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Quantification of betamethasone in human plasma by liquid chromatography–tandem mass spectrometry using atmospheric pressure photoionization in negative mode

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Abstract

Betamethasone is a synthetic corticosteroid designed to exert a marked glucocorticoid activity. As the free alcohol, betamethasone finds widespread clinical applications related to its anti-inflammatory and immunosuppressant activity.

In the present study, a fast, sensitive, robust method was developed for the determination and quantification of betamethasone in human plasma by liquid chromatography coupled with tandem mass spectrometry, using photospray ionization in negative mode.

Betamethasone was extracted from 0.5 ml human plasma by liquid–liquid extraction (LLE) using chloramphenicol as internal standard. The method has a chromatographic run of 2.5 min using a C_{18} analytical column (100 mm × 2.1 mm i.d.) and the linear calibration curve over the range was linear from 0.05 to 50 ng ml^{-1} ($r^2 > 0.993$). The between-run precision, based on the relative standard deviation replicate quality controls was 94.1% (0.15 ng ml^{-1}), 90.7% (4.0 ng ml^{-1}) and 97.2% (40 ng ml^{-1}). The between-run accuracy for the above-mentioned concentrations was 11.9, 9.0 and 9.8%, respectively. The method herein described was employed in a bioequivalence study of two formulations of dexchlorpheniramine/betamethasone 2 mg/0.25 mg tablets.
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Keywords: Betamethasone; Corticosteroid; Photospray; Human plasma

1. Introduction

Glucocorticoid drugs are synthetic analogues of hormones and these substances suppress inflammation in a wide variety of diseases, including allergic diseases, rheumatoid arthritis, inflammatory bowel disease and autoimmune diseases [1]. The most widespread use of glucocorticoids is in asthma and inhaled glucocorticoids have revolutionized treatment and now become the mainstay of therapy for patients with chronic disease, and are important drugs in therapy of acute lymphoblastic leukemia [2,3]. Betamethasone is a synthetic corticosteroid designed to exert a marked glucocorticoid activity. As the free alcohol, betamethasone finds widespread clinical applications related to its anti-inflammatory and immunosuppressant activity.

Several analytical methods mainly based on high-performance liquid chromatography (HPLC) [4–7], HPLC coupled to mass spectrometry (HPLC–MS) [8–10], high resolution gas chromatography coupled to mass spectrometry (HRGC–MS) [11,12], and recently HPLC coupled to tandem mass spectrometry (HPLC–MS–MS) [13–16] have been used for the quantification of betamethasone in plasma.

In 2000 it was introduced a new ionization mode to LC–MS system: atmospheric pressure photoionization (APPI) [17]. In APPI the ionization is initiated by 10 eV photons emitted by a krypton discharge lamp. The initial reaction in APPI is the formation of a radical cation of the dopant by 10 eV photons. For this reaction to occur, the ionization energy of the dopant has to be lower than the energy of the photons (for this reason toluene is normally used as dopant), and finally the dopant radical cations ionize the analytes through charge exchange [17,18].

Here we described a specific, sensitive and fast LC–MS–MS method using the APPI in negative mode for quantification

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of betamethasone in human plasma. The procedure requires a simple liquid–liquid extraction (LLE) and was developed for pharmacokinetics studies. This method was applied to a bioequivalence study in healthy volunteers ($n = 26$).

2. Experimental

2.1. Chemicals and reagents

Betamethasone and chloroamphenicol (internal standard) were obtained from USP (Rockville, MD, USA) and Sigma–Aldrich (St. Louis, MO, USA), respectively. Acetonitrile (HPLC grade) was purchased from J.T. Baker (Phillipsburg, NJ, USA) while the formic acid, analytical grade was purchased from Merck (Rio de Janeiro, Brazil). Ultrapure water was obtained from a Gradient Millipore system (São Paulo, Brazil). Blank blood was collected from healthy, drug-free volunteers. Plasma was obtained by centrifugation of blood treated with anticoagulant sodium heparin. Pooled plasma was prepared and stored at approximately −70 °C until needed.

