Research Article

Journal of MASS SPECTROMETRY

Received: 18 October 2010 Accepted: 12 January 2011 Published online in Wiley Online Library: 2011

(wileyonlinelibrary.com) DOI 10.1002/jms.1896

Determination of triamcinolone in human plasma by a sensitive HPLC–ESI-MS/MS method: application for a pharmacokinetic study using nasal spray formulation

Isabela Costa César,[a,b]* Ricardo Martins Duarte Byrro,[a,b] Fabiana Fernandes de Santana e Silva Cardoso,[a] Iram Moreira Mundim,[a] Leonardo de Souza Teixeira,[a] Weidson Carlo de Sousa,[a] Sandro Antônio Gomes,[a] Karini Bruno Bellorio,[a] Juliana Machado Brêtas[b] and Gerson Antônio Pianetti[b]

A liquid chromatography–electrospray ionization tandem mass spectrometry (HPLC–ESI-MS/MS) method for the quantitation of triamcinolone in human plasma after nasal spray application was developed and validated. Betamethasone was used as internal standard (IS). The analytes were extracted by a liquid–liquid procedure and separated on a Zorbax Eclipse XDB C_{18} column with a mobile phase composed of 2 mM aqueous ammonium acetate pH 3.2 and acetonitrile (55 : 45). Selected reaction monitoring was performed using the transitions *m/z* 435 → 415 and *m/z* 393 → 373 to quantify triamcinolone acetonide and betamethasone, respectively. Calibration curve was constructed over the range of 20–2000 pg/ml for triamcinolone acetonide. The lower limit of quantitation was 20 pg/ml. The mean RSD values were 4.6% and 5.7% for the intra-run and inter-run precision, respectively. The mean accuracy value was 98.5% and a recovery rate corresponding to 97.5% was achieved. No matrix effect was detected in the samples. The validated method was successfully applied to determine the plasma concentrations of triamcinolone acetonide in healthy volunteers, in a pharmacokinetic study with nasal spray formulation.

Keywords: triamcinolone; nasal spray; HPLC-ESI-MS/MS; plasma; pharmacokinetics

Introduction

Allergic rhinitis is a common inflammatory condition characterized by nasal congestion, rhinorrhea, sneezing, nasal itch and postnasal drainage and it has been shown to have a significant impact on quality of life.[1] Treatment of allergic rhinitis has been revolutionized by the introduction of topical nasal steroid sprays, which are the commonest prescription from otolaryngology departments. The regular use of nasal steroid sprays reduces nasal obstruction, rhinorrhea and overall nasal symptom scores.[2]

Triamcinolone acetonide (Fig. 1) is a synthetic glucocorticoid which has been formulated as both an aerosol and an aqueous metered-dose pump spray for nasal inhalation in the treatment of allergic rhinitis. Clinical trials with either formulation have shown that once-daily triamcinolone acetonide 110–220 μg reduces symptoms of allergic rhinitis within the first day of administration.[3,4] Triamcinolone acetonide treatment is well tolerated, with low incidence of adverse effects. In addition, the triamcinolone aqueous nasal spray presents high patient preference compared with other glucocorticoids[5] and may be considered a first-line therapy option in adults with moderately severe seasonal allergic rhinitis with predominantly nasal symptoms.[3]

A drug administered nasally and intended for local action has the potential to produce systemic activity, although plasma levels do not in general reflect the amount of drug reaching nasal sites of action. Systemic exposure following nasal administration can occur either from drug absorbed into the systemic circulation from the nasal mucosa, or after ingestion and absorption from the gastrointestinal tract. Although systemic absorption may contribute to clinical efficacy for certain corticosteroids and antihistamines, the consequences of systemic absorption (e.g. hypothalamic–pituitary–adrenal axis suppression by corticosteroids) are generally undesirable.[6,7] Hence, the approach recommended by Food and Drug Administration (FDA) for establishing the biovailability of suspension formulations of locally acting nasal drug products, both aerosols and sprays, is to conduct *in vivo* studies in addition to *in vitro* studies.[7]

* *Correspondence to: Isabela Costa César, Departamento de Produtos Farmacêuticos, Faculdade de Farmácia, Universidade Federal de Minas Gerais, Av. Pres. Antônio Carlos 6627, 31270-901 Belo Horizonte, MG, Brazil. E-mail: isaccesar@bol.com.br*

a *Instituto de Ciências Farmacêuticas, Alameda Coronel Eugênio Jardim 53, 74175-100 Goiânia, GO, Brazil*

b *Departamento de Produtos Farmacêuticos, Faculdade de Farmácia, Universidade Federal de Minas Gerais, Av. Pres. Antônio Carlos 6627, 31270-901 Belo Horizonte, MG, Brazil*

