Original Article

Determination of deflazacort in human plasma by liquid chromatography-mass spectrometry after liquid-liquid extraction and its application in human pharmacokinetics studies

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Abstract

Purpose: A sensitive liquid chromatography–mass spectrometric (LC/MS) has been developed and validated for the quantification of deflazacort in human plasma after liquid-liquid extraction (LLE). **Materials and Methods:** Best chromatographic resolution was achieved on a reverse-phase Phenomenex C_{18} column with the mobile phase of acetonitrile–water (30:70) and isocratic elution resulted in a total run time of about 3.5 min. The analyte was detected by using an electrospray positive ionization mass spectrometry in the selected ion monitoring (SIM) mode. Linearity was obtained in the concentration range studied (5–150 ng/ml) (r = 0.9974). **Results:** Lower limit of quantification (LLOQ) was found to be 5 ng/ml in 500µl plasma sample. Average recovery of the analyte was found to range from 86.80 to 88.19% in plasma at the concentrations of 15.0, 60.0 and 120.0 ng/ml. **Conclusions:** The present method was successfully applied in the pharmacokinetic study of deflazacort in human plasma.

Key words: Bioavailability, deflazacort, liquid chromatography–mass spectrometric, liquid-liquid extraction, pharmacokinetics

INTRODUCTION

Deflazacort[1] is chemically 11beta, 21-dihydroxy-2′-methyl-5′ betah-pregna-1,4- deino [17,16-d] oxazole-3,20-acetate. Deflazacort is a derivative of glucocorticoid; It is used for its anti-inflammatory properties in conditions responsive to corticosteroid therapy. λ_{max} of deflazacort is 208 nm, which shows its poor ultraviolet (UV) absorption characteristics. Hence determination of deflazacort using HPLC–UV will be a hard task, lacks sensitivity and requires long chromatographic run time in biological fluids. Due to the high polarity index of deflazacort the gas chromatographic (GC) method requires complex and time-consuming derivatization procedures. Limited analytical techniques were available in the assay of deflazacort such as liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS).[2-7] The present liquid chromatography–mass spectrometry method is relatively simple, rapid and highly sensitive in the determination of deflazacort in human plasma after liquid-liquid extraction (LLE) and has been successfully applied in the human pharmacokinetic study.

MATERIALS AND METHODS

Chemicals and reagents

Deflazacort (purity: 99.67%) and pantoprazole (internal standard, IS 99.01% purity) were obtained from M/s. Orchid pharmaceuticals (Chennai, India) and Cadila Pharm (Ahmedabad, India). Acetonitrile was of HPLC grade and obtained from E.Merck (Mumbai, India) and other chemicals used were

of analytical grade. Purified water from a Milli-Q-system (Millipore, Bangalore, India) was used through analysis.

Reference and test products

Reference Product: Defelzacort 6 mg

Test Product: A (Defelzacort 6 mg)

Human

Each product was subjected in to 12 volunteer. Human studies were approved by human ethical committee, Ooty, India.

Instrumentation

A Shimadzu 2010 A LC–MS (including two LC-10ADvp pumps, an online vacuum deaerator, a constant temperature automatic sampler, a quadruple mass spectrometer equipped with an electrospray ionization interface (ESI) source and LC–MS solution (Version 2.04) was used for data processing. A six-port switching valve was used to direct HPLC elute to a waste container in the first 1.5 min of the chromatographic run and afterwards to the ionization source.

Chromatographic conditions

Liquid chromatographic separations were achieved using a Phenomenex 5µ C_{18} column (100mm×4.6mm). The column and auto sampler tray temperature were kept constant at 40 and 4°C, respectively. The mobile phase consisted of a mixture of water (A) and acetonitrile (B) (70:30) and was delivered at a flow-rate of 0.3 ml min. The sample injection volume was 10 µl.

Mass spectrometric conditions

Samples were ionized by positive-ion electrospray ionization mode under the following source conditions: Gas flow:1.5 l min; curved desolvation line (CDL) voltage was fixed as in tuning, CDL temperature: 250°C; and block temperature:200°C. Mass spectra were obtained at a dwell time of 0.2 and 1 s for SIM and scan mode accordingly. Analysis was carried out using selected ion monitoring (SIM) for specific *m*/*z* 441.95 for deflazacort and *m*/*z* 384.0 for pantoprazole. Peak areas for all components were automatically integrated using LC/MS lab solution Version 2.04 (© 2010 A Shimadzu Corp.).