2.2. Calibration standards and quality controls

The stock solution of betamethasone was prepared in acetonitrile/water 1:1 at concentration of 1 mg ml^{-1}. Calibration curve for betamethasone was prepared in blank human plasma at concentrations of 0.050, 0.10, 0.20, 1.0, 5.0, 10, 20 and 50 ng ml^{-1} and performed in duplicate in each batch. Quality control samples were prepared in blank plasma at concentrations of 0.15; 4.0 and 40 ng ml^{-1} (QCA, QCB and QCC, respectively).

2.3. Sample preparation

Aliquots (0.50 ml) of human plasma were employed for liquid–liquid extraction with addition of IS solution (50 μl of the working standard solution). The tubes were vortex mixed for 20 s and allowed to stand at room temperature for 2 min. Four milliliters of diethyl ether-hexane (80:20, v/v) were added and the samples were vortex mixed for 40 s, the upper layer transferred to clean tubes and the solvent evaporated under N_2 (40 °C). The dry residue was re-dissolved with 200 μL of acetonitrile:water (50:50, v/v). The samples were transferred into glass microvials, capped and placed in an autosampler.

2.4. Liquid chromatography and mass spectrometry conditions

An HPLC system (LC10AD, Shimadzu, Japan) consisting of a pump and an autosampler was used for all analysis. The chromatographic system consisted on a C_{18} Genesis analytical column (100 mm × 2.1 mm id, 4 μm particle diameter, Grace Vydac, Hesperia, CA, USA) and the mobile phase was a mixture of acetonitrile/water (80:20, v/v, 10 mM of ammonium acetate) at a flow rate of 350 μL min^{-1}. The total run time was set for 2.5 min. The column was operated at room temperature and present a void time of 0.8 min. The temperature of the autosampler was maintained at 8.0 °C and was set up to make 40 μL sample injection. Mass spectrometry was performed in a Sciex API 3000 triple stage quadrupole mass spectrometry (Applied Biosystems, Foster City, CA, USA) equipped with an APPI source operating in negative mode (PS-). The source block temperature was set at 400 °C and the photoionization capillary voltage at 1.2 kV, nitrogen was used as the collision gas, and toluene was used as dopant at a flow of the 10 μL/min performed in a Harvard infusion pump 22 (Harvard Apparatus, Holliston, MA, USA). The ions monitored in Multiple Reaction Monitoring (MRM) and the conditions were described in Table 1. Data were acquired by Analyst software (1.3.4, Applied Biosystems) and calibrations curves for the analyte were constructed using the betamethasone and IS peak-area ration via a weighted ($1/x^2$) least-squares linear regression. Unknown sample peak-area ratios were then interpolated from the calibration curve to provide concentrations of betamethasone.

2.5. Specificity/selectivity

Each blank sample of five different pools of plasmas including a pool of lipemic and another of haemolyzed, were tested for interference using the proposed extraction procedure and analytical conditions.

2.6. Recovery

Preliminary experiments were conducted to evaluate the recovery with the extraction method described above. The percentage recovery was calculated as the ratio of the peak area for extracted blank plasma spiked at each standard concentration (0.15, 4.0 and 40 ng ml^{-1}) relative to peak area of the equivalent blank plasma samples spiked after the extraction.

2.7. Stability

Quality control samples prepared to test stability (0.15, 4.0 and 40 ng ml^{-1}) were subjected to short-term (6 h) room temperature, three freeze–thaw cycles and 24 h, long-term (21 days), autosampler (8 °C) stability tests. Subsequently, the bethametasone concentrations were measured in comparison with freshly prepared samples.

Table 1
Conditions of MRM in APPI in negative mode

Compound	Transition (m/z)	IS (V)	Temperature (°C)	DP (V)	FP (V)	CE (eV)	CXP (V)
Betamethasone	417.2/357.2	−1200	400	−80	−160	−26	−21
Chloroamphenicol	373.1/317.4	−1200	400	−20	−120	−26	−9

2.8. Precision and accuracy

To assess the precision and accuracy of the developed analytical method, three distinct concentrations in the range of expected concentrations where evaluated using eight determinations per concentration.

