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Determination of 21-hydroxydeflazacort in human plasma by high-performance liquid chromatography/atmospheric pressure chemical ionization tandem mass spectrometry. Application to bioequivalence study

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A liquid chromatographic atmospheric pressure chemical ionization tandem mass spectrometric method is described for the determination of 21-hydroxydeflazacort in human plasma using dexamethasone 21-acetate as an internal standard. The procedure requires a single diethyl ether extraction. After evaporation of the solvent under a nitrogen flow, the analytes are reconstituted in the mobile phase, chromatographed on a C_{18} reversed-phase column and analyzed by mass spectrometry via a heated nebulizer interface where they are detected by multiple reaction monitoring. The method has a chromatographic run time of less than 5 min and a linear calibration curve with a range of 1–400 ng ml^{-1} ($r > 0.999$). The between-run precision, based on the relative standard deviation for replicate quality controls, was $\leq$5.5% (10 ng ml^{-1}), 1.0% (50 ng ml^{-1}) and 2.7% (200 ng ml^{-1}). The between-run accuracy was $\pm$7.1, 3.8 and 4.8% for the above concentrations, respectively. This method was employed in a bioequivalence study of two DFZ tablet formulations (Denacen from Marjan Industria e Comercio, Brazil, as a test formulation, and Calcort from Merrell Lepetit, Brazil, as a reference formulation) in 24 healthy volunteers of both sexes who received a single 30 mg dose of each formulation. The study was conducted using an open, randomized, two-period crossover design with a 7-day washout interval. The 90% confidence interval (CI) of the individual geometric mean ratio for Denacen/Calcort was 89.8–109.5% for area under the curve $AUC_{(0-24\ h)}$ and 80.7–98.5% for C_{max}. Since both the 90% CI for $AUC_{(0-24\ h)}$ and C_{max} were included in the 80–125% interval proposed by the US Food and Drug Administration, Denacen was considered bioequivalent to Calcort according to both the rate and extent of absorption.

KEYWORDS: deflazacort; metabolite; quantification; liquid chromatography/mass spectrometry

INTRODUCTION

Deflazacort, 11β,21-dihydroxy-2′-methyl-5′β,*H*-pregna-1,4-dieno[17,16-*d*]oxazole-3,20-dione 21-acetate, (DFZ, Fig. 1) is an inactive prodrug derivative of prednisolone which is rapidly converted to the active metabolite 21-hydroxydeflazacort (21-OH-DFZ, Fig. 1) after oral administration. 21-OH-DFZ has anti-inflammatory and immunosuppressive activities. Deflazacort is employed in the treatment of rheumatoid arthritis, Ducchene dystrophy, systemic lupus erithematosus, uveitis and transplantation.[1] Although several method have been reported for the determination of 21-OH-DFZ in human plasma, all using high-performance liquid chromatographic (HPLC) separation and UV detection,[2–6] only the method proposed by Reynolds *et al.*[5] has the sensitivity required for pharmacokinetic studies, with low administration doses. The minimum quantification limit is 1 ng ml^{-1}. For routine analysis, this method has three inconveniences: it employs large plasma sample volumes (2 ml) and has a long extraction procedure (solid-phase extraction) and a long retention time for 21-OH-DFZ (10.6 min).

The quantification of drugs in biological matrices by liquid chromatography/tandem mass spectrometry (LC/MS/MS) is becoming more usual, improving the sensitivity and selectivity of methods.[7] This paper describes a fast, sensitive and specific LC/MS/MS method for the quantification of 21-OH-DFZ using dexamethasone 21-acetate (21-Ac-DXM, Fig. 1) as an internal standard (I.S.). The method was applied in a study of bioequivalence of two commercial 30 mg tablets formulations of DFZ:Calcort (Merrell Lepetit, Brazil) and Denacen (Marjan Industria e Comercio, Brazil) in 24 healthy volunteers.