Journal of MASS SPECTROMETRY

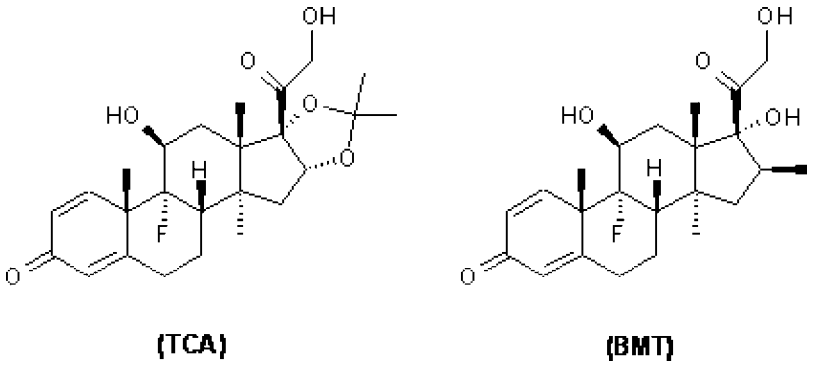

Figure 1. Chemical structures of triamcinolone acetonide (TCA) and betamethasone (BMT).

Few methods have been reported for determining triamcinolone acetonide in plasma, using high-performance liquid chromatography coupled to ultraviolet[8] or mass spectrometry detection.[9,10] However, these studies were performed after intramuscular,[8] oral[9] or intraocular[10] administration, so that there are no available studies reporting the pharmacokinetics of triamcinolone acetonide after the application of nasal sprays. Some studies were carried out aiming to evaluate the pharmacokinetics or bioequivalence of other drugs administered using nasal spray,[11–13] and the main required feature of the analytical methods seems to be the high sensitivity, considering the low systemic absorption provided by this formulation.

Hence, the aim of this work was to develop and validate a sensitive HPLC–ESI-MS/MS method for the quantitation of triamcinolone acetonide in human plasma, after nasal spray administration. The method was applied to a pharmacokinetic study in healthy volunteers who received triamcinolone acetonide nasal spray.

Experimental

Chemicals and reagents

Triamcinolone acetonide and betamethasone (internal standard) (Fig. 1) reference standards were purchased from the United States Pharmacopoeia (Rockville, MD, USA). Ultra-pure water was obtained from a Millipore System (Bedford, MA, USA). Acetonitrile (HPLC grade) was purchased from Tedia (Fairfield, OH, USA) and formic acid, ammonium acetate, methylene chloride, ethyl acetate and methyl-*t*-butyl ether (analytical grade) were obtained from J.T. Baker (Phillipsburg, NJ, USA).

Instrumentation and analytical conditions

The HPLC–ESI-MS/MS analyses were carried out on an Agilent 1200 system (Santa Clara, CA, USA), composed of a quaternary pump, an autosampler, a column oven and an API 5000 triple quadrupole mass spectrometer (MDS-SCIEX, Concord, Ontario, Canada), equipped with an electrospray ion source. Analyst v.1.4.2 software was used for data acquisition and analysis. LC separation was performed on a Zorbax Eclipse XDB C_{18} (50 mm × 4.6 mm i.d.; 1.8 µm particle size) from Agilent, at 30 °C. The mobile phase consisted of 2 mM aqueous ammonium acetate (pH 3.2) adjusted with formic acid and acetonitrile (55 : 45), at a flow rate of 1 ml/min. The run time was 1.6 min and the injection volume was 10 µl.

Mass spectrometric detection was performed using electrospray ion source in positive ionization mode. The turbo-gas temperature was 700 °C, with an ion spray needle voltage of 4000 V, declustering potential of 76, nebulizer gas setting of 50, curtain gas setting of 10 and collision gas setting of 8. The collision energies were optimized at 15 V for triamcinolone acetonide and 13 V for betamethasone. Selected reaction monitoring (SRM) was employed for data acquisition. The SRM fragmentation transitions were *m/z* 435 → 415 and *m/z* 393 → 373 for triamcinolone acetonide and betamethasone, respectively. The scan dwell time was set at 0.15 s for each channel.