Precision and accuracy was assessed at within-day basis (intra-batch), which defines those parameters during a single analytical run; and at between-day basis (inter-batch), which measure the between day variability, possibly involving different analysts, reagents, etc.

2.9. Bioequivalence study

The method was applied to evaluate the bioequivalence of two tablet formulations of dexchlorpheniramine/betamethasone 2 mg/0.25 mg in healthy volunteers ($n = 26$): dexchlorpheniramine/betamethasone (test formulation from EMS Indústria Farmacêutica Ltda, Brazil; lot no. MABETLG01, expiry date January 2005) and Celestamine® (standard reference formulation from Schering Plough; lot no. 14616A, expiry date November 2006).

Twenty-six healthy volunteers were selected for the study. The study was a single dose, two-way randomized crossover design with a 3-week washout period between the doses. Blood samples were collected before and 0.5, 0.75, 1, 1.15, 1.50, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 10, 12, 16, 24, 48, 72, 96, 120 and 144 h post-dosing.

The bioequivalence between the two formulations was assessed according to US-FDA methodology.

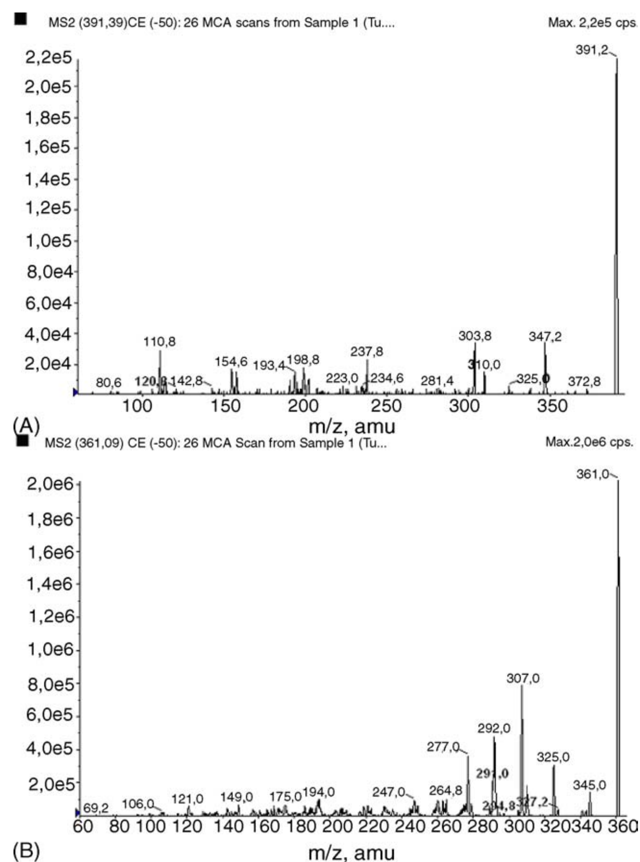

Fig. 1. Product ion mass spectrum of the betamethasone (A) and [bematehasone —CH_2O—H] (B).

3. Results and discussion

3.1. Method development

Photospray ionization is believed to be a near-universal and efficient ionization mode for many different classes of substances, including apolar substances such as polycyclic aromatic compounds. One advantage of this ionization system is that the molecule ionization is soft and produces minimal fragmentations and predominant molecular ion signal [19]. With betamethasone, it was observed in negative mode the formation of the molecular related ion $[M–H–CH_2O]^-$ of m/z 361 (Fig. 1), ten times higher than the deprotonated molecular ion $[M - H]^-$ of m/z 391. The formation of the ion $[M–H–CH_2O]^-$ is probably formed in the source by the loss of formaldehyde (CH_2O), involving the cleavage of $C_{20}–C_{21}$ (Fig. 2). This was previously observed by the collision-induced dissociation of the ion [M + acetate]⁻ in electrospray ionization (ESI) tandem mass spectrometry [20].