Preparation of stock and sample solutions

Stock solution of deflazacort was prepared by dissolving the accurately weighed reference compound in water and acetonitrile (1:1) to give a final concentration of 1 mg ml, stored at 4°C until it is used. The solution was then serially diluted with water and mixed with blank human plasma to achieve standard working solutions at concentration of 5.0, 10.0, 25.0, 50.0, 75.0, 100.0 and 150.0 ng ml for deflazacort, respectively. A 2500.0 ng ml internal standard working solution was prepared by diluting the 1 mg ml stock solution of internal standard with Millipore water.

Sample preparation

A 0.5 ml aliquot of human plasma sample was mixed with 0.1 ml of internal standard working solution (2500.0 ng/ml of pantoprazole) and 1.0 ml of borate buffer of pH 9.0 were added and mixed. The resulting solution was vortexed and extracted with ethyl acetate (3x2 ml). The upper organic layer was separated, evaporated and the drug was reconstituted using 0.5 ml of the mobile phase and analysed.

Assay validation

Sensitivity and specificity

The lower limit of quantification was determined as the minimum concentration that could be accurately and precisely quantified (lowest data point of the standard curve). The specificity of the assay for the analytes versus endogenous substances in the matrix was assessed comparing the lowest concentration in the calibration curves with reconstitutions prepared with drug-free plasma from five different humans.

Accuracy and precision

The accuracy and precision (presented as relative standard deviation, R.S.D.) of the assay were determined using quality control (QC) samples at 15.0, 60.0 and 120.0ng/ml. Accuracy (%) was determined by the percentage ratio of measured over spiked QC concentration (mean of measured/spiked×100%). Intra-day precision was determined by analyzing replicate aliquots of QCs (n = 5 per each concentration) on the same day. Inter-day precision was determined by repetitive analysis of QC samples (each concentration) on five consecutive days.

Recovery and ionization

To investigate the recovery of deflazacort by the LLE method, plasma samples were spiked with deflazacort at concentrations of 5, 15.0, 60.0 and 120.0 ng ml. The resulting peak–area ratios (analyte: Internal standard) were compared with that of the standards prepared

in mobile phase to provide the recovery values. Ion suppression of ionization was evaluated by comparing the absolute peak areas of control plasma extracted and then spiked with a known amount of drug, to neat standards injected directly in the same reconstitution solvent.

Stability

To evaluate sample stability after three freeze–thaw cycles and at room temperature, five replicates of QC samples at each of the low, medium and high concentrations were subjected to three freeze–thaw cycles or were stored at room temperature for 4 h before sample processing, respectively. Five replicates of QC samples at each of the low and high concentrations were processed and stored under autosampler conditions for 24 h. Stability was assessed by comparing the mean concentration of the stored QC samples with the mean concentration of freshly prepared QC samples.

Application of the assay

The developed LC/MS assay method was used in the pharmacokinetic study after oral (6.0 mg) administration of deflazacort to human volunteers. Volunteers were fasted for 12 h before dosing and 4 h afterwards, with free access to water. The venous blood samples (6 ml) were withdrawn via an indwelling cannula at predose and at 0.5,1.0,1 .5,2.0,2.5,3.0,4.0,6.0,8.0,12,18.0 and 24 h following drug administration in each period of the study. The samples were collected in pre-labeled vacutainers containing sodium citrate as the anti-coagulant and centrifuged at 4000 rpm for 10 min at 15°C and plasma was collected in pre-labeled sample collection tube. A wash out period of 7 days was observed between the two phases of the study. The samples were stored in the deep freezer at - 70 ± 5 °C until analyzed by a validated LC/MS method.

RESULT AND DISCUSSION

Method validation

Specificity

The full scan mass spectra[8] of deflazacort and Pantoprazole (IS) after direct injection in mobile phase are presented in Figure 1. The predominant protonated molecules found for deflazacort were m/z 441.95. The mass spectrometric parameters were optimized to obtain the higher signal for the selected ion 441.95, which also showed less internal interference. While using the isocratic programme, observed retention times were about 3.29 and 1.46 min for deflazacort and pantoprazole, respectively. No additional peaks due to endogenous substances that could have interfered with the detection of the compounds of interest were observed. The LC-MS chromatogram of blank plasma [Figure 2] and plasma spiked with deflazacort chromatograms are presented in Figures 3 and 4.

