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Bioanalytical Method Development and Validation of Doxercalciferol and 1 α , 25-Dihydroxy Vitamin D2 by High Performance Liquid Chromatography Tandem Mass Spectrometry Detection: Application to Pharmacokinetic Study

Thennati R, Shahi PK*, Chakra A, Patel H, Shah V and Bhokari A

Department of Pharmacokinetics, Sun Pharmaceutical Industries Limited, Tandalja, Vadodara, Gujarat, India

Abstract

The systemic circulating levels of Doxercalciferol and its active metabolite form were in the pg/mL range which represents a significant bioanalytical challenge for therapeutic monitoring. Hence Liquid chromatography with tandem mass spectrometry (LC–MS/MS) with derivatization technique was considered the most appropriate standard for the selective and sensitive determination of this molecule in biological matrices and a sensitive, selective, precise and well accurate liquid chromatography tandem mass spectro-metric (LC–MS/MS) assay method was developed for simultaneous determination of Doxercalciferol and its metabolite in human plasma at pg/ml lower limit of quantitation and in order to improve the accuracy and reliability of assay method for determination of parent compound, and metabolite, a derivatization procedure was adopted. Extraction procedure was optimized with solid phase extraction technique for better recovery, selectivity and low matrix effect. Prior to detection, doxercalciferol and metabolite were ionized using an ESI source in the multiple reaction monitoring (MRM) mode. The proposed method was extensively validated over the concentration range of 1 to 75.29 pg/mL for doxercalciferol and 1.5 to 75.07 pg/mL for its metabolite as per FDA guidelines and the results met the acceptance criteria and validated method was successfully applied for estimation of drug and metabolite concentration in the healthy male volunteers, bioequivalence and pharmacokinetic study of Doxercalciferol, 2.5 µg dose capsules under fasting condition.

Keywords: HPLC; Mass spectrometry; Estimation of doxercalciferol; Solid phase extraction; Bioequivalence

Introduction

The present paper describes a highly selective and sensitive method, which employs the derivatization procedure in order to improve the sensitivity by enhancing ionization and to attain the better signal to noise ratio. Solid phase extraction technique for sample preparation, and liquid chromatography with electrospray ionization detection was adopted for quantitation of doxerclciferol and metabolite in human plasma. The method was successfully applied to a pharmacokinetic study of Doxercalciferol in healthy male volunteers. The authenticity in the measurement of the study data was demonstrated through incurred sample reanalysis.

Doxercalciferol is a synthetic vitamin D_2 analog that undergoes metabolic activation *in vivo* to form 1α,25-dihydroxyvitamin D_2 (1α,25-$(OH)_2$ D_2) (DXM), a naturally occurring, biologically active form of vitamin D_2. It is available as soft gelatin capsules containing 0.5 mcg, 1 mcg or 2.5 mcg doxercalciferol (DXC). It is a colorless crystalline compound with a calculated molecular weight of 412.66 and a molecular formula of $C28H44O_2$. It is soluble in oils and organic solvents, but is relatively insoluble in water. Chemically, DXC is (1α, 3β, 5Z, 7E, 22E)-9, 10-secoergosta- 5, 7, 10 (19), 22-tetraene-1, 3-diol.

In healthy volunteers, peak blood levels of 1α,25-(OH)2 D_2, the major metabolite of DXC, are attained at 11-12 hours after repeated oral doses of 5 to 15 mcg of DXC and the mean elimination half-life of 1α,25-$(OH)_2$ D_2 is approximately 32 to 37 hours.

It is indicated for the treatment of secondary hyperparathyroidism in patients with chronic kidney disease on dialysis and in patients with stage 3 or stage 4 chronic kidney diseases.

Literature survey reveals that very few methods were reported for quantification of DXC and/or DXM in pharmaceutical dosage form and in human blood and plasma [1-18] which in turn not meeting the actual need of Bio-equivalence studies. These methods were developed for quantitation of degradants from DXC injection [1], quantitation of DXC from blood and cell culture [2], and for estimation of DXC and DXM from plasma samples [3-18].

In view of this, authors have attempted to develop an accurate and precise method for estimation of both DXC and DXM in human plasma with 1 pg and 2.5 pg/ml quantitation limit by using LC-MS/MS technique. This assay method was specific to differentiate the endogenous plasma constituents in the biological matrix and which further fulfilled the need of reliable measurement of doxercalciferol and metabolites as per the OGD requirement.

Experimental

DXC reference standard was obtained in-house from R&D center of Sun Pharmaceutical Industries Limited, India. DXM was obtained from Vivan Life Science, Mumbai, India. DXC D_6 and DXM D_6 were obtained from Clearsynth Labs Limited, Mumbai, India. LR grade ammonium trifluoroacetate was obtained from Sigma Aldrich (St. Lowis, USA). LC grade methanol and acetonitrile were purchased from J.T. Baker Inc. (Phillipsburg, NJ, USA). HPLC grade acetone was obtained from RFCL limited; ethanol was obtained from Spectrochem

***Corresponding author:** Pradeep Kumar Shahi, Department of Pharmacokinetics, Sun Pharmaceutical Industries Limited, Tandalja, Vadodara, Gujarat, India, Tel: 09924314799; E-mail: pradeep.shahi@sunpharma.com

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(Mumbai, India). Suprapur ammonia 25%, suprapur hydrochloric acid and HPLC grade dichloromethane were obtained from Merck (Mumbai, India). 4-[2-(6, 7-dimethoxy-4-methyl-3-oxo-3, 4-dihydroquinoxalyl) ethyl]-1, 2, 4-triazoline- 3, 5-dione (DMEQ TAD) was obtained from Arro Biochem (Mumbai, India). Milli-Q-water was used from Milli-Q –water purification system (Milli-pore, USA). Human plasma was collected into K2EDTA tubes from drug-free healthy volunteers, clinical pharmacology unit, Sun Pharmaceutical Industries Limited; Independence Ethics Committee approved these processes. Test product Doxercalciferol 2.5 mcg capsules was obtained from in-house and reference product Hectorol (doxercalciferol) 2.5 mcg capsules was obtained from Genzyme Corporation, USA.

