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LC–MS/MS determination of betamethasone and its phosphate and acetate esters in human plasma after sample stabilization

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ABSTRACT

Two specific liquid chromatography–mass spectrometric (LC–MS/MS) assays were developed and validated for the determination of betamethasone (BET), and its acetate (BA) and phosphate (BP) esters. The plasma and the blood used for the development and validation of these two methods were previously stabilized. Liquid–liquid extraction techniques were used after the addition of prednisolone as internal standard (IS). Samples were chromatographed using C8 column, while mass detection was carried out by electrospray ionization in the positive mode (ESI+). The method was proved linear over a working range 0.50–50.00 ng/ml for BET ($r^2 > 0.99$), while BA linear range was 1.0–20.0 ng/ml ($r^2 > 0.99$). Sensitivity was determined as 0.50 ng/ml for BET and 1.00 ng/ml for BA. Betamethasone phosphate LC–MS/MS method involved solid phase extraction after the addition of prednisolone phosphate as (IS). Separation was carried out using C18 column, while detection was by ESI+. The method showed good linearity over the working range 2.0–200.0 ng/ml ($r^2 > 099$). Both methods were applied to determine BET, BA and BP in plasma samples obtained for pharmacokinetics studies in human.

1. Introduction

Betamethasone, 9-fluoro-11β,17,21-trihydroxy-16β-methylpregna-1,4-diene-3,20-dione (Fig. 1), is a synthetic glucocorticoid. It is active in replacement therapy for adrenal insufficiency and as an anti-inflammatory and immunosuppressant. Betamethasone is used to treat many conditions including dermatitis, arthritis, inflammatory bowel disease, reactive airways disease, and respiratory distress syndrome in preterm infants and pruritus in corticosteroid-responsive dermatoses. Betamethasone is formed by hydrolysis of the phosphate or acetate esters after intravenous or intramuscular administration to human. Other esters and salts of betamethasone are available for other routes of administration or applications, e.g., valerate, butyrate, propionate benzoate.

There are several approved products formulated based on a fast releasing betamethasone phosphate ester or as a dual acting suspension formulation containing BP and BA esters [1,2]. Both esters are expected to be hydrolyzed in vivo to the active glucocorticoid BET [3].

Several analytical techniques have been published for the analysis of BET in different matrices, e.g., high performance liquid chromatography (HPLC) [3–5], gas chromatography with mass detection (GC–MS) [6,7] or liquid chromatography with mass detection (LC–MS/MS) [8–19]. The mentioned methods showed low sensitivity, e.g., 10, 50 and 300 ng/ml [3–5], implicated derivatization [6], were not fully validated or were not applicable to clinical or pharmacokinetic (PK) studies in human [12–19]. Nevertheless, some of these methods were applied to PK studies in animals, e.g., Samtani et al. [13] reported the use of LC–MS/MS for BET determination in sheep. Though the report did not include method's validation, yet was applied to PK determination in animals after IM dose of BP/BA suspension. The authors reported BET PK, while BP and PA were not measured. The authors suggested further PK studies are needed in human.

Surprisingly, though of its major importance, to date, BET, BA and BP pharmacokinetics in human after IM administration still is not well documented in the literature. One of the reasons is due to the absence of well-documented specific and sensitive validated methods of analysis. Therefore, the main aim of our work was to develop and validate highly specific and sensitive liquid chromatographic–mass spectrometric methods for the determination of BET, BA, and BP in human plasma. These methods were to be applied for PK determination in human after IM administration of BP/BA suspension. The first method was designed for BET and BA determination. The targeted working range for BET was 0.50–50.00 ng/ml, while for BA was 1.0–20.0 ng/ml. A second method was designed to determine BP in human plasma covering the linearity over the working range of 2.0–200.0 ng/ml.

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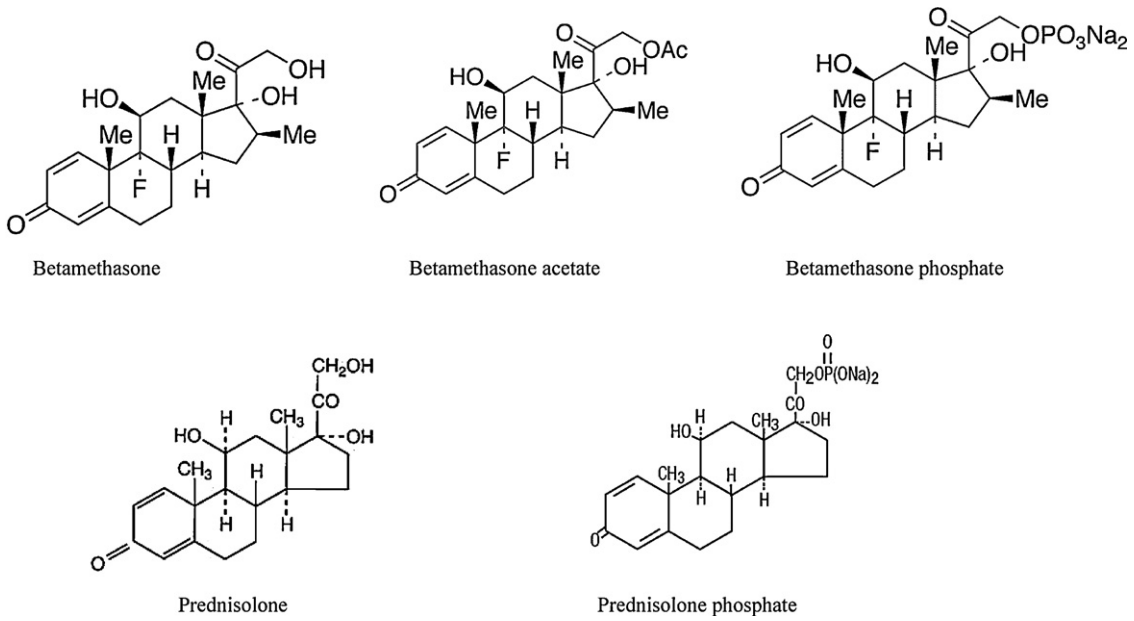

Fig. 1. Structures of BET, BA, BP and the internal standards prednisolone and prednisolone phosphate.

Furthermore, it was aim of our work to evaluate and validate arsenate and fluoride efficacy inhibiting the hydrolysis of BA and BP esters to BET in blood and plasma [3]. The methods were applied to determine the concentrations of the three analytes in the plasma obtained from healthy subjects participating in PK studies.

