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Quantitative liquid chromatographic and tandem mass spectrometric determination of vitamin D_3 in human serum with derivatization: A comparison of in-tube LLE, 96-well plate LLE and in-tip SPME☆

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ABSTRACT

Sensitive and selective methods based on high performance liquid chromatography (HPLC) with tandem mass spectrometric (MS/MS) detection were developed for the determination of vitamin D_3 in human serum. Derivatization of vitamin D_3 and its stable isotope labeled internal standard provided highly sensitive quantification and selective detection from endogenous compounds. Samples were prepared using the in-tube liquid–liquid extraction (LLE), 96-well plate LLE, and in-tip solid phase micro-extraction (SPME) in 96-well format. In all methods, the MS/MS detection was performed using Applied Biosystems-Sciex API 3000 tandem mass spectrometers interfaced with a heated nebulizer probe and operated in the positive ionization mode. Both tube and plate LLE methods achieved a lower limit of quantitation (LLOQ) of 0.5 ng/mL when 1.0 and 0.4 mL of human serum was processed, respectively, and were validated in the concentration range of 0.5–25 ng/mL; while for the in-tip SPME method, LLOQ was 5 ng/mL with only 0.1 mL of human serum required. Comparisons were made among three different methods, including precision and accuracy, sample throughput, recovery and matrix effects.

1. Introduction

It is well known that vitamin D plays a critical role in the control of calcium and phosphate metabolism in the human body by increasing calcium absorption in the intestine, mobilizing calcium from bone and decreasing its renal excretion. In human, there are two sources of vitamin D: dietary ergocalciferol (vitamin D_2), derived from ergosterol in plants, and cholecalciferol (vitamin D_3, **I**) generated in the skin from 7-dehydrocholesterol by the action of ultraviolet irradiation. Vitamin D_3 has no biological activity but is converted to biologically active metabolite, 25-hydroxyvitamin D_3 (calcifediol) by oxidation in the liver, and this metabolite is further converted to a series of other metabolites of varying activity in the kidney, the most of which is 1α,25-dihydroxyvitamin D_3 (calcitriol). The biosynthesis of vitamin D is illustrated in Fig. 1.

Determinations of vitamin D_3 and its metabolites at relatively high concentrations (≥2 ng/mL) have been reported in the literature [1–6]. These methods were mostly based on HPLC with ultraviolet (UV) detection, a highly complex sample preparation procedure, and were all potentially non-selective in the presence of metabolites and related compounds present in serum samples. For example, Holick et al. [7,8] used a LLE followed by solid phase extraction (SPE) and HPLC with UV detection to quantitate vitamin D_3 in human serum following oral doses of 12.5 mg or greater. The precision (% C.V.) of this method was 19% at the claimed LLOQ of 2 ng/mL, with poor and variable recovery (50–70%).

The development and validation of reliable quantification methods for vitamin D_3 in clinical samples at low sub-nanogram concentrations (0.1–1 ng/mL) using small volume of serum is difficult and has not been described in the literature. Concentrations of vitamin D_3 after microgram (μg) doses to human subjects are very low (≤0.5–5 ng/mL), and the determination of **I** in the presence of a number of metabolites circulating in human biofluids exhibiting similar chemical and HPLC behavior as the parent compound may be highly non-selective. In addition, both **I** and metabolites are potentially unstable in the presence of UV light and at elevated temperatures [9].

Similarly to the determination of the majority of drugs, metabolites and variety of other analytes in complex matrices, the HPLC with MS/MS detection has proven to be a powerful tool for the analysis of vitamin D related compounds in biological samples.

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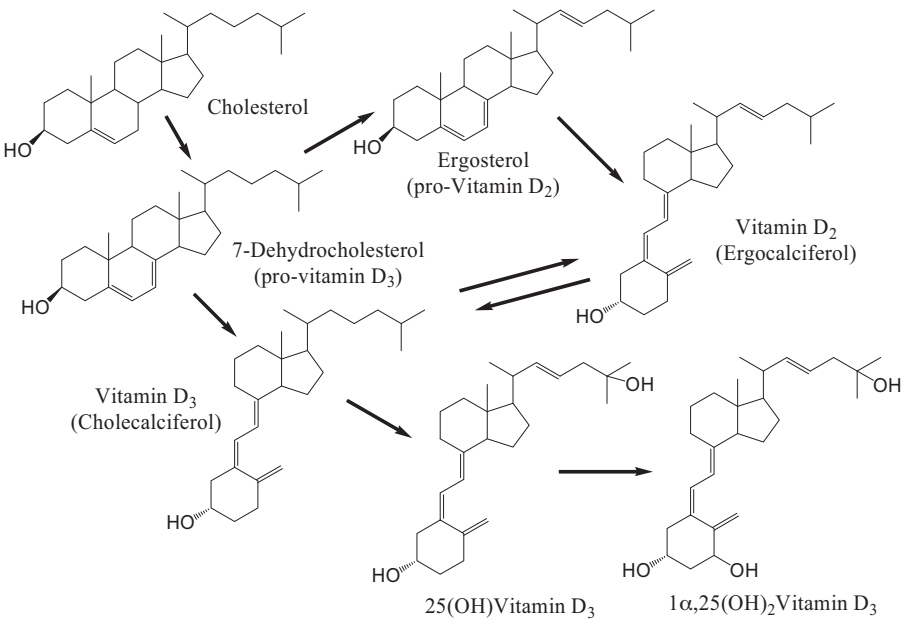

Fig. 1. Biosynthesis of vitamin D.

Atmospheric pressure ionization methods, such as electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) were used for structural identification by tandem mass spectrometry of vitamin D metabolites in biological fluids at relatively low concentrations and facilitated their quantification [10–13]. However, because vitamin D_3 and its metabolites have no easily ionizable polar functional groups in their molecules, the ionization efficiency of these compounds using either ESI or APCI is very limited, and the required sensitivity for trace determination of these compounds in biological fluids is difficult to achieve.

In recent years, a variety of analogs and metabolites of **I** have been derivatized with dienophiles to form a Diels-Alder cycloaddition products [14,15]. Higashi et al. [16–18], for example, described the determination of vitamin D_3 metabolites in plasma based on chemical derivatization. When reacted with 4-substituted 1,2,4-triazoline-3,5-dione (TAD) type compounds (dienophiles, Cookson reagents), the *s-cis*-diene moiety of the hydroxyvitamin D_3 analogs rapidly and quantitatively reacts with the derivatizing reagent to form Diels-Alder cyclo-addition products. The sensitivity of detection of metabolites of **I** was increased dramatically after introduction of the high proton-affinity atoms from these dienophilic reagents. For example, the derivatives of 25-(OH)D_3 with 4-(4-nitrophenyl)-1,2,4-triazoline-3,5-dione (NP-TAD) provided 30-fold higher sensitivity of detection compared to an underivatized compound in the negative ionization mode using APCI [18]. Although many applications have focused on identification and determination of vitamin D_3 metabolites, quantitative and selective analytical methods for the determination of vitamin D_3 by HPLC–MS/MS after derivatization with dienophiles directly in human plasma or serum at sub-nanogram/mL concentrations were not developed and described in the literature. The development of new, more sensitive, selective, reliable, and high throughput methods for the quantitative determination of vitamin D_3 was required to meet the high demand for pharmacokinetic (PK) data in support of clinical studies at very low (μg) oral doses of vitamin D_3. For this purpose, in our studies, the reaction between **I** and a dienophile, 4-phenyl-1,2,4-triazoline-3,5-dione (P-TAD), was utilized. This reaction was never used for the development of quantitative methods for **I** in plasma or serum.