Instrumentation

An UPLC system (Dionex Ultimate-3000RS, Thermo Scientific, Germany) connected with mass spectrometer API 5500 triple quadrupole instrument (ABI-SCIEX, Toronto, Canada) was used. Data processing was performed with Analyst 1.5.1 software package (Sciex).

Detection

The mass spectrometer was operated in the multiple reaction monitoring (MRM) modes. Sample introduction and ionization were optimized with electrospray ionization in the positive ion mode. Source dependent parameters optimized were as follows: nebulizer gas flow, 55 psi; curtain gas flow, 20 psi; ion spray voltage, 5500 V; temperature, 550^{0}C. The compound dependent parameters such as the declustering potential, entrance potential, collision energy, cell exit potential were optimized during tuning as 40, 4, 40, 21 eV for DXC+DMEQTAD and DXM+DMEQTAD; 40, 4, 40, 14 eV for DXC D_6+DMEQTAD; 40, 4, 39, 20 eV for DXM D_6 +DMEQTAD, respectively. The collision activated dissociation gas was set at 9 psi using nitrogen gas. Quadrupole 1 and quadrupole 3 were both maintained at a unit resolution and dwell time was set at 150-400 ms. After derivatization, mass transitions were selected as m/z 758.5/484.2 for DXC; 764.5/484.3 for DXC D_6; 774.5/484.2 for DXM; and 780.5/484.2 for DXM D_6.

Chromatography

The zorbax Eclipse XDB phenyl (150 x 3 mm, 3.5 µ) was selected as the analytical column. Column temperature was set at 52 ^{0}C. Gradient mobile phase composition and source flow rate is given in Table 1 with injection volume of 15 µL. DXC and DXC D_6 were eluted at 10.5 min while DXM and DXM D_6 were eluted at 6.5 min, with a total run time of 13.5 min for each sample.

Stock solutions of analytes and IS

The stock solutions of DXC, DXM, DXC D_6 and DXM D_6 were prepared in acetonitrile. Intermediate dilutions and working standard solutions were prepared from stock solutions using methanol and water (90:10, v/v) solvent mixture. These working standard solutions were used to prepare the calibration curve and quality control samples. Blank human plasma was screened prior to spiking to ensure it was free of endogenous interference at retention time (RT) of analytes and internal standard (IS). Eight point standard curve and six quality control samples along with diluted QC samples were prepared by spiking the blank plasma with an appropriate amount of analytes. Calibration samples were made at concentrations of 1.00, 2.01, 3.51, 8.28, 15.56, 33.13, 58.23 and 75.29 pg/mL and quality control samples at 2.99, 8.97, 16.58, 34.25, 61.17 and 171.17 pg/mL for DXC while calibration samples at 1.50, 3.00, 6.01, 12.01, 20.02, 34.03, 55.05 and 75.07 pg/mL and quality control samples were made at concentrations of 4.30, 12.89, 18.70, 32.85, 57.62 and 171.85 pg/mL for DXM.

Time (min)	Flow (mL/ min)	%A (2 mM ammonium trifluoroacetate in water)	%B (0.005% ammonia in methanol)
0	0.7	28	72
3.4	0.7	28	72
3.5	0.5	26	74
6.7	0.5	26	74
6.8	0.45	12	88
11.5	0.45	12	88
11.6	0.75	5	95
12.5	0.75	5	95
12.6	0.6	28	72
13.5	0.6	28	72

Table 1: Gradient programme for chromatography.

Calibration curve and quality control samples

Two separate stock solutions of analytes were prepared for bulk spiking of calibration curve and quality control samples for the method validation exercise as well as the subject sample analysis.

Sample preparation: Human K2 EDTA blank plasma was retrieved and thawed at room temperature. 735 µL of blank plasma free from significant interference at the RT of the analyte and the IS was aliquoted in respective processing tubes. 15 µL of combined analyte working solution of CS and QCs (except PB and ZS) was added. 50 µL of working IS (20.0 µg/mL DXC D_6 and 20.0 µg/mL DXM D_6) was added in all samples except PB and mixed well using a hand vortexer, added 100 µl of 0.1N HCl mixed well using a hand vortexer, added 1 mL acetonitrile in all the samples, vortexed for approximate 5 min at 2500 rpm using digital vortexer. All samples were centrifuged at 4000 rpm at <15°C for 5 min Supernatant was collected to which 2 ml of water added, vortexed for 5-10 sec using hand vortexer.

The first solid phase extraction was performed by using bond elute cartridges C18 (100 mg/3cc). These cartridges were conditioned twice with acetone, followed by methanol. The cartridges were equilibrated with water. The above processed samples were loaded in to the cartridges. The cartridges were washed with 0.5% ammonia in water. Then, cartridges were washed with 30% acetone in water followed by 40% acetonitrile in water and further followed by 70% methanol in water. The cartridges were dried for approximately 1 min at full pressure. The samples were eluted with acetone twice. All the samples were evaporated at 50°C in evaporator under nitrogen pressure up to dryness.

Samples were derivatized by DMEQ-TAD agent. 250 µL of DMEQ-TAD in acetone (300 µg/mL) was added in all the samples and mixed using vortexer for 5-10 sec, 250 µL of dichloromethane added to all above samples and vortexed using hand vortexer for 5-10 sec, all the samples were incubated at RT for 60 min, after incubation 250 µL of ethanol added to these samples to quench the reaction and vortexed again for 5-10 sec, followed by addition of 50 µL of 0.1N HCl.