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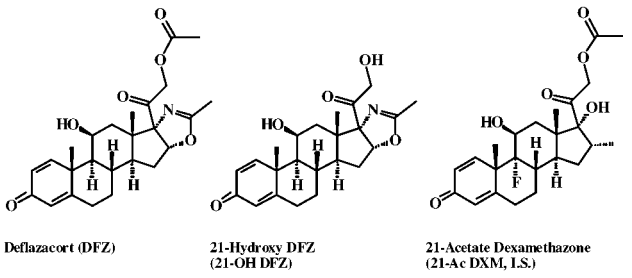

Figure 1. Structures of deflazacort (DFZ), 21-hydroxydeflazacort (21-OH-DFZ) and the Internal standard, dexamethazone 21-acetate (21-Ac-DXM).

EXPERIMENTAL

Materials

HPLC-grade solvents and analytical-grade reagents were purchased from Mallinckrodt (St Louis, MO, USA) and Nuclear (Sao Paulo, Brazil). 21-OH-DFZ was prepared from DFZ (acquired from Marjan Industria e Comercio) by treatment with methanol saturated with anhydrous hydrochloric acid. The reaction mixture was stirred for 1 h, keeping the temperature at 0 °C, and then the reaction mixture was stirred overnight at room temperature. After the solvent had been removed on a rotary evaporator, water was added and the mixture was adjusted to pH 10 with 1 M NaOH, and the aqueous layer was extracted three times with dichloromethane. The combined dichloromethane layers were washed with brine and dried over anhydrous sodium sulfate and evaporated in vacuum. The residue was purified by chromatography on a silica gel column (eluent diethyl ether–ethanol (9 : 1)). The crude 21-OH-DFZ was recrystallized from diethyl ether to yield a colorless crystalline solid. Homogeneity of the purified compound was assessed by analytical reversed-phase HPLC with a μBondpack C_{18} (125 Å) column, 10 mm, 3.9 × 300 mm, spherical, and the purity was >98%. The melting point (216–217 °C) was determined using a Kofler melting point apparatus and is uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AMX 500 MHz spectrometer with $CDCl_3$ as solvent. The NMR data were consistent with the structure of 21-OH-DFZ. 21-Ac-DXM was purchased from Sigma (St Louis, MO, USA). Blank human blood was collected from healthy, drug-free volunteers. Plasma was obtained by centrifugation of blood treated with the anticoagulant sodium heparin. Pooled plasma was prepared and stored at approximately −70 °C until needed.

Calibration standards and quality control

Solutions of 21-OH-DFZ and 21-Ac-DXM were prepared in methanol–water (50 : 50) at a concentration of 10 μg ml^{-1}. Calibration standards of 21-OH-DFZ were prepared at concentrations of 400.0, 200.0, 100.0, 50.0, 20.0, 10.0, 7.5, 5.0, 3.5, 2.0 and 1.0 ng ml^{-1} with an initial dilution of 0.4 ml of 10 μg ml^{-1} to 9.6 ml of blank plasma (final concentration 400 ng ml^{-1}). The subsequent concentrations were obtained by serial dilutions with the same blank plasma. An I.S. solution was prepared at 2000 ng ml^{-1} by diluting the 10 μg ml^{-1} 21-Ac-DXM solution with mobile phase.

The quality control concentrations were fixed at 10, 50 and 200 ng ml^{-1} (QCA, QCB and QCC, respectively) and were taken directly from calibration standards.

Sample preparation

Aliquots (200 μl) of human plasma were employed for extraction with addition of I.S. solution (50 μl). The tubes were vortex-mixed briefly and allowed to stand at room temperature for 5 min. Diethyl ether–dichloromethane (3 ml, 80 : 20) was added and the samples were vortex-mixed for 30 s. The tubes were centrifuged at 2000 rpm for 10 min at 4 °C. The upper organic layer was removed and transferred to new tubes. The solvent was evaporated under a nitrogen flow in a dry-bath at 37 °C. The samples were reconstituted with mobile phase (200 μl) and transferred into microvials, capped and placed in a Shimadzu Avp10 autosampler rack.