In order to map out the concentration-time profile following a single low oral dose of 70 μg of vitamin D_3 to human subjects, a method (method A) based on chemical derivatization of **I** with P-TAD, after conventional in-tube LLE, and HPLC with MS/MS detection has been initially developed and validated in our laboratory. This method required processing 1 mL of serum and achieved the LLOQ of 0.5 ng/mL. However, the method had a relatively low sample throughput due to the labor intensive and time-consuming sample preparation steps required. Later, a higher throughput, highly sensitive and selective HPLC–MS/MS method B was also developed. In method B, both the LLE and derivatization of vitamin D_3 and its stable isotope-labeled internal standard (D_6-**I**, IS, **II**) were both performed in the 96-well plate format. This method required only 0.4 mL of serum for processing and achieved the same LLOQ (0.5 ng/mL) as in method A. As part of a series of studies conducted in our laboratory to explore the SPME technique in high throughput drug analysis, we developed a new approach of using in-tip SPME [19] in 96-well format for determination of various drugs in biofluids. Compared with traditional extraction methods such as LLE and SPE, SPME has the advantage because of its simplicity and solventless characteristic. In order to further demonstrate the feasibility of using in-tip SPME approach in high throughput clinical sample analysis and to explore the potential advantages and limitations of SPME technique in routine bioanalysis, we developed another method for the quantification of vitamin D_3 in human serum using in-tip SPME (method C) followed by chemical derivatization of **I** and the IS (**II**) with P-TAD requiring only 0.1 mL of human serum but with a higher LLOQ of 5 ng/mL. In all three methods, the derivatives formed (**III** and **IV**, respectively, see Fig. 2) were determined using HPLC–MS/MS in the positive ionization mode. Head-to-head comparisons were made among three different methods including precision and accuracy, sample throughput, recovery and matrix effects, and the advantages and limitations of each method were discussed.

2. Experimental

2.1. Materials

Vitamin D_3 (**I**) was purchased from Sigma Chemical Company (St. Louis, MO, USA), and its deuterated internal standard (D_6-**I**, **II**) was synthesized at Merck Research Laboratories (Rahway, NJ, USA). All solvents were HPLC or analytical grade and

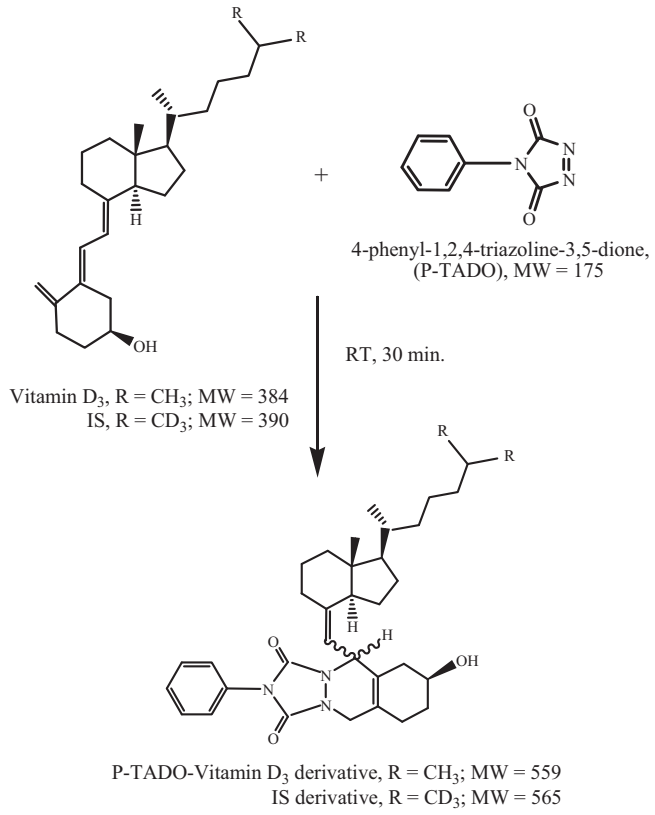

Fig. 2. Derivatization of vitamin-D_3 (**I**) and D_6-**I** (**II**) with P-TAD and the formation of P-TAD-vitamin-D_3 (**III**) and P-TAD-D_6-vitamin D_3 (**IV**).

were purchased from Fisher Scientific (Fair Lawn, NJ, USA). The derivatizing reagent (4-phenyl-1,2,4-triazoline-3,5-dione; P-TAD), ethylene glycol dimethacrylate (EGDMA), and dimethoxy-α-phenylacetophenone (DMPA) were purchased from Aldrich Chemical Company (Milwaukee, WI, USA). Ammonium acetate (HPLC grade) purchased from J.T. Baker (Phillipsburg, NJ, USA), formic acid (95%) and 1-decanol obtained from Sigma (Milwaukee, WI, USA), were used as received. Oasis HLB with particle size 60 μm was obtained from Waters (Milford, MA, USA). Deionized water was obtained by passing in-house water through a Millipore Milli-Q plus system (Bedford, MA, USA). Nitrogen (99.999%) was supplied by West Point Cryogenics (West Point, PA, USA). 96-Well collection plates (1.2 and 2.4 mL) and mats were purchased from Marsh Biomedical (Rochester, NY, USA). Different lots of human control serum were obtained from Biological Specialties Corp. (Lansdale, PA, USA) and stored at −20 °C before use.

2.2. Instrumentation

A Perkin-Elmer (Norwalk, CT, USA) binary 250 pump and a Varian (Palo Alto, CA, USA) ProStar 96-well plate autosampler were used in this work. The chromatographic separation of analytes was performed on a Phenomenex Synergi Polar-RP column (50 mm × 2 mm, 4 μm) with a 0.5 μm in-line filter. Mobile phase consisted of methanol:10 mM ammonium acetate (88:12, v/v) and was pumped at a flow rate of 0.3 mL/min. The total run time was 5 min. Two millilitres of methanol was used as a washing solvent for needle and flow path cleaning of the autosampler after each injection.

An Applied Biosystems-Sciex API 3000 triple quadruple mass spectrometer (Foster City, CA, USA) equipped with a heated nebulizer (HN) source operating in the positive ionization mode was used for all HPLC–MS/MS analysis. Multiple reaction-monitoring (MRM) was utilized for quantification. In HN experiments, the heated nebulizer probe temperature was maintained at 400 °C, and the nebulizing gas (air) pressure was set at 80 psi. The settings for the curtain and collision gases were 8 and 4 psi on the API 3000 mass spectrometer, respectively.