The second solid phase extraction was performed by using cartridges MCX (30 mg/1cc). These cartridges were conditioned with methanol followed by equilibrated with water. The above processed samples were loaded in to the cartridges. The cartridges were washed with water twice. The samples were eluted with 100 µL of methanol twice, and samples were collected in prelabelled sample collecting tubes to which 50 µL of water was added, vortexed well. These samples were transferred in to polypropylene vials for analysis

Selectivity: Selectivity was performed by analyzing the human blank plasma samples from eight different sources (4 normal, 2 hemolysed, 2 lipemic groups) to test for interference at the RT of analytes and IS along with LLOQ sample in each source matrix.

Matrix effect: Matrix effect for analytes and IS were evaluated by comparing the peak area ratio in the post- extracted plasma sample from 8 different drug-free blank plasma samples and aqueous or neat reconstitution samples. Experiments were performed at LQC-A and HQC levels with eight different plasma lots.

Precision and accuracy and dilution integrity: It was determined by replicate analysis of quality control samples (n=6) at a lower limit of quantification (LLOQ), low quality control (LQC-A and B), medium quality control (MQC-A and B), high quality control (HQC), upper limit of quantification ULOQ), and diluted QC (DQC) levels. The % CV was less than 15%, and accuracy was within 15% except LLOQ where it was within 20%. Fivefold dilution of diluted QC was done by using pre-screened blank plasma.

Recovery: The extraction efficiencies of analytes and IS were determined by analysis of six replicates at three quality control concentration level. The percentage recovery was evaluated by comparing the peak areas of extracted and post extracted samples to the peak areas of unextracted samples (spiked into mobile phase).

Stability: Stock solution stability was performed by comparing the area response of analyte and IS in the stability sample, with the area response of sample prepared from fresh stock solution. Stability studies in plasma were performed at the LQC-A and HQC concentration levels using six replicates at each level. Analyte was considered stable if the change is less than 15% as per US FDA and EMA guidelines. The stability of spiked human plasma and blood samples stored at room temperature (bench top stability) was evaluated for 8 h and 2 h., respectively. The stability of spiked human plasma samples stored at 4^0C in auto-sampler was evaluated for 84 h QC samples were processed kept in dry state for 8 h at room temperature for the evaluation of dry extract stability. All the plasma stability was evaluated against comparing with nominal value. The reinjection reproducibility was evaluated by comparing the extracted plasma samples that were injected immediately (time 0 h), with the samples that were re-injected after storing in the auto-sampler at 4^0C for 39 h. The freeze–thaw stability was conducted by comparing the stability samples that had been frozen at -20 ± 5°C, -35 ± 5°C and -65 ± 10°C and thawed four times against nominal values of QC samples. Six aliquots each of LQC-A and HQC concentration levels were used for the freeze–thaw stability evaluation. For long-term stability evaluation the concentrations (at -20 ± 5°C, -35 ± 5°C and -65 ± 10°C) obtained after 150 days were compared against nominal values of respective QC samples.

Application of method: The validated method has been employed for estimation DXC and DXM concentrations in human volunteer's bioequivalence study under fasting condition, after administration of a single dose capsule containing 2.5 µg doxercalciferol. The study was conducted according to current GCP guidelines. There were a total of 28 blood collection time points including the pre-dose samples (at -24.0, -16.0, -8.0, 0.0 and post dose samples (at 2.0, 3.0, 4.0, 5.0, 5.50, 6.0, 6.5, 7.0, 7.5, 8.0, 9.0, 10.0, 11.5, 13.0, 14.5, 16.0, 17.5, 19.0, 24.0, 48.0, 72.0, 96.0, 120.0 and 144.0 h) time intervals in separate vacutainers containing K2EDTA as an anticoagulant. The plasma from these samples was separated by centrifugation at 3300 rpm under refrigeration at 4°C ± 2°C for 15 min within 1.5 h after blood sampling collection. The plasma samples thus obtained were stored at ≤ -55°C until analysis. The pharmacokinetic parameters were computed using Win-Nonlin® (Pharsight Corporation, version 5.3) using non compartmental analyses and 90% confidence interval was computed using SAS software (SAS® Institute Inc., USA and version 9.2)

Results and Discussion

Method development

During the method development, mass parameters, chromatography conditions, mobile phase compositions, extraction conditions and derivatization procedure were optimized through several trials, to achieve high resolution and an increased intensity of the signals for all the analytes.

The electrospray ionization (ESI) was provided a maximum response over atmospheric pressure chemical ionization (APCI) mode, and was chosen for this method. The instrument was optimized to obtain better sensitivity and signal stability in positive polarity. Maximum response was obtained in positive ion mode as compare to the negative ion mode.

The derivitizing samples of DXC and DXM with DMEQ TAD (Cookson-type reagent) through Diels-Alder Reaction had given the more response as compared to without derivitizing the samples. Also make us enable to achieve the pg level sensitivity (Figure 1). The parent ion peaks in the ESI spectra of derivitizing samples of DXC and DXM were correspond to the ions at m/z 758.5 and 774.5, respectively as well as product ions of derivitizing samples of DXC and DXM were observed at m/z of 484.2 and 484.2, respectively (Figure 2). The isotopes labeled internal standards were used to compensate loss during sample preparation and avoid the matrix effect during the analysis.

Chromatography optimization

Initially, a different type of mobile phase in varying combinations was tried with aim to develop the method with better chromatography resolution, better signal and peak shape. The mobile phase containing ammonium trifluoroacetate as a buffer and ammonia in methanol gave the better response, but poor peak shape was observed. Isocratic mode and different gradient flow rate and composition were tried. The best signal along with a marked improvement in the peak shape was observed for DXC and DXM using a mobile phase and flow rate as stated in Table 1. Columns having short length, such as Acquity BEH C18 (100 x 2.1 mm, 1.7 µ), Acquity BEH C8 (100 x 2.1 mm, 1.7 µ) and columns having long length, such as Acquity BEH phenyl (150 x 3, 1.7 µ) and zorbax eclipse XDB phenyl (150 x 3 mm, 3.5 µ) were evaluated during the method development. Column having short lengths did not give proper separation of DXC and DXM. The best signal and good peak shape was obtained using the zorbax eclipse XDB phenyl (150 x 3 mm, 3.5 µ) column. DXM were eluted at 10.5 min and 6.5 min. Also utilization of stable isotope-labeled or suitable analog drugs as an IS was helpful to attain better accuracy and precision over the dynamic range.

