between −6.00% and 2.67%, and for BUD between −6.33% and −0.056%. Inter-day accuracy for CORT ranged between −1.85% and 0.660%, and for BUD between −5.44% and −1.22%. Intra-day precision for CORT ranged between 1.45% and 7.98%, and for BUD between 1.67% and 8.05%. Inter-day precision for CORT ranged between 5.45% and 6.46%, and for BUD between 4.45% and 6.48%. The method met the acceptance criteria for accuracy of %Deviation ± 15% for the QC-Low, QC-Mid, and QC-High samples, and for precision of %RSD ≤ 15% for QC-Low, QC-Mid, and QC-High samples. This indicated that the method was accurate and precise over the range of the assay.

3.3.2. Linearity and range

Linearity of the calibration curve was evaluated in four batches over the course of the validation and the results are presented in Table 2. The accuracy (%Deviation) of the calibration curve levels for CORT ranged between −2.79% and 5.10%, and for BUD between −3.44% and 3.21%. The precision (%RSD) of the calibration curve levels for CORT ranged between 1.13% and 7.43%, and for BUD between 0.755% and 5.65%. The coefficient of determination (mean ± SD, $n = 4$) for CORT was $r^2 = 0.997 \pm 0.002$, and for BUD $r^2 = 0.998 \pm 0.002$. The calibration curve met the acceptance criterion for linearity of $r^2 \geq 0.99$ after weighting with $1/x^2$. The range of the method was established as 1.0 ng/mL to 500 ng/mL where the calibration levels met the acceptance criteria of accuracy