2.3. Preparation of standard solutions and quality control samples

A stock solution of **I** (100 μg/mL) was prepared in methanol. This stock solution was further diluted with methanol to give a series of working standards with concentrations of 0.005, 0.01, 0.025, 0.05, 0.1, 0.15 and 0.25 μg/mL. The internal standard (**II**) was also prepared as a stock solution (100 μg/mL) in methanol. A working standard solution of 0.1 μg/mL of **II**, prepared by diluting stock solution with methanol, was used for serum samples analyses. All standard solutions were stored at 4 °C.

A stock solution for quality control (QC) samples of **I** was prepared separately by the same procedure using a separate weighing. QC samples were prepared by diluting the QC working solution with human control serum. QC samples at three concentrations (Low QC, Middle QC and High QC) were used to evaluate assay precision and accuracy. All QC samples were divided into 1.25 mL aliquots in separate cryo tubes and stored at −20 °C until analysis.

Because of light sensitivity, all standard preparation and sample extractions were performed under yellow light.

2.4. Sample processing

2.4.1. In-tube LLE (method A)

Standards, QC and subject serum samples were thawed at room temperature. One millilitre of serum samples was basified with pH 9.8 (1 mL) carbonate buffer, and then extracted twice with 7 mL MTBE using 15 mL conical disposable glass tubes. The tubes were placed in a dry ice/acetone bath until the aqueous layer was frozen. The organic extracts were manually transferred to a clean 15 mL centrifuge tube and were evaporated to dryness under heated N_2 stream. The dried residue was reacted with 0.2 mL of derivatizing agent, P-TAD (0.25 mg/mL in ACN) for 30 min. Upon completion of the reaction, the excess of P-TAD was reacted with 0.5 mL of methanol. The mixture was dried under heated N_2 stream and reconstituted in 150 μL of mobile phase. Fifteen microlitres of this solution was injected into the HPLC–MS/MS system. Serum standards were prepared the same way as serum samples by adding 100 μL of each working standard and 100 μL internal standard to 1 mL of human control serum. The resulting serum standard concentrations ranged from 0.5 to 25 ng/mL.

2.4.2. 96-Well plate LLE (method B)

Standards and QC serum samples were thawed at room temperature. Four hundred microlitres of subject or control serum samples was added individually into a 2 mL deep 96-well plate followed by 40 μL of 50% of methanol in water (v/v). Forty microlitres of the internal standard solution were added to each well, except to the well designated for the double blank serum. The plate containing samples was placed onto a Tomtec Quadra 96 Workstation (Hamden, CT, USA) for liquid transfer. After transferring 50 μL of 0.2 M sodium carbonate buffer solution (pH 11) and 1.28 mL of MTBE by Tomtec Workstation, the plate was sealed with mat made of molded PTFE/silicone liner and was roto-mixed for LLE. The plate was then centrifuged and the top organic layer was aspirated and dispensed by Tomtec Workstation into a 1.2 mL 96-well collection plate. The serum samples were extracted again with 1.28 mL of MTBE. The organic extract was evaporated to dryness under heated N_2 stream. The dried residue was reacted with 0.2 mL of derivatizing agent, P-TAD (0.125 mg/mL in ACN) for 30 min. Upon

completion of the reaction, the excess of P-TAD was reacted with 0.2 mL of methanol. The mixture was dried under heated N_2 stream and reconstituted in 150 μL of mobile phase. Fifteen 15 μL of this solution was injected into the HPLC–MS/MS system.

2.4.3. In-tip SPME (method C)

2.4.3.1. Preparation of in-tip SPME fibers. Methacrylate-based monoliths in-tip SPME fibers were prepared using photopolymerization. Briefly, polyethylene (PE) frits (25 μM, 6.3 mm in diameter) purchased from Innovative Microplate (Chicopee MA, USA), and non sterilized polypropylene pipette tips purchased from Tomtec Inc (Hamden, CT, USA) were used to prepare the in-tip SPME fibers. A piece of GC capillary tubing (0.02′ outer diameter, 6.5 cm length) was cut and inserted in the middle of the PE frit, the PE frit with the capillary tubing was carefully inserted to the pipette tip so that the PE frit sat tightly at the top of the pipette tip with the capillary exposed just outside the tip about 2–3 mm. The distance from the PE frit to the top of the pipette tip was about 11 mm. Up to 96 tips were prepared in the same way and were loaded on Tomtec Quadra 96 Workstation. A polymerization mixture of DMPA (0.008 g), EDMA (0.8 g), and 1-decanol (1.2 g) was prepared and then a sample of this mixture (4 μL) was vortex-mixed thoroughly with Oasis HLB particles (1 mg), about 100 μL of the mixture slurry were transferred to each well in a 96-well plate with 600 μL 12 × 8 removable tube strips from ArcticWhite LLC (Bethlehem, PA, USA). The plate was placed at one position on the deck of Tomtec Workstation and two UV light lamps were placed at another position for photopolymerization. The Tomtec Workstation was programmed as such that 10 μL of mixture solution was aspirated simultaneously from the 96-well plate to 96 pipette tips with capillary tubing inside and then moved to the UV lamp position, the 96 tips were irradiated at 365-nm for 10 min. After photopolymerization, the 96 tips were un-loaded from the Workstation, the capillary tubing was removed from the bottom of each tip and all the tips were immersed into a reservoir with methanol overnight and dried under vacuum. The freshly prepared tips were ready for SPME experiments and were used as disposable tips.

2.4.3.2. SPME conditions. Standard and QC serum samples were thawed at room temperature. One hundred microlitres of subject serum samples were added individually into a 2.4 mL deep 96-well plate spiked with 100 μL of methanol:water (50:50, v/v). Standard curve samples were prepared by spiking 100 μL of appropriate standard into 100 μL of human control serum. Internal standard solution (100 μL) was added to each well of the plate. After adding 50 μL of 0.2 M sodium carbonate buffer solution (pH 11) to all wells on the plate, the plate was sealed with mat made of molded PTFE/silicone line and vortex-mixed thoroughly on a VWR multitube vortexer for 2 min. In-tip SPME extraction and desorption process was fully automated on a Tomtec Quadra 96 Workstation (Hamden, CT, USA). Briefly, in-tip SPME fibers in 96-well format were loaded at position 1 (tip plate) on the deck of the Tomtec Quadra 96 Workstation. Washing solvent, water and 5% methanol (5% methanol:95% water, v/v) reservoirs were placed at positions 2 and 3, and eluting solvent methanol was at position 4, respectively. An empty 1.2 mL deep 96-well plate was placed at position 5 for desorption. The mat was carefully removed from the 2.4 mL sample plate and the plate was placed at position 6 for extraction. The Tomtec Quadra 96 Workstation was programmed as follows: after tips were picked up and washed subsequently with 50 μL of methanol and 50 μL of water, 300 μL of the solution containing serum was repeatedly aspirated and dispensed for 40 min from extraction plate at position 6. When extraction was completed, in-tip SPME fibers were washed once with 50 μL of water and 10% methanol, respectively, then 50 μL (twice) of methanol was aspirated from methanol reservoir with 50 μL air gap and dispensed into the empty 1.2 mL desorption plate. The 1.2 mL deep 96-well plate was evaporated to dryness under heated N_2 stream and the dried residue was reacted with 0.2 mL of derivatizing agent, P-TAD (0.125 mg/mL in ACN) for 30 min. Upon completion of the reaction, the excess of P-TAD was reacted with 0.2 mL of methanol. The mixture was dried under heated N_2 stream, reconstituted in 150 μL of mobile phase and 15 μL was injected into the HPLC–MS/MS system.