Extraction optimization

Initially different extraction procedures like protein precipitation (PPT), liquid–liquid extraction (LLE) and solid phase extraction (SPE) were tried to obtain better recovery and low matrix effect but ion suppression effect was encountered with protein precipitation method for both the drug and IS. Hence further method was optimized with SPE and LLE technique and finally concluded that double SPE technique was more suitable for extraction of the drug and IS with better recovery and low matrix effect.

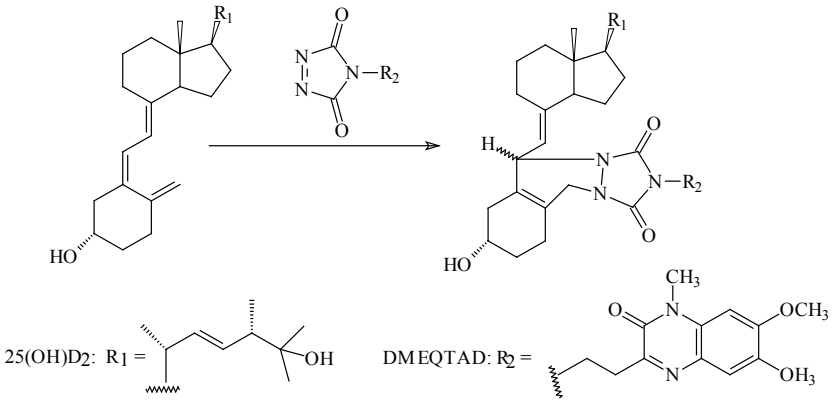

Figure 1: Derivatization process of DXC and DMEQ TAD (Diels-Alder Reaction).

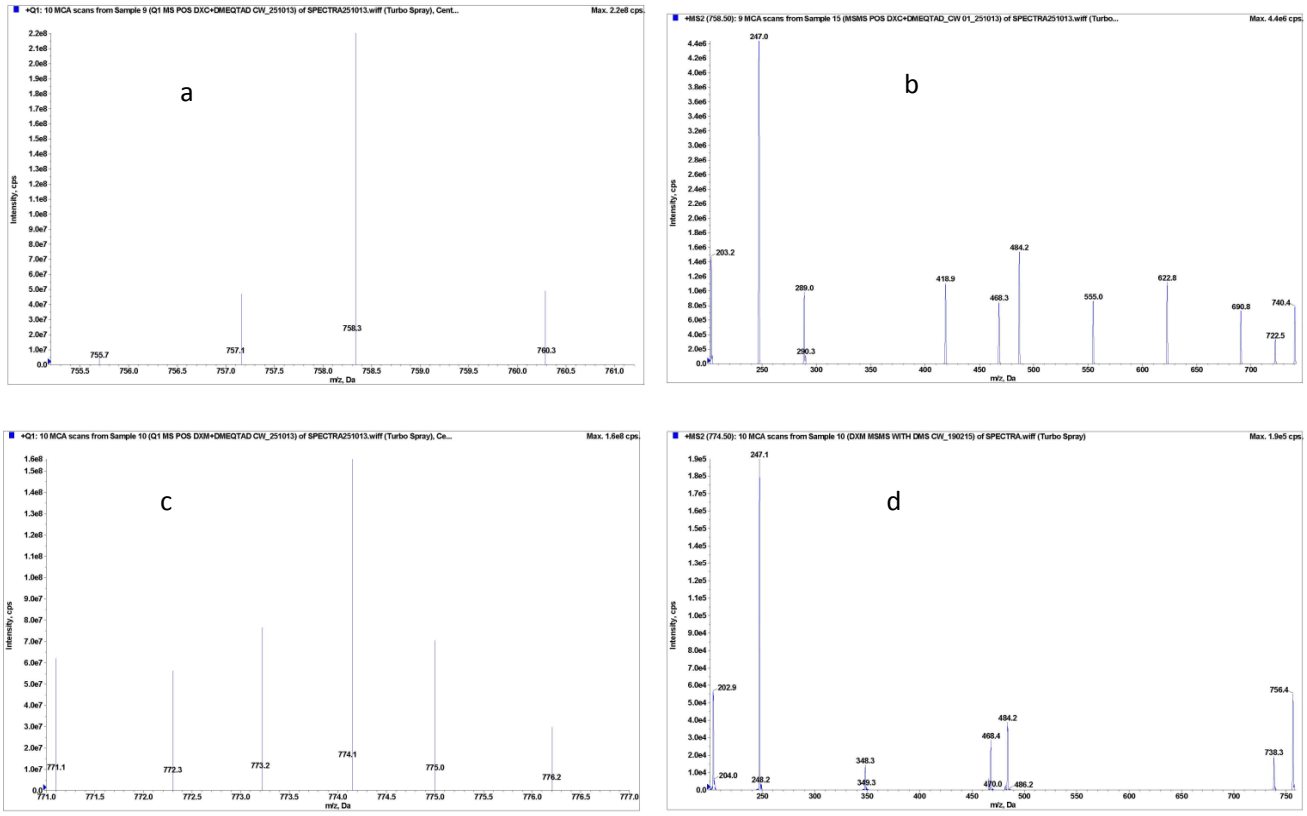

Figure 2: Mass spectra (a) DXC Parent ion, (b) DXC Product ion, (c) DXM Parent ion, and (d) DXM Product ion.