Preparation of standard solutions

Stock solutions of triamcinolone acetonide and betamethasone were prepared by dissolving the accurately weighed reference substances in acetonitrile. Working solution of triamcinolone acetonide was prepared by diluting the stock solution with acetonitrile and water (50 : 50) to a final concentration of 1 µg/ml.

The working solution of betamethasone (IS) was prepared by diluting the stock solution with acetonitrile to a final concentration of 25 ng/ml. All stock solutions were prepared immediately before the use.

Preparation of calibration and QC samples

Six calibrations samples were prepared by spiking appropriate amounts of the working solution of triamcinolone acetonide in blank plasma. The volume of the working solutions added did not exceed 1% of the total plasma volume. The blank plasma samples were obtained from healthy volunteers, who were not taking any medication, using heparin as anticoagulant. The concentration of the calibration samples in plasma was 20, 100, 500, 1000, 1500 and 2000 pg/ml of triamcinolone acetonide. Quality control (QC) samples in plasma were prepared in a similar way, at high, middle and low concentrations: 60, 800 and 1600 pg/ml.

Sample preparation

A 50-µl aliquot of the IS solution (25 ng/ml of betamethasone in acetonitrile) was added to 500 µl of plasma sample. The sample was vortex mixed for 10 s. A 3-ml aliquot of ethyl acetate, methylene chloride and methyl-*t*-butyl ether (4 : 3 : 3) was added and vortex mixed for 60 s. Then, the samples were centrifuged at 3400 rpm for 6 min. A 2.4 ml aliquot of the organic layer was transferred and evaporated to dryness using an evaporator at 40 °C under a stream of nitrogen. The dried extract was reconstituted in mobile phase (200 µl) and a 10-µl aliquot was injected into the chromatographic system.

Method validation

The validation process was carried out according to Guidance for Industry – Bioanalytical Method Validation, recommended by US Food and Drug Administration.[14]

The selectivity of the method was evaluated by assaying human blank plasma samples from six different donors, including one lipemic and one hemolyzed plasma. These samples were compared with those containing triamcinolone acetonide at the lower limit of quantitation (LLOQ) or betamethasone at 2.5 ng/ml. In addition, plasma samples spiked with caffeine (1 μg/ml), chlorpheniramine (76 ng/ml), metamizole (5 μg/ml) and acetaminophen (20 μg/ml) were also evaluated to ensure no interference in the method.

Linearity was assessed by six-point calibration curves in human plasma in duplicate in three consecutive days. The curves were constructed by plotting the peak area ratio of triamcinolone acetonide to the IS versus the concentration. The concentration range evaluated was 20–2000 pg/ml. The curves were evaluated by residuals and fitted by weighted linear regression. The LLOQ was established as the lowest concentration of calibration curve at which precision was within 20% and accuracy was within 20%, by means of the analyses of five replicates. In addition, the analyte response at this concentration should be at least five times the baseline noise.

To evaluate the precision and accuracy of the method, QC samples at three concentration levels (60, 800 and 1600 pg/ml of triamcinolone acetonide) were analyzed in six replicates on three different days. Intra-run and inter-run precision were calculated and expressed as relative standard deviation (RSD%).

The extraction recovery of the method was determined by comparing the peak areas obtained from the plasma samples with those of direct injected standards, at the same concentration. It was evaluated by analyzing five replicates containing 60, 800 and 1600 pg/ml of triamcinolone acetonide. The recovery of IS was determined in a similar way, at the working concentration (2.5 ng/ml of betamethasone).

The matrix effect was evaluated to verify whether the potential ion suppression or enhancement due to the co-elution matrix components existed in the analysis. The peak areas of triamcinolone acetonide and IS from the spike-after-extraction samples were compared with those of the standard solutions in the mobile phase, at the same concentrations. This experiment was carried out with blank plasma samples from six different donors, at low and high QC concentrations of triamcinolone acetonide and working concentration of betamethasone (2.5 ng/ml).

The stability of the analyte in plasma was evaluated under a variety of storage and handling conditions using the low and high QC samples, in six replicates. Freeze–thaw stability was evaluated after three complete freeze/thaw cycles (−70 to 23 °C) on consecutive days. Short-term temperature stability was assessed by analyzing samples that were kept at ambient temperature (23 °C) for 11 h. Long-term stability was performed at plasma samples that were stored at −70 °C, for 11 months. To evaluate the post-preparative stability, QC samples were extracted and kept in the autosampler (10 °C) for 34 h before the injection. The stabilities of the working solutions of triamcinolone and IS at 4 °C for 4 days were also evaluated. The analytes were considered stable when 85–115% of the initial concentrations were found.

