Research Article

International Journal of Bioassays
ISSN: 2278-778X
www.ijbio.com

A LIQUID CHROMATOGRAPHY/ELECTROSPRAY IONIZATION TANDEM MASS SPECTROMETRIC METHOD FOR THE QUANTIFICATION OF CALCITRIOL IN HUMAN PLASMA: APPLICATION TO PHARMACOKINETIC STUDY IN HUMAN SUBJECTS

Siddheshwar Patankar[1, 2] *, Ashutosh Pudage[2], Varad Pradhan[2], Neeshad Joshi[1] and Vikas Vaidya[1]
[1]Ramnarain Ruia College of Science, Matunga, Mumbai-400019, Maharashtra, India
[2]Accutest Research Laboratories (I) Pvt. Ltd, A-31, MIDC, TTC Industrial Area, Khairane, Navi Mumbai-400709, Maharashtra, India

Received for publication: August 21, 2013; **Revised**: September 10, 2013; **Accepted**: October 04, 2013

Abstract: Calcitriol is a synthetic vitamin D analog which is active in the regulation of the absorption of calcium from the gastrointestinal tract and its utilization in the body. Circulating concentrations of endogenous calcitriol complicate the analysis of pharmacokinetic parameters and the determination of bioequivalence (BE) when the drug is administered exogenously. The main objective of this study is to evaluate the significance of endogenous concentration and baseline correction in the bioequivalence assessment of drug Calcitriol. An ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) method has been developed for the determination of Calcitriol in human plasma using Calcitriol d6 as the internal standard by means of baseline correction approach. The plasma samples were prepared by solid phase extraction on Phenomenex Strata-X cartridges using 500µL human plasma sample. Chromatography was carried out on Waters Acquity UPLC BEH C18 (100mm × 2.1mm, 1.7µ) analytical column under gradient conditions using a mobile phase consisting of acetonitrile–4.0 mM ammonium trifluoroacetate. The precursor to product ion transition for the analyte and IS was monitored on Waters Xevo TQ-S triple quadrupole mass spectrometer, operating in the multiple reaction monitoring and positive ionization mode. The method was validated over a wide dynamic concentration range of 5–200 pg/mL for Calcitriol. Stability was evaluated under different conditions including bench top, processed sample, freeze and thaw and long term. The method was applied to support a bioequivalence study of 0.5 mcg fixed dose formulation in 10 healthy Indian subjects. Assay reproducibility was demonstrated by reanalysis of 120 incurred samples.

Keywords: Calcitriol, LC-MS/MS, Plasma, Baseline correction, Derivatization

INTRODUCTION

Calcitriol (fig. 1) is a white, crystalline compound which occurs naturally in humans. Chemically, calcitriol is 9,10-seco(5Z,7E)-5,7,10(19)-cholestatriene-1α,3β,25-triol and has the following structural formula: $C_{27}H_{44}O_3$ M.W. 416.65

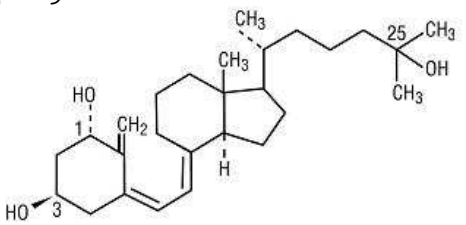

Fig.1: Chemical structure of Calcitriol

The other names frequently used for calcitriol are 1α, 25-dihydroxycholecalciferol and 1, 25-dihydroxyvitamin D3. Calcitriol is a secosteroid hormone that plays an important role in bone formation and mineralization by increasing plasma levels of calcium and phosphate [1, 2]. Calcitriol also has been shown to have anti-cancer activities [2–5]. Calcitriol or its analogues have been shown to produce additive or synergistic antineoplastic activity with a broad range of agents including dexamethasone [6, 7], retinoids [8, 9], tamoxifen [10–12], and radiation [13, 14], and several chemotherapy drugs, including docetaxel [15], paclitaxel [16], platinum compounds [17, 18], mitoxantrone [19], doxorubicin [20], and etoposide [21], are enhanced by calcitriol or its analogues.

Determination of Calcitriol in human plasma is challenging due to its protein binding and endogenous nature. Nevertheless, it shows very low circulating concentrations in plasma, which are usually in picomolar levels [22]. Literature search reveals different methods for quantitation of Calcitriol or Vitamin D metabolites in human plasma. Most of the methods have already been discussed by Shujing Ding [23]. A large amount of the LC-MS/MS methods use Cookson type derivatizing agents (eg. triazolinediones and triazolinediones-related reagents) for analysis of vitamin D metabolites [23, 24] and one of them have also used multiplexing approach of the derivatized analytes [25]. Other than derivatization, some of the authors have used lithium adduct [26, 27], Solid phase extraction [28], XLC-MS/MS (Extraction Liquid Chromatography-tandem Mass Spectrometry) [29] for the determination of Calcitriol and vitamin D metabolites. However these techniques lacked the sensitivity required to detect Calcitriol at low picogram

***Corresponding Author:**
Siddheshwar V Patankar,
Accutest Research Laboratories (I) Pvt. Ltd,
A-31, MIDC, TTC Industrial Area, Khairane,
Navi Mumbai-400709, Maharashtra, India.

level. The methods which were sensitive enough had labor-intensive and time-consuming sample extraction procedures. In this paper, we describe a simple and sensitive assay using UPLC-MS/MS after PTAD derivatization and by means of baseline correction approach for the quantitative determination of Calcitriol in human plasma.

MATERIALS AND METHODS

Chemical and reagents:

Calcitriol and Calcitriol d6 were purchased from Splendid labs (Mumbai) and Syncom (UK) respectively. The HPLC grade solvents viz. methanol, acetonitrile, chloroform were purchased from J.T. Baker (India). Whereas, ammonium trifluoroacetate, formic acid and acetic acid were purchased from Fluka (India). 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) was obtained from Sigma-Aldrich (India). HPLC grade water was obtained from Merck (India). Fig. 02 shows MS/MS spectra of PTAD derivatized Calcitriol and Calcitriol d6.