Auto sampler wash solution was optimized from 50% methanol to avoid any carry over effect. The sample volume was selected as 750 µL to attain pg level sensitivity for intended application. These optimized detection parameters, chromatographic conditions and extraction procedure resulted in accurate and precise detection of DXC and DXM in human plasma.

Method validation

A thorough and complete method validation of DXC and DXM in human plasma was performed as per the requirements of US FDA and EMA guidelines. The method was validated for selectivity, sensitivity, matrix effect, linearity, precision and accuracy, dilution integrity, recovery, reinjection reproducibility and stability.

Selectivity and sensitivity

Representative DXC and DXM chromatograms obtained from blank plasma and plasma spiked with a lower limit of quantification (LLOQ) sample are shown in Figure 3. The % interference observed at the RT of analytes and IS between eight different lots of human plasma, including hemolyzed and lipedemic plasma containing K2EDTA as an anticoagulant was nil for DXC and DXC D_6 respectively, as well as no interference were observed for DXM and DXM D_6 respectively, which was within acceptance criteria.

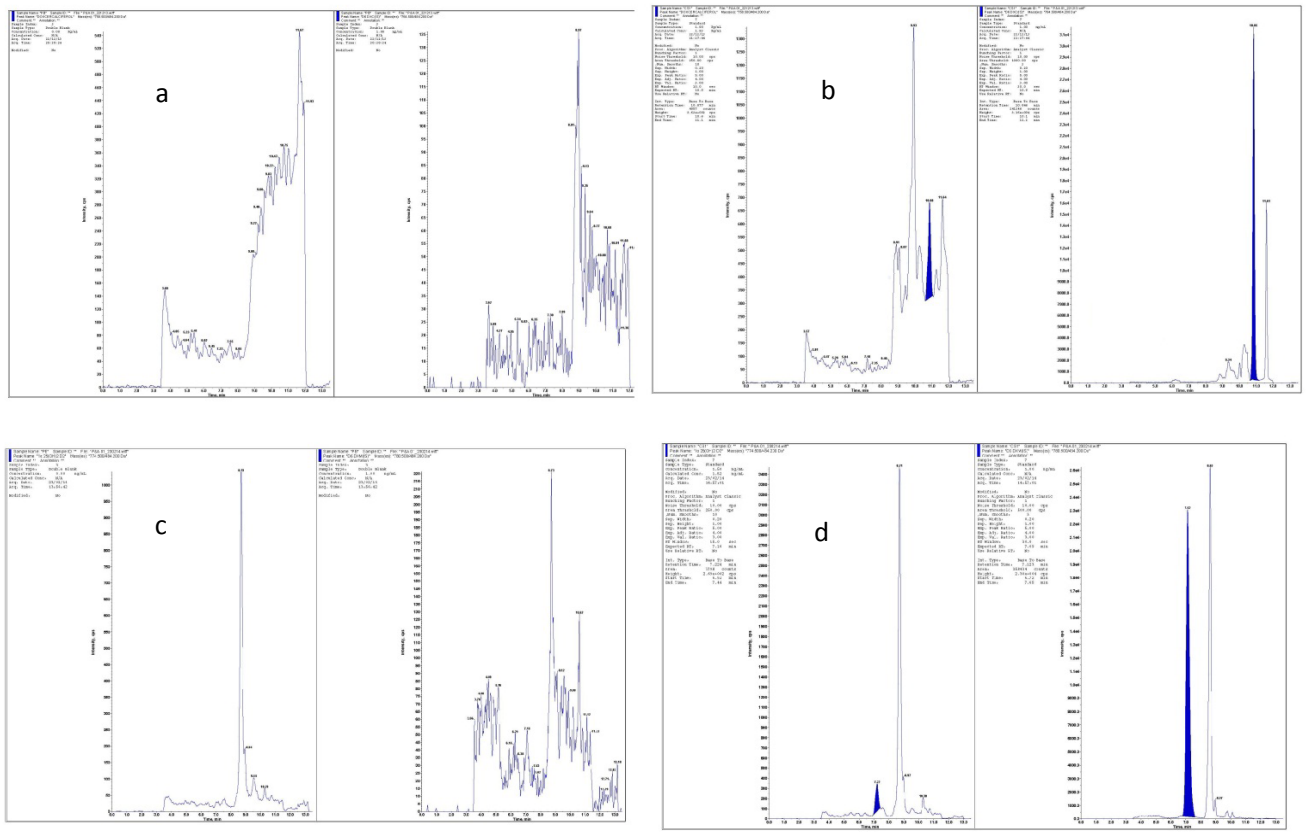

Figure 3: Human plasma chromatograms of (a) Blank of DXC and DXC D_6 (b) LLOQ of DXC and DXC D_6 (a) Blank of DXM and DXM D_6 and (a) LLOQ of DXM and DXM D_6.

Six replicates of extracted samples at the LLOQ level in one of the plasma sample having least interference at the RT of DXC and DXM were prepared and analyzed as part of P and A batch. The % CV of concentration of these six replicates of samples was 9.6% for DXC and 4.7% for DXM while % accuracy for both analytes was within 101.9-118.2%, confirming that interference does not affect the quantification at the LLOQ level. The LLOQ for DXC and DXM was 1 and 1.5 pg/mL, respectively. All the values obtained below LLOQ were considered as zero for statistical analysis.

Matrix effect

% CV of IS normalized matrix factor for DXC was found to be 12.0% for LQC-A and 2.5% for HQC and for DXM was found to be 8.0% for LQC-A and 2.1% for HQC indicating that the matrix effect on the ionization of analyte is within the acceptable range under these conditions.