Chromatographic conditions

An aliquot (80 μl) of the plasma extract was injected into a Supelcosil C_{18} column, 150 × 4.6 mm i.d., 5 μm (Supelco, Bellefonte, PA, USA) using a Shimadzu AVP LC system. Separation and elution were achieved using 70% of acetonitrile (ACN) and 30% of 0.5 mM acetic acid in water as the mobile phase at a flow-rate of 0.60 ml min^{-1}. The oven temperature was 40 °C and the time between injections was 5 min.

Mass spectrometric conditions

Mass spectrometric detection was performed using a MicroMass (Manchester, UK) Quatro LC triple-quadrupole mass spectrometer, equipped with a heated nebulizer as the APCI source. The temperatures of the atmospheric pressure chemical ionization (APCI) probe and source block were 350 and 120 °C, respectively. Nitrogen was used as nebulizer gas (70 l h^{-1}) and desolvation gas (699 l h^{-1}). The corona was set to 2.9 kV. The APCI source was operated in the positive ionization mode (AP+), and multiple reaction monitoring (MRM), m/z 400.12 → 124.13 and m/z 435.19 → 397.29, was used for quantification of 21-OH-DFZ and 21-Ac-DXM respectively. The dwell time, the cone voltage, the collision energy and collision gas (argon) pressure were 0.1 s, 45 V, 40 eV and 3×10^{-3} bar, respectively, for 21-OH-DFZ, and 0.1 s, 25 V, 10 eV and 3×10^{-3} bar, respectively, for 21-Ac-DXM. Data were acquired by MassLynx software (version 3.2, MicroMass) and calibration curves for the analyte were constructed using the calibration samples using 21-OH-DFZ to I.S. peak-area ratios (PARS) via a weighted ($1/x$) least-squares linear regression. Unknown sample PARS were then interpolated from the calibration curve to provide concentrations of 21-OH-DFZ.

Bioequivalence study

The method was applied to evaluate, in human volunteers, the performance of one DFZ tablet formulation

(Denacen, tablets, 30 mg, Marjan Brazil, Lot Number 0068) against one standard DFZ tablet formulation (Calcort, tablets, 30 mg, by Merrell Lepetit Brazil, Lot Number 9808521/3). The comparison was done through the quantification of 21-OH-DFZ, the active metabolite of DFZ. The bioequivalence between the two formulations was assessed by calculating individual C_{max}, $AUC_{(0-24\ h)}$, $AUC_{(0-\infty)}$ and $C_{max}/AUC_{(0-24\ h)}$ ratios (test/reference) together with their mean and 90% confidence intervals (CI) after logarithmic transformation of the data (additive model[8]). The inclusion of the 90% CI for the ratio in the 80–125% range was analyzed by a parametric (analysis of variance (ANOVA)) method.

Twenty-four healthy volunteers of both sexes (12 females and 12 males) who were between the ages of 18 and 35 years (mean ± SD, 25.2 ± 5.5 years), who had heights between 153.5 and 181.0 cm (mean ± SD, 164.9 ± 7.8 cm) and who weighed between 55.2 and 82.6 kg (mean ± SD, 64.0 ± 9.2 kg) and within 15% of their ideal body weight were enrolled in the study. All subjects gave written informed consent, and the Ceara Federal University Hospital Ethics Committee of Clinical Investigation approved the clinical protocol.

The volunteers were free from significant cardiac, hepatic, renal, pulmonary, neurological, gastrointestinal and hematological disease, as assessed by physical investigation, EGC and the following laboratory tests: blood pressure, urea, creatinine, AST, ALT, alkaline phosphatase, γ-GT, total bilirubin, uric acid, total cholesterol, triglycerides, albumin and total protein, hemoglobin, hematocrit, total and differential white cell counts, erythrocyte sedimentation rate and routine urinalysis. All subjects were negative for HIV, HBV and HCV.