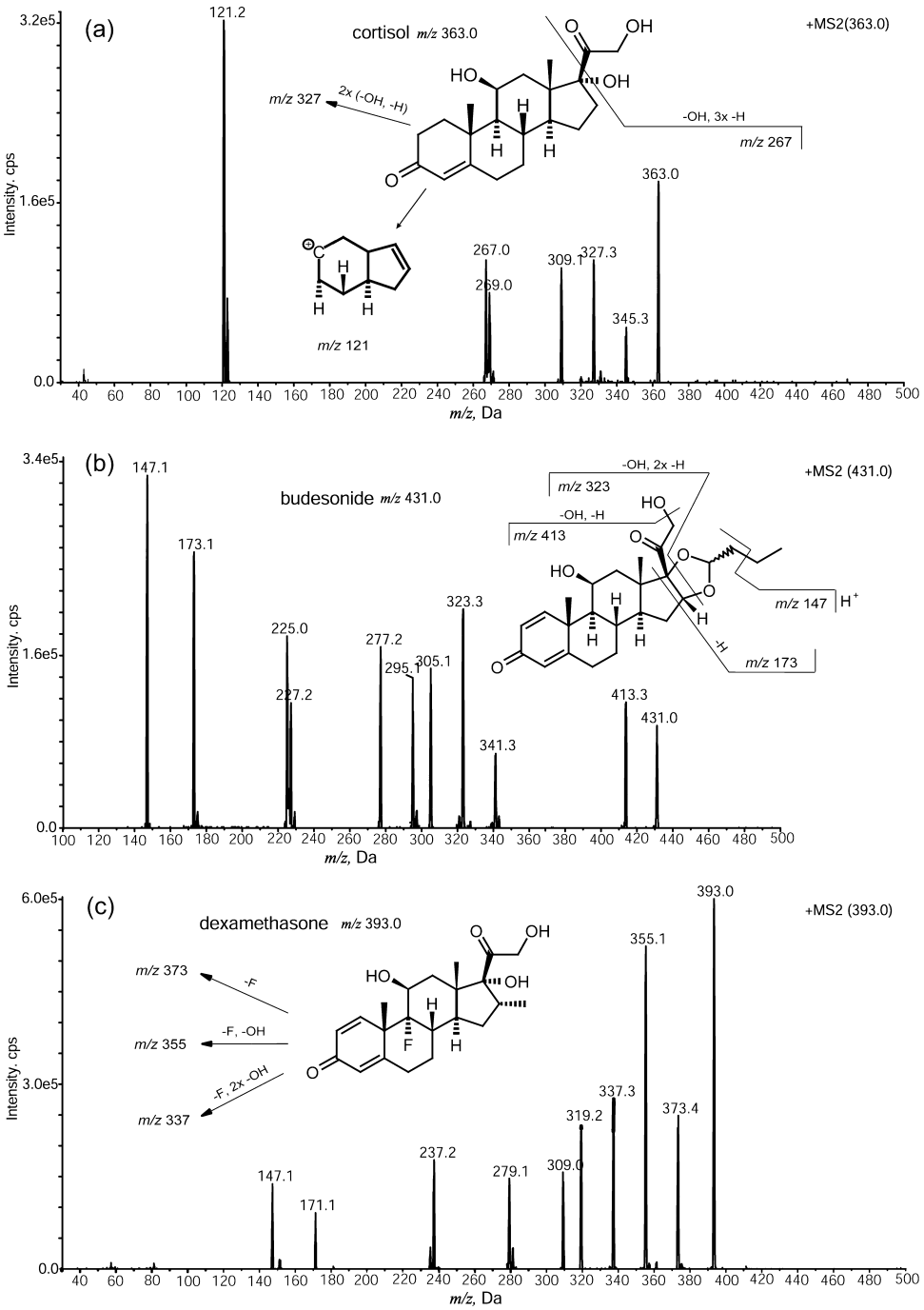

Fig. 1. Representative product ion mass spectra and proposed fragmentation pattern of (a) cortisol, (b) budesonide, and (c) dexamethasone obtained with Turbo Spray, positive ionization mode. The collision energy (CE) values were for cortisol, CE 30, for budesonide, CE 28, and for dexamethasone, CE 20.

(%Deviation) ± 15%, and precision (%RSD) ≤ 15%. The results indicated that the calibration curve was linear, accurate, and precise over the range of the method.

3.3.3. Selectivity and LLOQ

The selectivity met the acceptance criteria of S/N 5-times the response compared to the blank sample for CORT and BUD, and there was no significant interference at the retention times of CORT and BUD and DEX when the blank human plasma samples were compared to the LLOQ samples. The results indicated that the method was selective for these analytes. The LLOQ was determined in six replicate samples of the 1.0 ng/mL calibration standard. The S/N (mean ± SD, $n = 6$) for CORT was 30.9 ± 8.06, and for BUD 25.5 ± 6.94. The accuracy (%Deviation, $n = 6$) for CORT ranged between −5.44% and 1.31% and for BUD between −12.3% and 0.293%. The precision (%RSD, $n = 6$) for CORT was 2.37% and for BUD 5.64%. Results for the determination of LLOQ met the acceptance criteria of S/N 5-times the response compared to the blank sample, with an accuracy (%Deviation) ± 20% and precision %RSD ≤ 20%, and the CORT and BUD peaks were identifiable, discrete, and reproducible. The method was accurate and precise at the established LLOQ of 1.0 ng/mL of CORT and BUD requiring 100 μL of sample. Representative chromatograms of selectivity and LLOQ (1.0 ng/mL) in human plasma samples are presented in Fig. 3. Several CORT and BUD concentrations were evaluated for LLOQ in human plasma. CORT and BUD levels of 0.05, 0.1, 0.25, 0.5 ng/mL,

Table 1
Accuracy (intra- and inter-day) and precision (intra- and inter-day) for cortisol and budesonide.

