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Simultaneous quantification of beclomethasone dipropionate and its metabolite, beclomethasone 17-monopropionate in rat and human plasma and different rat tissues by liquid chromatography–positive electrospray ionization tandem mass spectrometry

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Abstract

A sensitive, rapid and selective liquid chromatography–positive electrospray ionization tandem mass spectrometry (LC–(ESI+)-MS–MS) method has been developed and validated for the simultaneous quantification of beclomethasone dipropionate (BDP) and its active metabolite, beclomethasone 17-monopropionate (17-BMP) in rat plasma and different tissues using fluticasone propionate (FP) as the internal standard. The method was validated over a linear range from 0.05 to 5 ng/ml for both analytes. A solid-phase extraction procedure was used for plasma samples and a liquid–liquid extraction procedure for tissues samples (lung, liver and kidney). The between-day and within-day coefficients of variation for all compounds were ≤20% at the concentrations of lower limit of quantification (LLOQ) and ≤15% at other quality control concentrations. The same method was also partially validated for human plasma. The utility of this assay was demonstrated by monitoring BDP and 17-BMP plasma and tissue concentrations in an animal study designed for evaluation of pulmonary targeting after intratracheal administration of BDP dry powder.

Keyword: Beclomethasone propionates

1. Introduction

Beclomethasone dipropionate (BDP), 9-chloro-11β-hydroxy-16β-methyl-pregna-1, 4-diene-3,20-dione (Fig. 1), was the first inhaled corticosteroid used for the treatment of asthma [1]. While BDP was introduced more than 30 years ago, little was known about its pharmacokinetics until recent years [2] because of the lack of sensitive analytical assays. Even though various assays [2–5] have been utilized in recent clinical studies by either drug companies or clinical research organizations, a full assay description and validation has generally not been reported. While this assay was developed in our laboratory, a more sensitive assay was independently developed in another study involving equine plasma and urine [6]. It is believed, however, that our report is the first detailed description of the assay involving rat and human plasma samples and different rat tissue samples.

This report describes the analytical procedure suitable to simultaneously measure BDP and its active metabolite, beclomethasone 17-monopropionate (17-BMP), in rat plasma and various tissues, such as lung, liver and kidney, using liquid chromatography–electrospray ionization tandem mass spectrometry. The assay was also cross-validated in human plasma.

2. Experimental

2.1. Materials

Micronized beclomethasone dipropionate (Fig. 1) was kindly provided by 3M (Saint Paul, MN, USA). Beclomethasone 17-monopropionate (Fig. 1) was purchased from European Directorate for the Quality of Medicine (EDQM). The internal standard, fluticasone propionate

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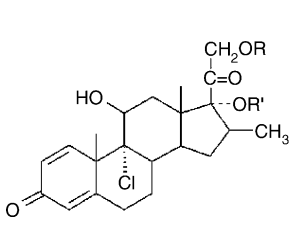

	R	R'
BDP	COC_2H_5	COC_2H_5
17-BMP	H	COC_2H_5
21-BMP	COC_2H_5	H
BOH	H	H

Fig. 1. Molecular structures of BDP, 17-BMP, 21-BMP and BOH.

(FP), was kindly provided by Glaxo Group Research (Herts, UK). Double distilled deionized water was prepared in our lab (Corning AG-3 Still, Corning, NY, USA) and filtered through 0.2 μm filter. Methanol, ethanol, formic acid, ammonium acetate, ethyl acetate and heptane were of HPLC grade and purchased from Fisher Scientific (Springfield, NJ, USA). Rat and human blank plasma were obtained from the Civitan regional blood system (Gainesville, FL, USA). The solid-phase LC18 (3 ml) cartridges for sample extraction were obtained from Supelco (Bellefonte, PA, USA).

The project was approved by the Animal Care Committee, University of Florida, an AAALAC approved facility. Specific pathogen-free, non-adrenalectomized male F-344 rats, weighing 200–250 g, were obtained from Harlan Spraque Dawley Inc. (IN, USA) and were housed 12 h in a light/dark, constant temperature environment prior to the experiment.