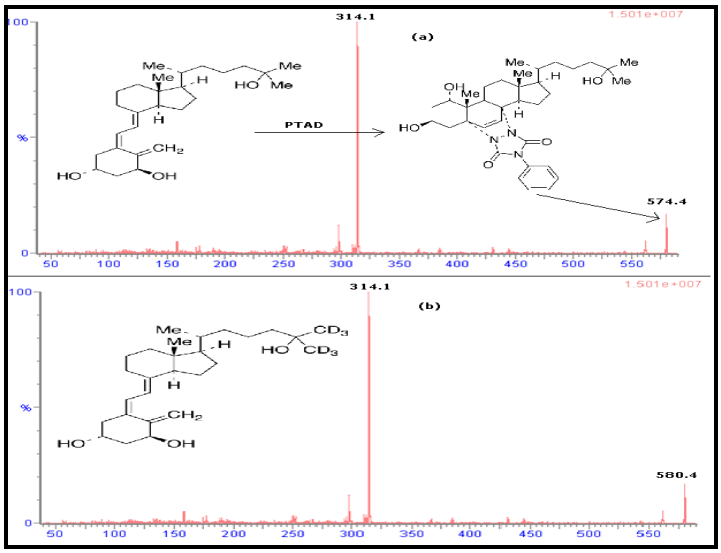

Figure.02: Representative Product ion spectra of $[M+H-H_2O]^+$ of a) PTAD derivatized Calcitriol b) PTAD derivatized Calcitriol d6

Preparation of standards and quality control samples:

Stock solutions of Calcitriol and IS were prepared by dissolving the test compounds in acetonitrile to obtain 200 µg/mL concentration for both. Working solutions at concentrations of 100, 200, 300, 400, 800, 1600, 2400, 3400 and 4000 pg/mL for Calcitriol were prepared through serial dilution of stock solution with diluent. The stock solutions and working solutions were stored at 2-8°C.

These working solutions were further diluted with surrogate matrix (water-acetonitrile 50:50, v/v) to give calibration standards in the range of 5pg/mL to 200pg/mL. Surrogate Calibration standards were prepared fresh daily from the working solutions.

Quality control (QC) samples were independently prepared in baseline corrected blank plasma at four different concentrations 5, 15, 70, and 150 pg/mL (LLQC, LQC, MQC and HQC respectively) for Calcitriol. Quality control samples were stored at -70°C until analysis.

Sample preparation:

Calibration standards were prepared in surrogate matrix i.e. (water-acetonitrile 50:50, v/v) for baseline computation experiments, whereas, after baseline correction, calibration standards and quality control samples were prepared in human plasma based matrix for validation runs and subsequent sample analysis. Sample preparation was carried out under sodium vapor lamp, as Calcitriol is light sensitive in nature.

For matrix based samples: Prior to analysis, all frozen subject samples and quality control samples were thawed and allowed to equilibrate at room temperature. To 500 µL of plasma sample, 25 µL of internal standard (100 $ngml^{-1}$ of D6- calcitriol) was added and vortexed for 30s. Further, 500 µL of 0.1% (v/v) formic acid was added and vortexed for another 30 s. Samples were then centrifuged at 14000 rpm for 5 min at 10°C and thereafter loaded on Phenomenex Strata-X (30 mg/1 cc) cartridges, after conditioning with 1 mL methanol followed by 1 mL of water. Washing of samples was done with 1 mL water followed by 1 mL of 20% acetonitrile in water. Subsequently, the cartridges were dried for 30 s by applying nitrogen (30psi). Elution of analyte and IS was done using 2 mL of chloroform into pre-labeled vials and briefly vortexed for 15 s. The samples were then evaporated to dryness under gentle stream of nitrogen at 30°C and derivatized with PTAD.

For calibration standards in surrogate matrix: Working solutions of Calibration standards were allowed to equilibrate at room temperature. To 475 µL of surrogate matrix, 25 µL of working solution of calcitriol and 50 µL of internal standard was added and vortexed for 30s. The samples were then evaporated to dryness under gentle stream of nitrogen at 30°C and derivatized with PTAD.

Derivatizaion reaction: The derivatizing agent PTAD (4-phenyl-1,2,4-triazoline-3,5-dione) was dissolved in acetonitrile to prepare 500 µg/mL concentration. 200 µL of derivatizing agent was added to each sample after evaporation step. The samples were vortexed for 30 s and kept at room temperature for two hours. After two hours the derivatized samples were vortexed for 30 s and evaporated to dryness under gentle stream of nitrogen at 30°C. The dried residues were then reconstituted with 300 µL of acetonitrile-4mM ammonium trifluoroacetate, (60:40, v/v) and an aliquot of 10 µL was then injected into the LC-MS/MS.

Baseline Concentration determination: Baseline correction approach was chosen to rule out the interference from the endogenous levels of Calcitriol present in plasma. For the same, calibrants in surrogate matrix were run along with zero standards and the low as well as high level quality control sample of each matrix lot in three replicates. The Baseline concentration thus obtained was added to the nominal concentration of low as well as high quality control samples. The baseline corrected quality control samples from individual lots which were found to be within acceptance in terms of accuracy and precision were selected.

Accordingly matrix lots selected and pooled for preparation of bulk spiked calibrants and quality control samples in specified range. Baseline concentration of pooled matrix was evaluated against the calibration standards in surrogate matrix and added to the nominal concentration of calibrants and quality control samples for further analysis.