2. Experimental

2.1. Reagents

Betamethasone, its acetate and phosphate esters, prednisolone and its phosphate ester were obtained from TRC (Toronto, Canada). Analytical grade for HPLC acetonitrile, methanol, formic acid and diethyl ether were supplied by Merck (Darmstadt, Germany). Sodium arsenate dibasic heptahydrate, potassium fluoride anhydrous, ammonium formate, ammonium hydroxide were obtained from Sigma (UK). MCX solid phase cartridges we supplied by Waters (Milford, USA). Water was purified using a Milli-Q® (Millipore, France). Six different batches of lithium heparin blood were obtained from healthy blood donors who were proved to be HIV, hepatitis B & C negative.

2.2. Plasma pretreatment

In order to prevent the in vitro hydrolysis of BP or BA esters to BET during blood collection and plasma sample handling, the blood samples were collected into pre-chilled plastic heparinized tubes containing 10 μl 2 M sodium arsenate solution per ml blood [3]. Blood samples (kept over ice) were harvested to plasma within a maximum of 15 min by centrifugation for 5 min at 1789 × *g* and 5 °C. Plasma was siphoned into pre-chilled plastic tubes containing 10 μl of 50% (w/v) potassium fluoride solution per ml plasma [3]. Plasma samples were immediately frozen and stored at −70 °C.

2.3. Preparation of stock solutions

Stock solution of BET and BA were prepared using equivalent amounts of 10.0 mg of BET or BA in 100 ml methanol to produce a concentration of 100.0 μg/ml of either analytes. Stock solutions were stored at −20 °C. Working solutions of BET or BA were prepared by diluting in methanol to a final concentration of 10.0 μg/ml and stored at −20 °C. Serial solution of both working solutions were prepared in methanol at 10.0, 20.0 40.0, 100.0, 200.0, 400.0, 600.0 and 1000.0 ng/ml of BET, and 20.0, 40.0, 80.0, 120.0, 160.0, 240.0, 320.0 and 400.0 ng/ml of BA.

Quality control (QC) serial solutions were prepared in methanol at 10.0, 30.0, 500.0 and 840 ng/ml of BET and 20.0, 60.0, 200.0 and 340.0 ng/ml of BA.

Prednisolone (IS) working solution was prepared in methanol at 150 ng/ml and stored at −20 °C.

Stock (100.0 μg/ml) and working solution (20.0 μg/ml) for BP were prepared in methanol. BP serial dilution of 40.0, 80.0, 200.0, 400.0, 1200.0, 2400.0, 3400.0 and 4000.0 ng/ml was also prepared in methanol. Quality control samples were prepared at 40.0, 120.0, 2000.0 and 3200.0 ng/ml. The IS (prednisolone phosphate) working solution was prepared with a final concentration of 500 ng/ml. All solutions were stored at −20 °C.

2.4. Calibration curves

Calibration curve standards were prepared by spiking 50 μl of each one of the abovementioned working solutions in 1.0 ml of stabilized plasma to produce the calibration curve standards equivalent to 0.50, 1.00, 2.00, 5.00, 10.00, 20.00, 30.00 and 50.00 ng/ml of BET and 1.00, 2.00, 4.00, 6.00, 8.00, 12.00, 16.00 and 20.00 ng/ml of BA.

A double blank plasma sample (no IS) and a single blank plasma prepared containing 30.0 ng/ml of IS were used as part of each run. Neither the double blank sample nor the single blank was used to construct the calibration function. Calibration curves were run daily together with quality control samples.

Betamethasone phosphate calibration curve standards were prepared by spiking 1.0 ml stabilized plasma with 50 μl of each one of the abovementioned working solutions, producing the calibration curve standards equivalent to 2.0, 4.0, 10.0, 20.0, 60.0, 120.0, 170.0 and 200.0 ng/ml. As in the above case of BET and BA, double and single blank samples were used. The single blank contained 100.0 ng/ml of IS. Calibration curves were run daily together with quality control samples.

2.5. Quality control samples

Quality control samples were prepared at lower limit of quantification (LLOQ), low QC (three times the LLOQ), mid QC and a high QC (80% of the ULOQ). Quality control samples were prepared daily by spiking 50 μl of each one of the abovementioned working solutions in 1.0 ml of stabilized plasma to produce QC samples equivalent to 0.50, 1.50, 25.00 and 42.00 ng/ml of BET and 1.00, 3.00, 10.00 and 17.00 ng/ml of BA.

Quality control samples of BP were prepared at final concentrations of 2.0, 6.0, 100.0 and 160.0 ng/ml.

2.6. Extraction

For BET and BA analysis, all QC, calibration curve, single blank and double blank plasma samples were extracted using a liquid–liquid extraction technique. 500 μl plasma sample were spiked with 100 μl of IS working solution (150.0 ng/ml) and vortexed for 10 s. Six ml of extraction solvent (diethyl ether) were added to each plasma samples, vortexed for 60 s and centrifuged for 5 min at 1789 × *g*. The organic layer was evaporated under nitrogen stream at 40 °C. Finally, samples residues were reconstituted with 200 μl mobile phase, vortexed for 30 s, transferred to 250 μl glass insert tubes then centrifuged for 5 min at 1789 × *g*. Only 50 μl of the supernatant were injected into the LC–MS/MS system.

Betamethasone phosphate QC samples, calibration curve standards, single blank and double blank plasma samples were extracted using a solid phase extraction (SPE) technique. 500 μl stabilized plasma were spiked with 100 μl of IS (prednisolone phosphate) working solution (500.0 ng/ml) and vortexed for 10 s. 500 μl of 2% formic acid in water were added to the plasma sample and vortexed for 10 s, centrifuged at 1789 × *g* for 5 min, and then applied to MCX solid phase extraction cartridge. The SPE cartridges were previously conditioned by adding 1.0 ml methanol and 1.0 ml deionized water. After the addition of plasma samples, the SPE cartridges were washed with 1.0 ml 2% formic acid in deionized water and 0.5 ml of deionized water. The samples were eluted with 1.0 ml 5% ammonium hydroxide in methanol. Samples were then evaporated under nitrogen stream at 40 °C. Finally, samples residues were reconstituted with 200 μl mobile phase, vortexed for 30 s and transferred to 250 μl glass insert tubes then centrifuged for 5 min at 1789 × *g*. Only 50 μl of the supernatant were injected into the LC–MS/MS system.

2.7. Chromatographic conditions

After several attempts using different columns and mobile phase combinations, the separation and determination of the BET, BA and IS (prednisolone) were carried out by using XTera MS C_8 5 μm 2.1 mm × 100 mm column (Waters, Ireland), connected to Agilent 1100 HPLC series (Agilent, Germany). The analytical column was thermostated at 30 °C. The combination of the mobile phase, prepared by mixing ammonium formate buffer (35%) and methanol (65%), with a flow rate of 0.3 ml/min was found to be adequate for the samples analysis. Samples were kept in the autosampler at 5 °C.