2.2. HPLC–MS–MS conditions

The high performance liquid chromatography (HPLC) was performed isocratically at ambient temperature using a Waters C_{18} 3.5 μm column (Symmetry, 50 mm × 2.1 mm i.d., Milford, MA, USA) preceded by a Whatman 3.5 μm ODS C_{18} guard column cartridge (20 mm × 2.0 mm i.d., Clifton, NJ, USA). The mobile phase consisted of 33 mM ammonium acetate/0.33% formic acid buffer (pH 3.4)–methanol (30:70, v/v), delivered by a flow rate of 0.3 ml/min by a LDC Analytical constaMetric® 3500 solvent delivery system (LDC/Milton Roy, Riviera Beach, FL, USA). 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. Positive 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/internal standard (I.S.)) versus analyte concentration with a weighting factor of the reciprocal of the analyte nominal concentration.

2.3. Preparation of calibration standards and quality control samples

2.3.1. Plasma samples

Primary stock solutions were prepared by dissolving the compounds or internal standard in methanol. Appropriate dilutions of the stock solutions with drug-free rat or human plasma were made subsequently in order to prepare the working solutions for BDP and 17-BMP. Two different series of stock solutions were prepared from different weightings for calibration standards (CCs) and quality control samples (QCs). The CCs and QCs ranged from 0.05 to 5 ng/ml for both analytes. Aliquots of 1 ml sample were transferred into thick wall glass tubes with enzyme inhibitor (17 mg $Na_2EDTA + 17.5$ mg NaF), capped and stored at approximately −69 °C. The internal standard stock solution was diluted with methanol to produce a working solution of 5 ng/ml.

2.3.2. Tissue samples

Immediately after decapitation of the rat, the lung, without trachea, a lobe of the liver and the two kidneys were resected and placed on ice. The weighed tissue was added into appropriate amount (1 g in 10 ml of buffer for liver and 1 g in 4 ml of buffer for lung, kidney and brain) of ice-cooled incubation buffer (10 mM Tris–HCl, 10 mM sodium molybdate, 2 mM 1,4-dithiothreitol and 2 mM phenylmethylsulfonyl fluoride (PMSF)) and homogenized in a Virtis 45 homogenizer at 40% of full speed, for three periods of 5 s each with a 30 s cooling period between each step.

The primary stock solution for CCs was diluted with methanol to produce the working solutions of 1, 2, 4, 10, 20, 40 and 100 ng/ml. Fifty microliters of each working solution was added to 1 ml blank rat tissue homogenate (liver, lung or kidney), prepared as described earlier, to generate 0.05, 0.1, 0.2, 0.5, 1, 2 and 5 ng/ml CCs. For QCs, the primary stock solution for QCs was diluted with methanol to produce the working solutions of 1, 2, 10 and 40 ng/ml which then were diluted in 1 ml blank rat tissue homogenate (liver, lung or kidney) to generate 0.05, 0.1, 0.5, and 2 ng/ml QCs. Fifty microliters of I.S. working solution was added into each CC or QC sample. After vortexing, 4 ml of ethyl acetate was added to inhibit the metabolism of BDP. The samples were capped and stored at approximately −69 °C.

2.4. Sample preparation

2.4.1. Plasma samples

BDP and 17-BMP were extracted from rat or human plasma by solid-phase extraction [7]. Plasma samples (1 ml) were thawed at room temperature. After addition of 50 μl of I.S. working solution to 1 ml plasma, they were then vortexed and 1 ml of 30% (v/v) ethanol was added into the plasma sample. After 15 min of incubation at 4 °C, the mixture was centrifuged at 4000 rpm (3148 × *g*) for 15 min to

remove the protein precipitate. The supernatant was then transferred onto the SPE extraction column preconditioned with ethanol and water. The column was drained under necessary vacuum to ensure that the sample was aspirated at a drop wise flow rate. The column was then washed with: (a) one column volume of 25% ethanol solution; (b) one column volume of water solution; and (c) 2 ml of 2:98 (v/v) ethyl acetate/*n*-heptane mixture. Finally, the sample was eluted with 3 ml of 35:65 (v/v) ethyl acetate/*n*-heptane mixture at a drop wise flow rate, evaporated in a vacuum centrifuge, and reconstituted in 50 μl of mobile phase. A volume of 30 μl was injected into the HPLC–MS–MS system.