Instrumentation and Analytical Conditions:

Waters Acquity UPLC (Waters Corp., Milford, MA, USA) coupled to Waters Xevo TQ-S (Waters Corp., Milford, MA, USA) was used. A Waters Acquity UPLC BEH C18 (100 mm × 2.1 mm, 1.7 µ) analytical column (Waters Corp., Milford, MA, USA) protected by an Acquity UPLC column in-line filter unit (0.2 µm in-line frit) was used for the separation of Calcitriol. The following gradient was used: Initial, B 80%; 0–4.0 min, B 80%-20%; 4.01–5.50 min, B 20%; 5.50–7.0 min, B 20%-80%; where mobile phase A was Acetonitrile and B was 4mM Ammonium Trifluoroacetate. The flow-rate of the mobile phase was set at 0.2 mL/min. Flow from the UPLC was diverted to the waste till 4.00 minutes and from 4.01 minutes onwards to the source till 5.5 minutes. After 5.5 min, flow was again diverted to waste till the end of the run. This was done to minimize the source contamination. Data was acquired by MassLynx V4.1 software and processed for quantification with QuanLynx V4.1 (Waters Corp., Milford, MA, USA). The MS/MS system was operated with an ESI interface in positive ionization mode (ESI+). Cone and desolvation gas flow rates, obtained from a nitrogen generator NM32L Peak Scientific (Scotland), were 150 L/h and 1100 L/h, respectively. MS parameters were defined with Waters IntelliStart software (automatic tuning and calibration of the waters Xevo TQ-S) and manually optimized as follows: capillary voltage of 3.8 kV, source temperature at 150 ◦C and desolvation temperature at 500 ◦C. Cone voltage was 2 V and 4 V, and collision energy was 14 eV and 18 eV for Calcitriol and I.S respectively. Quantification was determined using multiple reaction monitoring (MRM) mode for the transitions m/z 574.4 > 314.158 for Calcitriol, and 580.4 > 314.136 for I.S. The dwell time was set at 0.100s.

Method validation:

US FDA guidelines were followed for the validation of the developed method [30].

Selectivity and matrix effect: Selectivity was not performed for this assay due to the endogenous nature of the analyte. Matrix effect was evaluated by analyzing 6 sources of normal, 2 sources of hemolysed and 2 sources of lipemic human blank plasma at LQC and HQC level. Baseline concentration of these lots was evaluated against surrogate calibration standards and was subsequently added in the nominal concentration of LQC and HQC. Back calculated concentrations of the LQC's and HQC's in each lot were compared against the corrected nominal to arrive at % accuracy of the QCs.

The imprecision had not to exceed 15%, and the inaccuracy had to be within ±15% of the nominal value.

Linearity: Linearity was assessed by analyzing at least three calibration curves in surrogate matrix as well as in human plasma with nine levels on three different days. The curves were constructed from a linearly weighted ($1/X^2$) least squares regression obtained by plotting peak area ratios of the analyte to IS against the nominal concentration of analyte. The ratio of area response for analyte to IS was used for regression analysis. Each calibration curve was analyzed individually by using least square weighted ($1/X^2$) linear regression. The calculation was based on the peak area ratio of analyte versus the area of internal standard. The concentration of the analyte were calculated from calibration curve ($y = mx + c$; where y is the peak area ratio) using linear regression analysis with reciprocate of the drug concentration as a weighing factor ($1/X^2$). Several regression types were tested and the linear regression (weighted with $1/\text{concentration}^2$) was found to be the simplest regression.

Precision and Accuracy:

The intra-batch and inter-batch accuracy and precision were determined by replicate analysis of the four quality control levels on three different days. In each of the precision and accuracy batches, six replicates at each quality control level were analysed. Mean and standard deviation (SD) were obtained for calculated drug concentration over these batches. Accuracy and precision were calculated in terms of % accuracy and coefficient of variation (% CV) respectively.

Carry over effect:

Carryover effect was evaluated to ensure that the rinsing solution used to clean the injection needle and port is able to avoid any carry forward of injected sample in subsequent runs.Carry over effect was checked by processing blank samples, LLOQ and ULOQ

samples in duplicate using surrogate matrix and after ULOQ samples same blank samples were injected.

Recovery:

In this type of endogenous assay, absolute or relative recovery at low quality control level was not possible due to the presence of endogenous calcitriol. Hence, recovery was performed by preparing separately six replicates of LQC, MQC and HQC which were extracted as per the method. All these quality control samples were analysed against freshly prepared calibration curve in surrogate matrix in a single run. Back calculated concentration of quality control samples were compared with the corrected concentration by considering the baseline concentration.

Batch size determination:

This exercise was evaluated by analyzing one set of calibration curve standards and four sets of quality control samples (comprising 6 quality control samples in each set) at each level LQC, MQC and HQC. It was intended to assess the inter batch variability in longer run especially during subsequent sample analysis.

Dilution integrity:

The dilution integrity experiment was intended to validate the dilution test to be carried out on higher analyte concentrations (above ULOQ), which may be encountered during real subject samples analysis. It was performed at 1.6 times the ULOQ concentration. Six replicates samples of ½ and ¼ th concentration were prepared and the concentrations were calculated by applying the dilution factor of 2 and 4 respectively against the freshly prepared calibration curve.

Stabilities:

Stability experiments were conducted to evaluate different conditions that plasma samples may encounter during sample shipment as well as pre- and post-processing such as several freeze-thaw cycles and short term storage of plasma samples at room temperature. The stability of Calcitriol in human plasma was investigated at two QC levels LQC and HQC against the calibration standards in surrogate matrix. All stability results were evaluated by measuring the area response (analyte/IS) of stability samples against freshly prepared comparison standards with identical concentration. Stock solutions and working solutions of analyte and IS were checked for short term stability at room temperature and long term stability at 2–8°C. The solutions were considered stable if the deviation from nominal value was within ±10.0%. Autosampler stability, processed sample stability, dry extract stability, bench top (at room temperature), and freeze–thaw stability at 3 and 5 freeze thaw cycles were performed at LQC and HQC using six replicates at each level. Long term stability of spiked plasma samples stored at –70° C was also studied at both these levels. The samples were considered stable if the deviation from the mean calculated concentration of freshly thawed quality control samples was within ±15.0%.