The study was conducted in a open, randomized, two-period crossover fashion with a 7-day washout period between doses. During each period, the volunteers were hospitalized at 11:00 p.m., having already had a normal evening meal, and after an overnight fast they received at 7:00 a.m. a single 30 mg dose of the appropriate DFZ formulation along with 200 ml of tap water. No food was allowed during 4 h following drug administration, after which a standard lunch was consumed and an evening meal was provided 10 h after dosing. No other food was permitted during the 'in-house' period. Liquid consumption was permitted *ad libitium* after lunch but xanthine-containing drinks, including tea, coffee, and cola, were avoided.

Blood samples (10 ml) from a suitable antecubital vein were collected in EDTA-containing tubes before and 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0, 10.0, 12.0 and 24.0 h after the administration of each dose of DFZ. The blood samples were centrifuged at 4000 rpm for 10 min at room temperature and the plasma was decanted and stored at −20 °C until assayed. All samples from a single volunteer were analyzed on the same day to avoid inter-assay variation.

RESULTS AND DISCUSSION

Method development

Full-scan positive-ion mass spectra of 21-OH-DFZ and the I.S. showed the protoned molecular ion $[M + H]^+$ at *m/z* 400 and 435, respectively. The most abundant ion in the product ion spectra was at *m/z* 124 for 21-OH-DFZ and at *m/z* 397 for 21-Ac-DXM (Fig. 2). From these results, the mass spectrometer was set as follows: *m/z* 400 for 21-OH-DFZ and *m/z* 435 for 21-Ac-DXM as the precursor ions and *m/z* 124 for 21-OH-DFZ and *m/z* 397 for 21-Ac-DXM as product ions in the MRM mode.

