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Sensitive analysis of 1α,25-dihydroxyvitamin D_3 in biological fluids by liquid chromatography–tandem mass spectrometry

Anne-Marie Kissmeyer*, Kim Sonne

Department of Pharmacokinetics and Metabolism, Leo Pharmaceutical Products, DK-2750 Ballerup, Denmark

Abstract

A liquid chromatographic–tandem mass spectrometric assay using 5% bovine serum albumin as the calibration matrix has been developed for the quantitative analysis of 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] in biological fluids. The analyte was extracted from the matrix after protein precipitation using an automated solid-phase extraction procedure involving both a reversed-phase and normal-phase procedure on a single C_{18} cartridge. The analytical chromatography was performed using a Symmetry C_8 50×2.1 mm, 3.5 μm column. The mobile phase was a linear gradient from 75 to 99% methanol with a constant concentration of 2 m*M* ammonium acetate. 1α,25$(OH)_2D_3$ and the internal standard [$^2H_6$]1α,25$(OH)_2D_3$ were detected by using MS–MS. The ion source was operated in the positive electrospray ionisation mode. The assay is specific, sensitive, and has a capacity of more than 100 samples per day, with a limit of quantitation of 20 pg ml^{-1} for a 1.0-ml sample aliquot. The assay has been used for the analysis of 1α,25$(OH)_2D_3$ in serum from rats and pigs simultaneously with the analysis of the vitamin D analog seocalcitol. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Validation; Stability studies; Vitamins; Seocalcitol; Calcitriol

1. Introduction

1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] is the physiologically active form of vitamin D_3. Vitamin D_3 is formed in the skin from its precursor 7-dehydrocholesterol after UV irradiation or it is absorbed from the diet [1]. In the 1920s it was discovered that vitamin D_3 had a positive effect on rickets [2,3]. But it took about 50 years before it was demonstrated that 1α,25$(OH)_2D_3$ is the active form of vitamin D_3 [4,5]. The activation to 1α,25$(OH)_2D_3$ occurs initially in the liver, where vitamin D_3 is hydroxylated to 25-hydroxy vitamin D_3 (25OHD_3) [6], and subsequently 25OHD_3 is hydroxylated to 1α,25$(OH)_2D_3$ in the kidney [4,7]. The physiological level of 1α,25$(OH)_2D_3$ in the blood steam is in the low pg ml^{-1} range [8].

The classical function of 1α,25$(OH)_2D_3$ is to maintain the extracellular concentration of calcium and phosphorus in the physiological range and to stimulate bone mineralisation [9]. However, in the 1980s it was discovered that 1α,25$(OH)_2D_3$ also exerts other biological activities. These effects include cell growth regulation (inhibition of proliferation and induction of differentiation) of various cell types, for example cancer cells, skin cells, and cells of the immune system [9]. The effect of 1α,25$(OH)_2D_3$ is mediated via a specific intracellular receptor, which belongs to the superfamily of

*Corresponding author. Tel.: +45-44-923-800; fax: +45-44-845-880.

E-mail address: anne-marie.kissmeyer@leo-pharma.com (A.-M. Kissmeyer).

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steroid receptors [10]. After binding to $1\alpha,25(OH)_2D_3$, the receptor–ligand complex acts as a transcription factor that binds to the vitamin D responsive elements (VDREs) on the genome and regulates the expression of a number of genes involved in calcium homeostasis or in the control of cell growth and differentiation, depending on the target cell.