Subject analysis:

The analytical method developed was applied to evaluate comparatively the calcitriol plasma concentration from two formulations of calcitriol capsule 0.5 mcg, in normal, Healthy, Adult, Human subjects under fed conditions. Ten healthy male volunteers were selected for study after assessment of their health status by clinical evaluation (physical examination, ECG) and routine laboratory tests to achieve minimum eight subject's pharmacokinetic and statistical data. Each subject was judged to be in good health through medical history, physical examination and routine laboratory tests. Written consent was taken from all the subjects after informing them about the objectives and possible risks involved in the study. The study was conducted strictly in accordance with guidelines laid down by International Conference on Harmonization, E6 Good Clinical Practice [32]. The subjects were administered the study drug in sitting position with 240 mL of water at ambient temperature in each study period, 30 minutes after start of the breakfast. A total of 24 blood samples (6 mL each) were collected during each period of the study at -18.00, -12.00, -6.00, pre-dose (within 5 minutes before dosing) and then 0.50, 1.00, 1.50, 2.00, 2.50, 3.00, 3.50, 4.00, 4.50, 5.00, 5.50, 6.00, 7.00, 8.00, 10.00, 12.00, 24.00, 36.00, 48.00 and 72.00 hrs post dose. The drug concentration of calcitrol in plasma for each subject, each sampling time and each product was noted. For each subject, an average baseline calcitrol concentration was determined and subtracted from the plasma concentrations determined on the pharmacokinetic sampling day. A baseline concentration was determined for each dosing period. Bioequivalence between the two formulations was assessed by calculating individual test/reference ratios for the peak of concentration (C_{max}), Area under the curve (AUC) of plasma concentration and the area under the curve between the first sample (pre-dose) and infinite (AUC0-inf).C_{max} and the time taken to achieve this concentration (T_{max}) were obtained directly from the curves. The pk parameters were calculated for baseline corrected and uncorrected Calcitriol. Pharmacokinetic and statistical analysis was done using software SAS® version 9.2.

Incurred sample reanalysis:

The assay reproducibility was demonstrated by reanalysis of 5% of total subject samples on completion of subject sample analysis. An incurred sample reanalysis (assay reproducibility test) was conducted by random selection of subject samples. The selection criteria included samples which were near the Cmax

and the elimination phase in the pharmacokinetic profile of the drug. The results obtained were compared with the data obtained earlier for the same sample using the same procedure. The percent change in the values should not be more than ±20% [33].

RESULTS AND DISCUSSIONS

Method development:

Plasma extraction: As Calcitriol is tightly bound to plasma proteins and is endogenously present in the human plasma different methods such as protein precipitation; solid phase extraction (SPE) and liquid liquid extraction (LLE) were employed in order to achieve acceptable recovery and reproducibility. Protein precipitation was tried with acetonitrile and methanol, but it lacked the required reproducibility. Liquid-liquid extraction was tried with a combination of many extraction solvents. However, the recovery and sensitivity was inconsistent and poor with some ion suppression in most of the solvent systems. Solid phase extraction showed consistent recovery and reproducibility with phenomenex strata X polymeric reversed phase (30 mg/ 1cc) cartridges. The derivatizing agent PTAD was also tested for different concentrations and time, and it was concluded that 500µg/mL of PTAD for two hours was required for complete derivatization of Calcitriol and IS. A deuterated internal standard was used to minimize any analytical variation due to solvent evaporation, integrity of the column and ionization efficiency of the analyte. Calcitriol d6 (IS) had similar chromatographic behavior and was quantitatively extracted with the proposed extraction procedure. Also, there was no effect of IS on analyte recovery, sensitivity or ion suppression.

Chromatography:

Chromatographic separation of the analyte was initiated under gradient conditions to obtain adequate response, sharp peak shape and a short analysis time. To find the best eluting solvent system, various combinations of methanol/acetonitrile along with buffers (ammonium trifluoroacetate/acetic acid, ammonium formate/formic acid, ammonium acetate/acetic acid, ammonium bicarbonate/ammonium hydroxide) having different ionic strengths (1–10 mM) in the pH range of 3.0–10.0 and volume ratios were tested. With methanol as organic solvent, moderate peak tailing was observed. Out of all the mobile phase compositions, mobile phase of ammonium bicarbonate/ammonium hydroxide with acetonitrile showed comparable results with the present method. But it was excluded due to frequent high column back pressures and high pH. The mobile phase consisting of acetonitrile–4.0 mM ammonium trifluoroacetate in gradient run was found most suitable for baseline separation of Calcitriol and IS. Different column temperatures were also tested from 25°C to 45°C, and concluded that the resolution improved with 40°C column temperature. Fig. 03 shows the representative MRM chromatograms of Calcitriol and Calcitriol d6.

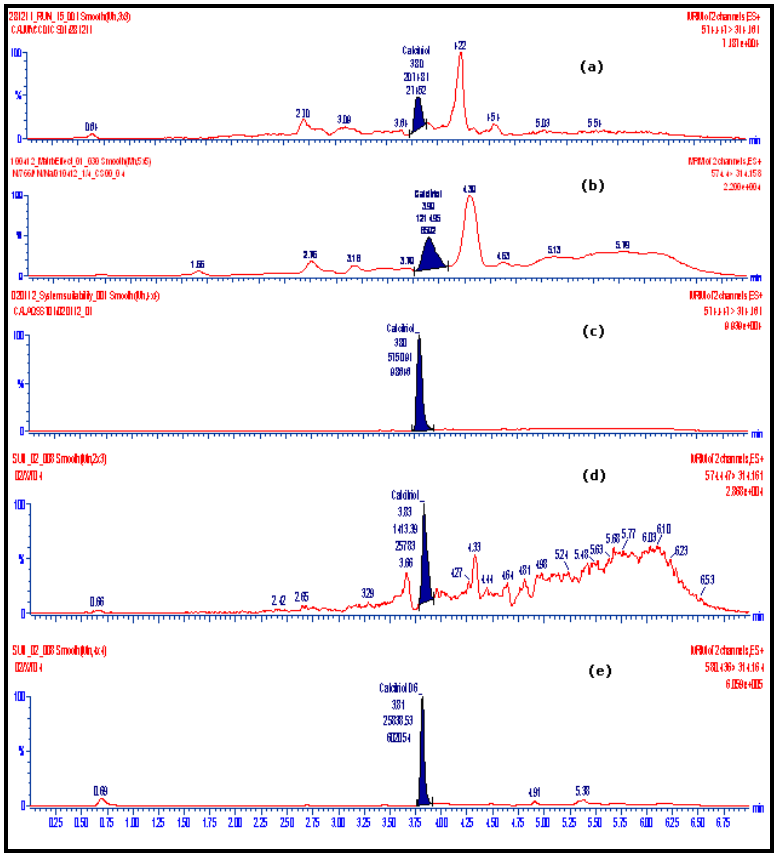

Fig. 03: Representative MRM chromatograms of blank human plasma samples (a) Calcitriol at LLOQ level (5.0 pg/mL), (b) Blank plasma sample, (c) Calcitriol at MQC level (60.0pg/mL), (d) Unknown sample of Calcitriol at Cmax level from a pharmacokinetic study and (e) Calcitriol d6, IS (200pg/mL)