Application to a pharmacokinetic study

The validated method was used to determine the plasma concentration of triamcinolone acetonide in a pharmacokinetic study using a nasal spray formulation (Nasacort®, Aventis Pharma). The administration of the drug was performed in a room isolated from external contamination and an adequate cleaning in the nostrils of the volunteers was carried out before the administration of the product. Sixty subjects received either four sprays, two per nostril, alternately, corresponding to 220 μg of triamcinolone acetonide. Then, the volunteers received 200 ml of water and sat with the neck turned back for an hour. During the administration procedure, the volunteers used disposable clothes that were subsequently discarded.

The blood samples (10 ml) were collected into heparinized tubes at 0, 5, 10, 20, 30, 40, 50 min and 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 12 and 24 h after drug administration. Plasma samples were obtained by centrifugation and frozen at −70 °C until analyses. The study protocol was approved by the Ethics Committee of Universidade Estadual de Campinas.

Results and Discussion

Conditions for HPLC–ESI-MS/MS

The first step of the mass spectrometric detection consisted in recording the MS spectra of both triamcinolone acetonide and betamethasone. From full-scan mass spectra the protonated molecules, $[M + H]^+$, with *m/z* 435 and 393 for triamcinolone acetonide and betamethasone, respectively, were selected as precursor ions and fragmented in MS/MS mode. A collision energy optimization was performed to achieve intense product ions. At collision energy of 15 eV, triamcinolone acetonide presented the most intense and selective product ion at *m/z* 415. For betamethasone, the major product ion was *m/z* 373, at a collision energy of 13 eV (Fig. 2). The HPLC–ESI-MS/MS conditions provided high signal intensities for both triamcinolone acetonide and betamethasone. The addition of ammonium acetate buffer and formic acid in the mobile phase was important to improve the signal intensities in positive-ion mode.

Nasally administered drugs, as triamcinolone acetonide, provided considerably reduced plasma levels, due to the low systemic absorption provided by these formulations. The pharmacokinetic evaluation of drugs administered using nasal sprays requires ultra sensitive methods, able to quantify low amounts of the drug in the biological matrix. The method presented in this work provided a limit of quantitation of 20 pg/ml, allowing reliable analytical measurements and adequate characterization of the plasma concentration–time profiles of triamcinolone acetonide after the nasal spray administration.

Pharmacokinetic and bioequivalence studies involve the analysis of a large number of biological samples. That is the reason why high-throughput methods are needed in order to perform this type of analysis.[15] Besides the low limit of quantitation, the developed method presents a major advantage compared with most of the assays for triamcinolone acetonide, when considering its short run time. The retention times of betamethasone (IS) and triamcinolone acetonide were 0.95 and 1.17 min, respectively, and the total run time was 1.6 min (Fig. 3). Hence, the method may certainly be applied to high-throughput assays.