Accuracy	Cortisol				Accuracy	Budesonide			
	Intra-day			Inter-day		Intra-day			Inter-day
	Day 1 ($n=6$)	Day 2 ($n=6$)	Day 3 ($n=6$)	Days 1–3 ($n=18$)		Day 1 ($n=6$)	Day 2 ($n=6$)	Day 3 ($n=6$)	Days 1–3 ($n=18$)
QC-Low (3.0 ng/mL)					**QC-Low** (3.0 ng/mL)				
Mean (ng/mL)	2.96	2.82	3.06	2.94	Mean (ng/mL)	2.92	3.00	2.97	2.96
SD[a] (ng/mL)	0.145	0.086	0.085	0.143	SD[a] (ng/mL)	0.194	0.171	0.149	0.165
%Deviation[b]	**−1.44**	**−6.00**	**1.89**	**−1.85**	%Deviation[b]	**−2.56**	**−0.056**	**−1.06**	**−1.22**
QC-Mid (80 ng/mL)					**QC-Mid** (80 ng/mL)				
Mean (ng/mL)	81.8	77.7	82.1	80.5	Mean (ng/mL)	78.3	76.9	77.4	77.5
SD[a] (ng/mL)	3.21	1.48	4.35	3.69	SD[a] (ng/mL)	3.74	4.26	1.82	3.28
%Deviation[b]	**2.25**	**−2.94**	**2.67**	**0.660**	%Deviation[b]	**−2.19**	**−3.83**	**−3.25**	**−3.09**
QC-High (400 ng/mL)					**QC-High** (400 ng/mL)				
Mean (ng/mL)	397	380	407	395	Mean (ng/mL)	378	382	375	378
SD[a] (ng/mL)	9.58	7.47	18.0	16.4	SD[a] (ng/mL)	16.6	15.5	8.12	13.4
%Deviation[b]	**−0.667**	**−5.04**	**1.63**	**−1.36**	%Deviation[b]	**−5.54**	**−4.46**	**−6.33**	**−5.44**
Precision	Cortisol				Precision	Budesonide			
	Intra-day			Inter-day		Intra-day			Inter-day
	Day 1 ($n=6$)	Day 2 ($n=6$)	Day 3 ($n=5$)	Days 1–3 ($n=17$)		Day 1 ($n=6$)	Day 2 ($n=6$)	Day 3 ($n=5$)	Days 1–3 ($n=17$)
QC-Low (3.0 ng/mL)					**QC-Low** (3.0 ng/mL)				
Mean (ng/mL)	2.97	2.86	2.80	2.88	Mean (ng/mL)	2.80	2.99	2.97	2.92
SD[a] (ng/mL)	0.237	0.041	0.102	0.160	SD[a] (ng/mL)	0.102	0.157	0.239	0.182
%RSD[c]	**7.98**	**1.45**	**3.64**	**5.57**	%RSD[c]	**3.64**	**5.25**	**8.05**	**6.25**
QC-Mid (80 ng/mL)					**QC-Mid** (80 ng/mL)				
Mean (ng/mL)	70.4	74.4	80.5	75.1	Mean (ng/mL)	75.0	76.8	71.7	74.5
SD[a] (ng/mL)	2.52	2.60	2.13	4.85	SD[a] (ng/mL)	2.25	3.91	1.20	3.31
%RSD[c]	**3.58**	**3.49**	**2.64**	**6.46**	%RSD[c]	**2.99**	**5.10**	**1.67**	**4.45**
QC-High (400 ng/mL)					**QC-High** (400 ng/mL)				
Mean (ng/mL)	381	380	402	388	Mean (ng/mL)	396	392	363	384
SD[a] (ng/mL)	9.24	22.5	23.8	21.1	SD[a] (ng/mL)	16.2	26.1	19.1	24.9
%RSD[c]	**2.42**	**5.92**	**5.92**	**5.45**	%RSD[c]	**4.11**	**6.65**	**5.26**	**6.48**

[a] Standard deviation.
[b] $\left[\left(\frac{\text{measured amount}}{\text{added amount}}\right)\times 100\right]-100\,(\%)$.
[c] Relative standard deviation (%).

were tested and found that in the 0.05, and 0.1 ng/mL samples, the S/N values were less than 3 for CORT and BUD, therefore, these results were rejected. For the 0.25 and 0.5 ng/mL samples, the S/N values were higher than 5, but the results had high variance and failed the acceptance criteria of accuracy and precision set by the FDA [20]. When 1.0 ng/mL CORT and BUD were analyzed, the results met these acceptance criteria, consistently, therefore, 1.0 ng/mL was accepted as the LLOQ for CORT and BUD. The limit of detection was 0.25 ng/mL, with S/N 6.6 for CORT, and 0.25 ng/mL with S/N 5.2 for BUD.