2.5. Validation procedures

2.5.1. Precision and accuracy

The precision of the method was determined by the replicate analysis ($n = 5$) of **I** in five different sources of human serum at all concentrations utilized for the construction of calibration curves. The linearity of each calibration curve was confirmed by plotting the peak height ratio of the derivatized drug (**III**) to the derivatized internal standard (**IV**) *vs.* drug concentration. Calibration curve calculations were made by subtracting the mean peak height ratio of derivatized drug (**III**)/derivatized IS (**IV**) of triplicate analysis of the control blank serum samples used for the construction of standard lines from the respective peak height ratios of each standard concentration. The unknown sample concentrations were calculated from the equation, $y = mx + b$, as determined by weighted ($1/x$) linear regression of the standard line. The accuracy of the method was determined as the percentage between the mean concentration observed and the nominal concentration. The intra-day accuracy was required to be within ±15% at all concentrations. The precision, expressed by the coefficient of variation (%C.V.), was required to be <15% at the LLOQ and <10% at all other concentrations.

2.5.2. Selectivity

The selectivity of the method was confirmed by processing control drug-free human serum samples from six different sources to determine whether endogenous peaks interfered at the mass transitions chosen for the derivatized analyte and/or the internal standard. In addition, the "cross-talk" between MS channels used for monitoring the analyte and the internal standard was evaluated.

2.5.3. Recovery and matrix effect

Extraction recovery for **I** and **II** was evaluated using standards spiked at three concentrations of analytes. Recovery was determined by comparing the absolute peak heights of the standards in control human serum extracted and derivatized according to the described sample processing procedures to control serum extracted in the same manner and then spiked post extraction with the analytes and derivatized. Matrix enhancement/suppression of ionization or "absolute" matrix effect was evaluated by comparing the absolute peak heights of the standards in post spiked extraction samples, to neat derivatized standards injected directly in the same reconstitution solvent. Since a stable isotope labeled internal standard was used, a potential "relative" matrix effect on ionization should not have any adverse effect on the quantitation of **I** in different serum lots. This was evaluated and confirmed by the determination of slopes of the calibration curves in five different lots of control serum as suggested in the literature [20].

2.5.4. Stability

The stability of **I** and its internal standard was investigated in the stock and working solutions. Analytes and internal standards were considered stable in the stock and working solutions when 95–105% of the original concentration was recovered. Storage stability of **I** in human serum and the influence of freeze-thaw cycles were also examined by processing a set of QC samples. The calculated mean values should not deviate by greater than 15% of the nominal value. Intraday precision for QC replicates should not exceed 10%.

3. Results and discussion

3.1. Derivatization of vitamin D_3 and optimization of MS/MS conditions

Vitamin D_3 that is formed from cholesterol by opening the B-ring of the secosteroid, is generated in the skin from 7-dehydrocholesterol under UV radiation (Fig. 1). Therefore, it is necessary to evaluate the potential interference from 7-dehydrocholesterol in the determination of vitamin D_3 in human serum. MS/MS detection of un-derivatized vitamin D_3 neat standard solution yields precursor and product ion pair at m/z $385 \rightarrow 367$. After extraction of spiked vitamin D_3 standard sample from control human serum, a co-eluting interference peak was observed at the same MS/MS transition as the vitamin D_3. The interference peak was identified as 7-dehydrocholesterol which had the same molecular weight as vitamin D_3 and fragmented to the same product ion (m/z 367). Therefore, HPLC separation was required to selectively quantify vitamin D_3 in the presence of 7-dehydrocholesterol. As reported in the literature, because a cisoid diene system is a feature common to most vitamin D related compounds, all these compounds may react with dienophilic reagents such as triazolinediones to form Diels-Alder cycloaddition products at room temperature. These derivatized products will greatly enhance the sensitivity of their MS/MS detection. At the same time, these derivatives may also distinguish themselves from other interferences due to a significant increase in the molecular mass and their detection in the higher mass range together with a different fragmentation in the ion source. A variety of analogs and metabolites of vitamin D compounds have been derivatized with different triazolinediones [14–18,21,22] for structural confirmation, among them P-TAD. However, the P-TAD was never used as a derivatizing reagent for quantitative determination of **I** in plasma or serum samples.

In MS/MS infusion experiments, a methanol-free methylene chloride was chosen as reaction solvent as P-TAD is very sensitive to air and moisture. 1 μg/mL of the P-TAD-vitamin D_3 derivative was prepared as follows: a P-TAD solution (0.29 M) in methylene chloride was reacted at room temperature with 0.1 M of **I** also in methylene chloride at 1:1 molar ratio. The reaction was stopped by adding water and the post-reaction mixture was extracted with methylene chloride. The organic extract was evaporated to dryness and the residue was reconstituted in methanol. The derivatized vitamin D_3 was detected in the positive ionization mode using a heated nebulizer probe and a major product ion at m/z 298 was observed. The same experiment was performed using 7-dehydrocholesterol, however, a fragment ion at m/z 298 was not observed. Therefore, MRM transition at m/z $560 \rightarrow 298$ was selected for selective quantification of vitamin D_3 (Fig. 3). The different fragmentation patterns between the derivatized vitamin D_3 (**III**) and 7-dehydrocholesterol demonstrated that the derivatization enhanced not only sensitivity but also selectivity of the determination of vitamin D_3. Precursor – product ion at m/z $566 \rightarrow 298$ was chosen for the derivatized internal standard (**IV**, Fig. 2). Optimization of source and MS parameters were obtained by infusing neat solutions of the synthesized derivatives at a flow rate of 20 μL/min into a mobile phase pumped at 0.5 mL/min using the heated nebulizer interface at 400 °C.

It is important that during derivatization of the human serum extracts, an excess concentrations of the P-TAD reagent is provided since many endogenous components may be co-extracted and they may consume the P-TAD reagent affecting the derivatization yield of an analyte of interest and the sensitivity and reliability of the method. Therefore, the amount of derivatizing reagents used needs to be optimized.

3.2. Automated in-tip SPME method evaluation and optimization

As an innovative technique that allows the integration of sampling and extraction in a single step, SPME has the advantages of (1) less solvent used, (2) easy handling, (3) little equipment needed, and (4) faster speed of analysis. However, the technique has not yet been accepted as an alternative approach for quantitative determination of analytes in pharmaceutical bioanalysis for many reasons. First of all, because of the fiber and in-tube configurations, SPME suffers from low-throughput and lack of automation which limits its application in analysis of a large number of similar samples. Secondly, as pointed out by Ulrich [23], there are some major disadvantages of SPME that include longer desorption time, carry over effects due to repeated use of the same fiber and method being prone to errors due to considerably lower recovery in comparison with more classical extraction methods. These issues have not been completely addressed and resolved since SPME invention.