The analysis of betamethasone phosphate and IS (prednisolone phosphate) was carried out by injecting the samples into XTera MS C_{18} 5 μm 3.9 mm × 150 mm column (Waters, Ireland), using Agilent 1100 series. The analytical column was thermostated at 30 °C. The mobile phase was prepared by mixing ammonium formate buffer (60%), methanol (20%) and acetonitrile (20%). Flow rate was 1.0 ml/min, while samples were kept in the autosampler at 5 °C.

2.8. Mass spectrometric conditions

BET and BA monitoring and quantitation were achieved using a API4000 triple quadrupole mass spectrometer (Applied Biosystems, MDS, SCIEX, Canada) set at unit resolution in the multiple reaction monitoring (MRM) mode using turbo ion spray with positive mode ionization. The analyses were run by Analyst 1.5 software.

The mass spectrometric conditions were optimized to obtain maximum sensitivity for BET, BA and prednisolone (IS). To achieve this, 100 ng/ml solution from each one of these solutions were prepared separately in mobile phase (35% ammonium formate buffer, 65% methanol) and infused into mass detector using syringe pump in the infusion mode. The best mass detector specific parameters for each compound were found to be for BET *m*/*z* 393.10 > 373.10 with the declustering potential (DP): 26, collision energy (CE): 13 and collision cell exit potential (CXP): 20, while for BA were *m*/*z* 435.10 > 415.10 with DP: 51, CE: 13 and CXP: 14. Prednisolone (IS) parameters were *m*/*z* 361.10 > 343.10 with DP: 51, CE: 13 and CXP: 10. Other parameters were: curtain gas (CUR): 10, ion spray voltages: 5500, temperature: 600, collision gas (CAD): 4, ion source GS1: 35, ion source GS2: 35, interface heater: ON and entrance potential (EP): 10.

Betamethasone phosphate monitoring and quantitation were achieved using a API4000 triple quadrupole mass spectrometer set at unit resolution in the multiple reaction monitoring (MRM) mode using turbo ion spray with positive ionization. Betamethasone phosphate parameters were *m*/*z* 473.20 > 435.20 with (DP): 51, (CE):15 (CXP): 12; while for prednisolone phosphate (IS) were: *m*/*z* 441.00 > 423.00 with (DP): 66, (CE):15 and (CXP): 12. Other parameters were (CUR): 10, (IS): 5500, temperature: 600, collision gas: 4, ion source (GS1): 35, ion source (GS2): 35, interface heater: ON and entrance potential (EP): 10.

The detection in MS/MS technique is highly specific and sensitive, nevertheless, endogenous substances can exist in much higher concentration than the analytes of interest and may co-elute with those affecting the ionization of the analytes leading to high imprecision and loss of sensitivity [20]. In order to determine ion suppression matrix effect profiles, analytes were infused into the mobile phase through a T-connection between the column and the interface while injecting the extracted blank plasma samples. The purpose of this post-column infusion with the analytes is to raise the background level so the suppression matrix will appear as negative peaks.

2.9. Stability testing

In order to prevent the in vitro hydrolysis of BP or BA esters to BET during blood collection and plasma sample handling and storage stages, 50 μl of 2 M sodium arsenate solution/ml blood was used as stabilizer during blood collection, while 10 μl of 50% (w/v) potassium fluoride/ml plasma was used during plasma handling and storage stages. This method of stabilization was first reported by Petersen et al., yet no validation data were submitted [3].

Stability of BET, BA and BP was studied in blood and plasma covering the interval from the time the blood samples are withdrawn from the volunteers until the moment the samples are stored in the freezer after centrifuged to plasma.

Different blood aliquots, stabilized with sodium arsenate solution and kept over ice, were spiked with 50 ng/ml BA or 160 ng/ml BP. Blood were harvested to plasma and siphoned into potassium fluoride containing tubes at zero, 30 and 60 min of storage. The obtained plasma samples were analyzed for BET, BA and BP using the corresponding LC–MS/MS method.

Additional blood aliquots, stabilized with sodium arsenate solution, were spiked with 50 ng/ml of BET. Blood samples were

harvested to plasma and siphoned into potassium fluoride containing tubes at zero, 30 and 60 min of storage over ice. The obtained plasma samples were analyzed for BET, BA and BP using the corresponding LC–MS/MS method.

Another set of experiments were conducted to determine the analytes stability in plasma. For that purpose, enough volumes of arsenate stabilized blood were harvested to plasma and collected into plastic tubes containing potassium fluoride. These plasma tubes were spiked with 50 ng/ml of BET, BA or 160 ng/ml of BP, and were kept at bench top (room temperature) during 1 h simulating extreme extraction conditions. Samples were analyzed for BET, BA and BP using the corresponding LC–MS/MS method.

Moreover, the stability of the three analytes in the same stabilized plasma aliquot was studied for 3 h over the bench top. The aim of this last experiment was to assure no interference would have place between the three analytes present in subjects' plasma samples at their highest possible concentrations (42 ng/ml BET, 17 ng/ml BA and 160 ng/ml BP). After 3 h storage, samples were analyzed for BET, BA and BP using the corresponding LC–MS/MS method.

2.10. Data treatment

The linearity of the method of determination of BET and BA was tested over the range of concentrations 0.50–50.00 ng/ml for BET and 1.0–20.0 ng/ml for BA; while for BP linearity was tested over the range 2.0–200.0 ng/ml. Calibration curves were constructed by determining the best-fit of peak area ratios (peak area analyte/peak area internal standard) vs. concentrations, and fitted to the equation $y = bx + a$ by least-squares regression. Different runs have been made and the best fit was obtained by using $1/X$ weighing.

3. Results and discussion

3.1. Separation and specificity

ESI is a soft ionization technique that produces high mass-to-charge [M+1]+ precursor ions with minimal fragmentation of the analyte. BET, BA, BP and internal standards gave protonated precursor [M+1]+ in the MS mode. The major ions observed were m/z 393.1 for BET, 435.1 for BA and 361.1 for prednisolone. The major ion observed for BP was 473.2, while was 441.0 for prednisolone phosphate. The most intense product ions observed in the MSMS spectra were m/z 373.1 for BET, 415.1 for BA and 343.1 for the prednisolone. Betamethasone phosphate most intense product ion was 435.2, while was 423.0 for prednisolone phosphate. The corresponding spectra of all analytes are depicted in Fig. 2.