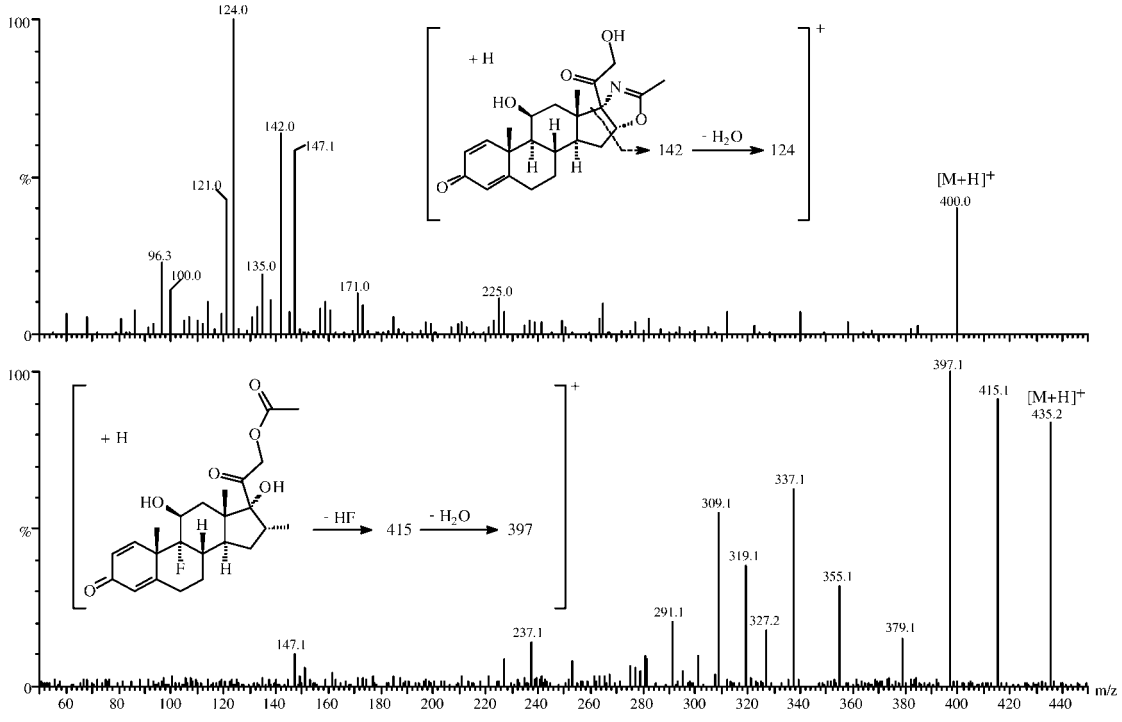

Figure 2. APCI product ion spectra of the protonated molecular ion of 21-OH-DFZ (top) and 21-Ac-DXM (bottom). The structure of the molecular ion and neutral losses are shown, proposing an explanation of base peaks.

Neutral losses of 20 and 18 Da were found for 21-Ac-DXM, as in a previous report on the collision-induced dissociation of betamethasone (geometric isomer of DXM[9]). From Fig. 2, we propose, for 21-OH-DFZ, a cleavage point and subsequent loss of water generating the base peak.

Diethyl ether, chloroform, hexane, dichloromethane and binary mixtures of these solvents in different proportions were tested for extraction. The best results were obtained with diethyl ether–dichloromethane (80 : 20). The recoveries of 21-OH-DFZ, based on peak area ratios of extracted plasma mobile phase, both previously spiked at final concentrations of 10, 50 and 200 ng ml^{-1}, were 68 ± 8.6, 70 ± 6.2 and $71 \pm 7.8\%$ (mean $\pm$ RSD, $n = 3$), respectively. For the I.S. (2000 ng ml^{-1}) the recovery was $73 \pm 6.8\%$ (mean $\pm$ RSD, $n = 3$).

No peak was observed in the mass chromatogram of human blank plasma serum under the LC/APCI-MS/MS conditions described above, as shown in Fig. 3(a) and (b).

The mass chromatograms of a sample are shown in Fig. 4(a) and (b), in which the retention times of 21-OH-DFZ and 21-Ac-DXM were 3.3 and 3.9 min, respectively.

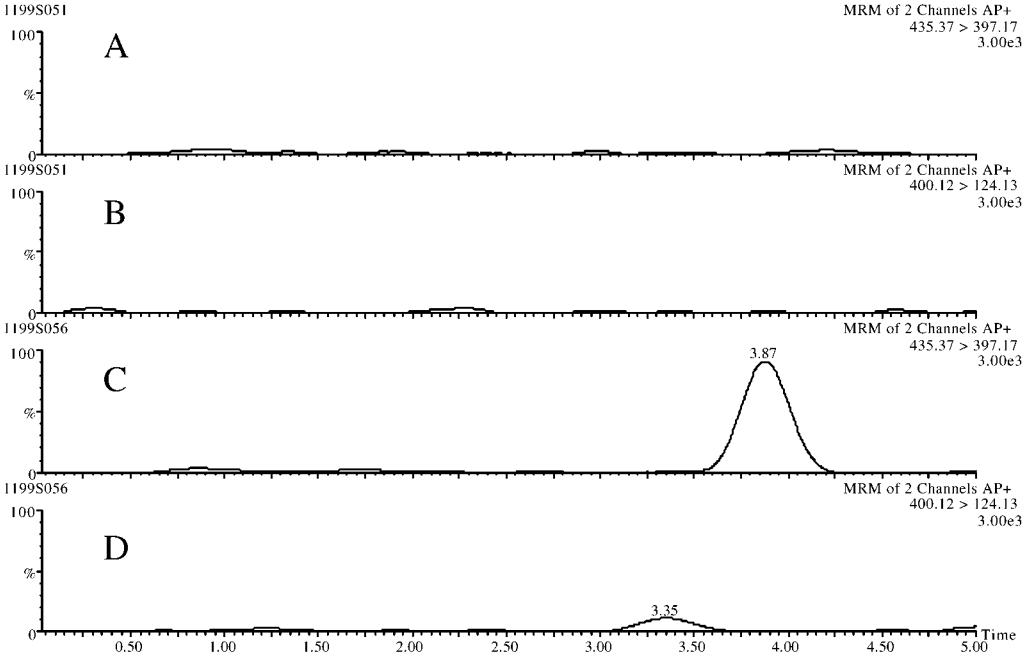

Figure 3. MRM chromatograms of blank human plasma: (A) 21-Ac-DXM and (B) 21-OH-DFZ. MRM chromatograms of spiked blank human plasma at a final concentration of 1 ng ml^{-1}: (C) 21-Ac-DXM and (D) 21-OH-DFZ.