Linearity

The peak area ratios of calibration standards were proportional to the concentration of DXC and DXM in each assay over the nominal concentration range of 1.0-75.29 pg/mL for DXC and 1.5-75.07 pg/mL for DXM. The calibration curves appeared linear and were well described by least-squares linear regression lines. As compared to the 1/x weighing factor, a weighing factor of 1/x2 achieved the best result and was chosen to achieve homogeneity of variance. The correlation coefficient was >0.9900 for both DXC and DXM. The observed mean back-calculated concentration with accuracy and precision (% CV) of four linearity run's analyzed during method validation is given in Table 2. The deviations of the back calculated values from the nominal standard concentrations were less than 15%. This validated linearity range justifies the concentration observed during real sample analysis.

Precision and accuracy

The inter-run precision and accuracy were determined by pooling all individual assay results of replicate (n = 6) quality control over the four separate batch runs, analyzed on at least two different days. The inter-run, intra-run precision (% CV) was below 15% (except LLOQ where it was within 20%) and inter-run, intra-run accuracy was in between 85 and 115% (except LLOQ where it was within 80-120%) for both DXC and DXM. All these data is presented in Table 3 for DXC and Table 4 for DXM indicate that the method is precise and accurate.

Recovery

Six aqueous replicates (samples spiked in reconstitution solution) at low, medium and high quality control concentration levels for both DXC and DXM were prepared for recovery determination, and the areas obtained were compared with the areas obtained for extracted samples of the same concentration levels. The mean recovery for both DXC and DXM was 86.3-93.8% with a precision of below 15%, and the mean recovery for DXC D_6 was 92.8% and for DXM D_6 was 92.3% with a precision of below 15%. This indicates that the extraction efficiency for analytes and IS was consistent, precise and reproducible.

Doxercalciferol				1α, 25-Dihydroxy Vitamin D2			
Nominal concentration (pg/mL)	Obtained mean concentration (pg/mL)	%CV	% Accuracy	Nominal concentration (pg/mL)	Obtained mean concentration (pg/mL)	%CV	% Accuracy
1	0.973	6	97.3	1.5	1.582	5.9	105.5
2.01	1.872	4.2	93.1	3	2.768	5.5	92.3
3.51	3.855	3	109.8	6.01	5.872	6.5	97.7
8.28	8.686	3.9	104.9	12.01	11.634	2.5	96.9
15.56	16.328	6.4	104.9	20.02	19.77	5.4	98.8
33.13	33.194	2	100.2	34.03	34.348	3.2	100.9
58.23	57.558	4.6	98.8	55.05	54.1	2.4	98.3
75.29	71.998	1.3	95.6	75.07	79.268	4.5	105.6

Table 2: Calibration curve details for Doxercalciferol and 1α, 25-Dihydroxy Vitamin D2 concentration.

Within run				Between run		
Nominal concentration (pg/mL)	Obtained mean concentration (pg/mL)	%CV	% Accuracy	Obtained mean concentration (pg/mL)	%CV	% Accuracy
1	1.182	9.6	118.2	0.979	18.3	97.9
2.99	2.845	11.7	95.2	2.991	9	100
8.97	8.762	3.6	97.7	9.26	5.5	103.2
16.58	16.955	5.5	102.3	17.101	5.1	103.1
34.25	33.577	2.8	98	34.333	3.8	100.2
61.17	57.763	3.8	94.4	59.587	5	97.4
75.29	68.665	2.6	91.2	70.454	4.2	93.6
171.17	163.318	3.2	95.4	170.592	3.6	99.7

Table 3: Within-run and between-run precision and accuracy of Doxercalciferol.

Within run				Between run		
Nominal concentration (pg/mL)	Obtained mean concentration (pg/mL)	%CV	% Accuracy	Obtained mean concentration (pg/mL)	% CV	% Accuracy
1.5	1.528	4.7	101.9	1.469	6.7	97.9
4.3	4.087	4.4	95	4.067	7.7	94.6
12.89	12.552	2	97.4	12.458	4.6	96.6
18.7	17.922	4.6	95.8	18.024	5.9	96.4
32.85	33.678	1.1	102.5	33.463	4.2	101.9
57.62	54.83	3.5	95.2	55.537	3.7	96.4
75.07	74.712	4.3	99.5	75.705	4.4	100.8
171.85	167.597	1.5	97.5	163.613	4.7	95.2

Table 4: Within-run and between-run precision and accuracy of 1α, 25-Dihydroxy Vitamin D2.

Reinjection reproducibility

Reinjection reproducibility exercise was performed to check whether the instrument performance remains unchanged after hardware deactivation due to any instrument failure during real subject sample analysis. The change was within 15% at three QC concentration levels from initial values; hence batch can be reinjected in the case of instrument failure during real subject sample analysis.

Stabilities

Stock solution and working solution stability was performed to check stability of analytes and IS in stock solutions prepared in acetonitrile and in working prepared in 90% methanol, respectively and stored at -35°C in a deep freezer. The freshly prepared stock and working solutions were compared with stock solutions prepared before 18 days and 17 days, respectively. The % change stock and working solution for analytes and IS was within-1.8-3.8% which indicates that stock and working solutions were stable at least for 17 days. Bench top and auto-sampler stability for analyte and metabolite was investigated at LQC-A and HQC levels. The results revealed that both analyte and metabolite was stable in plasma for at least 8 h at RT, and 84 h in an auto sampler at 4°C. It was confirmed that repeated freezing and thawing (four cycles) of plasma samples spiked with analyte and metabolite at LQC-A and HQC levels did not affect their stability. The long-term stability results also indicated that analyte and metabolite was stable in a matrix up to 150 days at a storage temperature of at -20 ± 5°C, -35 ± 5°C and -65 ± 10°C. The results obtained from all these stability studies are tabulated in Table 5.