2.4.2. Tissue samples

BDP and 17-BMP were extracted from rat tissue homogenates by liquid–liquid extraction. At the time of analysis, the frozen tissue samples were thawed at room temperature and then were shaken on a shaker for 15 min. After centrifugation (5 min, 3148 × *g*), the upper organic layer was transferred into a new tube, evaporated in a vacuum centrifuge, and reconstituted in 50 μl of mobile phase. A volume of 30 μl was injected into the HPLC–MS–MS system.

2.5. Validation

2.5.1. Selectivity

Drug-free plasma and tissue samples from six different rats or drug-free plasma from six batches of human plasma were extracted and analyzed to assess the potential endogenous interferences. Any apparent response at the retention times of BDP, 17-BMP and I.S. was compared to the response at the lower limit of quantification for BDP, 17-BMP and to the response at the working concentration for I.S.

The application of ESI and the relative fast chromatographic separation led to the questions about the influence of matrix effects on the analyte determination.

To show the potential matrix effects caused by matrix components, a post-column infusion experiment [11] was carried out. Continuous infusion of all compounds (5 μg/ml for all compounds at 10 μl/min) was performed via an infusion pump and T-piece after the analytical column, and blank matrix extract was injected into the HPLC system. The obtained chromatograms were compared with that obtained after injecting mobile phase to detect any “invisible” endogenous interferences and their retention times.

2.5.2. Recovery

The recovery of BDP and 17-BMP was evaluated in triplicate at three concentrations (0.1, 0.5 and 2 ng/ml) by comparing the peak areas of BDP and 17-BMP to the peak areas of corresponding compounds in samples prepared by spiking extracted drug-free plasma or tissue 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.

2.5.3. Calibration and sample quantification

Duplicate seven point standard curves ranging from 0.05 to 5 ng/ml of BDP and 17-BMP were run on three separate days for rat plasma and tissue samples but only 1 day for human plasma. Calibration curves ($y = ax + b$), represented by the plots of the peak-area ratios (y) of the analyte to I.S. versus the nominal concentration (x) of the calibration standards, were generated using linear least square regression. 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.

2.5.4. Accuracy and precision

Within-day accuracy and precision were evaluated by analysis of quality controls at concentrations of 0.05, 0.1, 0.5 and 2 ng/ml ($n = 6$ at each concentration) on the same day. The same experiment was repeated on three different days to assess the between-day accuracy and precision. For human plasma samples, the validation was only conducted on 1 day. 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 ≤20% on a day-to-day basis [8].

2.5.5. Stability

Freeze and thaw stability was determined by analyzing triplicate quality control samples at the concentrations of 0.1, 0.5 and 2 ng/ml following one cycle of freezing at −69 °C and thawing. Short-term temperature stability was evaluated at the same concentrations after the samples (plasma or tissue samples) were thawed and kept at room temperature for 6 h. The bench top stability at the same concentrations after extraction and reconstitution was evaluated over 5 h. Stability was expressed as the percentage ratio of measured concentration to the nominal concentration. Stability study was only conducted for rat plasma and tissue samples.

2.6. Application

The assay developed was utilized to monitor BDP and 17-BMP plasma and tissue concentrations in an ex vivo receptor binding rat model [9] designed for evaluation of pulmonary targeting after intratracheal administration of micronized BDP dry powder. Each rat was weighed and placed in a rat holder for intraperitoneal administration of the anesthetics (fresh preparation of the combination of 1.5 ml of 10% ketamine, 1.5 ml of 2% xylazine, and 0.5 ml of 1% acepromazine at the dose 1 ml/kg). The depth of the anesthesia was checked by tail pinch or pedal withdrawal reflex. For the intratracheal administration, after loss of reflexes, one inch of a special round-tipped canula attached to a delivery device for administration of dry powders (Penn-Century, Philadelphia, PA, USA) was introduced into the trachea through the

mouth. A mixture of lactose–BDP mixture (100 μg/kg of BDP) or lactose alone (placebo) was placed in the chamber of the device and instilled in the lungs with insufflation of 3 ml of air. Then the rats (one rat per time point) were decapitated with a guillotine at 0.5, 1, 2, 4, 7 and 12 h after drug administration and 6 h after lactose administration. Blood was collected in tubes containing heparin and suitable enzyme inhibitors (Na_2EDTA and NaF) and centrifuged at 4000 rpm (3148 × *g*) for 10 min. The plasma was separated and stored at approximately −69 °C until analyzed. Meanwhile, lungs, kidneys and livers were immediately processed as described in Section 2.3.2 for concentration measurement and receptor binding studies. The experiment was repeated on three different days. The measured plasma and tissue concentrations of BDP and 17-BMP and the receptor binding results in different tissues will be used for pharmacokinetic/pharmacodynamic (PK/PD) modeling of BDP after intratracheal administration in a future paper.