Linearity and lower limit of quantification

The linear regression analysis of deflazacort constructed by plotting the peak–area ratio of deflazacort to the internal standard (y) versus analyte concentration (µg ml) in spiked plasma samples (x). The calibration curves were fluctuated in the range of 5.0–150ng ml. The average regression equation of these curves and their correlation coefficients (r) were calculated it showed good linear relationship between the peak areas and the concentrations. The lower limit of quantitation was 5 ng ml for determination of deflazacort in plasma. The limit has already been sufficient for pharmacokinetic studies of deflazacort.

Precision

The intra-day precision (presented as relative standard deviation) is shown in Table 1. The accuracy, defined as (measured concentration/spiked concentration) ×100%, reached from 93.07 to 99.65% through out the

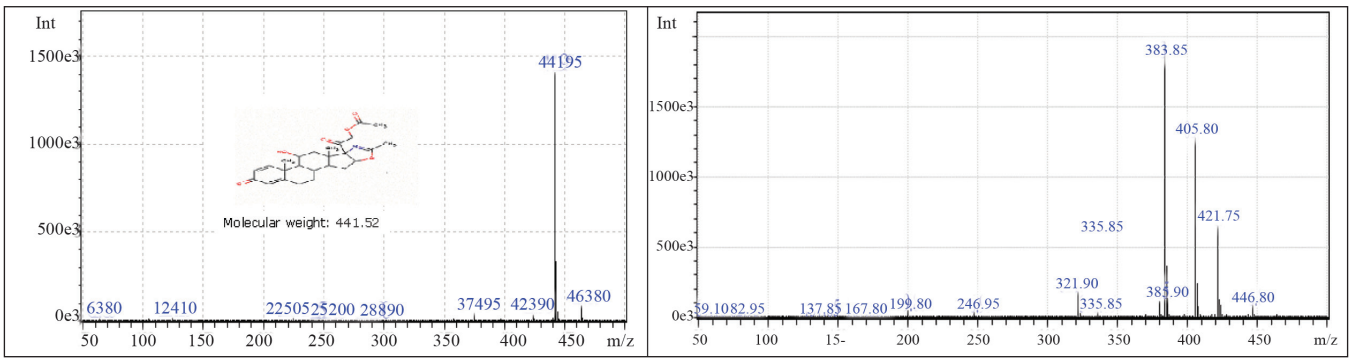

Figure 1: Mass spectrum of deflazacort and pantoprazole at positive mode scan

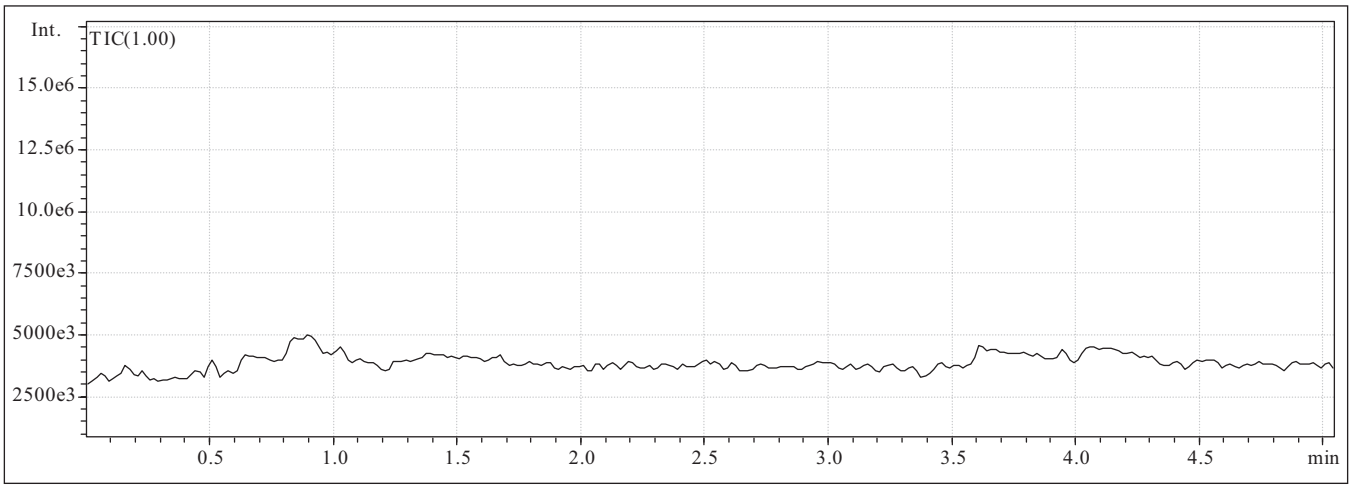

Figure 2: Blank plasma

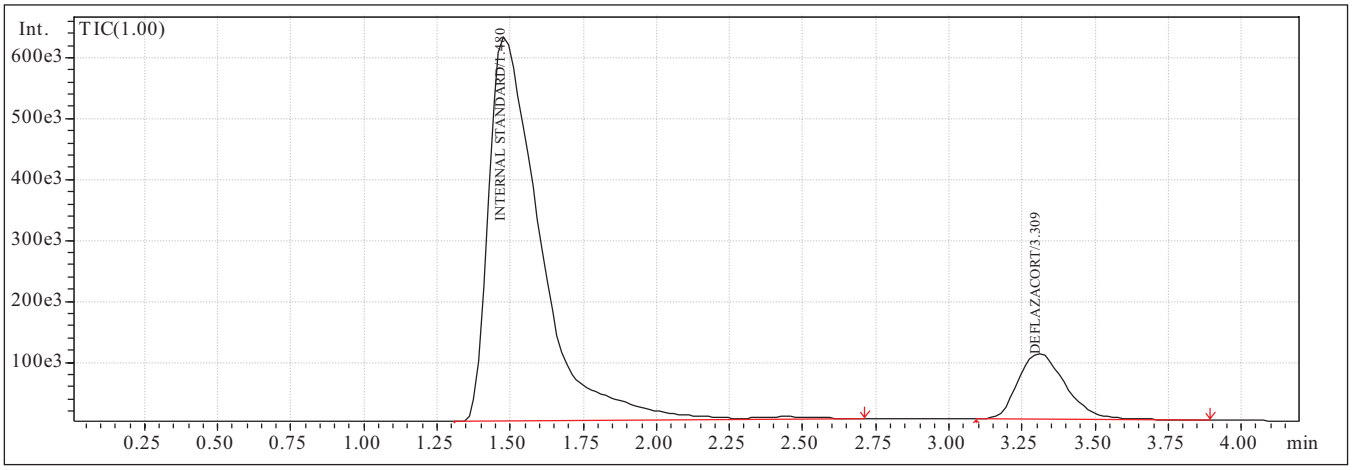

Figure 3: Blank plasma spiked with deflazacort

four concentrations examined. The inter-day precision was studied over five days.

Recovery and stability