The clinical usefulness of $1\alpha,25(OH)_2D_3$ is mainly limited by its effect on calcium metabolism, with the risk of inducing hypercalcemia and soft tissue calcifications. On the other hand, the promising effects on cell growth regulation have encouraged the search for vitamin D analogs with strong effects on the cell growth regulation combined with a reduced activity in the calcium metabolism. Thus, analogs for the treatment of psoriasis, cancer, and immunological diseases have been or are under development. This includes, for example, calcipotriol [11], tacalcitol [12,13], 22-oxacalcitriol [14], seocalcitol [15], and lexacalcitol [16]. Other analogs, e.g. F_6-$1\alpha,25(OH)_2D_3$ [17], 19-nor-$1\alpha,25(OH)_2D_2$ [18], 22-oxacalcitriol [19], and ED 71 [20] have been or are under development for the treatment of secondary hyperparathyroidism, renal osteodystrophy, and osteoporosis. In addition, to the above synthetic analogs, 1α-hydroxyvitamin D_3 ($1\alpha OHD_3$) is a prodrug of $1\alpha,25(OH)_2D_3$ that has been clinically used for many years for the treatment of renal osteodystrophy [21], and 1α-hydroxyvitamin D_2 ($1\alpha OHD_2$) has recently been approved for the treatment of secondary hyperparathyroidism [22]. Both prodrugs are converted in the liver to $1\alpha,25(OH)_2D_3$ and $1\alpha,24(OH)_2D_2$ or $1\alpha,25(OH)_2D_2$, respectively [23,24].

$1\alpha,25(OH)_2D_3$ and vitamin D analogs are active after oral administration of very low doses or after topical application. For example, the anticancer analog seocalcitol is orally administered to humans in doses from 1 to 25 μg per individual [25,26]. Consequently, highly sensitive assays in the picogram range or lower are required in order to study pharmacokinetics in humans, but also to investigate toxicokinetics in animals.

Various sensitive assays for the analysis of $1\alpha,25(OH)_2D_3$ or its analogs in serum have been published over the past 5–10 years [27–32]. However, as discussed recently [33] none of these assays is suitable for routine analysis or applicable for metabolites or analogs of vitamin D in general. The introduction of the latest generation of triple quadrupole mass spectrometers, e.g. PE/Sciex API 3000, in the late 1990s has now made it possible to obtain highly sensitive liquid chromatography–tandem mass spectrometric assays for metabolites and analogs of vitamin D. Recently, two sensitive assays for the analysis of seocalcitol and 22-oxacalcitriol, respectively, in serum were independently reported [33–35]. Both assays include two solid-phase extraction procedures followed by reversed-phase LC–electrospray ionisation (ESI)-MS–MS, and they are both reported to be suitable for routine analysis. Vitamin D analogs are known to suppress the level of endogenous $1\alpha,25(OH)_2D_3$ [25,28,29,36]. It would therefore be valuable, if the level of $1\alpha,25(OH)_2D_3$ could be determined simultaneously with the investigation of the serum concentrations of an analog. Hence, the present paper is a follow-up on the first LC–MS–MS assay of seocalcitol in serum [33], and it describes how this assay is also applicable to the analysis of $1\alpha,25(OH)_2D_3$ separately or simultaneously with seocalcitol (Fig. 1).

2. Experimental

2.1. Materials

$1\alpha,25(OH)_2D_3$ and $[26,27\text{-}^2H_6]$-$1\alpha,25(OH)_2D_3$ [internal standard (I.S.)] (Fig. 1) were synthesised at Leo Pharmaceutical Products (Ballerup, Denmark). All other chemicals and reagents were commercially available. All solvents were HPLC-grade. Ammonium acetate was analytical reagent-grade and nitrogen gas was 99.999% pure.

2.2. Samples for validation of the $1\alpha,25(OH)_2D_3$ assay

Serum from the relevant species (e.g., rats, pigs, and humans) contains endogenous $1\alpha,25(OH)_2D_3$, and in rats and pigs the level is in the 50–100 pg ml^{-1} range, however, it is somewhat lower in humans. Therefore, it was decided to use a 5% bovine serum albumin (BSA) solution as the matrix for the validation of the $1\alpha,25(OH)_2D_3$ assay. Prior