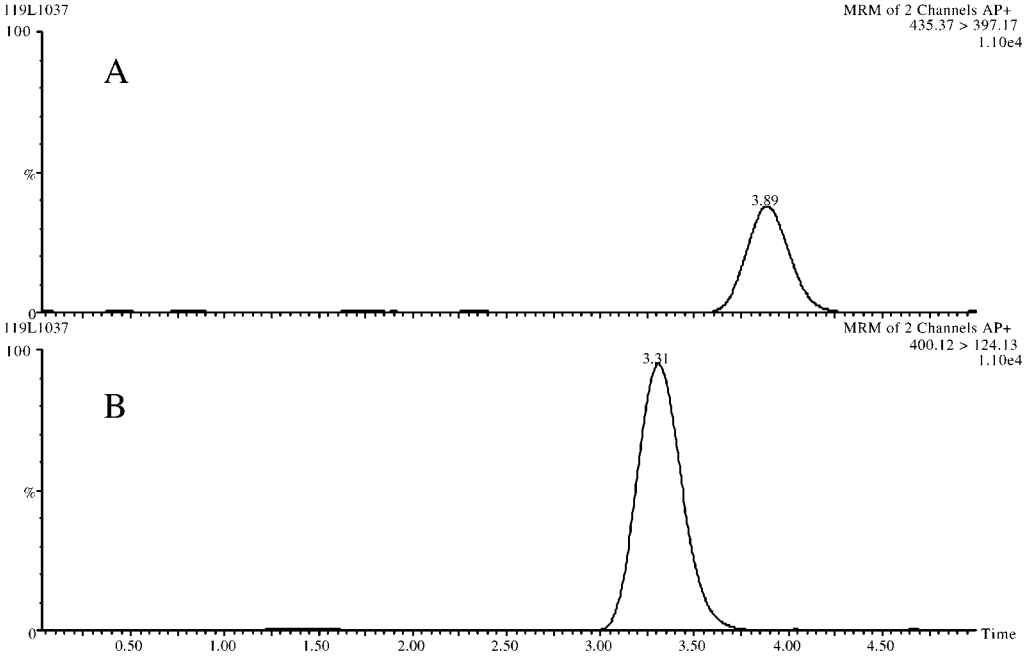

Figure 4. MRM chromatograms of plasma sample collected 1 h after administration of DFZ: (A) 21-Ac-DXM and (B) 21-OH-DFZ.

Table 1. Between-run plasma calibration quality report

Concentration (ng ml^{-1})	1.00	2.00	3.50	5.00	7.50	10.0	20.0	50.0	100	200	400	Slope	Intercept	*r*
Day 1	1.25	2.48	3.59	5.04	7.14	11.1	20.0	47.5	98.4	206	397	0.99751	0.18110	0.99984
Day 2	0.99	2.43	3.90	5.25	7.51	10.6	19.4	53.7	101	198	396	0.98866	0.82410	0.99994
Day 3	1.02	1.95	3.40	5.27	7.56	10.0	21.1	49.0	102	207	391	0.98752	0.96504	0.99966
Day 4	1.10	2.08	3.57	4.98	7.36	10.3	20.6	50.7	99.1	200	399	0.99763	0.16925	0.99999
Day 5	1.27	1.84	3.22	4.77	7.21	9.80	19.8	49.6	101	198	402	1.00303	−0.23020	0.99998
Day 6	1.08	2.09	3.41	5.02	7.47	9.99	19.7	51.9	98.5	201	401	1.00223	0.01984	0.99998
Mean	1.11	2.14	3.51	5.05	7.37	10.3	20.1	50.4	100	202	398	0.99610	0.32152	0.99990
SD	0.12	0.26	0.23	0.19	0.17	0.48	0.63	2.19	1.63	3.66	3.80	0.00661	0.46992	0.99989
RSD (%)	10.5	12.0	6.60	3.68	2.32	4.69	3.14	4.34	1.62	1.81	0.96			
RE (%)	11.8	7.17	0.38	1.07	−1.71	3.02	0.43	0.80	0.10	0.86	−0.60			