3.3.4. Recovery and matrix effect

The results of the %Recovery and %Matrix effect are presented in Table 3. The mean %Recovery values ($n=4$) for CORT for QC-Low, QC-Mid, and QC-High were 90.0%, 103% and 106%, respectively, and for BUD 93.1%, 89.9% and 86.5%, respectively. The results indicated that CORT and BUD were extracted relatively uniformly over the concentration range of the assay. The mean %Matrix effect values ($n=4$) for CORT for QC-Low, QC-Mid, and QC-High were −7.99%, −0.470% and −0.783%, respectively, and for BUD −10.4%, −12.1%, and −13.1%, respectively. The results met the acceptance criterion

Table 2
Linearity and range of cortisol and budesonide calibration standards.

Cortisol					Budesonide			
Nominal concentration (ng/mL)	Measured concentration (ng/mL)	Accuracy (%Deviation)[a]	SD[b] (ng/mL)	Precision (%RSD)[c]	Measured concentration (ng/mL)	Accuracy (%Deviation)[a]	SD[b] (ng/mL)	Precision (%RSD)[c]
1.0	0.995	−0.502	0.011	1.13	1.00	−0.002	0.008	0.755
2.5	2.51	0.464	0.073	2.91	2.49	−0.497	0.141	5.65
5.0	5.15	2.89	0.382	7.43	5.01	0.187	0.267	5.34
10.0	9.83	−1.70	0.235	2.39	10.3	3.21	0.395	3.82
20	19.6	−2.21	0.640	3.27	19.3	−3.44	0.432	2.24
50	49.4	−1.25	3.58	7.26	49.8	−0.418	1.70	3.41
100	105	5.10	2.16	2.06	103	2.55	2.38	2.32
500	486	−2.79	5.91	1.22	492	−1.59	21.4	4.35

[a] $\left[\left(\frac{\text{measured amount}}{\text{added amount}}\right)\times 100\right]-100\,(\%)$.
[b] Standard deviation.
[c] Relative standard deviation (%).
The coefficient of determination (r^2) was for cortisol 0.997 ± 0.002 and for budesonide 0.998 ± 0.002, (mean ± SD, $n=4$).

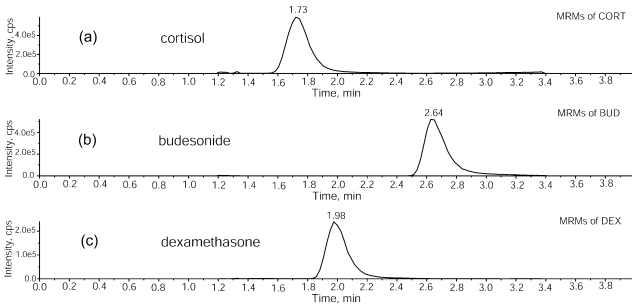

Fig. 2. A representative chromatogram of a QC-Mid (80 ng/mL) sample prepared in charcoal stripped human plasma. The retention times were (a) cortisol 1.73 min, (b) budesonide 2.64 min, and (c) dexamethasone 1.98 min. The signals for the compounds represent the TIC of the MRM channels.