Mass spectrometry:

In order to develop a sensitive assay for calcitriol different ionization source were tried including Electrospray ionization source (ESI) and Atmospheric pressure chemical ionization source (APCI). Since ESI source is suitable to analyze the polar compounds and APCI to analyze the non-polar compounds, the derivatized and underivatized Calcitriol was tested in both the modes. In both the modes the intensity of underivatized calcitriol was very less. Hence from thorough literature search it was concluded to derivatize the molecule with 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) [23]. After PTAD derivatization, the intensity of calcitriol was improved in ESI source but no significant change was observed in APCI source. But there was a shift of molecular weight from 416.65 to 574.0 thus reducing the interference from low m/z background ions and following the same $(M+H-H_2O)^+$ precursor ions as reported earlier [23]. The product ion observed was predominantly 314 as reported by

Shujing Ding et. al. [23], but the addition of methyl amine as reported by the same author was avoided because it did not show any significant improvement in the intensity of Calcitriol. The advantages of using ESI source were demonstrated in our experiments with less background noise and better sensitivity. Calcitriol d6 was used as internal standard in the present study, which showed same precursor to product ion spectrum as Calcitriol. Fig. 2 shows the product ion spectrum of Calcitriol and Calcitriol d6 respectively.

Baseline correction[34]:

The quantitative determination of endogenous (i.e. naturally occurring) compounds in biological samples is more complicated, both analytically and from a validation point of view. It is often difficult, if not impossible, to obtain analyte-free samples of the authentic biological matrix or samples with accurately-known analyte concentrations, so the preparation of reference samples has to be addressed in a different way and, as a consequence, validation also becomes less straightforward. An endogenous compound have been measured in clinical laboratories for many years and with a variety of techniques, but interest in the determination of these compounds, now often referred to as biomarkers, as part of the development process of new drugs, is relatively recent. Often, critical decisions about the continuation of a drug-development program are based on concentrations of one or more well-selected biomarkers, as these give crucial information about the efficacy and the safety of a drug. To ensure adequate confidence in the results, the availability of accurate analytical methods is essential and, for low molecular-weight analytes, this has led to an increasing desire to apply chromatographic techniques rather than traditional ligand-binding assays, because of their better analytical performance. The validation of chromatographic methods for endogenous analytes has so far been hampered by the absence of official guidelines. any researchers would want to apply the method-validation principles for drug assays, in particular those issued by the US Food and Drug Administration [30] also to their methods for endogenous analytes, in order to ensure results with a comparable level of quality, but it has to be noted that these principles were not primarily meant for endogenous compounds and, in many cases, cannot be directly applied. An essential part of method development is the selection of a proper way to prepare calibration and QC samples. Ideally, these samples are aliquots of the authentic biological matrix containing an accurately known concentration of the authentic analyte. For endogenous compounds, the authentic biological matrix typically contains an unknown concentration of the analyte, making it unsuitable for the preparation of reference samples. A well-known approach to circumvent this problem is the application of the method of standard addition. By adding increasing concentrations of the analyte to individual aliquots of the sample of interest, a calibration curve is created and the endogenous concentration in the sample is determined from the intercept of this calibration curve. If no analyte-free samples of the authentic matrix are available, calibration standards can be prepared by spiking the analyte in some sort of artificial or surrogate matrix. Surrogate matrices can vary widely in complexity. In its simplest form, a surrogate matrix may be pure water or a buffer, such as phosphate-buffered saline (PBS), which is frequently used for plasma and serum analyses because of its similar pH (7.4) and ionic strength (150 mM). Often, bovine (BSA) or human serum albumin (HSA) is added to PBS at a concentration of 40–60 g/l to take the protein content of the biological matrix into account and increase the solubility of hydrophobic analytes. Plasma has a more complex composition – because of its variety of proteins and lipids – and is more difficult to mimic. Several commercial suppliers offer ready-to-use synthetic surrogate matrices, the exact nature of which is often kept unknown.

Alternatively, many endogenous analytes can be removed from the authentic biological matrix – along with other small organic compounds – by stripping with activated carbon. A typical procedure for this is to add activated carbon to the matrix and mix it for a several hours to days, followed by centrifugation and subsequent filtering of the matrix through micro-porous membranes. Special care should be taken that all carbon particles are effectively removed before the stripped matrix is spiked, because added analytes will readily bind to remaining traces of carbon, and that will result in decreased concentrations of dissolved analyte. A disadvantage of the carbon-stripping method is that it is not universally applicable. In addition, batch-to-batch variations may occur, which could very well be related to the analyte concentration before stripping. Obviously, it is advisable to start with a sample having a relatively low concentration of endogenous analyte.

During method development of Calcitriol, we adopted both the techniques, i.e. standard addition approach as well as use of surrogate matrix. Initial development was mainly focused on use of surrogate matrix to produce reliable and precise results. Surrogate matrices used include phosphate buffer saline, charcoal stripped human plasma, water and water:acetonitrile, 50:50% v/v. Use of phosphate buffer saline decreased the response of calcitriol, making it difficult to quantify at low picogram level. Use of water was not compatible with derivatizing agent, PTAD, which obviously resulted in incomplete or nil derivatization reaction and thus obstructing the precise quatification capabilities of the assay. Charcoal stripping of human plasma was also tried. But, the experiment showed that, calcitriol was not easy to

remove from the bound plasma proteins and required around 24 hours of shaking, with relatively high amount of activated carbon, which was practically impossible to set up a rapid assay. Finally, the surrogate matrix of water: acetonitrile, 50:50% v/v was used due to its not interfering ability with drug or IS, simplicity of use and prospects to quantify calcitriol at low picogram level. For validation experiments and sample analysis surrogate matrix was used for baseline calculation of individual human plasma lots and prepared fresh daily.