Assay performance

The calibration curves showed good linearity throughout the range 1.0–400.0 ng ml^{-1}. The slopes, intercepts and coefficients of determinations from the validation analyses are reported in Table 1.

The between-run precision and accuracy were determined from the calibration standards and quality controls samples. The within-run precision and accuracy were determined from the quality control samples. Calibration curve experiments were performed in duplicate. A quality control sample was analyzed after a sequence of 10 unknown samples.

The assay precision was determined as the relative standard deviation, RSD (%) = 100(SD/M), and accuracy as the percentage relative error, RE (%) = $(E - T)(100/T)$, where M is the mean, SD is the standard deviation of M, E is the experimentally determined concentration and T is the theoretical concentration.

Table 1 summarizes the between-run accuracy and precision for calibration standards. The calibration curve between-run precision was estimated as 0.96–12.0%, and accuracy as 0.10–11.8% over the calibration range.

Table 2 summarizes the accuracy and precision for quality controls. The within-run accuracy was ±11.0, 2.2 and 7.4% for QCA, QCB and QCC, respectively, and the within-run precision was ≤8.7, 5.9 and 6.7%, respectively. The between-run precision was ≤7.1, 3.8 and 4.8% for QCA, QCB and QCC, respectively, and the between-run accuracy was ±5.6, 1.0 and 2.8%, respectively.

The limit of quantification was 1 ng ml^{-1} (RSD = 10.5% and RE = 11.8%) [Fig. 3(c) and (d)].

Table 2. Within-run and between-run plasma quality control report

Sample		*n*	Mean	SD	RSD (%)	RE (%)
QCA	Day 1	7	11.3	0.97	8.72	11.0
(10 ng ml^{-1})	Day 2	5	11.0	0.87	7.92	9.70
	Day 3	6	10.4	0.42	4.06	4.30
	Day 4	7	10.3	0.67	6.53	2.90
	Day 5	8	10.3	0.62	5.96	3.25
	Day 6	3	10.1	0.32	3.22	0.80
	Between-run	36	10.6	0.75	7.12	5.56
QCB	Day 1	7	51.1	3.04	5.94	2.17
(50 ng ml^{-1})	Day 2	5	49.7	1.70	3.42	−0.59
	Day 3	6	50.6	1.64	3.25	1.25
	Day 4	7	50.4	2.36	4.68	0.90
	Day 5	8	50.6	1.21	2.39	1.22
	Day 6	3	50.1	0.79	1.59	0.12
	Between-run	36	50.5	1.92	3.80	1.01
QCC	Day 1	7	215	10.7	4.96	7.42
(200 ng ml^{-1})	Day 2	5	204	8.01	3.92	2.04
	Day 3	6	202	2.18	1.08	0.87
	Day 4	7	204	4.38	2.15	1.83
	Day 5	8	206	13.9	6.74	3.24
	Day 6	3	196	1.01	0.51	−1.88
	Between-run	36	206	9.90	4.81	2.79

Bioequivalence study

Both DFZ formulations were well tolerated at the dose administered. No adverse effects were reported, and the biochemical parameters remained unchanged and within the reference range. The maximum concentration reached (C_{max}) and the areas under the curves ($AUC_{(0-24\ h)}$) were compared. For Denacen 30 mg tablets the C_{max} geometric mean ratio was 89.2% (90% CI = 80.7–98.5) of Calcort. The Denacen 30 mg tablets $AUC_{(0-24\ h)}$ geometric mean ratio was 98.2% (90% CI = 89.8–109.5) of Calcort. Pharmacokinetic parameters are given in Table 3 and mean plasma concentrations of 21-OH-DFZ are shown in Fig. 5. These results are consistent with those reported by Mollmann *et al.*[4] in a pharmacokinetic/pharmacodynamic evaluation of DFZ in comparison with methylprednisolone and prednisolone.