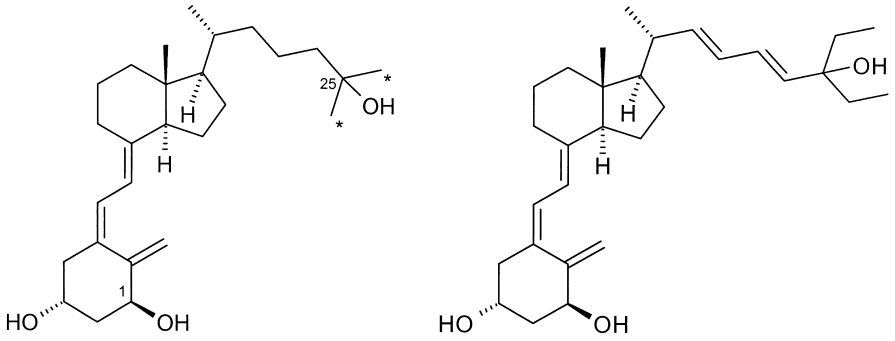

Fig. 1. The chemical structure of $1\alpha,25(OH)_2D_3$ (left) and seocalcitol (right). The asterisks show the deuterium position in the internal standard $[^2H_6]1\alpha,25(OH)_2D_3$.

to validation it was verified that the slope of a $1\alpha,25(OH)_2D_3$ spiked 5% BSA calibration curve was identical to the slope of the spiked serum curves. The 5% BSA solution was also chosen instead of pure water as vitamin D analogs in aqueous solutions easily adsorb to glassware etc. when neither an organic solvent nor some protein is present.

2.2.1. Standards and quality control samples (calibration curves and precision/accuracy)

To perform a standard curve, 1.00 ml of 5% bovine serum albumin was spiked with 50 μl of calibration standards [33]. The concentration of $1\alpha,25(OH)_2D_3$ in the calibration samples was 0, 20, 40, 60, 80, and 100 pg ml^{-1}, respectively, and the concentration of the I.S. was approximately 1000 pg ml^{-1}. Quality control (QC) samples were prepared correspondingly at 20, 50, and 100 pg ml^{-1}.

2.2.2. Selectivity samples

One millilitre ($N=6$) of serum from rat, pig or human was precipitated with two volumes of acetonitrile with and without the I.S. Subsequently, the precipitated samples were handled as above. In addition, serum samples collected from rats and pigs with normal and completely suppressed levels of endogenous $1\alpha,25(OH)_2D_3$ were used to investigate selectivity.

2.2.3. Recovery samples

One millilitre of 5% BSA was precipitated with two volumes of acetonitrile. Thereafter, the precipitated samples were handled in the same way as the standards until reconstitution. The samples were reconstituted in calibration standards containing 20, 50, or 100 pg ml^{-1} of $1\alpha,25(OH)_2D_3$ and 1000 pg ml^{-1} of the I.S.

2.2.4. Freeze–thaw cycle stability

The stability following three freeze–thaw cycles was determined in authentic serum from rats and pigs. Three samples from each pool were immediately analysed. The pool from each species was frozen at −18°C for 24 h and thawed unassisted at room temperature. When the pools were completely thawed, three samples from each pool were collected for immediate analysis. The remaining pools were returned to the freezer. The cycle of thawing and freezing was repeated two more times, and the samples were collected and analysed after each cycle.

2.2.5. Autosampler stability

The effect of 24 h storage in the autosampler was investigated by using samples identical to the QC samples and authentic rat serum. The samples at each level were analysed in duplicate at time 0 and at 24 h.

2.3. Extraction procedures

All samples were extracted as previously described [33]. Briefly, 1 ml of sample was protein precipitated with two volumes of acetonitrile after the I.S. had been added. The supernatant was diluted with water and loaded to an Isolute MF C_{18} solid

phase extraction (SPE) column (100 mg; 1 ml) (IST, Mid-Glamorgan, UK). Following a washing procedure, the analytes were eluated with heptane–2-propanol (93:7). The organic solvent was evaporated and the residues were reconstituted in 200 μl of methanol–1 *M* ammonium acetate–water (500:2:500) before injection to the LC–MS–MS system.