Application

The validated method has been successfully applied, to quantify analyte and metabolite concentrations in human bioequivalence study under fasting condition, after administration of Doxercalciferol 2.5 mcg capsules as an oral dose. The pharmacokinetic parameters evaluated were C_{max} (maximum observed drug concentration), AUC_{0-t} and AUC_{0-inf} (area under the plasma concentration–time curve measured t time and infinite time, using the trapezoidal rule), t_{max} (time to observe maximum drug concentration), Kel (apparent first order terminal rate constant calculated from a semi-log plot of the plasma concentration versus time curve, using the method of the least square regression) and $t_{1/2}$ (terminal half-life as determined by the quotient 0.693/Kel, (Table 6).

The plasma concentrations of DXC was corrected for baseline endogenous levels by subtracting the mean value of four pre-dose levels at -24, -16, -8 and 0 hour baseline time points from each subsequent DXC concentration obtained after dosing and used for all pharmacokinetic calculations. Any negative values obtained from baseline correction at time 0 hour, was considered as zero (0) and any subject with pre-dose concentration more than 5% of their C_{max}

Stability experiments	Doxercalciferol			1α, 25-Dihydroxy Vitamin D2		
conditions	QC level	%Mean accuracy (n=6)	% CV (n=6)	QC level	%Mean accuracy (n=6)	% CV (n=6)
Bench top stability (room temperature, 8h)	LQC-A	107.4	4.6	LQC-A	91.2	2
	HQC	98	1.5	HQC	105.7	1.4
Auto sampler stability (4°C, 84 h)	LQC-A	98	5	LQC-A	97.7	7.1
	HQC	98.4	2.3	HQC	103.8	1.7
Freeze–thaw stability (-20 ± 5°C, cycle 4)	LQC-A	96	7.4	LQC-A	93.1	4.4
	HQC	98.3	2.1	HQC	102	2
Freeze–thaw stability (-35 ± 5°C, cycle 4)	LQC-A	96.4	5	LQC-A	95.2	3.5
	HQC	98.3	1.3	HQC	102.3	1.6
Freeze–thaw stability (-65 ± 10°C, cycle 4)	LQC-A	96.5	5.5	LQC-A	98.7	5.9
	HQC	100.3	2.3	HQC	102.6	2.4
Dry extract stability (room temperature, 8 h)	LQC-A	103	7.3	LQC-A	90.3	9.4
	HQC	96.2	1.2	HQC	104	2.4
Long-term stability (-20 ± 5°C, 150 days)	LQC-A	96	14.6	LQC-A	98.6	5.6
	HQC	106.5	2.4	HQC	105	3.2
Long-term stability (-35 ± 5°C, 150 days)	LQC-A	93.3	4.9	LQC-A	102	4.8
	HQC	109.6	2.5	HQC	106.3	2.2
Long-term stability (-65 ± 10°C, 150 days)	LQC-A	94.4	9.6	LQC-A	101.9	4.2
	HQC	105.3	2.3	HQC	104.6	2.9

Table 5: Stability results for Doxercalciferol and 1α, 25-Dihydroxy Vitamin D2.

	Doxercalciferol		1α, 25-Dihydroxy Vitamin D2	
Pharmacokinetic parameters	Test	Reference	Test	Reference
AUC_{0-t} (pg.h/mL)	181.14 ± 112.30	152.75 ± 90.09	1247.62 ± 520.69	1156.16 ± 484.14
AUC_{0-inf} (pg.h/mL)	204.39 ± 118.52	170.9 ± 97.46	1357.32 ± 536.61	1265.79 ± 487.39
C_{max} (pg /mL)	19.06 ± 9.53	17.27 ± 8.13	23.78 ± 10.42	22.61 ± 10.26
T_{max} (h)	6.65 ± 0.61	6.71 ± 0.99	12.8 ± 3.47	13.26 ± 3.86
K_{el} (h-1)	0.12 ± 0.07	0.14 ± 0.05	0.02 ± 0.01	0.02 ± 0.01
$t_{1/2}$ (h)	8.45 ± 6.23	6.46 ± 4.42	32.21 ± 9.35	31.88 ± 7.65

Table 6: Mean pharmacokinetic parameters of Doxercalciferol and 1α, 25-Dihydroxy Vitamin D2 in 45 healthy volunteers after oral administration of 2.5 µg (4 x 2.5 µg) test and reference products under fasting condition.

	Doxercalciferol			1α, 25-Dihydroxy Vitamin D2		
PK Variables	Ratio of LSM[1] (%)	Reference CV %[2]	95 % Upper Confidence Bound[3]	Ratio of LSM[1] (%)	Reference CV %[2]	90% Geometric C.I.[4]
AUC_{0-t}	111.58	33.55	-0.028	107.01	26.89	99.82 - 114.71
$AUC_{0-inf\#}$	115.1	32.08	-0.014	106.47	24.04	99.50 - 113.93
C_{max}	105.89	30.61	-0.036	105.91	24.1	99.30 - 112.96

Table 7: Statistical summary of Doxercalciferol and 1α, 25-Dihydroxy Vitamin D2 in 45 healthy volunteers after oral administration of 2.5 µg (4 x 2.5 µg) test and reference products under fasting condition.