In the current study, a simple, flexible, low-cost, and reproducible procedure for the preparation of in-tip SPME fibers with polymer monoliths using photo-polymerization technique was developed. The porous polymers have many advantages including the simplicity of their fabrication and the wide range of chemistries for reactions. More importantly, the relative large surface areas of polymer monolith in-tip SPME fibers dramatically increase the absolute extraction recoveries (up to 30%) while in general, the recoveries reported for SPME are considerably lower (<1%). In addition, in-tip SPME is very easily coupled with commercial available liquid handling systems for automated sample preparation without introducing any additional equipment while maintaining its simplicity.

Factors that were involved in fiber fabrication included concentration of the initiator, total monomer to total porogen ratio, porogen type, and optimized photopolymerization time. The optimized reagent composition of 40 wt% of EGDMA and 60 wt% of 1-decanol with 1 wt% of DPA was obtained for preparing monolith in terms of rigidity and homogeneity. The scanning electron microscopy (SEM) micrographs of the monolithic structure indicated that the UV irradiation time of 10 min was sufficient of initiating monolithic polymerization of the solution in the tips.

For the SPME automation to be successful in parallel extraction format, good reproducibility of the amount of analyte extracted by different fibers is necessary. In this study, 96 in-tip SPME fibers were fabricated at the same time by photopolymerization using a Tomtec Quadra 96 Workstation. Fiber-to-fiber reproducibility was found to be within %C.V. of 15.4% using absolute peak area counts of a standard analyte. However, the precision increased significantly to 5.6% C.V. with the use of a stable isotope labeled internal standard that was extracted simultaneously with the analyte. This indicated that the inter fiber reproducibility is not critical in quantitative analysis in terms of accuracy and precision as long as an internal standard is able to compensate for the inter fiber variability in extraction capacity.

During in-tip SPME method development, parameters such as fiber coating, sample volume, extraction conditions (pH or ionic strength), extraction and desorption time, desorption solvents and calibration method were thoroughly investigated. For in-tip SPME, samples were aspirated/dispensed across the solid phase media and the extraction equilibrium was accomplished through multiple aspirate/dispense cycles. Unlike conventional SPME methods, where agitation is a big issue especially in automated SPME-LC, the uniformity of agitation could be easily achieved for in-tip SPME using automated liquid handling system such as Tomtec Workstation. Extraction conditions were based on those from LLE and serum sample volume was chosen at 100 μL. In order to achieve the maximum recovery in a reasonable period of time, 480 cycles were chosen which required about 40 min. Because of the small

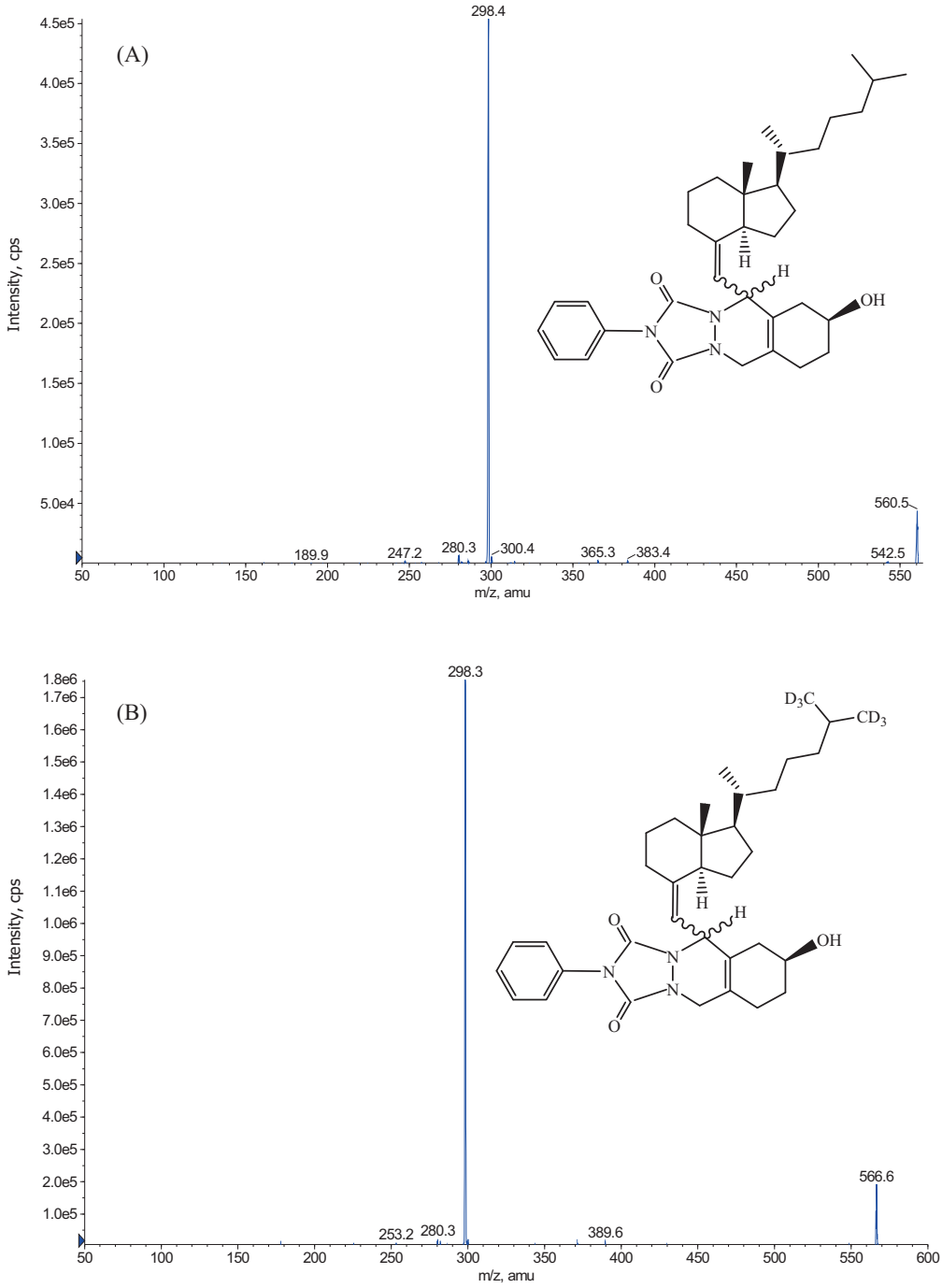

Fig. 3. The product ion mass spectra of the protonated molecules of P-TAD-vitamin-D_3 (**III**, chromatogram A) and P-TAD-D_6-vitamin D_3 (**IV**, chromatogram B) derivatives $(M+H)^+$, $m/z = 560$ and 566, respectively, under the optimized MS/MS conditions used in the assay.

monolithic bed volume of the in-tip SPME fiber, extracted analytes could be released efficiently from the extraction sorbent with minimal elution volume. An elution scheme of $2\times$ 50 μL of solvent was sufficient for the quantitative release of analytes. Carryover effect was eliminated as polymer monolith in-tip SPME fibers were used as disposable.