2.4. Liquid chromatography–tandem mass spectrometry

The LC conditions have previously been described [33]. Briefly, the analytical column was a Symmetry C_8 50×2.1 mm (I.D.) (3.5-μm particle size) (Waters, Milford, MA, USA). The mobile phase was a linear gradient from 75 to 99% methanol with a constant concentration of 2 m*M* ammonium acetate with a flow-rate of 0.3 ml min^{-1}. The injection volume was 150 μl.

The mass detector was a PE/Sciex API 3000 Mass Spectrometer using a PE/Sciex TurboIonSpray ion source (Concord, Canada), which was operated in the positive ESI mode at 250°C. The settings were the same as described for seocalcitol [33] except for the multiple reaction monitoring (MRM) transitions of 1α,25$(OH)_2D_3$ and the I.S. The retention time for 1α,25$(OH)_2D_3$ and the I.S. was approximately 4.2 min.

2.5. Calculation of validation parameters

The method was validated over the concentration range of 20–100 pg ml^{-1}. The calibration curve was based on six spiked samples in singlet and QC samples in duplicate at three levels in three separate runs. A fixed amount of 1000 pg ml^{-1} of [$^2H_6$]1α,25$(OH)_2D_3$ was used as the I.S. for all samples. Calibration curves were constructed using 1/concentration weighted linear regression (peak height ratios vs. the concentrations). Peak heights, regression parameters, concentrations, and intra-run accuracy and precision were calculated using TurboQuan Version 1.0.

3. Results and discussion

The main objective of this work was to develop and validate an assay to determine the level of endogenous 1α,25$(OH)_2D_3$ in serum in connection with toxicokinetic investigations of seocalcitol in rats and pigs, as vitamin D analogs at high doses are known to suppress the level of endogenous 1α,25$(OH)_2D_3$ [25,28,29,36]. Due to the presence of endogenous 1α,25$(OH)_2D_3$ in serum, most of the validation has been performed using 5% BSA as the matrix. The assay was set up to be used separately or simultaneously with seocalcitol.

3.1. MS–MS and selectivity

The ESI mass spectrum of 1α,25$(OH)_2D_3$ and the product ion mass spectrum of 1α,25$(OH)_2D_3$ are shown in Fig. 2. The most intense ion formed from 1α,25$(OH)_2D_3$ was *m/z* 434, corresponding to the ammonium adduct ion ($[M+NH_4]^+$) (small amounts of, e.g., the $[M+Na]^+$ is also observed). The most intense product ion was *m/z* 399 corresponding to a neutral loss of 35 mass units, e.g., water (H_2O) and ammonia (NH_3). The energy in the skimmer zone, the collision energy and collision gas setting have optimal settings identical to the settings for seocalcitol [33], which makes it possible to determine seocalcitol and 1α,25$(OH)_2D_3$ simultaneously. Other energy settings and higher temperature in the ion source were also investigated in order to obtain a more sufficient decomposition of $[M+NH_4]^+$. However, a more complete degradation of $[M+NH_4]^+$ did not increase the intensity of the base peak [$M+NH_4$–35] in the MS–MS spectrum nor did other abundant product ions appear.

A neutral loss of 35 is usually not considered to be specific. However, by using the ''phase-switching'' solid-phase extraction clean-up procedure combined with reversed-phase HPLC a very high degree of selectivity was achieved. Typical selected reaction monitoring (SRM) chromatograms from pig serum are shown in Fig. 3. The left panel is a chromatogram of serum from an untreated pig with a significant signal corresponding to approximately 60 pg ml^{-1} 1α,25$(OH)_2D_3$. The right panel is a chromatogram of serum from a pig treated with seocalcitol showing no signal corresponding to 1α,25$(OH)_2D_3$ (similar observations were done in rat serum). This is an indirect demonstration of the selectivity of the assay as we know that seocalcitol, at calcemic doses,