Journal of MASS SPECTROMETRY

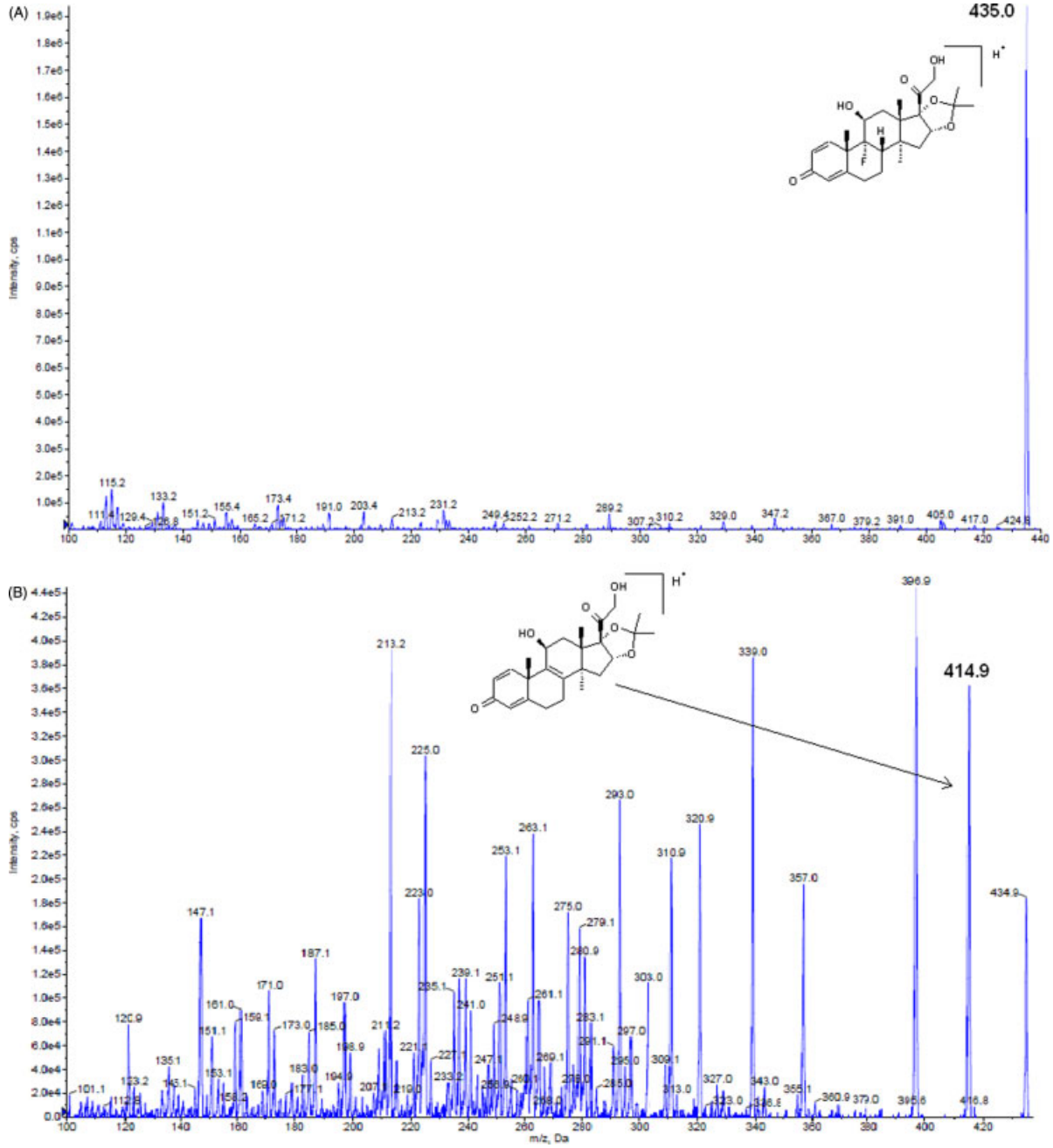

Figure 2. Mass spectra of triamcinolone acetonide (A) and fragmentation spectra of ion $m/z = 435$ (B), obtained by electrospray ionization in positive ion mode.

Sample extraction

Various organic solvents were tested to develop an efficient liquid–liquid extraction procedure. The combination of ethyl acetate, methylene chloride and methyl-*t*-butyl ether (4 : 3 : 3) yielded the highest recoveries for both triamcinolone acetonide and IS from plasma. In addition, the extraction procedure provided clean injection extracts and was shown to be robust. The high recovery rates achieved, close to 100%, allowed the quantitation of very small amounts of the analyte in plasma samples, along with the other analytical conditions.

Betamethasone presented similar recovery, chromatographic and ionization properties of triamcinolone acetonide, so that it was shown to be an adequate internal standard for the analysis.

Method validation

No significant interference was detected at the retention times of the analytes, in the six different blank plasma chromatograms (Fig. 3). The plasma samples spiked with caffeine, chlorpheniramine, metamizole or acetaminophen did not interfere at the ion transitions selected for the analytes quantitation.

The triamcinolone acetonide calibration curves were shown to be linear over the range 20–2000 pg/ml. A typical standard curve was $y = 1.05 \times 10^{-3} \times +4.74 \times 10^{-3}$, with a weighted factor $1/x$. The mean regression coefficient was 0.99863 ± 0.00083 ($n = 3$). Linearity results obtained in the three consecutive days are presented in Table 1. All back-calculated standard concentrations were within 15% deviation from the nominal value, except at the