Despite of chloramphenicol be chemically unrelated with betamethasone, it was chosen as internal standard due to similar retention time (1.1 min for betamethasone and 1.2 min for chloramphenicol), response factor, and recuperation. In the case of chloramphenicol the deprotonated molecule $[M–H]^-$ was the base peak in the mass spectrum (Fig. 3). The most intense transition was m/z 321 → m/z 152, probably due to the cleavage of the carbon-carbon bond on the alkyl branch as shown in Fig. 4. Other intense fragment ions observed are the m/z 121 and m/z 257. The fragment ion of m/z 121 may be the nitrophenyl fragment and the m/z 257 might be explained by a charge migration hydrogen shift with a concerted loss of HCl and CO. The fragmentation of the chloroamphenicol in APPI is similar to the previously reported analysis by electrospray negative mode tandem mass spectrometry [21], and the literature data about the use of LC–MS in chloramphenicol residue analysis in food show that the mechanism of APCI with the negative ion mode is that the chloramphenicol moiety captures electrons generated from the corona discharge and loses a proton to become negatively charged due to a high electron affinity. In APPI, a similar mechanism or, alternatively, the ion-molecule reacting with the ion in the mobile phase, which leads to the formation of the observed $[M–H]^-$ ion seems to occur [22].

Due to the high intensities of the m/z 361 → 307 (betamethasone) and m/z 321 → 152 (IS) transitions and no detectable interference in blank human plasma samples these transitions were used in the present method.

The limit of quantification (LOQ) was validated for 50 pg ml^{-1} and with a run time of the 2.5 min. The mass chromatograms of a blank and LOQ samples are shown in Fig. 5.

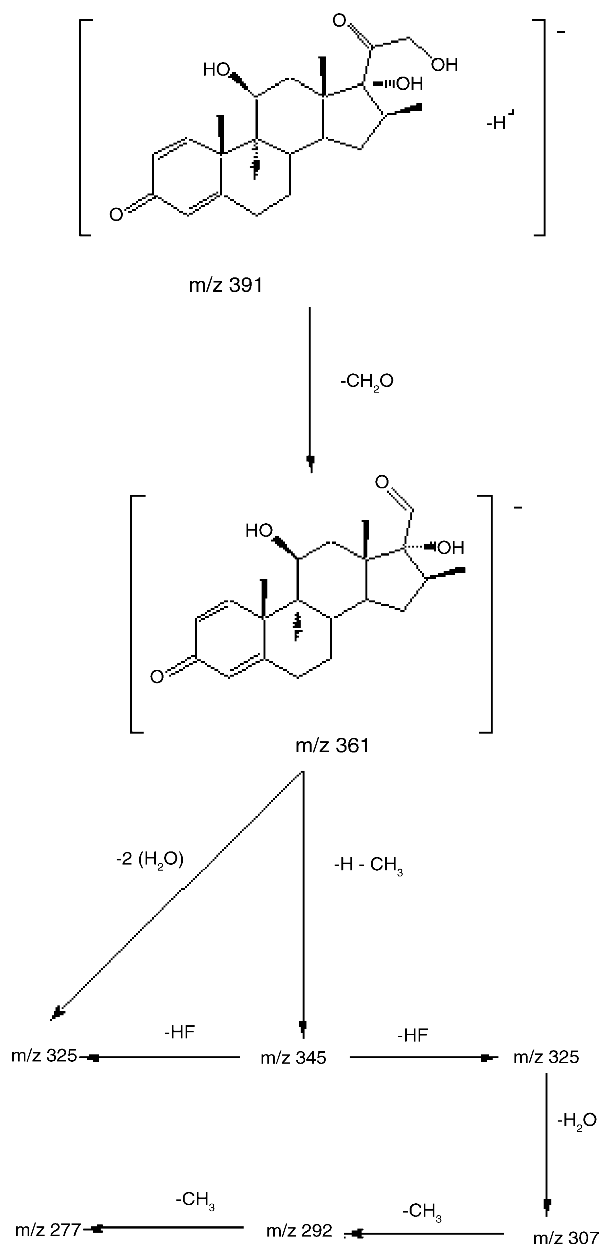

Fig. 2. Proposed mass fragmentation pathways for the [bematehasone —CH_2O—H].