The absolute recoveries of deflazacort at concentrations of 15.0, 60.0 and 120.0 ng ml (n = 5) were 86.60±5.75, 86.57±8.59 and 88.19±9.15%, respectively. Stability of deflazacort during sample handling (freeze–thaw and short-term temperature) and the stability of processed samples were evaluated and deflazacort was stable for at least 4 h at room temperature in plasma samples, for 24 h in autosampler conditions and in plasma samples following three freeze–thaw cycles.

Ionization

It was shown that LLE improves the sample clean-up to remove internal substances from plasma and thereby decrease the amount of matrix injected onto the column, thus the ion suppression effect was minimized. The results indicated that there was no significant difference between the signals of analytes extracted from human plasma and the mobile phase, which proves that there were no matrix effects.

Pharmacokinetic study

The assay was conducted to obtain pharmacokinetic data for deflazacort in human plasma after oral administration (6.0 mg) application of the LC/MS method developed here to *in vivo* pharmacokinetic studies in humans. The area under the plasma concentration (AUCs curve) of deflazacort after oral administrations were 2830.15±380.84 and 2854.16±657.50 ng h ml, respectively. The mean C_{max} value was 66.66±4.10 and 69.60±3.46 ng/mL corresponding mean t_{max} value was 1.88±0.43 and 1.75±0.26 h. The mean plasma elimination half-life was 2.40±0.31 and 2.58±0.28 h. for test and