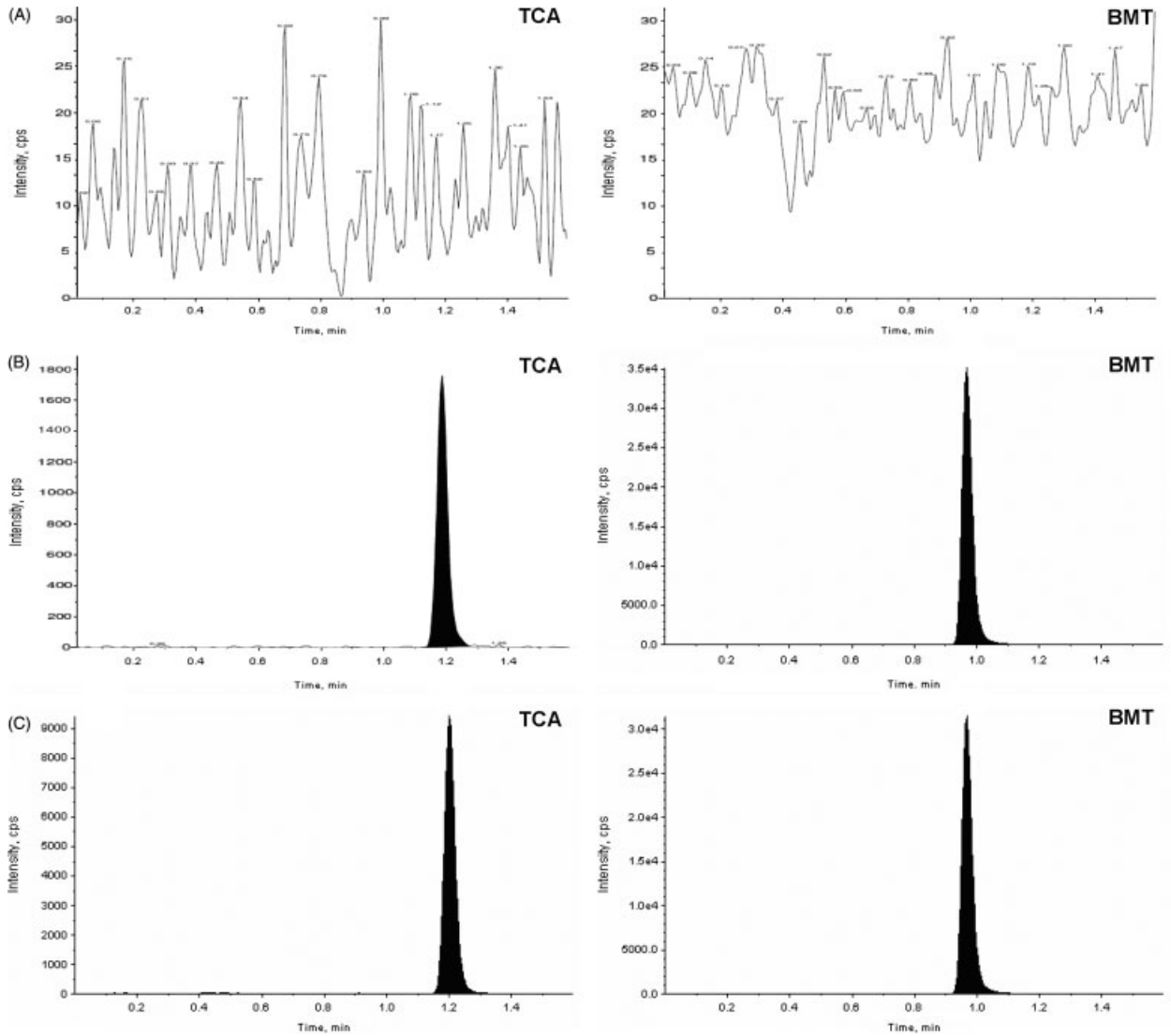

Figure 3. SRM chromatograms of (A) blank plasma sample, (B) blank plasma spiked with triamcinolone acetonide (TCA) at LLOQ (20 pg/ml) and betamethasone (BMT) at 2.5 ng/ml and (C) volunteer plasma collected 10 min after the administration of nasal spray (251 ng/ml of TCA).

Table 1. Precision and accuracy data of back-calculated concentrations of calibration samples for triamcinolone acetonide in plasma

Nominal concentration (ng/ml)	Observed concentration (ng/ml, mean ± SD)	Precision (%RSD)	Accuracy (%)
20	21.3 ± 1.3	6.2	106.5
100	99.9 ± 6.8	6.8	99.9
500	473 ± 12.3	2.6	94.5
1000	990 ± 52.9	5.3	99.0
1500	1450 ± 40.4	2.8	96.8
2000	2080 ± 64.6	3.1	104.2

SD, standard deviation; RSD, relative standard deviation.