The combination of HPLC (under the isocratic conditions described) with ESI-MS/MS leads to short retention times and yields both high selectivity and sensitivity. The SRM chromatograms obtained from extracted double blank plasma samples are depicted in Fig. 3, indicating no endogenous peaks at the retention times (t_R) of analytes or internal standard. No interferences of the analytes were observed due high selectivity of the MSMS technique. Fig. 4 shows the chromatogram of blank plasma samples. No ion suppression effects were observed.

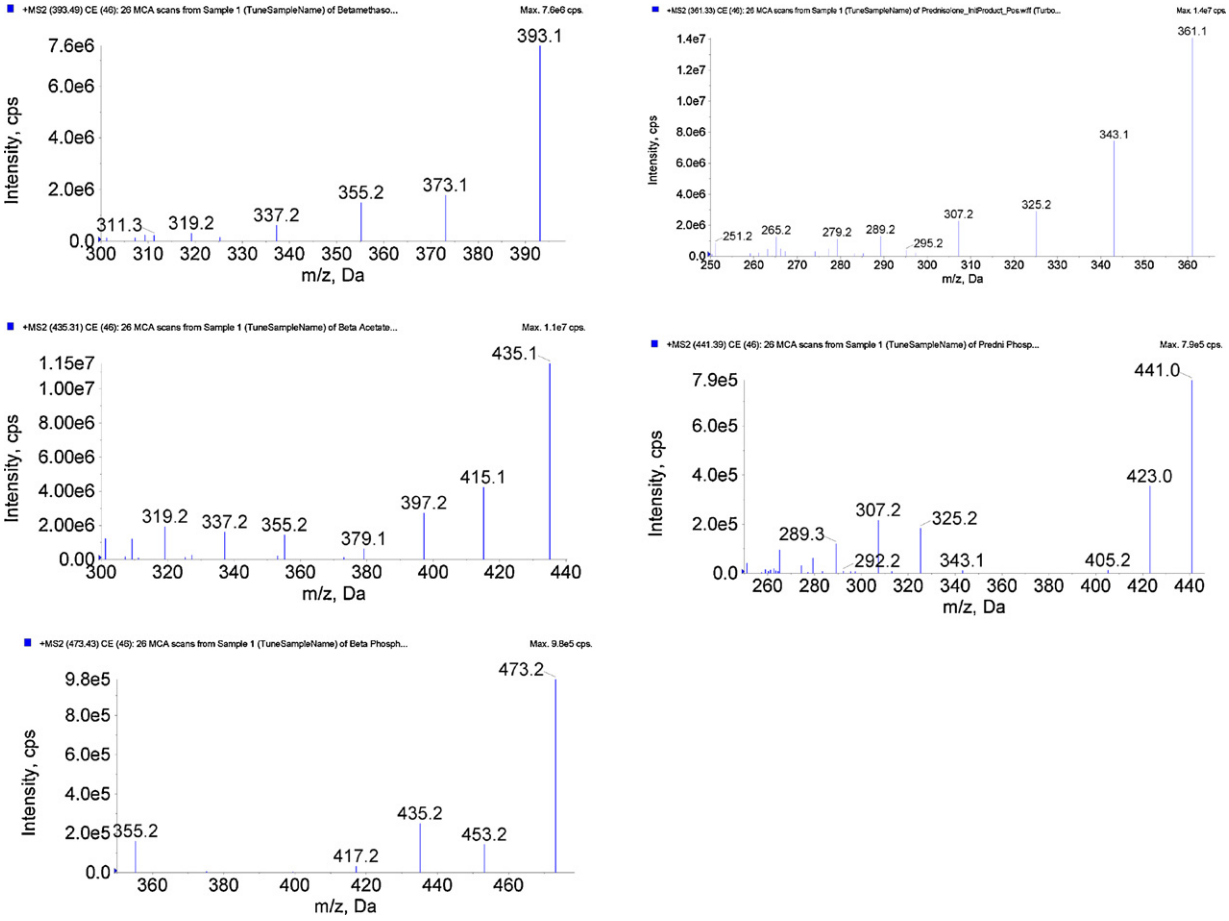

Fig. 2. Mass spectra of BET, BA, BP, prednisolone and prednisolone phosphate listed top to bottom and left to right.

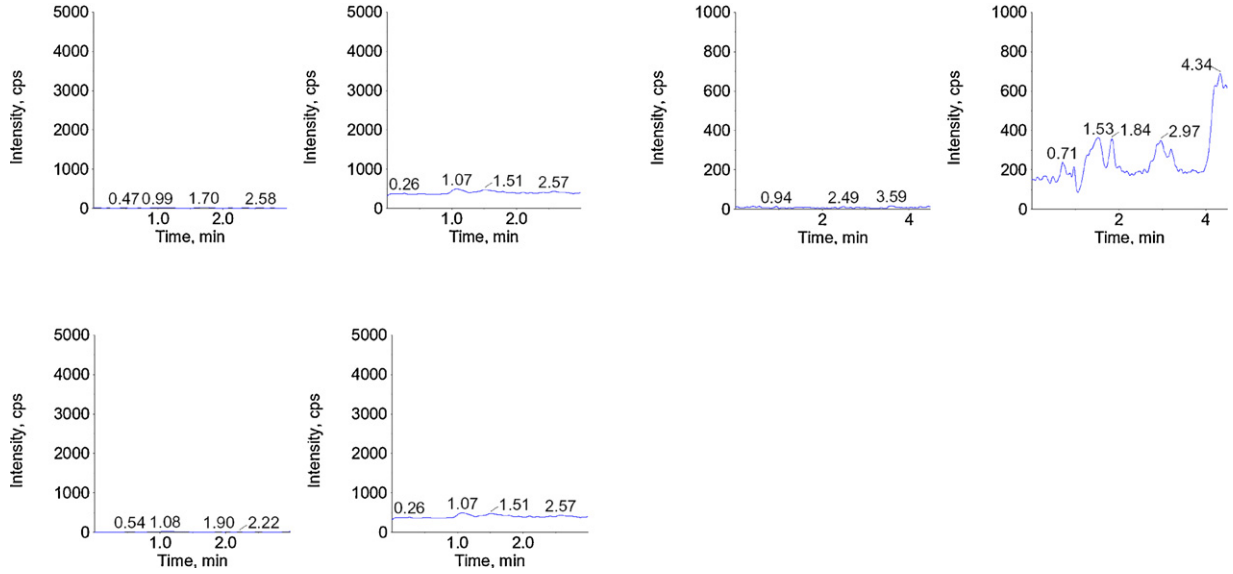

Fig. 3. LC–MS/MS chromatograms of BET, BA and BP double blank plasma sample obtained from one of the volunteers listed top to bottom and left to right.