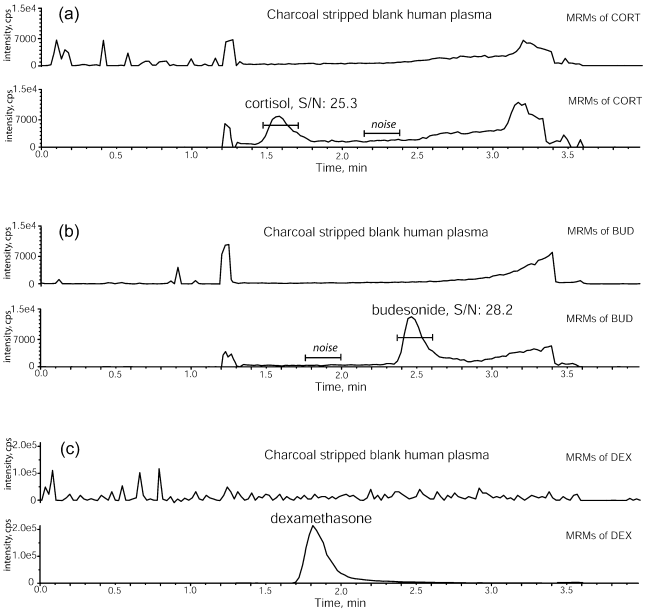

Fig. 3. Selectivity and lower limit of quantification (LLOQ, 1.0 ng/mL) in charcoal stripped human plasma. (a) TIC of the MRM signals of cortisol in blank human plasma (top), and in the LLOQ sample (bottom); (b) TIC of the MRM signals of budesonide in blank human plasma (top), and in the LLOQ sample (bottom) and (c) TIC of the MRM signals of dexamethasone in blank human plasma (top), and in the LLOQ sample (bottom). The signal-to-noise (S/N) values are reported on the top of the cortisol and budesonide peaks.

Table 3
Recovery and matrix effect of cortisol and budesonide.

	%Recovery		%Matrix effect	
	Cortisol	Budesonide	Cortisol	Budesonide
QC-Low (3.00 ng/mL)				
Mean (%)	90.0	93.1	−7.99	−10.4
SD[a] (%)	5.22	7.28	1.56	5.31
$n = 4$				
QC-Mid (80 ng/mL)				
Mean (%)	103	89.9	−0.470	−12.1
SD[a] (%)	6.84	6.06	4.00	0.330
$n = 4$				
QC-High (400 ng/mL)				
Mean (%)	106	86.5	−0.783	−13.1
SD[a] (%)	6.39	7.58	5.71	4.35
$n = 4$				

[a] Standard deviation.

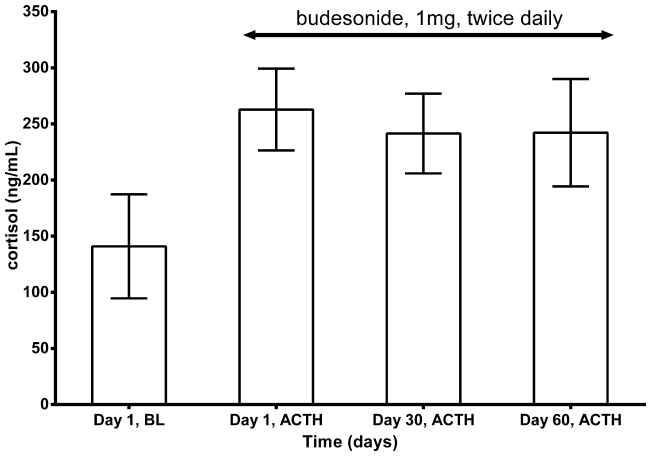

Fig. 4. Representative plot of cortisol concentrations vs. time obtained from five volunteers following adrenocorticotropic hormone (ACTH) stimulation (day 1, 30, 60, ACTH), and budesonide administration, intranasally (1 mg, twice a day, for 60 days). Budesonide levels remained below LLOQ (1.0 ng/mL), consistently, during the study period. Data are presented as mean ± SD, BL = baseline.

of %Matrix effect of no more than 15%. This indicated that the matrix effect from charcoal stripped human plasma contributing to the ionization suppression or enhancement of CORT and BUD remained within the acceptable range.

3.3.5. *Dilution integrity and evaluation of carry-over*

The results of the dilution integrity are presented in Table 4. After the 10-fold dilution of the six aliquots of the QC-High samples, the accuracy (%Deviation, $n = 6$) for CORT was −2.69%, and for BUD −9.93%, and the precision (%RSD, $n = 6$) for CORT was 4.35% and for BUD 3.15%. The results met the acceptance criteria of accuracy (%Deviation) ± 15% from the actual value (40 ng/mL), and precision (%RSD) ≤ 15%. This indicated that samples exceeding the calibration curve concentrations could be diluted 10-fold to bring them into the range of the assay with acceptable accuracy and precision. Carry-over was evaluated by three injections of a QC-High sample, immediately followed by three injections of a blank human plasma sample. The carry-over for BUD was 13.0% of the peak area counts observed in the LLOQ sample, and there was no detectable carry-over for CORT and DEX. This met the acceptance criteria for carry-over of no more than 20% peak area counts for CORT and BUD, and 5% for DEX, compared to the LLOQ sample.