LLOQ, for which the maximum acceptable deviation was set at 20%. The residuals had no tendency of variation with concentration. The obtained LLOQ was 20 pg/ml, with a precision of 10.7% and 103.8% in terms of RSD and accuracy, respectively.

The intra-run and inter-run precision and accuracy were calculated by analyzing six replicates of QC samples at three concentration levels, in three different days. The obtained data are shown in Table 2. The mean RSD values were 4.6% and 5.7% for the intra-run and inter-run precision, respectively. The mean accuracy values were 98.1% and 98.8% for the intra-run and inter-run accuracy, respectively. These data indicated reproducible results, and that the assay was accurate and reliable.

The mean recovery rate of triamcinolone acetonide ($n = 15$), determined at three concentrations, was 97.5%. The recovery of the IS was shown to be 100.7% ($n = 15$). The recovery data are shown in Table 3.

The matrix effect was evaluated by comparing the mean peak areas of triamcinolone acetonide and IS from the spike-after-extraction samples with those of the standard solutions in the mobile phase. The observed variation did not exceed the range 85–115%, so that in the present HPLC–MS/MS method, the matrix effects for the analytes were not significant.

The results of stability experiments showed that triamcinolone acetonide plasma samples were stable for up to 11 h at 23 °C, for

Journal of MASS SPECTROMETRY

Table 2. Precision and accuracy data for triamcinolone acetonide by HPLC–ESI-MS/MS

Assay	Nominal concentration (ng/ml)	Observed concentration (ng/ml, mean ± SD)	Precision (%RSD)	Accuracy (%)
Intra-run	60	57.7 ± 3.0	5.2	96.1
($n = 6$)	800	794 ± 53.0	6.7	99.2
	1600	1580 ± 32.3	2.0	99.0
Inter-run	60	59.5 ± 4.6	7.7	99.1
($n = 18$)	800	783 ± 42.5	5.4	97.8
	1600	1590 ± 65.7	4.1	99.6

SD, standard deviation; RSD, relative standard deviation.

Table 3. Recovery data for triamcinolone acetonide and betamethasone by HPLC–ESI-MS/MS

Analyte	Nominal concentration (ng/ml)	Recovery (%)	%RSD
Triamcinolone acetonide ($n = 5$)	60	94.1	4.1
	800	99.8	3.5
Betamethasone (IS) ($n = 15$)	1600	98.7	3.7
	2.5	100.7	5.1

11 months at −70 °C, for 34 h after extraction in the autosampler and after three complete freeze/thaw cycles on consecutive days, as the mean changes in analyte content were within ±15% of initial concentration, at low and high QC. Working solutions of triamcinolone acetonide and IS were stable for at least 4 days at 4 °C.

Application to a clinical pharmacokinetic study

The validated method was applied to a pharmacokinetic study in healthy volunteers. Fifty-three volunteers completed the study. The sensitivity and the specificity of the method showed to be adequate to accurately characterize the pharmacokinetics of triamcinolone acetonide after nasal spray administration. The mean plasma concentration–time curve of triamcinolone acetonide is shown in Fig. 4.

The main pharmacokinetic parameters of the drug were calculated, using WinNonLin 5.1 software. The mean C_{max}, 350.3 ± 134.4 pg/ml, was reached 0.28 ± 0.17 h (T_{max}) after drug administration. The mean values of AUC_{0-t} and $AUC_{0-\infty}$ obtained were 712.7 ± 355.5 pg h/ml and 823.2 ± 387.3 pg h/ml, respectively. The extrapolated area of plasma concentration versus time was not higher than 20% of $AUC_{0-\infty}$, demonstrating the suitability of the method and experimental design. The elimination half-life of the drug was 2.8 ± 1.0 h. Triamcinolone acetonide was detected in plasma 5 min after the administration in the most of subjects, demonstrating a rapid nasal absorption.

Conclusion

To our knowledge, this is the first HPLC–ESI-MS/MS method for the quantitation of triamcinolone acetonide in human plasma after nasal spray administration in human subjects. The method provided sensitive and reliable results and demonstrated high-throughput capability because of the short run time required for analysis. In addition, the established LLOQ was considerably low (20 pg/ml), so that the method may be successfully applied to clinical pharmacokinetic and bioequivalence studies of triamcinolone acetonide administered using nasal spray formulations.