Method validation:

Matrix effect: Each matrix lot was processed in replicate (n=9) times to get triplicate sample of zero standard which was used to evaluate the baseline concentration in respective matrix lot and for spiking LQC (n=3) and HQC (n=3) concentration respectively. Please refer Table No.1 for details. Data for lower quality control sample is shown, as there was not much matrix effect at higher quality control sample.

A calibration curve in surrogate matrix (Acetonitrile : Water :: 50:50) and three replicates each of zero standard, low quality control samples as well as high quality control samples from each source of normal controlled matrix were processed freshly and analyzed in a single run. Baseline concentration for each processed lot was calculated using calibration curve standards. Mean of back calculated baseline concentration for each lot was used to arrive at the nominal concentration of LQC's and HQC's in respective lot. Out of eight heparinised normal lots tested only one lot showed % accuracy > 15%. Out of two heparinised Hemolysed lots tested one lot showed % accuracy > 15%.

Table.1: Matrix Effect at LQC Level (Actual Concentration of LQC: 14.031 pg/mL)

Plasma Lot used for Baseline calculation	Back calculated Baseline Conc. (pg/mL)	Average Baseline conc. (pg/mL)	Base line corrected conc. at LQC level (pg/mL)	Back calculated conc. From each lot at LQC level (pg/mL)	% Accuracy	%CV	Results
Heparinised Normal Human plasma	34.285			41.526	85.94		
	34.564	34.291	48.322	53.241	110.18	13.62	PASS
	34.025			43.472	89.96		
Heparinised Normal Human plasma	61.426			72.933	96.00		
	63.012	61.939	75.970	76.521	100.73	9.32	PASS
	61.379			63.693	83.84		
Heparinised Normal Human plasma	63.203			90.584	110.65		
	72.041	67.835	81.866	92.192	112.61	1.09	PASS
	68.262			92.429	112.90		
Heparinised Normal Human plasma	41.990			59.348	101.90		
	47.121	44.208	58.239	69.295	118.98	12.31	PASS
	43.513			54.554	93.67		
Heparinised Normal Human plasma	43.742			67.627	105.62		
	55.129	49.996	64.027	77.410	120.90	12.33	PASS
	51.118			60.576	94.61		
Heparinised Hemolysed Human plasma	63.801			91.856	118.28		
	67.554	63.631	77.662	88.800	114.34	1.84	FAIL
	59.538			91.494	117.81		
Heparinised Hemolysed Human plasma	75.358			93.420	107.28		
	69.857	73.053	87.084	91.896	105.53	1.98	PASS
	73.945			89.795	103.11		
Heparinised Lipemic Human plasma	68.873			88.375	102.28		
	73.271	72.378	86.409	82.818	95.84	4.61	PASS
	74.989			80.925	93.65		
Heparinised Lipemic Human plasma	71.784			69.202	79.71		
	70.436	72.783	86.814	74.814	86.18	4.13	PASS
	76.130			73.836	85.05		

Baseline corrected concentration:

Baseline corrected concentration was evaluated by using surrogate matrix (Acetonitrile: Water:: 50:50 v/v). Zero standards (n=6) prepared from pooled screened plasma were run against calibrants prepared in surrogate matrix to evaluate the baseline concentration. Mean of back calculated baseline concentration was added to nominal concentration of all the calibration curve standards as well as quality control samples. The inter day and intraday precision and accuracy data were obtained by measuring three different standard curves on three different days for inter day and one batch for intraday using both the

matrices. Representative chromatograms obtained from the LLOQ and extracted zero sample are presented in Fig.3. calcitriol and IS retention times were around 3.2 ± 0.5 min and 3.1 ± 0.5 min respectively.

Recovery:

The mean recovery of Calcitriol for the LQC, MQC and HQC QCs were 110.66%, 108.56% and 113.09%, respectively. The mean overall recovery of Calcitriol was 110.77% with a precision of 2.05%.

Accuracy and precision:

Intra-batch precision and accuracy of the assay was measured for Calcitriol at each QC level (5.021, 14.032, 70.159, 150.340 pg/mL) in surrogate matrix as well as in baseline corrected human plasma. The results were within the acceptance criteria for precision and accuracy (± 15% and ± 20% for LLOQ). Please refer Table 01 for details.

Table.1: Accuracy and precision data for Calcitriol quantification in human plasma.

QC samples	Nominal conc. (pg/mL)	Baseline Corrected conc. (pg/mL)	Accuracy		Precision	
			Intra-batch assay[a]	Inter-batch assay[b]	Intra-batch assay [a]	Inter-batch assay [b]
QC-LLOQ	5.021	91.033	97.65	103.81	6.19	7.15
QCL	14.032	100.044	100.30	99.75	0.82	3.19
QCM	70.159	156.171	93.46	93.25	1.81	1.98
QCH	150.340	236.352	100.13	100.38	1.07	1.98

[a] (n=6), expressed as (found concentration/nominal concentration)x100.

[b] Values obtained from all four runs (n=24).

Hemolysis effect:

The precision and accuracy of quality control samples at LQC and HQC levels were found to be within acceptance, hence it was concluded that hemolysis effect is absent.

Stability of Calcitriol:

The stability test of Calcitriol in human plasma showed no significant degradation, when kept on bench at room temperature for 18 hrs before processing. The sample was also stable for 5 freeze-thaw cycles. Extracted samples of Calcitriol were stable for 55 hrs when kept in auto sampler at 10°C. Dry extracted samples were stable for 25 h at 2-8°C. Stock solution of Calcitriol was found to be stable for 23 h at room temperature, whereas it was stable for 9 days at 2-8°C. Calcitriol was stable in plasma at -70°C for 91 days. Please refer Table No. 02 for details.