3. Results and discussion

3.1. Mass spectrometry/chromatography

Both atmosphere pressure chemical ionization (APCI) and electrospray ionization modes were tested for all compounds. Positive ESI (ESI+) was chosen for the better sensitivity. The parent mass spectra and daughter ion mass spectra of the two analytes and of the I.S. under multiple reaction monitoring (MRM) mode 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. Figs. 3–5 showed the typical chromatograms for blank

Table 1
The transition channels for two analytes and the internal standard

Compound	Parent ion	Daughter ion
Beclomethasone dipropionate (BDP)	521	319
Beclomethasone 17-monopropionate (17-BMP)	465	279
Fluticasone propionate (I.S.)	501	313

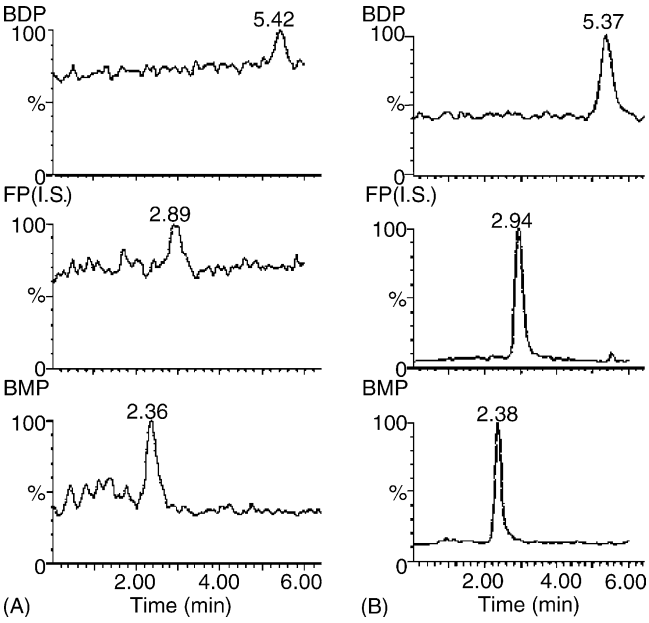

Fig. 3. Chromatograms of blank human plasma (A) and human plasma spiked with 0.1 ng/ml analytes and 1 ng/ml I.S (B). Relative peak height and different scales in (A) and (B).

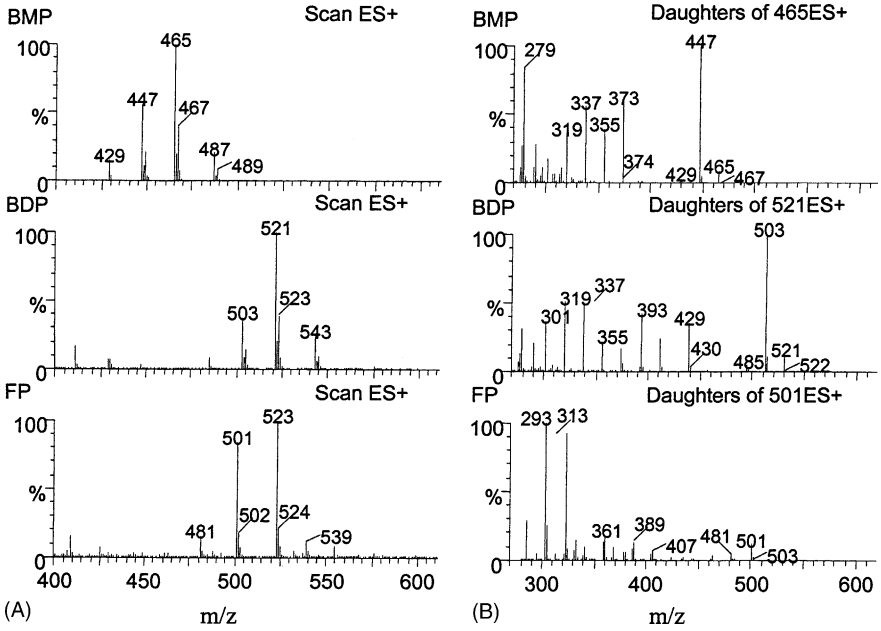

Fig. 2. Full scan (A) and daughter scan (B) spectra of 17-BMP, BDP and I.S.