3.2. Method validation

The two LC–MS/MS methods have been validated according to currently accepted US Food and Drug Administration (FDA) bioanalytical method validation guidance [21]. The following parameters were considered.

To test the specificity, six double blank and six blank samples were prepared using six different batches of human plasma. Samples were tested for interference using the proposed extraction procedure and chromatographic/mass spectrometric conditions and were compared with those at LLOQ. No significant interference at the retention time of the drug or internal standard was observed as illustrated in the chromatograms presented in Figs. 3 and 4.

Betamethasone LC–MS/MS method was proved linear for BET determination over the range 0.50–50.00 ng/ml with backcalculated accuracy of 95.33–100.83% (CV% 1.92–4.53). The method was linear for BA over the range 1.0–20.0 ng/ml with backcalculated accuracy of 95.00–103.33% (CV% 2.25–5.77). Betamethasone phosphate LC–MS/MS method was linear over the range 2.0–200.0 ng/ml with backcalculated accuracy of 97.00–102.38% (CV% 1.86–8.54). In all 18 calibration curves, the coefficients of determination were better than 0.99.

Betamethasone intra-day accuracy results obtained by analyzing six plasma samples at LLOQ, low, mid and high QC ranged between 98.67 and 101.08% (CV% 1.69–5.58); while for BA ranged 97.78–99.17% (CV% 1.76–1.48). Betamethasone phosphate intra-day accuracy ranged 96.39–102.04% (CV% 4.29–1.86). The inter-day precision and accuracy results obtained by analyzing six spiked samples of BET, BA and BP at LLOQ, low, mid and high QC over three consecutive days are depicted in Table 1. The limits of detection were 0.04 ng/ml for BET, 0.07 ng/ml for BA and 0.32 ng/ml for BP, while the lower limits of quantitation chromatograms of the three analytes are depicted in Fig. 5. Results are presented in Table 1.

For the two methods, the obtained results were within the acceptance criteria of no more than 20% deviation at LLOQ and no more than 15% deviation for standards above this point (LLOQ).

The matrix suppression was determined by injecting pure authentic standard solution of BET, BA or BP and internal

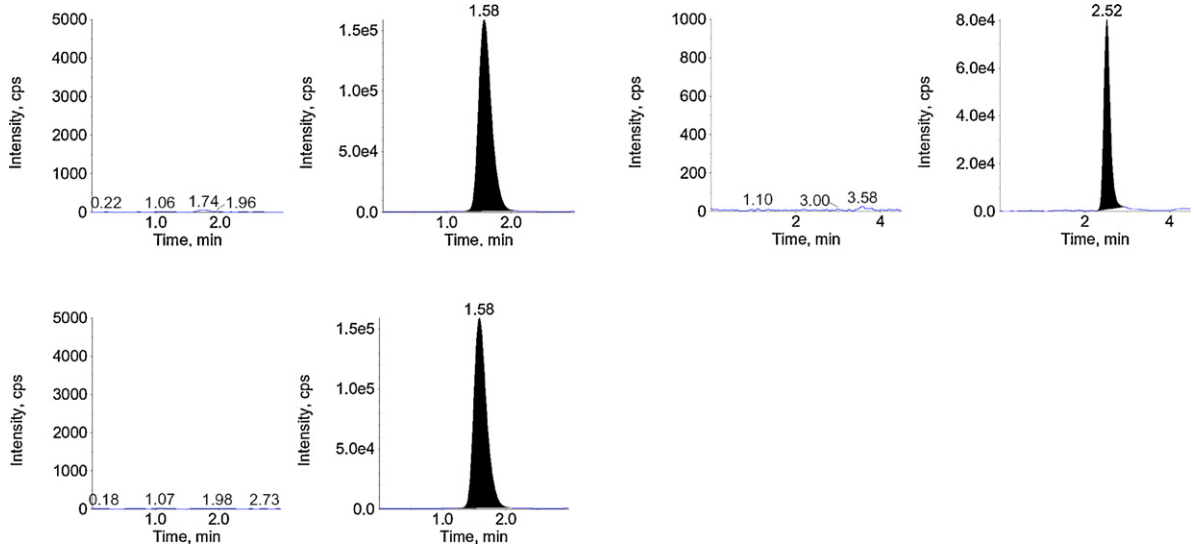

Fig. 4. LC–MS/MS chromatograms of BET, BA and BP blank plasma sample obtained from one of the volunteers listed top to bottom and left to right.

Table 1
Interday accuracy and precision.

	Betamethasone				Betamethasone acetate				Betamethasone phosphate			
	LLOQ	Low	Mid	High	LLOQ	Low	Mid	High	LLOQ	Low	Mid	High
N	18	18	18	18	17	18	18	18	18	18	18	17
Ratio per level	100%	100%	100%	100%	94%	100%	100%	100%	100%	100%	100%	94%
Mean of accuracy (%)	92.89	98.30	99.90	99.10	107.35	95.19	103.78	102.42	96.11	98.89	99.22	95.36
SD	10.20	5.33	2.25	4.71	7.10	4.39	3.52	2.14	10.37	3.62	1.56	3.24
CV (%)	10.98	5.42	2.25	4.76	6.61	4.61	3.39	2.09	10.79	3.66	1.57	3.40

N: number of QC samples included in the calculations of mean accuracy, SD and CV%.