Table.02: Stability of Calcitriol under various conditions

	LQC (100.044 pg/mL)	*HQC* (236.352 ng/mL)
Bench top stabiliy (room temperature, 18 h), N=6		
RSD%	4.70	1.89
Accuracy%	94.27	98.24
Freeze-Thaw stability (5 Cycles, -20°C), N=6		
RSD%	9.18	2.33
Accuracy%	96.47	98.97
Autosampler stability (10°C, 55 H), N=6		
RSD%	6.18	3.12
Accuracy%	93.70	99.92
Processed samples stability (2-80C, 25 H), N=6		
RSD%	5.86	2.74
Accuracy%	95.53	100.30
Dry Extract stability (2-80C, 25 H), N=6		
RSD%	6.75	2.70
Accuracy%	92.42	100.12
Long term stability (-700C, 91 D), N=6		
RSD%	6.25	3.11
Accuracy%	94.5	94.2

Comparative pharmacokinetic parameters:

The validated method was successfully applied to the assay of Calcitriol in 10 healthy Indian male subjects. Fig. 4 shows the mean plasma concentration vs. time profile for Calcitriol under fasting condition. Table 03 summarizes the mean pharmacokinetic parameters after oral administration of 0.5mcg Calcitriol test and reference formulations. About 650 samples including the calibration and QC samples along with subject samples were analyzed during a period of 8 days and the precision and accuracy for calibration and QC samples were well within the acceptable limits. The % change for assay reproducibility in 10% incurred samples was within ±10%. This authenticates the reproducibility of the proposed method.

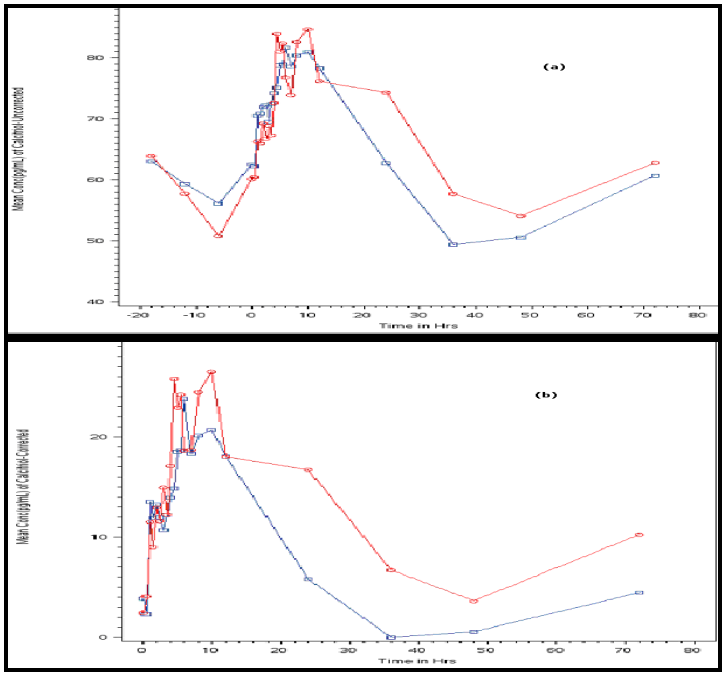

Fig.04: Mean plasma conc (pg/mL) of Calcitriol-Baseline Corrected and Baseline Uncorrected vs Time in hrs.

Table.3: Mean pharmacokinetic parameters obtained from 10 volunteers after administration each of 0.5 mcg Calcitriol capsule formulation.

Parameters	Test Formulation		Reference Formulation	
	Mean	SD	Mean	SD
C_{max} (pg/mL)	95.47	35.22	89.37	24.91
t_{max} (h)	7.30	3.60	13.25	21.19
AUC_{o-inf} (ngh/mL)	4720.30	1583.04	4411.10	1313.59

After Baseline Correction

Parameters	Test Formulation		Reference Formulation	
	Mean	SD	Mean	SD
C_{max} (pg/mL)	37.30	22.25	29.10	9.23
t_{max} (h)	7.30	3.60	13.25	21.19
AUC_{o-inf} (ngh/mL)	819.74	446.13	602.13	343.53

CONCLUSION

This work describes a fast, sensitive and robust method to quantify Calcitriol in human plasma using D6-calcitriol as the internal standard. Extracted samples were analyzed by UPLC-ESI-MS/MS. This method agrees with the requirements proposed by the US Food and Drug Administration and ANVISA of high selectivity and high sample throughput in comparative pharmacokinetic assays such as bioequivalence studies. The lowest concentration quantified was 5.003pg/mL with suitable accuracy and precision. The intra-assay precision ranged from 0.82% to 6.19% while inter-assay precision ranged from 1.98% to 7.15%. The intra-assay accuracy ranged from 93.46% to 100.30% while inter-assay accuracy ranged from 93.25% to 103.81%. The described method for separation and quantification of calcitriol in human plasma was successfully applied in bioequivalence study of two 0.5 mcg calcitriol capsule formulations using an open, randomized, two-period, cross over design.