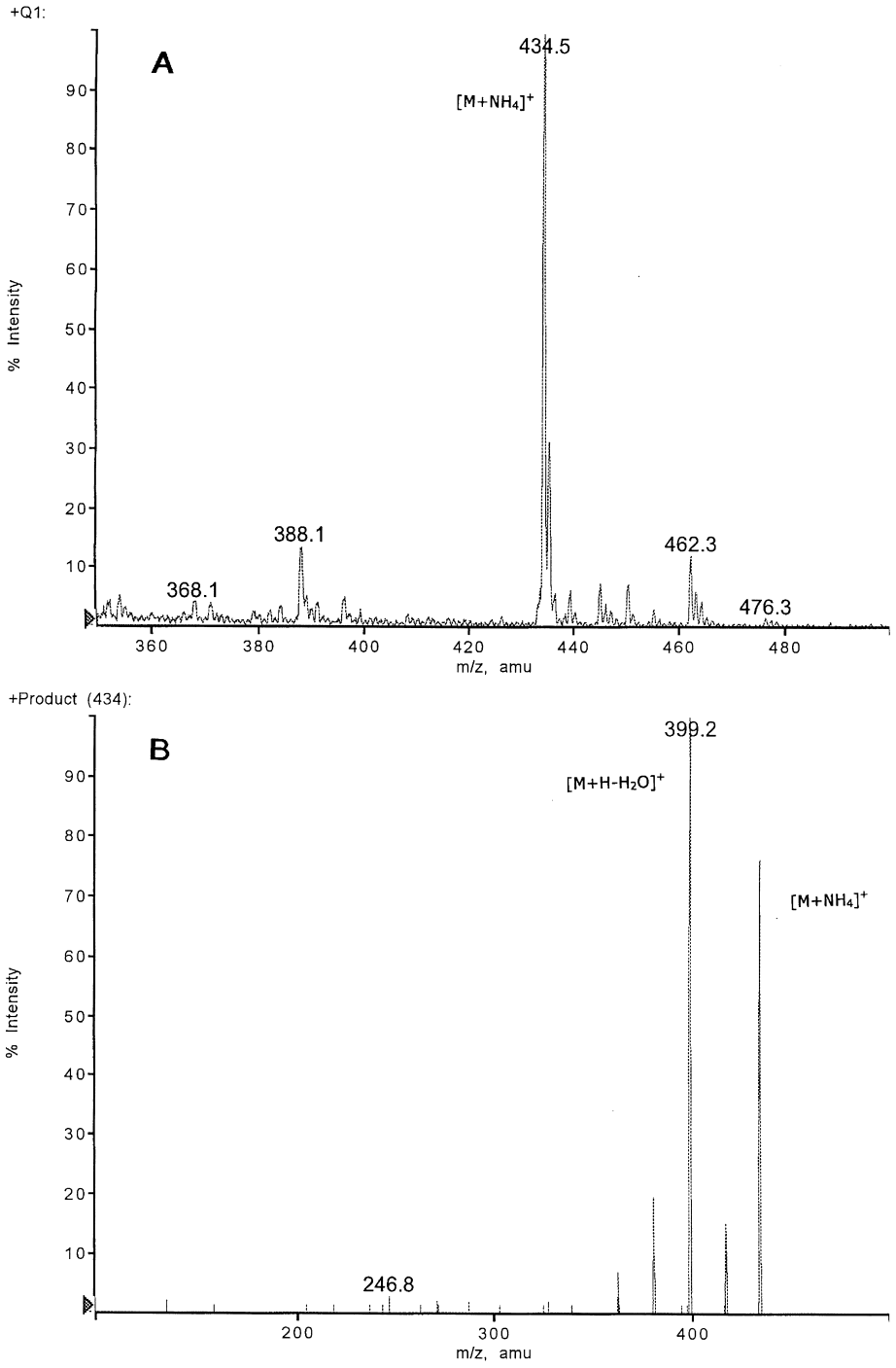

Fig. 2. Full scan positive ESI ion mass spectrum of 1α,25(OH)$_2$D$_3$([M+NH$_4$]+ m/z of 434.5) (A). Product ion mass spectrum (MS–MS) of 1α,25(OH)$_2$D$_3$ ([M+NH$_4$]+ m/z of 434.5) (B).