3.3.6. *CORT and BUD levels in human volunteers*

The MAD is a pump-driven spray used to deliver concentrated topical anti-inflammatory medication to the deepest recesses of the paranasal sinuses for the treatment of CRS [27]. Alternatively, corticosteroids can be administered in a saline suspension irrigated through the nostrils, which is the standard care for many clinicians [1,28]. The MAD is believed to be more effective than irrigation [27]; however, the safety of the MAD needs to be assessed before it can be adopted in the standard care.

The objective of the clinical study was to investigate the difference between the plasma levels of BUD delivered by nasal irrigation vs. MAD, and whether BUD via MAD causes suppression of CORT levels which may be due to adrenal insufficiency. A representative plot of CORT concentrations vs. time obtained from five volunteers is presented in Fig. 4. Plasma CORT levels were as follows (mean ± SD, $n = 5$). Baseline level prior to ACTH stimulation, day 1, 141 ± 46.3 ng/mL, following ACTH stimulation, day 1, 263 ± 36.4 ng/mL, day 30, 241 ± 35.5 ng/mL, and day 60, 242 ± 47.8 ng/mL. The five volunteers received BUD administered intranasally (1 mg, twice a day, for 60 days), and their BUD levels

Table 4
Dilution integrity of cortisol and budesonide.

Diluted QC-High sample (ng/mL)[a]	Analyte	Measured concentration (ng/mL)	SD[b] (ng/mL)	Accuracy (%Deviation)[c]	Precision (%RSD)[d]
40.0	Cortisol	38.9	1.69	−2.69	4.35
40.0	Budesonide	36.1	1.14	−9.93	3.15

[a] QC-High (400 ng/mL) samples were diluted 10-fold to yield 40 ng/mL concentrations ($n = 6$).
[b] Standard deviation.
[c] $\left[\left(\frac{\text{measured amount}}{\text{added amount}}\right) \times 100\right] - 100\,(\%)$.
[d] Relative standard deviation (%).

remained below LLOQ (1.0 ng/mL), consistently. For CORT, similar results were reported in the literature [1,29]. In a 30 day trial of the safety of BUD administered via impregnated nasal saline irrigation (0.5 mg/day), the mean CORT levels were 33.9 μg/dL (95% confidence interval, 30.5–37.3 μg/dL) following ACTH stimulation [1]. A limited number of studies are available on the BUD plasma levels following nasal administration [30]. In one example, BUD was cleared from plasma 6 h after the nasal administration of 400–800 μg of BUD [30], but no study has been reported on the doses of BUD via a pump-driven spray, which resulted in BUD plasma levels, which were high enough to be clinically relevant for the treatment of severe CRS. In the study, there was an 8-h interval between the last BUD dose (1 mg) and the blood sampling, which may explain why most of the BUD was cleared from the systemic circulation during this time period. This resulted in the BUD plasma concentrations below LLOQ (i.e., 1 ng/mL), consistently.

4. Conclusions

A sensitive and selective UHPLC/MS/MS method was developed and validated for the quantification of trace levels CORT and BUD in charcoal stripped human plasma. The validation was performed according to regulatory guidelines [20]. The assay was applied for the measurement of CORT and BUD levels in plasma samples of human volunteers; however, the BUD were consistently below the LLOQ (1 ng/mL) in the samples. To our knowledge, this is the first validated LC/MS/MS method for the simultaneous quantification of CORT and BUD in human plasma using charcoal stripped blank human plasma as the validation matrix.

Acknowledgements

This work was supported by the St. Paul's Hospital Foundation, Vancouver, BC, Canada; reference 01866152.

The authors wish to thank Dr. Frank S. Abbott, Professor and Dean Emeritus, Faculty of Pharmaceutical Sciences, The University of British Columbia, Vancouver, BC, for his comments.