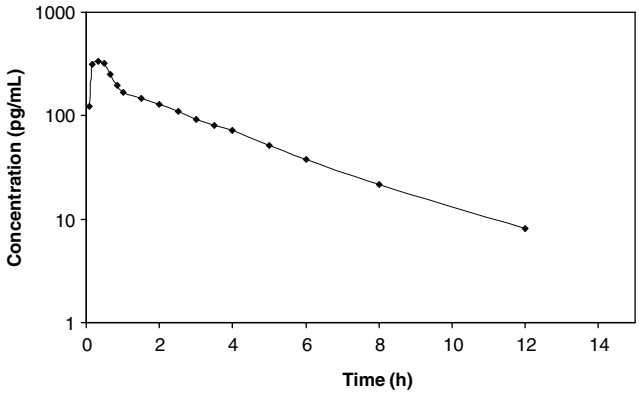

Figure 4. Plasma concentration–time curve of triamcinolone acetonide after the administration of nasal spray in healthy volunteers ($n = 53$).

References

[1] H. Herman. Once-daily administration of intranasal corticosteroids for allergic rhinitis: a comparative review of efficacy, safety, patient preference, and cost. *Am. J. Rhinol.* **2007**, *21*, 70.

[2] A. N. Waddell, S. K. Patel, A. G. Toma, A. R. Maw. Intranasal steroid sprays in the treatment of rhinitis: is one better than another? *J. Laryngol. Otol.* **2003**, *117*, 843.

[3] W. Jeal, D. Faulds. Triamcinolone acetonide. A review of its pharmacological properties and therapeutic efficacy in the management of allergic rhinitis. *Drugs* **1997**, *532*, 57.

[4] J. Condemi, R. Schulz, J. Lim. Triamcinolone acetonide aqueous nasal spray versus loratadine in seasonal allergic rhinitis: efficacy and quality of life. *Ann. Allergy Asthma Immunol.* **2000**, *84*, 533.

[5] C. Bachert, T. El-Akkad. Patient preferences and sensory comparisons of three intranasal corticosteroids for the treatment of allergic rhinitis. *Ann. Allergy Asthma Immunol.* **2002**, *892*, 92.

[6] P. T. Daley-Yates, A. C. Price, J. R. Sisson, A. Pereira, N. Dallow. Beclomethasone dipropionate: absolute bioavailability, pharmacokinetics and metabolism following intravenous, oral, intranasal and inhaled administration in man. *Br. J. Clin. Pharmacol.* **2001**, *51*, 400.

[7] Food and Drug Administration. *FDA Guidance for Industry: Bioavailability and Bioequivalence Studies for Nasal Aerosols and Nasal Sprays for Local Action*. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, Rockville, **2003**.

[8] S. A. Doppenschmitt, B. Scheidel, F. Harrison, J. P. Surmann. Simultaneous determination of triamcinolone acetonide and hydrocortisone in human plasma by high-performance liquid chromatography. *J. Chromatogr. B* **1996**, *682*, 79.

[9] 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.* **2004**, *50*, 2345.

[10] 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.* **2007**, *79*, 3786.

[11] D. P. Wermeling, J. L. Miller, S. M. Archer, M. K. Ravens, A. C. Rudy. Pharmacokinetics, bioequivalence, and spray weight reproducibility of intranasal butorphanol after administration with 2 different nasal spray pumps. *J. Clin. Pharmacol.* **2005**, *45*, 969.

[12] N. Uemura, T. Onishi, A. Mitaniyama, T. Kaneki, K. Ninomiya, K. Nakamura, M. Tateno. Bioequivalence and rapid absorption of zolmitriptan nasal spray compared with oral tablets in healthy japanese subjects. *Clin. Drug Invest.* **2005**, *25*, 199.
[13] C. Joukhadar, B. Schenk, S. T. Kaehler, C. J. Kollenz, P. Bauer, M. Muller, H. G. Eichler. A replicate study design for testing bioequivalence: a case study on two desmopressin nasal spray preparations. *Eur. J. Clin. Pharmacol.* **2003**, *59*, 631.
[14] Food and Drug Administration. *FDA Guidance for Industry: Bioanalytical Method Validation*. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, Rockville, **2001**.
[15] L. Vlase, B. Kiss, D. Muntean, S. E. Leucut. Rapid high-performance liquid chromatography–tandem mass spectrometry method for determination of pentoxifylline and its active metabolites M1 and M5 in human plasma and its application in bioavailability study. *Talanta* **2010**, *82*, 945.