3.3. Method validation

Methods A and B were validated in human serum in the concentration range of 0.5–25 ng/mL of **I**, whereas the in-tip SPME method was validated in the concentration range of 5–250 ng/mL. The assessment of the intraday variability of each method was conducted in five different lots of human control serum spiked with **I**. The resulting method precision and accuracy data are presented in Table 1. For the in-tube LLE method A, the intra-day precision (%C.V.) was 2.8% at LLOQ, and was equal to or lower than 6.0% at all other concentrations used for the construction of the calibration curve. Method A accuracy was found to be within ±2% of the nominal concentration for all the standards evaluated. In 96-well plate LLE method B, the intra-day precision (%C.V.) was 5.9% at LLOQ, and was equal to or lower than 5.2% at all other concentrations used

Table 1
Intraday precision and accuracy data for the determination of vitamin D_3 in five different lots of human control serum using in-tube LLE (method A), 96-well plate LLE (method B), and in-tip SPME (method C), respectively.

Nominal Conc. (ng/mL)	Accuracy (%)[a] [%C.V.] ($n=5$)	Accuracy (%)[a] [%C.V.] ($n=5$)	Nominal conc. (ng/mL)	Accuracy (%)[a] [%C.V.] ($n=5$)
	Method A	Method B		Method C
0.5	100.0 [2.8]	102.0 [5.9]	5	103.6 [10.9]
1	100.0 [2.4]	97.3 [4.2]	10	99.2 [8.7]
2.5	98.8 [6.0]	100.6 [5.2]	25	100.4 [5.6]
5	101.6 [3.5]	99.8 [1.6]	50	92.8 [2.8]
10	99.1 [0.9]	101.2 [2.3]	100	101.3 [2.2]
15	99.7 [3.0]	98.9 [2.2]	150	104.8 [3.2]
25	100.3 [2.0]	100.2 [3.1]	250	98.0 [6.4]

[a] Expressed as [(mean calculated concentration)/(nominal concentration)] × 100%.

for the construction of the calibration curve. Method accuracy was found to be within ±3% of the nominal concentration for all standards evaluated. In in-tip SPME method C, the intra-day precision (%C.V.) was 10.9% at LLOQ, and was equal to or lower than 8.7% at all other concentrations used for the construction of the calibration curve. Method accuracy was found to be within ±8% of the nominal concentration at all standard concentrations evaluated. The correlation coefficient for the mean standard curves constructed from five different lots of human serum was 0.9997, 0.9996, and 0.9980 for methods A, B, and C, respectively.

3.4. Selectivity

Assessment of the selectivity of the method is critical and needs to be confirmed in the presence of in vivo metabolites of the analyte. Some metabolites may be converted to parent drug during sample preparation and/or undergo partial fragmentation in the ion source at high temperatures giving the same molecular ion as for the parent drug. The major metabolites 25-hydroxyvitamin D_3 and 1,25-dihydroxyvitamin D_3 of **I** were evaluated for the "cross-talk" in channels used for monitoring both **I** and the internal standard. No interference or "cross-talk" from these metabolites was observed. In addition, the "cross-talk" between channels used for monitoring both **I** and the internal standard was evaluated by the analysis of standard samples containing individual compounds separately at the concentrations of 25 and 10 ng/mL for **I** and internal standard, respectively, and monitoring the response in other MS/MS channel used for quantification. No response was observed in the channel of the other analytes at their retention times. Also, no interference or "cross-talk" was observed from these compounds in the channels used for monitoring derivatized **I** and the internal standard (**III** and **IV**, respectively). Fig. 4 shows the representative extracted ion chromatograms obtained from human control serum blank (Fig. 4(1a) and (1b)), human control serum spiked with 10 ng/mL of **II** (Fig. 4(2a) and (2b)), human control serum spiked with 0.5 ng/mL of **I** and 10ng/mL of **II** (Fig. 4(3a) and (3b)), and human control serum spiked only with 40 ng/mL of **I** (Fig. 4(4a) and (4b)). No "cross-talk" between channels used for the determination of **I** and **II** was observed. The small peak (<10% of the LLOQ) in the channel used for monitoring derivative **III** in blank serum sample was due to the endogenous **I** present in the serum sample. In clinical samples, when necessary and if present in pre-dose human serum in any quantifiable concentrations, this endogenous peak was subtracted from the total concentration of **I** determined in that sample.

3.5. Recovery and assessment of the matrix effect

Extraction recovery and the effect of the serum matrix on ionization and derivatization efficiency were evaluated for **I** and the internal standard using standards spiked at concentrations of 0.5, 10, and 25 ng/mL for **I**, and at 10 ng/mL for **II** in both methods A and

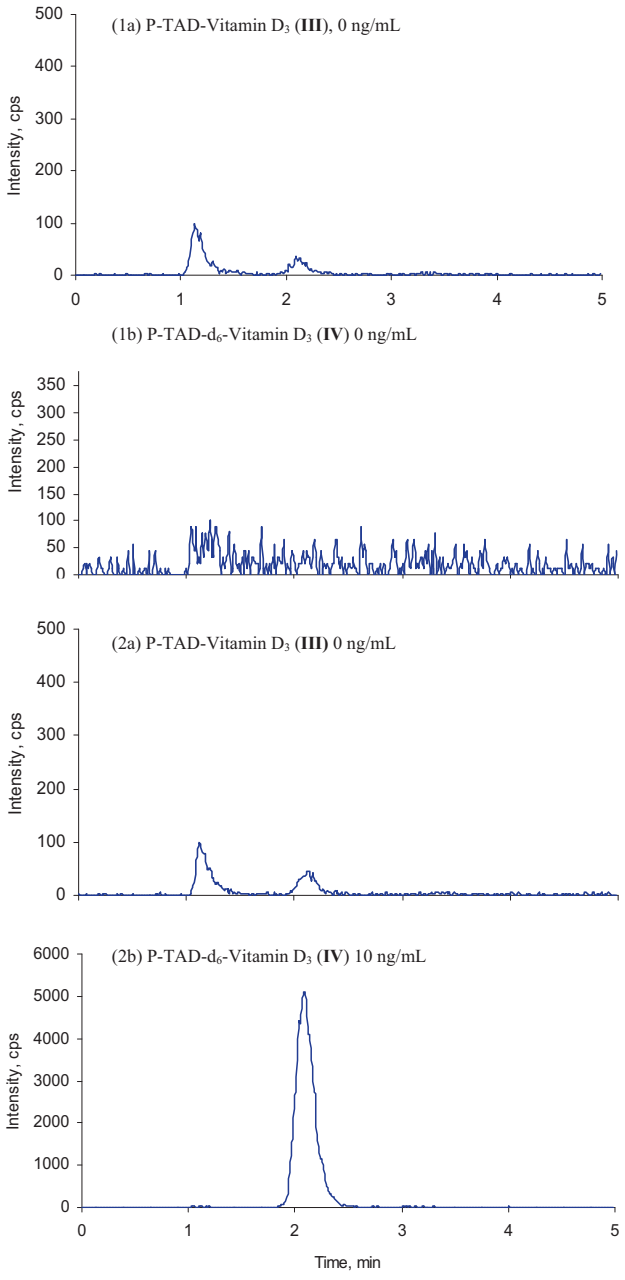

Fig. 4. Representative chromatograms of human control serum obtained by multiple reaction monitoring at m/z 560 → 298 for derivatized vitamin D_3 and m/z 566 → 298 for derivatized D_6-vitamin D_3 using 96-well plate LLE.