References

[1] N.S. Sachanandani, J.F. Piccirillo, M.A. Kramper, S.E. Thawley, A. Vlahiotis, The effect of nasally administered budesonide respules on adrenal cortex function in patients with chronic rhinosinusitis, Arch. Otolaryngol. Head Neck Surg. 135 (2009) 303–307.
[2] D.M. Poetker, D.D. Reh, A comprehensive review of the adverse effects of systemic corticosteroids, Otolaryngol. Clin. N. Am. 43 (2010) 753–768.
[3] S.J. Szefler, Pharmacodynamics and pharmacokinetics of budesonide: a new nebulized corticosteroid, J. Allergy Clin. Immunol. 104 (1999) 175–183.
[4] G. Passalacqua, M. Albano, G.W. Canonica, C. Bachert, P. Van Cauwenberge, R.J. Davies, S.R. Durham, K. Kontou-Fili, F. Horak, H.J. Malling, Inhaled and nasal corticosteroids: safety aspects, Allergy 55 (2000) 16–33.
[5] D.J. Clark, A. Grove, R.I. Cargill, B.J. Lipworth, Comparative adrenal suppression with inhaled budesonide and fluticasone propionate in adult asthmatic patients, Thorax 51 (1996) 262–266.
[6] C. Moller, H. Ahlstrom, K.A. Henricson, L.A. Malmqvist, A. Akerlund, H. Hildebrand, Safety of nasal budesonide in the long-term treatment of children with perennial rhinitis, Clin. Exp. Allergy 33 (2003) 816–822.
[7] F. Arioli, M. Fidani, A. Casati, M.L. Fracchiolla, G. Pompa, Investigation on possible transformations of cortisol, cortisone and cortisol glucuronide in bovine faecal matter using liquid chromatography–mass spectrometry, Steroids 75 (2010) 350–354.
[8] P.J. Taylor, S.P. van Rosendal, J.S. Coombes, R.D. Gordon, M. Stowasser, Simultaneous measurement of aldosterone and cortisol by high-performance liquid chromatography–tandem mass spectrometry: application to dehydration–rehydration studies, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 878 (2010) 1195–1198.
[9] I.A. Ionita, D.M. Fast, F. Akhlaghi, Development of a sensitive and selective method for the quantitative analysis of cortisol, cortisone, prednisolone and prednisone in human plasma, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 877 (2009) 765–772.
[10] A. Cuzzola, A. Petri, F. Mazzini, P. Salvadori, Application of hyphenated mass spectrometry techniques for the analysis of urinary free glucocorticoids, Rapid Commun. Mass Spectrom. 23 (2009) 2975–2982.
[11] Y. Wang, Y. Tang, H. Moellmann, G. Hochhaus, Simultaneous quantification of budesonide and its two metabolites, 6beta-hydroxybudesonide and 16alpha-hydroxyprednisolone, in human plasma by liquid chromatography negative electrospray ionization tandem mass spectrometry, Biomed. Chromatogr. 17 (2003) 158–164.
[12] K. Deventer, P. Mikulcikova, H. Van Hoecke, P. Van Eenoo, F.T. Delbeke, Detection of budesonide in human urine after inhalation by liquid chromatography–mass spectrometry, J. Pharm. Biomed. Anal. 42 (2006) 474–479.
[13] J. Qu, Y. Qu, R.M. Straubinger, Ultra-sensitive quantification of corticosteroids in plasma samples using selective solid-phase extraction and reversed-phase capillary high-performance liquid chromatography/tandem mass spectrometry, Anal. Chem. 79 (2007) 3786–3793.
[14] J. Algorta, M.A. Pena, S. Francisco, Z. Abajo, E. Sanz, Randomised, crossover clinical trial, in healthy volunteers, to compare the systemic availability of two topical intranasal budesonide formulations, Trials 9 (2008) 34.
[15] K. Raaska, M. Niemi, M. Neuvonen, P.J. Neuvonen, K.T. Kivisto, Plasma concentrations of inhaled budesonide and its effects on plasma cortisol are increased by the cytochrome P4503A4 inhibitor itraconazole, Clin. Pharmacol. Ther. 72 (2002) 362–369.