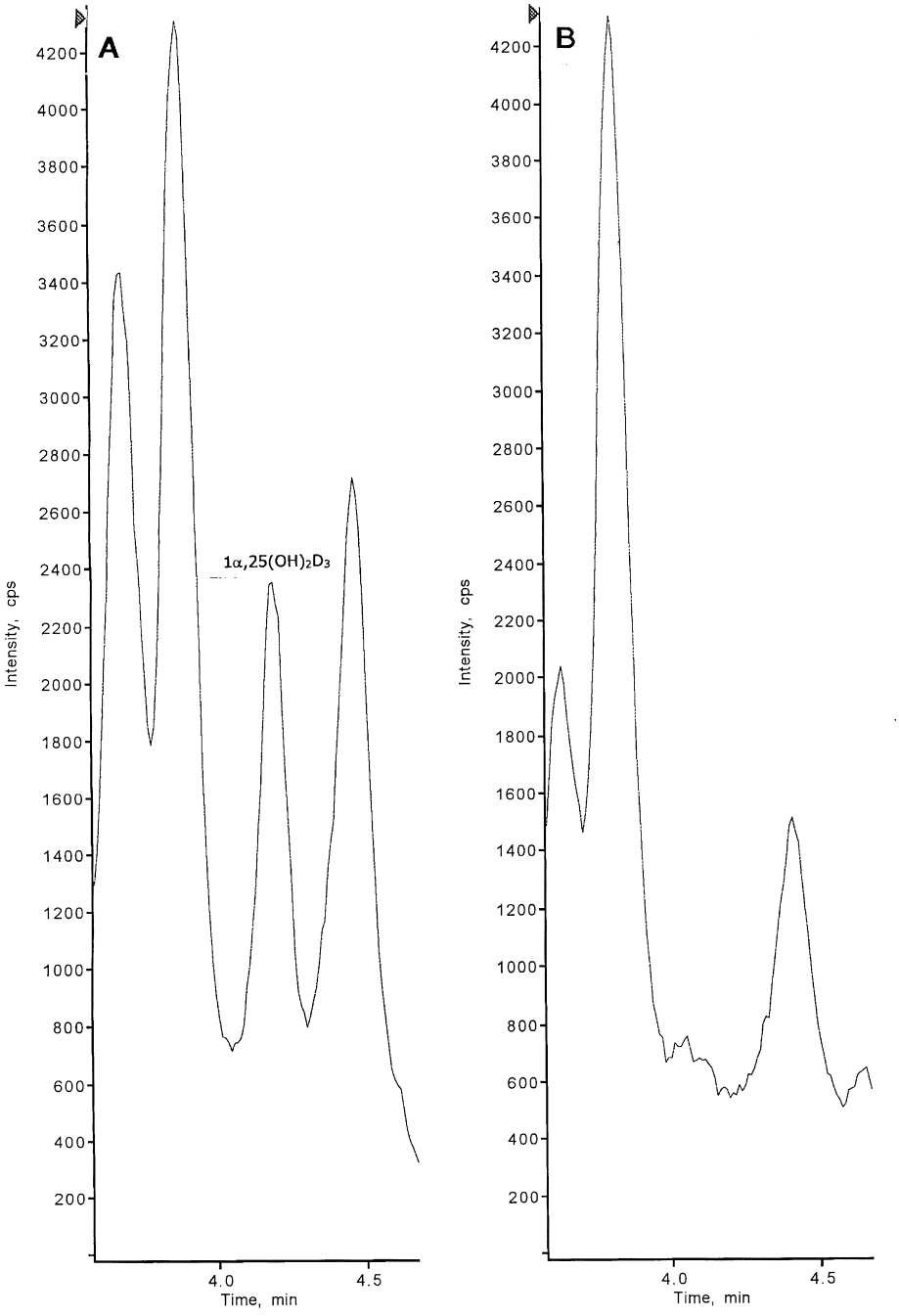

Fig. 3. Selected reaction monitoring (SRM) ion chromatogram of pig serum before treatment with seocalcitol. Transitions: m/z 434.5→399.2 for 1α,25$(OH)_2D_3$ (A). Selected reaction monitoring (SRM) ion chromatogram of pig serum after 8 days treatment with seocalcitol (0.3 μg/kg once daily). Transitions: m/z 434.5→399.2 for 1α,25$(OH)_2D_3$ (B).

suppresses the level of endogenous 1α,25$(OH)_2D_3$ completely [25]. In addition, no interference was observed with the blank serum samples from rats, pigs, humans, and identical samples spiked with the [$^2H_6$]-1α,25$(OH)_2D_3$ (the I.S.). Consequently, the transitions m/z 434→399 and 440→405 could be used as the MRM settings.