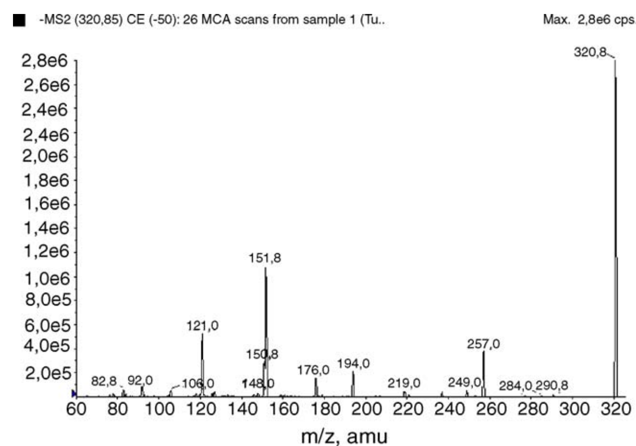

Fig. 3. Product ion mass spectrum of the chloroamphenicol.

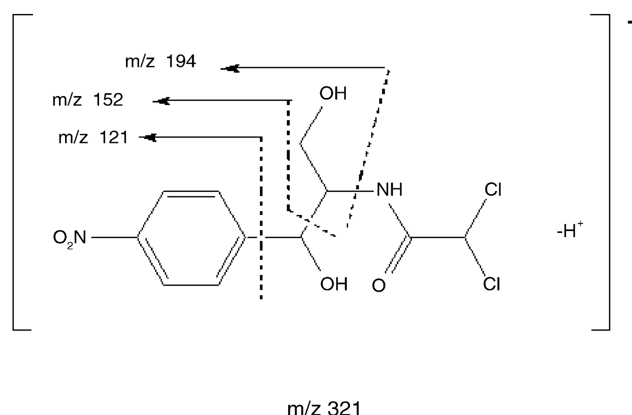

Fig. 4. Typical mass fragmentation pathways for the chloroamphenicol.

3.2. Assay performance

The optimized method was validated by assessment of recovery, linearity, quantification limit, precision and accuracy. Coefficients of variation and relative errors of less than 15 % were considered acceptable, except for the quantification limit (LOQ), whose values were extended to 20%, as recommended by Shah et al. [23] and Bressole et al. [24] for the analysis of biological samples for pharmacokinetic studies. The method was linear

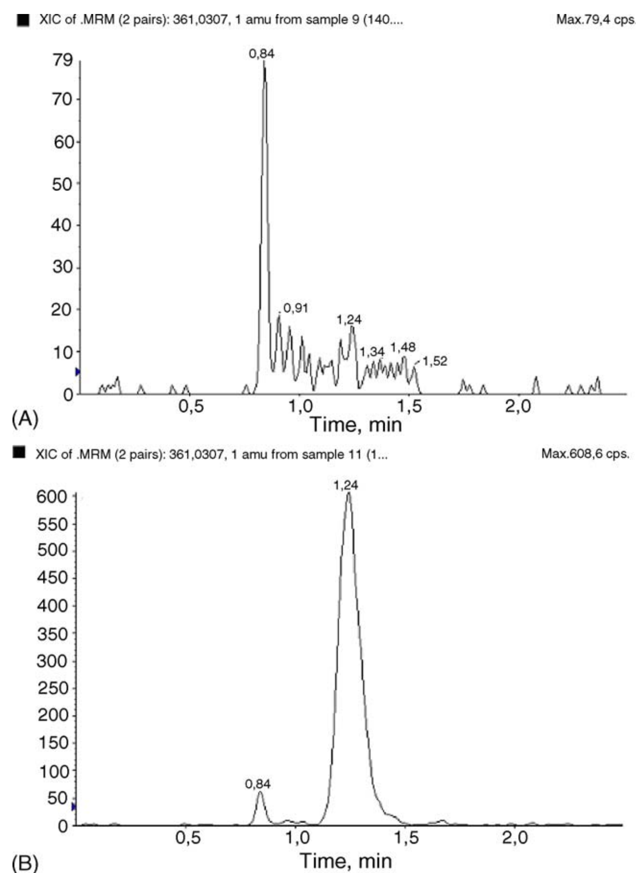

Fig. 5. (A) MRM chromatogram of blank pooled human plasma for the betamethasone (*m/z* 361) and (B) MRM chromatogram of the betamethasone spiked in human plasma at a final concentration of 50 pg ml^{-1}.