[16] K.L. Clearie, P.A. Williamson, K. Meldrum, M. Gillen, L.G. Carlsson, M. Carlholm, J. Ekelund, B.J. Lipworth, Pharmacokinetic and pharmacodynamic comparison of hydrofluoroalkane and chlorofluorocarbon formulations of budesonide, Br. J. Clin. Pharmacol. 71 (2011) 504–513.
[17] X. Cui, B. Shao, R. Zhao, Y. Yang, J. Hu, X. Tu, Simultaneous determination of seventeen glucocorticoids residues in milk and eggs by ultra-performance liquid chromatography/electrospray tandem mass spectrometry, Rapid Commun. Mass Spectrom. 20 (2006) 2355–2364.
[18] B. Shao, X. Cui, Y. Yang, J. Zhang, Y. Wu, Validation of a solid-phase extraction and ultra-performance liquid chromatographic tandem mass spectrometric method for the detection of 16 glucocorticoids in pig tissues, J. AOAC Int. 92 (2009) 604–611.
[19] R.L. Taylor, S.K. Grebe, R.J. Singh, Quantitative, highly sensitive liquid chromatography–tandem mass spectrometry method for detection of synthetic corticosteroids, Clin. Chem. 50 (2004) 2345–2352.
[20] FDA, Guidance for Industry, Bioanalytical Method Validation, Rockville, 2001 (accessed 20.11.13) http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidance/ucm070107.pdf
[21] K. Phillips, J. Oborne, S. Lewis, T.W. Harrison, A.E. Tattersfield, Time course of action of two inhaled corticosteroids, fluticasone propionate and budesonide, Thorax 59 (2004) 26–30.
[22] R.K. Cydulka, C.L. Emerman, Adrenal function and physiologic stress during acute asthma exacerbation, Ann. Emerg. Med. 31 (1998) 558–561.
[23] R.I. Dorin, C.R. Qualls, L.M. Crapo, Diagnosis of adrenal insufficiency, Ann. Intern. Med. 139 (2003) 194–204.
[24] S.Q. Zhang, Y.M. Fan, Determination of andrographolide sodium bisulphite in Beagle dog plasma by LC–MS/MS and its application to pharmacokinetics, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 907 (2012) 173–177.
[25] W.J. Jusko, N.A. Pyszczynski, M.S. Bushway, R. D'Ambrosio, S.M. Mis, Fifteen years of operation of a high-performance liquid chromatographic assay for prednisolone, cortisol and prednisone in plasma, J. Chromatogr. B: Biomed. Appl. 658 (1994) 47–54.
[26] R. Difrancesco, V. Frerichs, J. Donnelly, C. Hagler, J. Hochreiter, K.M. Tornatore, Simultaneous determination of cortisol, dexamethasone, methylprednisolone, prednisone, prednisolone, mycophenolic acid and mycophenolic acid glucuronide in human plasma utilizing liquid chromatography–tandem mass

spectrometry, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 859 (2007) 42–51.
[27] S.J. Kanowitz, P.S. Batra, M.J. Citardi, Topical budesonide via mucosal atomization device in refractory postoperative chronic rhinosinusitis, Otolaryngol. Head Neck Surg. 139 (2008) 131–136.
[28] L. Rudmik, Z.M. Soler, R.R. Orlandi, M.G. Stewart, N. Bhattacharyya, D.W. Kennedy, T.L. Smith, Early postoperative care following endoscopic sinus surgery: an evidence-based review with recommendations, Int. Forum Allergy Rhinol. 1 (2011) 417–430.
[29] R.K. Bhalla, K. Payton, E.D. Wright, Safety of budesonide in saline sinonasal irrigations in the management of chronic rhinosinusitis with polyposis: lack of significant adrenal suppression, J. Otolaryngol. Head Neck Surg. 37 (2008) 821–825.
[30] L. Thorsson, O. Borga, S. Edsbacker, Systemic availability of budesonide after nasal administration of three different formulations: pressurized aerosol, aqueous pump spray, and powder, Br. J. Clin. Pharmacol. 47 (1999) 619–624.