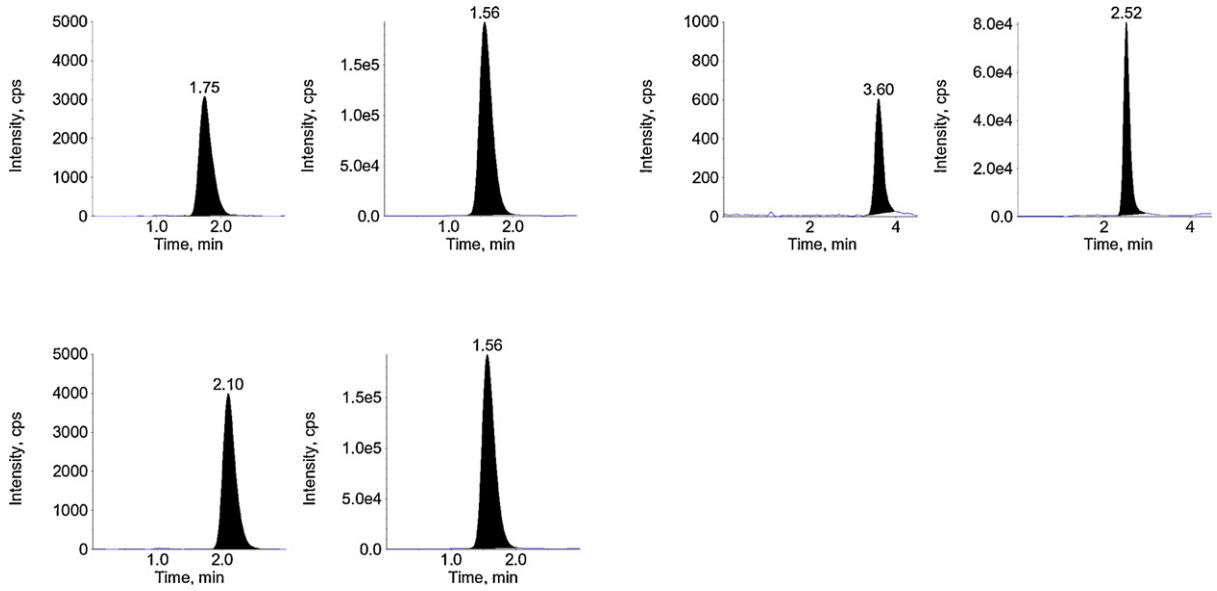

Fig. 5. LC–MS/MS chromatograms showing plasma samples spiked with BET, BA and BP at LLOQ, chromatograms are listed top to bottom and left to right.

standard at low, mid and high QC and compared to the same solution reconstituted in extracted blank human plasma from 6 different batches.

Matrix mean factor was found to be 5.17% (2.38 CV%) for BET and 4.35% (3.20 CV%) for BA; while for BP was found to be 1.90% (3.35 CV%).

The results of stability testing in blood proved that the arsenate stabilizer prevented the in vitro hydrolysis of BA to BET as this last was not detected (<0.2 ng/ml), while BA was found stable after 1 h (+4.18%). BP hydrolysis in blood was also prevented by the addition arsenate solution to blood as BET was determined to be +4.61% after 1 h of storage, while BP showed an increase of +3.24%. Both positive values reflected within criteria variability of the method rather than stability problem. The analysis of the stabilized blood spiked with BET showed that the analyte was stable after 1 h (+4.03%).

The addition of fluoride solution to plasma samples prevented the hydrolysis of BA and BP to BET as BET was not formed (<LLOQ) after leaving the stabilized plasma samples spiked with BA or BP for 1 h over the bench at room temperature.

Moreover, it was proved that the presence of the three moieties in the same sample did not show interference between the analytes. The analytes were stable for 1 h over the bench at room temperature (95.81% BET, 97.15% BA and 99.18% BP).

Table 2 shows the data representing the stability of BET, BA and BP in plasma samples over three cycles of freeze (−70 °C) and thawing (room temperature). The tests indicated that the analytes were stable in human plasma for three cycles of freeze and thaw when stored at −70 °C and thawed to room temperature.

The post-preparative stability of QC samples kept in the autosampler for 42 h (BET and BA) and for 17 h (BP) at 5 °C, was also assessed. The mean recoveries at low and high QC were 110. 61–111.11 for BET; 96.27–112.22 for BA and 91.94–100.37% for BP. Precision ranged between 1.28 and 5.20%. The results indicated that analytes can remain at the autosampler at 5 °C for at least 42 h (BET

Table 2
Freeze and thaw stability after 3 cycles at −70 °C.

Sample no.	Betamethasone		Betamethasone acetate		Betamethasone phosphate	
	QC lowAccuracy (%)	QC highAccuracy (%)	QC lowAccuracy (%)	QC highAccuracy (%)	QC lowAccuracy (%)	QC highAccuracy (%)
1	102.00	104.26	103.33	118.24	101.67	108.75
2	90.67	94.24	90.00	95.29	113.33	118.69
3	100.00	94.50	96.67	100.00	108.33	107.75
4	94.00	94.31	96.67	93.53	98.33	117.19
5	92.00	103.50	93.33	95.29	85.00	109.19
6	90.67	96.57	96.67	100.00	106.67	112.38
Mean of accuracy (%)	94.89	97.90	96.11	100.39	102.22	112.32
CV (%)	5.19	4.82	4.61	9.10	9.71	4.13

Table 3
Short term stability at room temperature.

Sample no.	Betamethasone 6 h		Betamethasone acetate 6 h		Betamethasone phosphate 5 h	
	QC low Accuracy (%)	QC high Accuracy (%)	QC low Accuracy (%)	QC high Accuracy (%)	QC low Accuracy (%)	QC high Accuracy (%)
1	117.33	94.95	116.67	85.88	101.67	102.81
2	109.33	104.40	100.00	104.12	96.67	99.63
3	110.00	95.07	106.67	106.47	100.00	99.19
4	114.00	100.60	110.00	102.94	90.00	94.31
5	100.67	100.86	96.67	94.12	95.00	100.13
6	89.33	104.26	93.33	101.76	81.67	103.81
Mean of accuracy (%)	106.78	100.02	103.90	99.22	94.17	99.98
CV (%)	9.57	4.20	8.47	7.82	7.82	3.34

Table 4
Long term stability at −70 °C.

Sample no.	Betamethasone 248 days		Betamethasone acetate 248 days		Betamethasone phosphate 121 days	
	QC low Accuracy (%)	QC high Accuracy (%)	QC low Accuracy (%)	QC high Accuracy (%)	QC low Accuracy (%)	QC high Accuracy (%)
1	105.33	102.07	113.33	114.71	80.00	97.31
2	97.33	100.90	106.67	105.88	91.67	98.88
3	99.33	103.29	106.67	117.65	95.00	99.69
4	98.00	99.26	103.33	112.35	93.33	105.19
5	100.67	101.67	106.67	117.65	106.67	97.00
6	106.67	100.86	113.33	114.71	90.00	103.69
Mean of accuracy (%)	101.22	101.34	108.33	113.82	92.78	100.29
CV (%)	3.85	1.34	3.77	3.85	9.28	3.38

and BA) and for 17 h (BP), without showing significant loss in the quantified values, and that samples should be processed within this period of time.

For short-term stability determination, stored plasma aliquots were thawed and kept at room temperature for a period of time exceeding that expected to be encountered during the routine sample preparation. Samples were extracted and analyzed as abovementioned. Results are given below in Table 3. Short-term stability indicated reliable stability behavior under the experimental conditions of the regular runs.

Table 4 summarizes the long-term stability data of the three analytes in human stabilized plasma samples stored for a period of more than four months at −70 °C. The stability study of BET, BA and BP in human stabilized plasma showed reliable stability behavior as the mean of the results of the tested samples were within the acceptance criteria of ±15% of the initial values. These findings indicated that storage of BET, BA and BP stabilized plasma samples at −70 °C is adequate, and no stability-related problems would be expected during the samples routine analysis for pharmacokinetics or clinical studies.