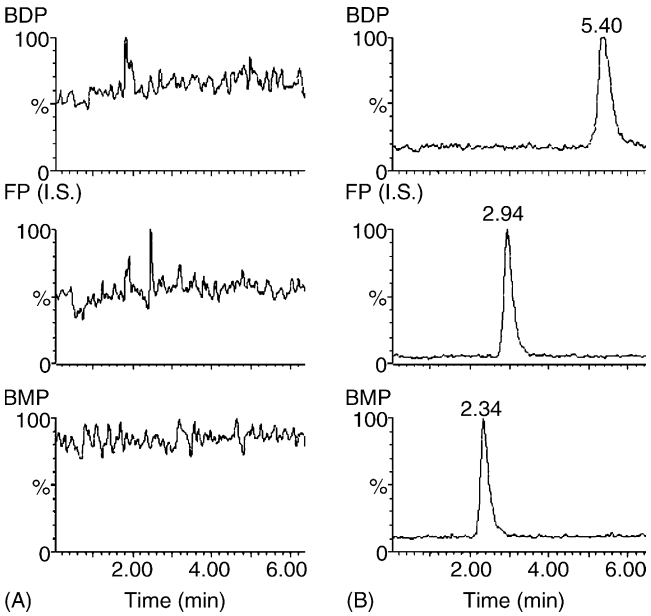

Fig. 4. Chromatograms of blank rat plasma (A) and rat plasma spiked with 0.1 ng/ml analytes and 1 ng/ml I.S (B). Relative peak height and different scales in (A) and (B).

human plasma, rat plasma and lung samples and their corresponding samples spiked with 0.05 ng/ml BDP, and 17-BMP and 0.25 ng/ml I.S. The analysis time was 7.0 min with 17-BMP eluted at 2.38 min, I.S. at 2.94 min and BDP at 5.37 min. The resulting calibration curves were linear with correlation coefficients >0.99. The metabolite of 17-BMP, beclomethasone (BOH) was also tested for assay development, but the preliminary data showed that almost no detectable BOH was found in rat plasma or tissue samples.

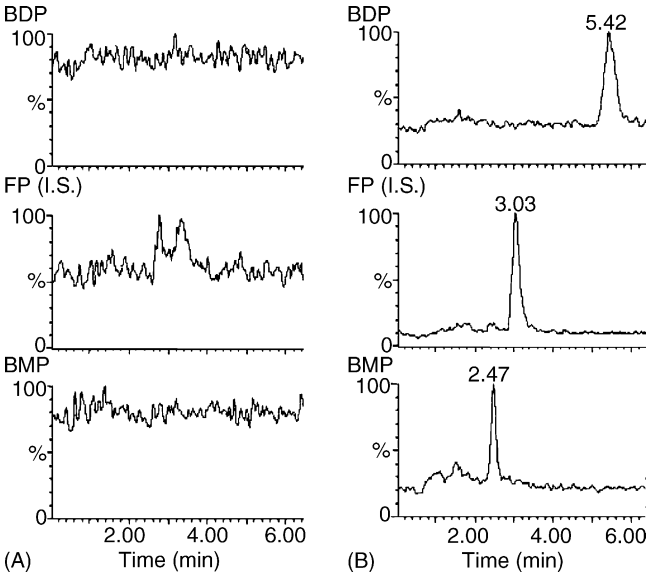

Fig. 5. Chromatograms of blank lung (A) and lung spiked with 0.1 ng/ml analytes and 1 ng/ml I.S (B). Relative peak height and different scales in (A) and (B).