Table 2
Validations with the quality controls (QC) having the results of the accuracy and precision of the betamethasone

	Parameter	Nominal concentration (ng ml^{-1})			
		0.050	0.15	4.0	40
Intra-batch	Mean found ($n=8$) (ng ml^{-1})	0.043	0.141	3.71	40.0
	Precision (%)	17.5	11.4	9.5	5.2
	Accuracy (%)	86.8	93.8	92.5	100.0
Inter-batch	Mean found ($n=3$) (ng ml^{-1})	0.046	0.138	3.63	38.9
	Precision (%)	13.0	8.7	9.0	9.8
	Accuracy (%)	93.8	92.3	90.7	97.2

for the betamethasone from 0.05 to 50 ng ml^{-1} ($r^2 > 0.9970$) on repeated calibration curves.

The recovery of Betamethasone and the IS were calculated from the peak area ratios of extracted human plasma previously spiked at final concentrations (for analyte and IS) of 0.15, 4.0 and 40 ng ml^{-1} ($n=15$ for each concentration) were to betamethasone 92.6; 99.8 and 97.2%, respectively and to chloramphenicol 102.2, 94.9 and 95.8%. Between- and within-run accuracy and precision for the quality controls are summarized in Table 2.

No matrix effect was observed, this was evaluate the ion suppression effect, based on post-column mixing of the analyte of interest with the eluate of a column to which a blank sample is injected, has been proposed by Bonfiglio et al. [25].

The stability tests performed indicated no significant degradation under the conditions described above, including in the freeze and thaw test, short-term room temperature test and long-term test (28 days). The human plasma spiked at final concentrations of 0.15, 4.0 and 40 ng ml^{-1} ($n=5$ for each concentration). In the latter case (long-term test) a variation of −2.0, −2.6 and −3.5%, respectively, were determined relative to freshly spiked samples.

3.3. Bioequivalence

The geometric mean and respective 90% confidence interval (Cl) of betamethasone/Celestamine® percent ratios were 107.61% (101.62–113.95%) for AUC_{last}, 106.93% 102.08–112.00% for $AUC_{0\text{-}inf}$ and 105.06% (96.56–114.31%) for C_{max}. After the oral administration of the dexchlorpheniramine/betamethasone tablets to the volunteers, the observed betamethasone peak plasma mean concentration (C_{max}) values

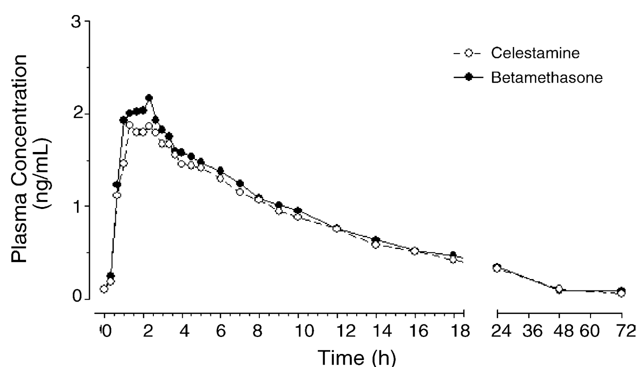

Fig. 6. Mean concentration data vs. time.

were in Fig. 6. In addition, the calculated 90% Cl for mean C_{max}, AUC_{last} and AUC_{inf} betamethasone/celestamine® individual ratios were within the 80–125% interval defined by the US Food and Drug Administration [26].

4. Conclusions

This is the first method to measured betamethasone using LC–MS–MS with the photoionization source in negative mode for the quantification of betamethasone in human plasma. This method offers advantages over those previously reported, in terms of a simple liquid–liquid extraction without clean-up procedures and a faster run time (2.5 min). The LOQ of 50 pg ml^{-1} is sufficient for pharmacokinetics studie (the estimate LOQ/$C_{max} < 3\%$) and could be further improved by sample concentration if required. The assay performance results indicate that the method is precise and accurate enough for the routine determination of the betamethasone in human plasma.

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