REFERENCES

1. Kanis JA. Vitamin D metabolism and its clinical application. J Bone Joint Surg Br 1982; 64 (5):542–60.
2. Morris HA. Vitamin D: a hormone for all seasons-how much is enough? Clin Biochem Rev 2005; 26 (1):21–32.
3. Guyton KZ, Kensler TW, Posner GH. Cancer chemoprevention using natural vitamin D and synthetic analogs. Annu Rev Pharmacol Toxicol 2001; 41: 421–42.
4. Getzenberg RH, Light BW, Lapco PE, Konety BR, Nangia AK, Acierno JS, et al. Vitamin D inhibition of prostate adenocarcinoma growth and metastasis in the Dunning rat prostate model system. Urology 1997; 50: 999–1006.
5. TomaszM. Beer,MilindJavle, Gilbert N. Lam, W. David Henner, Alvin Wong,and Donald L.Trump Clin Cancer Res 11(2005) 7794.
6. Bernardi RJ, Trump DL, Yu WD, McGuire TF, Hershberger PA, Johnson CS. Combination of 1a,25- dihydroxyvitamin D (3) with dexamethasone enhances cell cycle arrest and apoptosis: role of nuclear receptor cross-talk and Erk/Akt signaling. Clin Cancer Res 2001; 7:4164-73.
7. Yu WD, McElwain MC, Modzelewski RA, et al. Enhancement of 1,25-dihydroxyvitamin D3-mediated antitumor activity with dexamethasone. JNatl Cancer Inst1998; 90:134-41.
8. Guzey M, Sattler C, DeLuca HF. Combinational effects of vitamin D3 and retinoic acid (all trans and 9 cis) on proliferation, differentiation, and programmed cell death in two small cell lungcar cinoma cell lines. Biochem Biophys Res Commun1998;249:735-44.
9. KogaM, Sutherland RL. Retinoic acid acts synergistically with 1,25-dihydroxyvitamin D3 or antioestrogen to inhibitT-47Dhuman breast cancer cell proliferation. J Steroid BiochemMol Biol 1991;39:455 -60.
10. Anzano MA, Smith JM, Uskokovic MR, et al. 1a,25-Dihydroxy-16-ene-23-yne-26,27-hexafluorocholecalciferol (Ro24-5531), a new deltanoid (vitamin D analogue) for prevention of breast cancer in the rat. Cancer Res1994;54:1653-6.
11. Welsh J. Induction of apoptosis in breast cancer cells in response to vitamin D and antiestrogens. Biochem Cell Biol1994;72:537-45.
12. Abe-Hashimoto J, Kikuchi T, MatsumotoT, Nishii Y, Ogata E, Ikeda K. Antitumor effect of 22-oxa-calcitriol, a noncalcemic analogue of calcitriol, in athymic mice implanted with human breast carcinoma and its synergismwith tamoxifen. Cancer Res1993;53:2534-7.
13. Dunlap N, Schwartz GG, Eads D, et al. 1a,25-Dihydroxyvitamin D (3) (calcitriol) and its analogue, 19-nor-1a,25(OH)(2)D(2), potentiate the effects of ionisingr adiation on human prostate cancer cells. BrJ Cancer 2003;89:746-53.
14. Polar MK, Gennings C, Park M, Gupta MS, Gewirtz DA. Effect of the vitamin D3 analogILX 23^7553 on apoptosis and sensitivity to fractionated radiation in breast tumor cells and normal human fibroblasts. Cancer Chemother Pharmacol 2003; 51:415-21.
15. Beer TM, HoughKM, GarzottoM, LoweBA, Henner WD. Weekly high-dose calcitriol and docetaxel in advanced prostate cancer. Semin Oncol 2001;28: 49-55.
16. Hershberger PA, Yu WD, Modzelewski RA, Rueger RM, Johnson CS, Trump DL. Calcitriol (1,25-dihydroxycholecalciferol) enhances paclitaxel antitumor activity in vitro and in vivo and accelerates paclitaxel-induced apoptosis. Clin Cancer Res 2001;7:1043-51.
17. Moffatt KA, JohannesWU, Miller GJ. 1a,25-Dihydroxyvitamin D3 and platinumdrugs act synergistically to inhibit the growth of prostate cancer cell lines. Clin Cancer Res1999;5:695-703.
18. Light BW, YuWD, McElwainMC, Russell DM,Trump DL, Johnson CS. Potentiation of isplatin antitumor activity

usinga vitamin D analogue in a murine squamous cell carcinoma model system. Cancer Res 1997; 57:3759-64.

19. Ahmed S, Johnson CS, Rueger RM, Trump DL. Calcitriol (1, 25-dihydroxycholecalciferol) potentiates activity of mitoxantrone/dexamethasone in an androgen independent prostate cancer model. JUrol 2002; 168:756 - 61.

20. Sundaram S, Chaudhry M, Reardon D, Gupta M, GewirtzDA.The vitamin D3analogEB1089 enhances the antiproliferative and apoptotic effects of Adriamycin in MCF-7 breast tumor cells. Breast Cancer Res Treat 2000;63:1-10.

21. Torres R, Calle C, Aller P, Mata F. Etoposide stimulates1,25- dihydroxyvitamin D3 differentiation activity, hormone bindingand hormone receptor expression in HL-60 human promyelocytic cells. Mol Cell Biochem 2000;208:157-62.

22. Shimada K, Mitamura K, Higashi T (2001) J Chromatogr A 935:141–172.

23. Shujing Ding, Inez Schoenmakers, Kerry Jones, Albert Koulman, Ann Prentice, Dietrich A. Volmer, Anal Bioanal Chem (2010) 398:779–789.

24. Aronov PA, Hall LM, Dettmer K, Stephensen CB, Hammock BD. Metabolic profiling of major vitamin D metabolites using Diels-Alder derivatization and ultraperformance liquid chromatography-tandem mass spectrometry. Anal Bioanal Chem 2008;391:1917-30.

25. Brian Netzel, US patent No. US61/155050, 2009.

26. Yuan C, Kosewick J, He X, Kozak M, Wang S, Rapid Commun Mass Spectrom. 2011 May 15;25(9):1241-9

27. Casetta B, Jans I, Billen J, Vanderschueren D, Bouillon R. Eur J Mass Spectrom (Chichester, Eng). 2010;16(1):81-9.

28. Kissmeyer AM, Sonne K. Sensitive analysis of 1·,25-dihydroxyvitamin D3 in biological fluids by liquid chromatography-tandem mass spectrometry. J Chromatogr A 2001;935:93-103.

29. Mata-Granados JM, Ferreiro-Verab C, Luque de Castro MD, Quesada Gómez JM, Rev Osteoporos Metab Miner 2010 2;2:55-61.

30. Online document CDER (Center for Drug Evaluation and Research), Guidance for Industry: Bioanalytical Method Validation, US FDA, 2001, http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070107.pdf.

31. Ahmet C Gören*, Gökhan Bilsel, Mine Bilsel J. Chem. Metrl. 1:1 (2007) 1-9.

32. Guidance for Industry: ICH E6 Good Clinical Practice, U.S. Department of Health and Human Services, Food and Drug Administration, Centre for Drug Evaluation and Research (CDER), Centre for Biologics Evaluation and Research (CBER), 1996.

33. Yadav M, Shrivastav PS, Incurred sample reanalysis (ISR): a decisive tool in bioanalytical research Bioanalysis, 2011, 3, 1007-1024.

34. Nico C, Merbel VD, Quantitative determination of endogenous compounds in biological samples using chromatographic techniques, Trends in Anal. Chem, 2008, 27, 924-933.

Source of support: Nil
Conflict of interest: None Declared