3.2. Internal standard selection

Isotopically-labeled compounds were not available. Therefore, structural analogs were screened to find suitable compounds for internal standard. Fluticasone propionate was finally chosen as the internal standard for its better sensitivity and simultaneous good correction for both BDP and 17-BMP as shown by the data.

3.3. Sample preparation

Due to the relatively small number of samples but many different matrices (plasma and three tissues) it was not the goal to perform extensive method optimization but to provide sample analysis with sufficient accuracy, precision and robustness of the assays. It was preferred to apply the same sample preparation and analysis technique for all compounds and matrices with minor variations to save method development time and to allow a rapid switching from one assay to another. Therefore, a simple liquid–liquid extraction method modified from a previous study [10] was tried for all the matrices. Unfortunately, this method did not work well for plasma samples in terms of the sample cleanness even though it worked for the tissues. A solid-phase extraction method from [7] was adopted for plasma samples. Further optimization was tried, but the original method seemed to provide the best recovery balance between BDP and 17-BMP.

3.4. Mobile phase and buffer system selection

In order to achieve the maximum signal response under ESI+ condition and the shortest analysis time, the percentage of organic phase in the mobile phase was maintained as high as possible while still avoiding the early front peak which contained most of the hydrophilic response-suppressing endogenous interferences. Methanol and acetonitrile were screened for the organic phase. Methanol was selected due to the better sensitivity. Ammonium acetate buffer system was optimized in terms of the concentration and the composition of the buffer system. Acetate acid and formic acid were screened for the acid component. Formic acid was selected due to its better sensitivity and higher buffering capacity while achieving that sensitivity. Finally, the mobile phase consisted of 33 mM ammonium acetate/0.33% formic acid buffer (pH 3.4)–methanol (30:70, v/v).

3.5. Matrix effect

The application of ESI and the relative fast chromatographic separation led to the questions about the influence of matrix effects on the analyte determination. To show the potential matrix effects caused by matrix components, a post-column infusion experiment [11] was carried out. Continuous infusion of all compounds was performed via

Table 2
The recoveries of BDP, 17-BMP and I.S. from plasma and tissue samples[a]

	BDP			17-BMP			I.S.
	0.1 ng/ml	0.5 ng/ml	2 ng/ml	0.1 ng/ml	0.5 ng/ml	2 ng/ml	0.25 ng/ml
Human plasma	86.3 (8.9)	87.6 (5.2)	85.9 (3.2)	53.6 (3.4)	64.9 (6.8)	59.7 (1.8)	75 (5.7)
Rat plasma	83.8 (4.5)	83.0 (4.1)	82.6 (1.8)	48.3 (1.2)	59.5 (1.8)	60.0 (4.9)	70 (6.5)
Liver	83.5 (11.6)	83.2 (5.8)	81.1 (5.3)	49.9 (5.1)	49.5 (2.8)	51.1 (1.4)	65.3 (4.6)
Lung	85.0 (14.9)	83.4 (11.2)	83.0 (12.7)	76.4 (1.1)	73.1 (13.8)	71.2 (1.5)	92.8 (9.8)
Kidney	79.0 (13.3)	76.9 (7.9)	79.3 (3.5)	32.4 (5.9)	30.7 (4.1)	33.3 (5.6)	40.2 (5.6)

[a] Values are presented as mean (standard deviation) ($n = 6$).

an infusion pump and T-piece after the analytical column, and blank matrix extract was injected into the HPLC system. Suppressive matrix effect was observed at the retention times of 0.7 and 2.6 min after injection of all matrices compared to the injection of mobile phase. These suppressive effects were not compound-specific since they were observed in the MRM channels for both the analytes and I.S. However, the determination of the analytes was not affected because BDP, 17-BMP and I.S. were eluted at 5.37, 2.38 and 2.94 min. The infusion was run up to 2 h and no late eluting interferences were observed.

3.6. Selectivity

Drug-free plasma and tissue samples from six rats or drug-free plasma from six batches of human plasma were screened during method validation. Figs. 3–5 compared the chromatograms for extracted drug-free samples and samples spiked with 0.05 ng/ml BDP and 17-BMP and 0.25 ng/ml I.S. Even though minor peaks were observed at the retention times of BDP, 17-BMP and I.S. in human plasma samples (Fig. 3), the accuracy and precision of the assay were within the acceptable range (Table 3). No significant interferences were observed at the retention times of BDP, 17-BMP and I.S. in rat plasma and tissue samples (Figs. 4 and 5; liver and kidney chromatograms not shown).