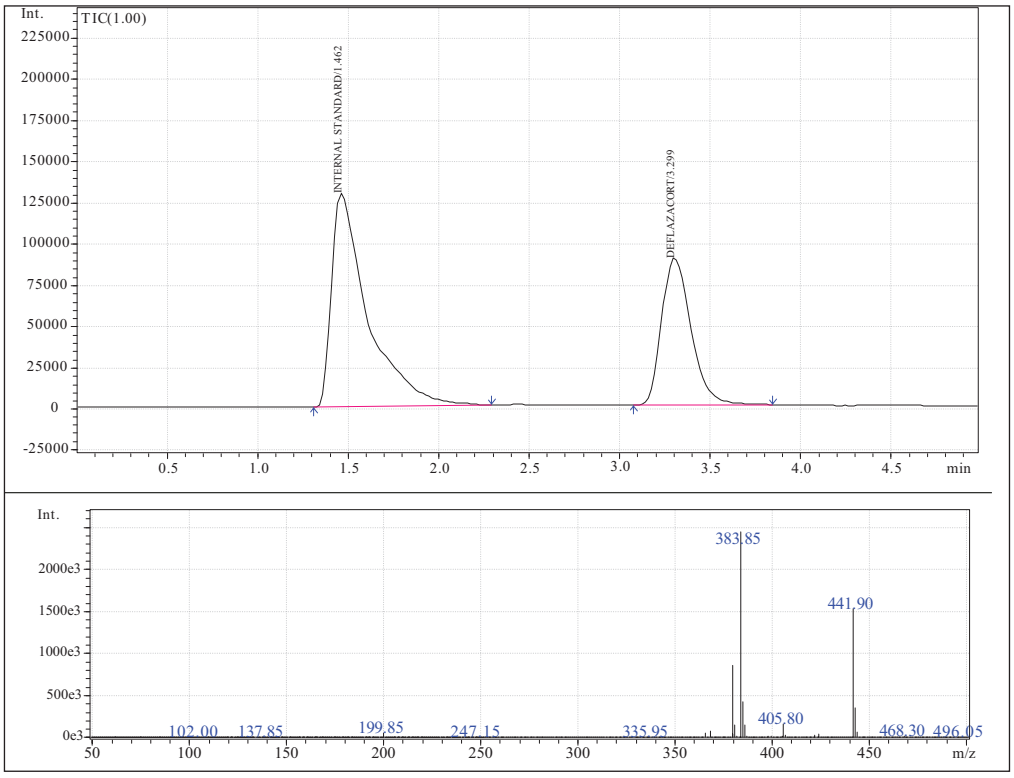

Figure 4: Plasma sample 3 h after oral administration of deflazacort tablets to human volunteers

Table 1: Precision of deflazacort in human plasma

Intra-day (*n*=5)			
(ng/mL)	15	60	120
Mean ±S.D.	14.73±0.43	59.10 ±0.60	119.58 ±0.41
Accuracy (%)	101.79	101.52	99.66
R.S.D. (%)	2.92	1.01	0.34
Inter-day (*n*=5)			
(ng/mL)	15	60	120
Mean ±S.D.	14.59±0.30	59.34±0.59	119.781±0.32
Accuracy (%)	97.29	98.90	99.82
R.S.D. (%)	2.04	0.99	0.27

Table 2: Mean pharmacokinetic parameters

Parameters	Test product	Reference product	% Ratio
AUC_{0-24}(ng.h/ml)	66.66	69.60	95.79
$AUC_{0-\infty}$ (ng.h/ml)	269.42	291.79	92.33
C_{max} (ng/ml)	293.73	314.91	93.27
t_{max} (h)	1.88	1.75	107.14
k_{eli} (h^{-1})	0.29	0.27	108.22
$T_{½}$ (h)	2.40	2.58	92.87

reference respectively [Figure 5, Table 2]. Other pharmacokinetic parameters in this study are shown in Table 2. The present method could be applied to pharmacokinetic studies after a lower dose administration of deflazacort (6 mg).

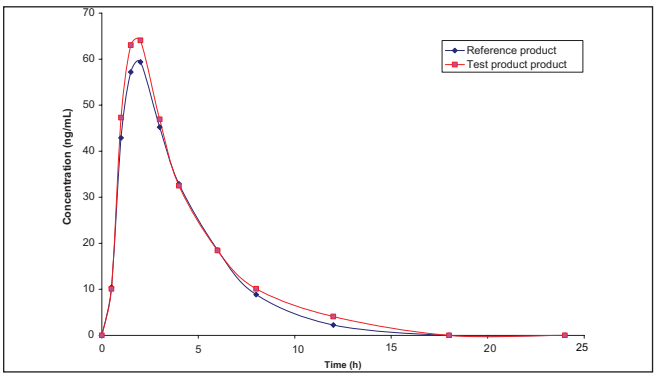

Figure 5: Mean plasma concentration of test and reference product

CONCLUSIONS

LLE of deflazacort from plasma was found to be more precise than the solid phase extraction. The current method guarantees a high precision, accuracy, recovery and a relatively short analysis time and will be a useful tool in the pharmacokinetic study of deflazacort in humans.

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