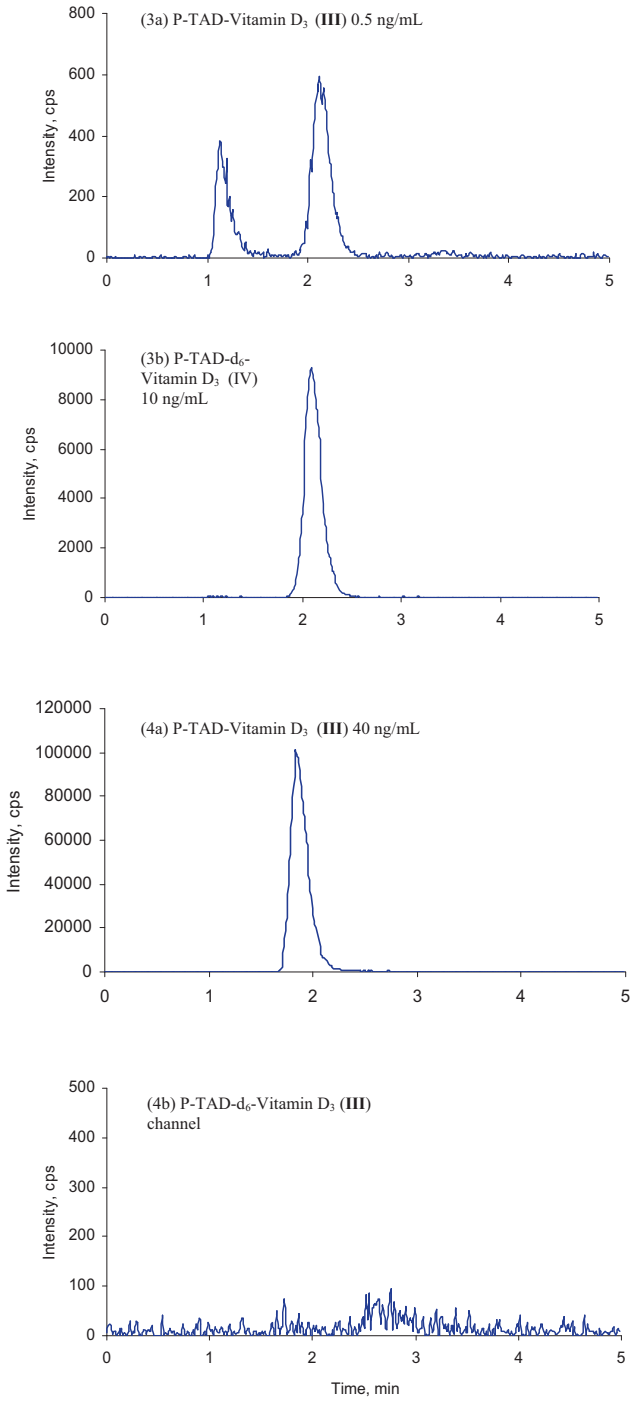

Fig. 4. *(Continued)*

B. In method C, this evaluation was performed at concentrations of 5, 100, and 250 ng/mL for **I**, and 100 ng/mL for **II**. Recoveries were determined by comparing the peak height of standards spiked into three different lots of human control serum and extracted as per sample preparation to human control serum extracted in the same manner and then spiked post-extraction with a known amount of the drug. The mean recoveries of **I** and **II** were 82% and 84%, respectively, in method A; 69% and 63%, respectively, in method B and 29% and 24%, respectively, in method C (Table 2). Recovery in all methods was consistent over the entire range of the standard curve indicating that extraction of the analytes in each method was independent on the concentration. In order to assess the "absolute" matrix effect on the ionization and derivatization process, the absolute peak heights of control human serum samples extracted and then spiked with a known amount of analytes and derivatized, were compared to neat standards derivatized and injected directly in the same solvent. Results are also shown in Table 2. Considering the experimental uncertainties, the "absolute" matrix effects in methods A and C were much less significant than those observed in method B with ion enhancement about 140%. However, the use of stable isotope labeled **I** as the internal standard fully compensated for any variation in matrix effect and/or recovery between different lots of human control serum. Therefore, an "absolute" matrix effect on ionization or any differences in derivatization efficiency of **I** and internal standard would not have any adverse effect on the precision and accuracy of any of the methods presented. The absence of a "relative" matrix effect [20,24,25] on ionization and derivatization efficiency was confirmed by an examination of the slopes of the calibration curves that were constructed in five different lots of human control serum. The high precision of these slopes (1.3, 2.0 and 4.2% C.V.) in methods A, B and C, respectively, confirms the absence of a "relative" matrix effect in all methods developed.

3.6. Analyte stability

Standard solution stability was confirmed for a period of 20 days when refrigerated. QC samples ($n = 5$ at each concentration) were subjected to three freeze–thaw cycles consisting of a thaw to reach room temperature and then refreezing at −20 °C. These samples, together with a set ($n = 5$ at each concentration) of human QC samples that were not subjected to additional freeze–thaw cycles, were then defrosted and analyzed. In all cases, the results for the samples that were subjected to additional freeze–thaw cycles were within ±9% of the nominal value (Table 3).

3.7. Method comparison

The LLOQ of 0.5 ng/mL was achieved using both in-tube and 96-well plate LLE methods, however, in 96-well plate LLE method B, only 0.4 mL of human serum needed to be processed instead of 1 mL required in the in-tube LLE method A. Both procedures were validated in the same concentration range of 0.5–25 ng/mL. In the in-tip SPME method, the LLOQ was 5 ng/mL but only 0.1 mL of serum was required. The method C was validated in the concentration range of 5–250 ng/mL. The linearity of the calibration curves, the intraday precision and accuracy were all satisfactory in all methods. Recoveries of analytes using in-tube LLE were above 80% compared to about 70% in the 96-well plate LLE. The recoveries in both LLE methods were much higher than those from the in-tip SPME (~30%). However, the overall sample preparation time was decreased from 9 h per 96 samples required in method A to about 3 h in method B and 2 h in method C with much less labor efforts involved. The matrix effects in different human serum samples from the three methods were also examined. Since a stable isotope-labeled internal standard was used in all methods, a potential "relative" matrix effect on ionization was shown not to have any adverse effect on the quantitation of vitamin D_3 in different serum lots. Based on the excellent intra-day precision and accuracy results obtained in all methods that were obtained using five different lots of control human serum, any differences in the "absolute" matrix effect on ionization or any difference in derivatization efficiency do not have any significant effect on the precision, accuracy and the reliability of the analysis. This was confirmed by the analysis of subjects' samples using methods A and B. Both methods generated very similar concentration data (Fig. 5).