3.7. Recovery

The recoveries (Table 2) from human plasma samples for BDP and 17-BMP were listed in Table 2. I.S. recovery was determined to be 70 ± 6.5% at the working concentration of 5 ng/ml. The recoveries from tissue samples for 17-BMP and I.S. were also listed in Table 2. Despite the relatively low values for 17-BMP in plasma and some tissue (liver and kidney) samples, the recoveries were fairly reproducible as shown by the data. Since the original extraction method [7] was developed for FP, the relative low recovery for 17-BMP was expected due to its relatively lower lipophilicity. Further optimization in sample extraction was tried, but improvement of 17-BMP recovery resulted in significant loss of BDP recovery that led to the failure of BDP assay validation. Therefore, the original extraction method was adapted.

3.8. Precision and accuracy

Table 3 lists the within and between-day precision and accuracy data for BDP and 17-BMP at four quality control levels in plasma and tissue samples. 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 (86.3–106.4% at

Table 3
Within-day (WT) and between-day (BT) accuracy/precision for BDP and 17-BMP in different matrices

		BDP				17-BMP			
		0.05 ng/ml	0.1 ng/ml	0.5 ng/ml	2 ng/ml	0.05 ng/ml	0.1 ng/ml	0.5 ng/ml	2 ng/ml
Human plasma	WT	95.6/16.4	89.7/6.5	98.3/7.8	104.9/3.4	90.6/15.6	96.1/12.9	100.3/6.8	98.7/4.5
Rat plasma	WT	89.3/13.8	85.7/4.8	101.7/5.9	102.4/4.0	95.5/8.8	102.9/14.9	109.3/6.2	102.3/5.0
	BT	92.2/14.3	101/8.2	95.9/10.2	96.2/6.5	89.2/11.2	102/13.2	97.3/10.2	96.5/6.6
Liver	WT	86.3/12.8	90.1/6.5	93.8/10.6	91.3/9.1	92.0/12.0	93.4/11.6	102.3/9.4	105.7/4.9
	BT	94.9/13.5	97.6/7.1	102.9/13	93.9/10.5	96/12.8	95.4/13.4	99.6/10.1	96.9/12.2
Lung	WT	105.3/11.8	95.1/7.5	96.5/6.4	97.3/10.5	93.7/14.1	90.6/10.4	102.5/7.4	102.5/3.1
	BT	92.2/13.8	92.5/10.3	97.9/12.3	95.5/10.2	95.9/13.3	92.8/11.9	96.8/7.8	96.9/13.7
Kidney	WT	90.6/12.5	98.3/7.9	94.2/8.9	89.3/10.1	97.3/10.8	85.4/11.3	98.9/6.1	102.7/2.1
	BT	95.5/12.2	95.7/9.4	92.2/11.1	102/11.5	95.2/11.7	98.6/12.6	93.2/9.5	95.6/7.0

Accuracy: 100% measured concentration/nominal concentration. Precision: coefficient of variation (100% standard deviation/mean). WT: $n = 6$ on 1 day. BT: $n = 18$ on 3 days.

Table 4
Stability (%) of BDP and 17-BMP in rat plasma samples under various conditions[a]

	BDP			17-BMP		
	0.1 ng/ml	0.5 ng/ml	2 ng/ml	0.1 ng/ml	0.5 ng/ml	2 ng/ml
Freeze–thaw (three cycles)	93.9 ± 2.0	95.1 ± 8.6	106.6 ± 5.6	99.1 ± 12.5	103.7 ± 5.4	107.8 ± 5.5
Short term (6 h)	90.1 ± 10.1	92.4 ± 13.1	101.9 ± 9.5	92.8 ± 9.4	93.87 ± 3.6	104.0 ± 4.1
Bench top (7 h)	98.5 ± 10.1	96.8 ± 9.5	101.1 ± 10.3	97.6 ± 10.2	102.9 ± 15.4	101.2 ± 4.9

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

LLOQ and 85.7–105.7% at other concentrations) and precision (<18% at LLOQ and <15% at other concentrations). LLOQs were set at 0.05 ng/ml for both analytes with an accuracy of 80–120% and a precision ≤20% on a day-to-day basis [8]. The linear range was 0.05–5 ng/ml of BDP and 17-BMP with correlation coefficients >0.99.