The recoveries were evaluated for BET, BA and BP by comparing peak areas of the extracted samples with the unextracted pure authentic standard solutions peak areas at two low and high QC. Results are presented in Table 5.

The stability of the stock solution of BET was tested and established for 20 days at −20 °C and was found to be 101.20% (CV% 1.54), while working solution kept at room temperature was stable for at least for 6 h with 100.98% recovery (1.12 CV%). Betamethasone acetate stock solution stability was evaluated as 99.02% (CV% 3.17), while working solution stability was 105.08% (1.14 CV%).

The stability of the stock solution of BP was tested for 36 days at −20 °C and was found to be 101.02% (CV% 1.19), while working solution at room temperature was stable for at least 5 h with 99.11% recovery (1.62 CV%).

4. Application

These two specific and sensitive methods were applied to analyze plasma samples obtained from six healthy volunteers who participated in a pilot pharmacokinetics study. Formal informed consent form was signed by each volunteer. The study protocol was approved by the institutional review board (IRB) and by Jordan FDA. Each subject received a single dose of 1 ml of Celestone® suspension containing 3 mg/3 mg betamethasone phosphate and acetate, administered by intramuscular injection. Each blood sample was split into 2 different tubes. The first blood portion (accurately 5 ml) was placed into a prechilled heparinized tube containing 50 μl of 2 M sodium arsenate solution as stabilizer. This stabilized portion

Table 5
Analytical recovery.*

	QC low	QC mid	QC high	QC low	QC mid	QC high	QC low	QC mid	QC high
N	12	12	12	12	12	12	12	12	12
Recovery (%)	73.49	69.76	69.87	78.99	72.71	71.65	79.98	78.06	78.34
Mean recovery (%)		71.04			74.45			78.79	
SD		2.13			3.97			1.04	
CV (%)		2.99			5.33			1.32	

N: number of QC samples included in the calculations of mean accuracy, SD and CV (%).
* Analytical recovery calculated by comparing peak area of (QC spiked plasma samples) with standard solution spiked in extracted blank plasma samples.

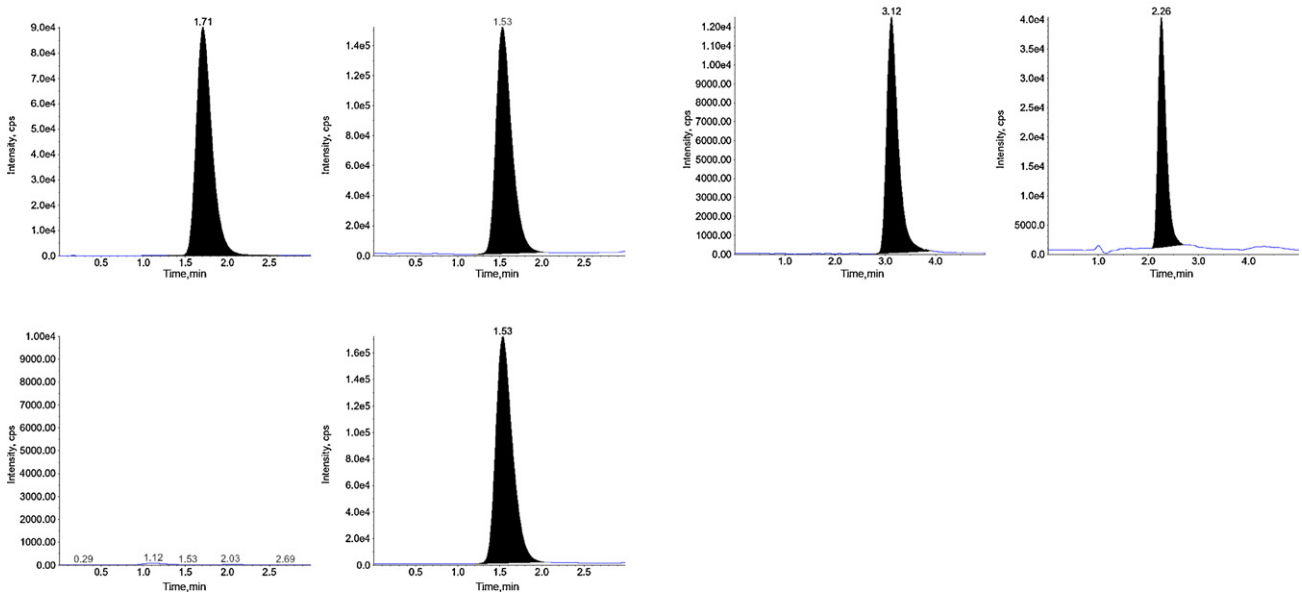

Fig. 6. Representative chromatograms of a plasma sample obtained from a volunteer after IM administration of 3 mg/3 mg BA and BP suspension. Backcalculated concentrations were 23.04 ng/ml for BET, below LLOQ for BA, while BP was 35 ng/ml. Chromatograms are listed top to bottom and left to right.

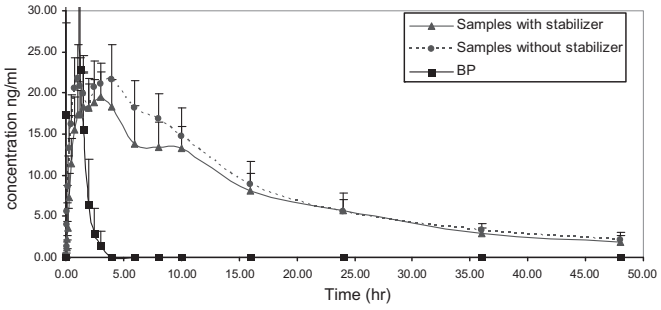

Fig. 7. Mean plasma concentration-time profiles of BET in six healthy subjects after a single-dose IM administration of a dual acting suspension containing 3 mg BA and 3 mg BP.

of the blood was centrifuged to plasma and siphoned into a plastic tube containing 10 μl of 50% (w/v) potassium fluoride solution per ml plasma. The second portion of blood, around 5 ml, was centrifuged to plasma and placed in a plain plastic tube. No stabilizer was added to this portion of blood or plasma. In both cases, the plasma samples were stored immediately at −70 °C until analysis. BET, BA and BP methods were applied to analyze both portions. Fig. 6 illustrates a representative chromatograms of a plasma sample obtained from a volunteer after IM administration of 3 mg/3 mg BA and BP suspension, while Fig. 7 shows mean BET pharmacokinetics profile of six subjects when their samples were analyzed with and without stabilizers. Pharmacokinetics parameters were as follows: C_{max}: 21.19 ± 2.29 with stabilizer; 22.09 ± 3.68 ng/ml without stabilizer; while AUC was 354.06 ± 76.28 stabilizer and 379.79 ± 56.25 ng/ml h without stabilizer. Betamethasone acetate was not detected in the plasma regardless the samples were stabilized or not, while, betamethasone phosphate was perfectly profiled in all subjects when samples were stabilized (Fig. 7).