Since the 90% CI for both C_{max} and AUC ratio were inside the 80–125% interval proposed by the US Food and Drug Administration,[10,11] it is concluded that Denacen 30 mg tablets are bioequivalent to Calcort 30 mg tablets for both the rate and the extent of absorption.

Table 3. Pharmacokinetic parameters from 24 human volunteers following oral administration of a single 30 mg DFZ tablet

	Calcort		Denacen	
Parameter	Mean	SD	Mean	SD
$AUC_{all(0-24\ h)}$	336	120	322	100
AUC_{∞}	344	122	343	103
$AUC_{all(0-24\ h)/\infty}$	97.8	—	93.9	—
$C_{max(ng\ ml^{-1})}$	143	44	129	40
$T_{max(h)}$ (range)	1.0	0.5–2.5	1.0	0.5–3.0
$T_{1/2(h)}$	1.3	0.3	1.7	1.5

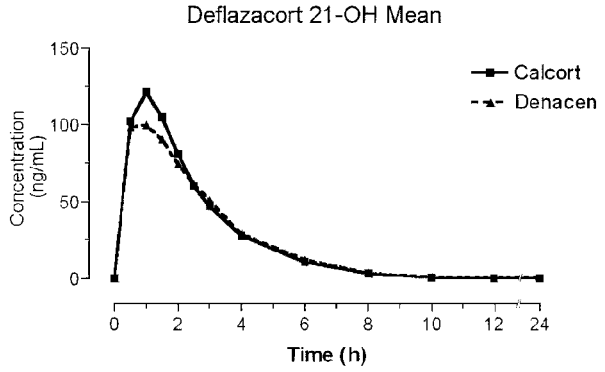

Figure 5. Mean plasma concentration of 21-OH-DFZ in 24 human volunteers following oral administration of a single 30 mg DFZ tablet.

CONCLUSION

A fast and reliable LC/APCI-MSMS method for the determination of 21-OH-DFZ in human plasma was developed and validated. The validation results indicated that the method is rugged, precise and accurate and is suitable for routine determination of 21-OH-DFZ in human plasma.

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REFERENCES

1. Markham A, Bryson HM. *Drugs* 1995; **50**: 317.
2. Rao N, Eller M, Arumugham T, Weir S. *Eur. J. Drug Metab. Pharmacol.* 1996; **21**: 241.
3. Rao N, Bhargava VO, Reynolds DL, Eller MG, Weir SJ. *Biopharm. Drug Dispos.* 1996; **17**: 753.
4. Mollmann H, Hochhaus G, Rohatagi S, Barth J, Derendorf H. *Pharm. Res.* 1995; **12**: 1096.
5. Reynolds DL, Burmaster SD, Eichmeier LS. *Biomed. Chromatogr.* 1994; **8**: 230.
6. Hirata H, Kasama T, Sawai Y, Fike RR. *J. Chromatogr. B* 1994; **658**: 55.
7. Huang EC, Wachs T, Conboy JJ, Henion JD. *Anal. Chem.* 1990; **62**: 713.
8. Marzo A. *J. Pharm. Pharmacol.* 1997; **49**: 1259.
9. Polettini A, Bouland GM, Montagna M. *J. Chromatogr B* 1998; **713**: 399.
10. *Federal Register Part 320: Bioavailability and Bioequivalence Requirements.* Food and Drug Administration: Washington, DC, 1985; 154.
11. Food and Drug Administration. *Pharmacopeial Forum* 1993; **19**: 6501.