Although method C did not meet the assay sensitivity requirement for clinical sample analysis after dosing with 70 μg of **I**, the

Table 2
Extraction Recovery and assessment of the "absolute" matrix effects during the determination of vitamin D_3 and internal standard in human control serum using methods A, B and C.[a]

Vitamin D_3 concentration (ng/mL)	%Extraction recovery[b]			%Matrix effect[c]		
Method	A	B	C	A	B	C
0.5	81	68		92	142	
5.0			26			121
10.0	78	68		89	141	
25.0	88	70		89	145	
100			31			114
250			30			110
Mean	82	69	29	90	143	115
D_6-**I**						
10.0 (**II**)	84	63		92	140	
100 (**II**)			24			116

[a] Determined in three different lots of control human serum.
[b] Extraction recovery was calculated by dividing the mean peak height of analyte spiked before extraction by the respective mean peak height of analyte spiked after extraction and multiplying by 100.
[c] Matrix effect was calculated by dividing the mean peak height of an analyte spiked after extraction by the mean peak height of the neat analyte standard and multiplying by 100.

Table 3
Intra-day analysis of serum quality control (QC) samples containing vitamin D_3.

	Low QC	Middle QC	High QC
In-tube LLE (method A)			
Nominal conc. (ng/mL)	1.5	12.5	22.0
Mean calculated conc. (ng/mL), $n=5$	1.4	11.5	21.3
Accuracy[a] (%)	96.0	92.0	96.8
%C.V.[b]	9.6	2.5	2.8
96-Well plate LLE (method B)			
Nominal conc. (ng/mL)	1.5	12.5	22.0
Mean calculated conc. (ng/mL), $n=5$	1.6	12.8	23.9
Accuracy[a] (%)	103.6	102.0	108.4
%C.V.[b]	3.0	6.0	3.3
In-tip SPME (method C)			
Nominal conc. (ng/mL)	15	125	220
Mean calculated conc. (ng/mL), $n=5$	16.0	132.0	237.0
Accuracy [a] (%)	106.7	105.6	107.7
%C.V.[b]	4.4	7.0	5.8

[a] Expressed as [(mean calculated concentration)/(nominal concentration)] × 100%.
[b] Expressed as coefficient of variation (%C.V.).

Table 4
Inter-day analysis of serum quality control (QC) samples from a clinical study.

	Low QC (ng/mL)	Middle QC (ng/mL)	High QC (ng/mL)
Daily results			
Run 1	1.38	11.8	20.8
	1.46	11.6	22.1
Run 2	1.59	13.1	22.1
	1.66	13.0	22.7
Run 3	1.40	12.3	21.8
	1.63	12.9	22.6
Run 4	1.45	12.3	21.5
	1.30	12.9	21.3
Initial mean ($n=8$)	1.48	12.5	21.9
Accuracy[a] (%)	98.9	99.9	99.4
%C.V.[b]	8.7	4.7	3.0

[a] Expressed as [(mean calculated concentration)/(nominal concentration)] × 100%.
[b] Coefficient of variation.

advantages of using SPME to support PK studies at higher doses of **I** were also evident. First of all, the procedure is simple and organic solvent consumption is far less than that of the two LLE methods A and B. Less than 500 μL of solvent was used during extraction and desorption process in method C, compared with more than 17 mL in method A and 2.56 mL in method B. Secondly, the sample preparation time was significantly reduced because of the use of a simple and automated SPME process. Our studies further demonstrated that as an alternative approach for routine analysis of **I**, SPME is more suitable for PK studies when dose levels are relatively high and the concept of coupling the in-tip SPME with automated liquid handling system has proven to be a suitable direction for SPME automation in liquid chromatography both in terms of automation of fiber coating procedure and sample analysis.

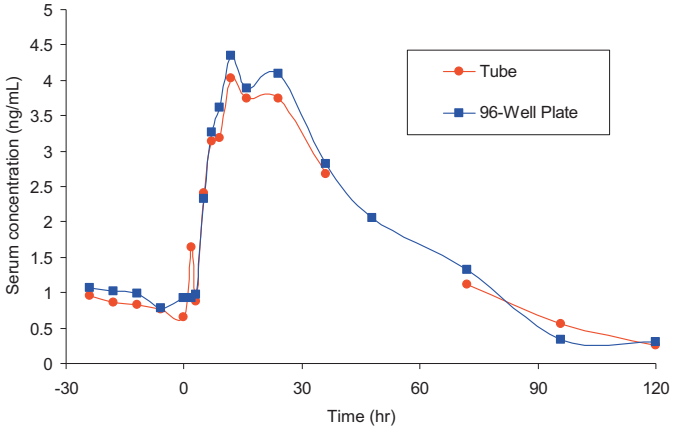

Fig. 5. Concentration-time profile of vitamin D_3 in serum of a healthy subject (A) after single-dose administration of 70 μg of vitamin D_3.

3.8. Clinical sample analysis

Both methods A and B were applied for the determination of **I** in more than 400 serum samples from a clinical study in which healthy subject received single oral dose of 70 μg of **I**. Inter-day precision and accuracy of the method for the clinical samples analysis were determined by analyzing QC samples at low, medium, and high concentrations. Table 4 demonstrates the mean values and method precision and accuracy for QC samples prepared before the analysis of the study samples and for QC samples analyzed in replicate with the daily runs of clinical samples. The precision for daily runs (%C.V.) was less than 8.7% with accuracy ranging from 98.9 to 99.9%.

4. Conclusions

Selective and sensitive HPLC–MS/MS methods using in-tube LLE, 96-well plate LLE, and in-tip SPME with derivatization were developed and validated for the determination of vitamin D_3 in human serum. The use of chemical derivatization was necessary to improve

analyte ionization efficiency, detection selectivity, and assay sensitivity in the presence of biological matrix and in the presence of metabolites. Among the three validated methods, the in-tube LLE method A, although tedious and time consuming, provided better accuracy and precision than the 96-well plate LLE and in-tip SPME, and was chosen for the determination of vitamin D_3 in human serum after dosing human subjects with low oral doses of 70 μg of vitamin D_3. The 96-well plate LLE method B increased sample throughput and provided comparable assay accuracy, precision, and the same LLOQ (0.5 ng/mL) as method A. Method B required lower sample volume (0.4 mL) in comparison with method A (1 mL). The in-tip SPME coupled with automated liquid handling system provided an alternative new approach for high throughput routine drug analysis. For the first time, a simple, fast and high throughput method was developed for preparing monolithic in-tip SPME fibers using photopolymerization. These disposable fiber tips completely eliminated the carryover effect and unnecessary pre-conditioning steps associated with the use of non-disposable fibers. The feasibility of using the automated SPME–HPLC–MSMS as an alternative approach in bioanalysis was demonstrated for supporting PK studies at higher doses of analytes.

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