5. Conclusion

The LC–MS/MS methods developed for the quantitation of BET, BA and PB in human plasma were proved accurate, precise, sensitive, specific and reproducible. Under the stabilization conditions used during blood and plasma samples processing, the hydrolysis of betamethasone acetate or phosphate to betamethasone was prevented. Betamethasone phosphate ester was only detected when blood and plasma samples were stabilized, while betamethasone PK profiling in human reflected the need for sample stabilization in order to predict the clinical efficacy properly.

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References

[1] Physician's Desk Reference (PDR), http://www.pdr.net/search/searchResult.aspx?searchCriteria=betamethasone.
[2] Martindale, The Complete Drug Reference, 34th edition, Pharmaceutical Press, London, UK, 2004 (Electronic version).
[3] M.C. Petersen, R.L. Nation, J.J. Ashley, Simultaneous determination of betamethasone, betamethasone acetate and hydrocortisone in biological fluids using high performance liquid chromatography, J. Chromatogr. 183 (1980) 131–139.
[4] W.S. Cheng, J.L. Chen, C.H. Chiang, Simultaneous determination of plasma betamethasone disodium phosphate and betamethasone in rabbit by high performance liquid chromatography, J. Food Drug Anal. 17 (2009) 348–356.
[5] M.N. Samtani, M. Schwab, P.W. Nathanielsz, W.J. Jusko, Stabilization and HPLC analysis of betamethasone sodium phosphate in plasma, J. Pharm. Sci. 93 (2004) 726–732.
[6] G.M. Rodchenkov, V.P. Uralets, V.A. Semenov, V.A. Gurevich, Gas chromatographic and mass spectral study of betamethasone synthetic corticosteroid metabolism, J. Chromatogr. 432 (1988) 283–289.
[7] D.G. Watson, C.N. McGhee, J.M. Midgley, G.N. Dutton, M.J. Noble, Penetration of topically applied betamethasone sodium phosphate into human aqueous humour, Eye (Lond.) 4 (1990) 603–606.
[8] J.J. Zou, L. Dai, L. Ding, D.W. Xiao, Z.Y. Bin, H.W. Fan, L. Liu, G.J. Wang, Determination of betamethasone and betamethasone 17-monopropionate in human plasma by liquid chromatography–positive/negative electrospray ionization tandem mass spectrometry, J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci. 873 (2008) 159–164.
[9] T.T. Qu, R. Zhang, B.J. Wang, X.Y. Liu, G.Y. Yuan, R.C. Guo, Determination of betamethasone in human plasma by liquid chromatography with tandem mass, Yao Xue Xue Bao 43 (2008) 402–407.
[10] D. Chen, Y. Tao, Z. Liu, H. Zhang, Z. Liu, Y. Wang, L. Huang, Y. Pan, D. Peng, Development of a liquid chromatography–tandem mass spectrometry with pressurized liquid extraction for determination of glucocorticoid residues in edible tissues, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 879 (2011) 174–180.
[11] D. Chen, Y. Tao, Z. Liu, Z. Liu, Y. Wang, L. Huang, Z. Yuan, Development of a liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the quantification of glucocorticoid residues in edible tissues of swine, cattle,

sheep, and chicken, Food Addit. Contam. A: Chem. Anal. Control Expo. Risk Assess. 10 (2010) 1363–1371.
[12] J.H. Andersen, L.G. Hansen, M. Pedersen, Optimization of solid phase extraction clean up and validation of quantitative determination of corticosteroids in urine by liquid chromatography–tandem mass spectrometry, Anal. Chim. Acta 617 (2008) 216–224.
[13] M.N. Samtani, M. Lohle, A. Grant, P.W. Nathanielsz, W.J. Jusko, Betamethasone pharmacokinetics after two prodrug formulations in sheep: implications for antenatal corticosteroid use, Drug Metab. Dispos. 33 (2005) 1124–1130.
[14] F. Oueslati, N. Ben Hamida, A. Toumi, H. Trabelsi, Effect of organic modifier and temperature on the resolution of betamethasone and dexamethasone using a porous graphitic carbon column: application to their identification and confirmation in human urine by LC–ESI-MS/MS, J. Sep. Sci. 18 (2007) 3137–3142.
[15] E.M. Malone, C.T. Elliott, D.G. Kennedy, L. Regan, Screening and quantitative confirmatory method for the analysis of glucocorticoids in bovine milk using liquid chromatography–tandem mass spectrometry, J. AOAC Int. 93 (2010) 1656–1665.
[16] C. Li, Y. Wu, T. Yang, Y. Zhang, Rapid simultaneous determination of dexamethasone and betamethasone in milk by liquid chromatography tandem mass spectrometry with isotope dilution, J. Chromatogr. A 1217 (2010) 411–414.
[17] E.M. Malone, G. Dowling, C.T. Elliott, D.G. Kennedy, L. Regan, Development of a rapid, multi-class method for the confirmatory analysis of anti-inflammatory drugs in bovine milk using liquid chromatography tandem mass spectrometry, J. Chromatogr. A 1216 (2009) 8132–8140.
[18] M. Li, X. Wang, B. Chen, M. Lin, A.V. Buevich, T.M. Chan, A.M. Rustum, Use of liquid chromatography/tandem mass spectrometric molecular fingerprinting for the rapid structural identification of pharmaceutical impurities, Rapid Commun. Mass Spectrom. 22 (2009) 3533–3542.
[19] M. Li, M. Lin, A. Rustum, Application of LC–MS(n) in conjunction with mechanism-based stress studies in the elucidation of drug impurity structure: rapid identification of a process impurity in betamethasone 17-valerate drug substance, J. Pharm. Biomed. Anal. 48 (2008) 1451–1456.
[20] R. Willoughby, E. Sheehan, S. Mitrovich, A global view of LC/MS, 2nd edition, Global View Publishing, Pittsburgh, PA, 2002.
[21] Guidance for Industry. Bioanalytical Method Validation, US Department of Health and Human Services Food and Drug Administration, Center for Drug Evaluation and Research (CDER), 2001.