3.9. Stability

BDP and 17-BMP are known to be the substrates for the carboxylesterase that is widely distributed in plasma and various tissues [12]. Na_2EDTA and NaF were added to the blood collecting tube for each plasma sample as esterase inhibitor. PMSF was added to the tissue buffer as esterase inhibitor, but certain metabolism was still observed for BDP during sample preparation. Therefore, 4 ml of ethyl acetate was added to the tissue homogenate right after the homogenization to further inhibit the hydrolysis reaction. Table 4 lists the data for the freeze–thaw stability, the short-term stability under room temperature and the bench top stability at three concentrations for rat plasma samples. Stability was expressed as the percentage ratio of measured concentration to the nominal concentration. The results showed that both analytes were stable under conditions investigated in this study since the measured concentrations were all within 85–115% of the nominal concentrations. Similar results were obtained for human plasma samples and different rat tissues samples.

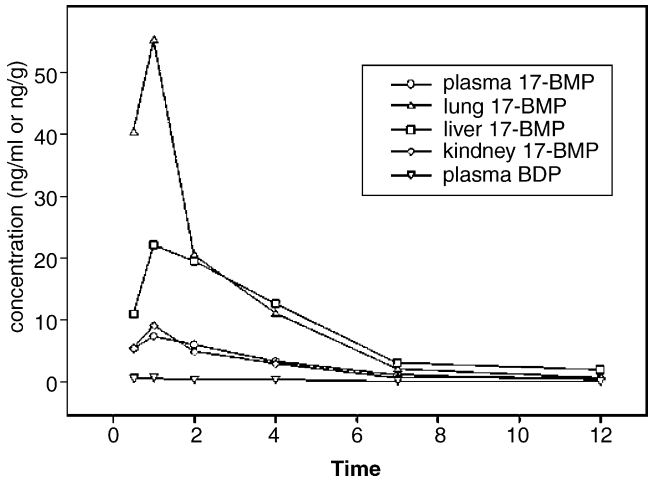

Fig. 6. BDP and 17-BMP plasma and tissue concentration profiles in an animal study designed for pulmonary targeting evaluation of intratracheally delivered BDP dry powder. Data represent results for on animal, as this figure is intended to demonstrate the assays suitability to follow BDP and BMP levels. PK estimates for the whole animal experiments will be published separately.

3.10. Application to experimental sample analysis

Plasma and tissue samples from a pulmonary targeting experiment for intratracheally delivered micronized BDP dry powder were analyzed and the concentration versus time profiles for BDP and 17-BMP are shown in Fig. 6. The results indicated that the assay is suitable to study the pharmacokinetics of BDP after intratracheal administration in the rat model [9] at the investigated dose.

4. Conclusions

A simple, sensitive and selective LC–(ESI+)-MS–MS method was developed for simultaneously quantifying BDP and its active metabolite, 17-BMP, in rat plasma or tissue samples and human plasma samples. This assay is more versatile than HPLC assays dependent on the use of radiotracers [13] and more sensitive than the previously described assay for BDP and BMP in biological fluids [14] which reported a sensitivity of 1 ng/ml.

While beclomethasone represents also a potential metabolite for BDP, the assay was not extended to the determination of beclomethasone, as previous pharmacokinetic studies suggested very low levels of this potential metabolite and activity much lower than that of beclomethasone monopropionate. The validation results have shown that the method is robust and is suitable to assess BDP and 17-BMP levels after intratracheally delivered BDP dry powder. The same method can also be utilized for human plasma samples even though only a cross-validation was conducted for human plasma. The LLOQ of 50 pg/ml and the linear range of 0.05–5 ng/ml for both BDP and 17-BMP were comparable to assay performance of “unpublished” assays employed in clinical studies [2–5] although a more sensitive assay with LLOQ of 25 pg/ml for both BDP and 17-BMP was developed in equine plasma and urine samples very recently [6]. In addition, the assay is also able to detect the analytes in biological tissues. Thus, a toll is now available that will allow detailed distribution studies of BDP and 17-BMP in biological tissues.

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