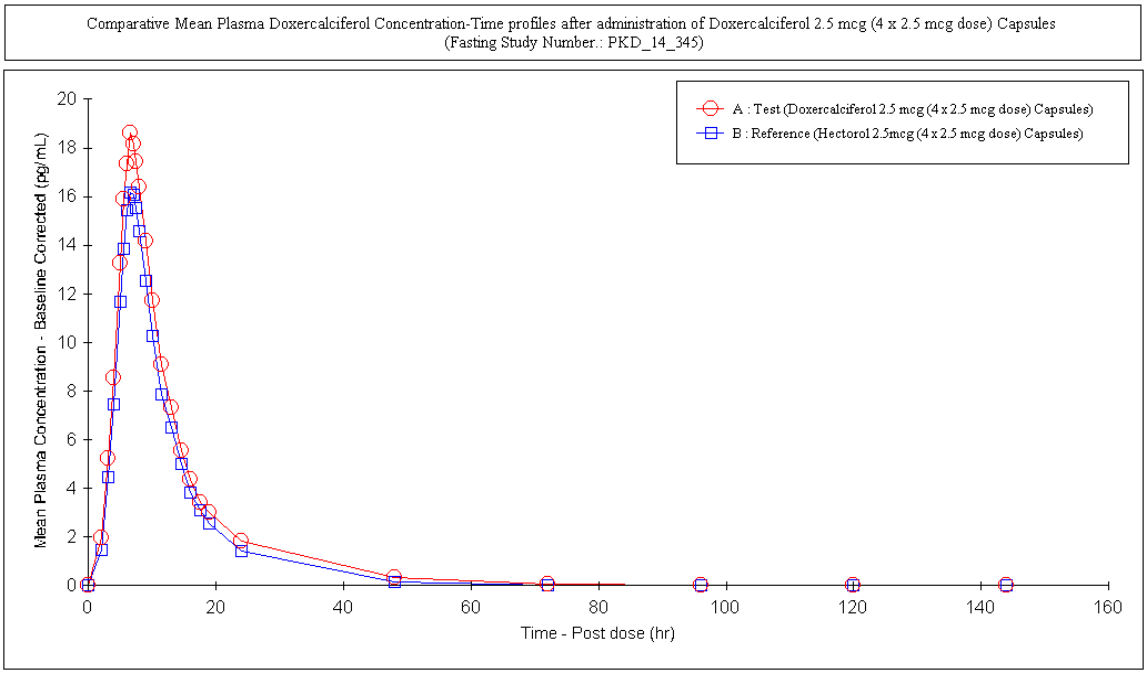

Figure 4: Mean Doxercalciferol plasma concentrations of test versus reference after oral administration of 2.5 µg (4 x 2.5 µg) capsules in 45 healthy volunteers.

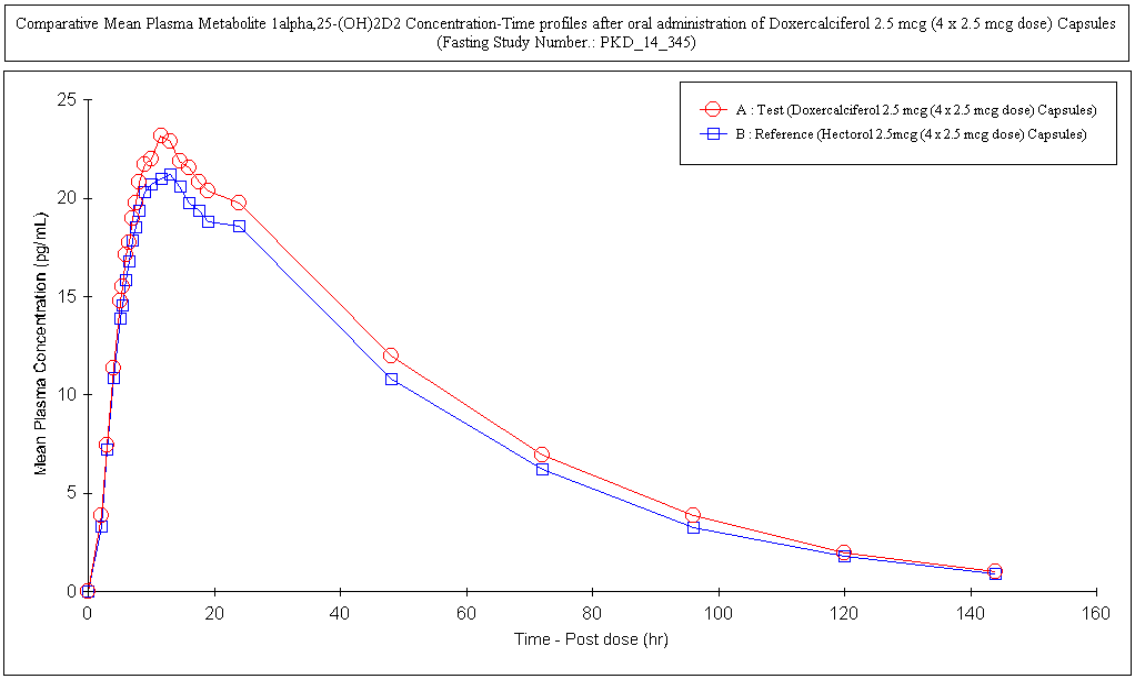

Figure 5: Mean 1α, 25-Dihydroxy Vitamin D2 plasma concentrations of test versus reference after oral administration of 2.5 µg (4 x 2.5 µg) capsules in 45 healthy volunteers.

should be excluded from BE statistical analysis and the 90% confidence intervals based on the remaining subjects.

For DXC, the Test/Reference ratios for C_{max}, AUC_{0-t}, and AUC_{0-inf} were 105.89, 111.58 and 115.10%, respectively. For DXC, intrasubject variability of C_{max}, AUC_{0-t} and AUC_{0-inf} were 30.61, 33.55, and 32.08%, respectively. Therefore, 95 % upper confidence of C_{max}, AUC_{0-t} and AUC_{0-inf} were -0.036, -0.028 and -0.028 were demonstrating the bioequivalence of the two formulations of DXC (Table 7). The mean concentration versus time profile of DXC and DXM in human plasma from 45 subjects that are receiving 4 x 2.5 µg oral dose of Doxercalciferol capsule as test and reference is shown in Figures 4 and 5, respectively. However metabolite data was provided as supportive evidence for comparability of therapeutic outcome of test and reference product (Tables 6 and 7).

Conclusion

The proposed bio-analytical method is highly sensitive, highly selective, precise, accurate, rugged and reproducible. This method was successfully applied in bioequivalence study to evaluate the plasma concentrations of DXC and DXM in study of healthy human volunteers.

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