All the vitamin analogs that we have investigated to date using the PE/Sciex API 3000 mass spectrometer form ammonium adduct ions as the base peak in single MS scans. Many of the analogs give the most intense product ions corresponding to a neutral loss of 35 mass units, e.g. water (H_2O) and ammonia (NH_3) in MS–MS scans. The signal-to-noise response might on the other hand be different, which is illustrated in Fig. 4 showing the SRM ion chromatogram of the 26-hydroxy and 26a-hydroxy derivatives of seocalcitol (these compounds are major or minor metabolites of seocalcitol) [37,38]. The different intensity of these six very similar compounds indicates that the sensitivity of the vitamin D analogs might not always be in the same range. Although many of the vitamin analogs investigated have a loss of 35 mass units there are also analogs which form completely different MS–MS spectra. For example one analog very similar to seocalcitol (one of the double bonds in the side chain is replaced by a triple bond), gives a MS–MS base peak of m/z 135. Finally, the most intense product ion of 22-oxacalcitol is reported to be m/z 297, which corresponds to a loss of 139 mass units [35].

3.2. Linearity of 1α,25$(OH)_2D_3$

The response function for the calibration curve covering the concentration of 20–100 pg ml^{-1} was determined with every run within the validation. The best calibration curves were obtained using 1/concentration weighted linear regression (peak height ratio of the analyte versus the I.S., plotted against the concentration). The slopes, intercepts, and coefficients of regression appear in Table 1 and the residuals are stated in Table 2. The highest concentration in the standard curve has been chosen to be 100 pg ml^{-1} because all serum samples are expected to contain less than 100 pg ml^{-1}. However, in connection with an extension of the assay it was subsequently demonstrated that the linearity can be extended several fold upwards.

3.3. Precision and accuracy of 1α,25$(OH)_2D_3$

The precision and accuracy were investigated on two conditions: within run (intra-run) and between run (inter-run). Six determinations at three levels covering the analytical range were used to determine the intra-run precision and accuracy. Between the runs, parameters were assessed from duplicate samples at each level on three separate days. The precision is calculated as RSD (%)=standard deviation/mean·100%, and the accuracy is calculated as the percentage of nominal concentration[(measured conc./nominal conc.)·100%]. The intra- and inter-run precision and accuracy are given in Table 3. The repeatability (intra-run) and reproducibility (inter-run) were <15% at all levels. The accuracy was within 85–115% at all levels. The intra- and inter-run precision of endogenous 1α,25$(OH)_2D_3$ in rat and pig serum was also <15%. Due to the very low levels of endogenous 1α,25$(OH)_2D_3$ in the human serum available, it has been impossible to get any precision data in human serum so far.

3.4. Lower limit of quantitation (LLOQ) of 1α,25$(OH)_2D_3$

The lower limit of quantitation is defined as the lowest quantified level with precision of ±20% and accuracy of 80–120%. From the results listed in Table 3 it can be seen that 20 pg ml^{-1} of 1α,25$(OH)_2D_3$ in 5% BSA fulfils the definition of LLOQ. Ten pg ml^{-1} was originally included in the validation, but it could not meet the LLOQ criteria.

3.5. Extraction recovery of 1α,25$(OH)_2D_3$

The extraction recovery was determined by analysis of six samples at three levels by comparison of the response from spiked and extracted samples and from extracted 5% BSA samples spiked with 1α,25$(OH)_2D_3$ and the I.S. after the extraction procedure. Recovery is given as the ratio between

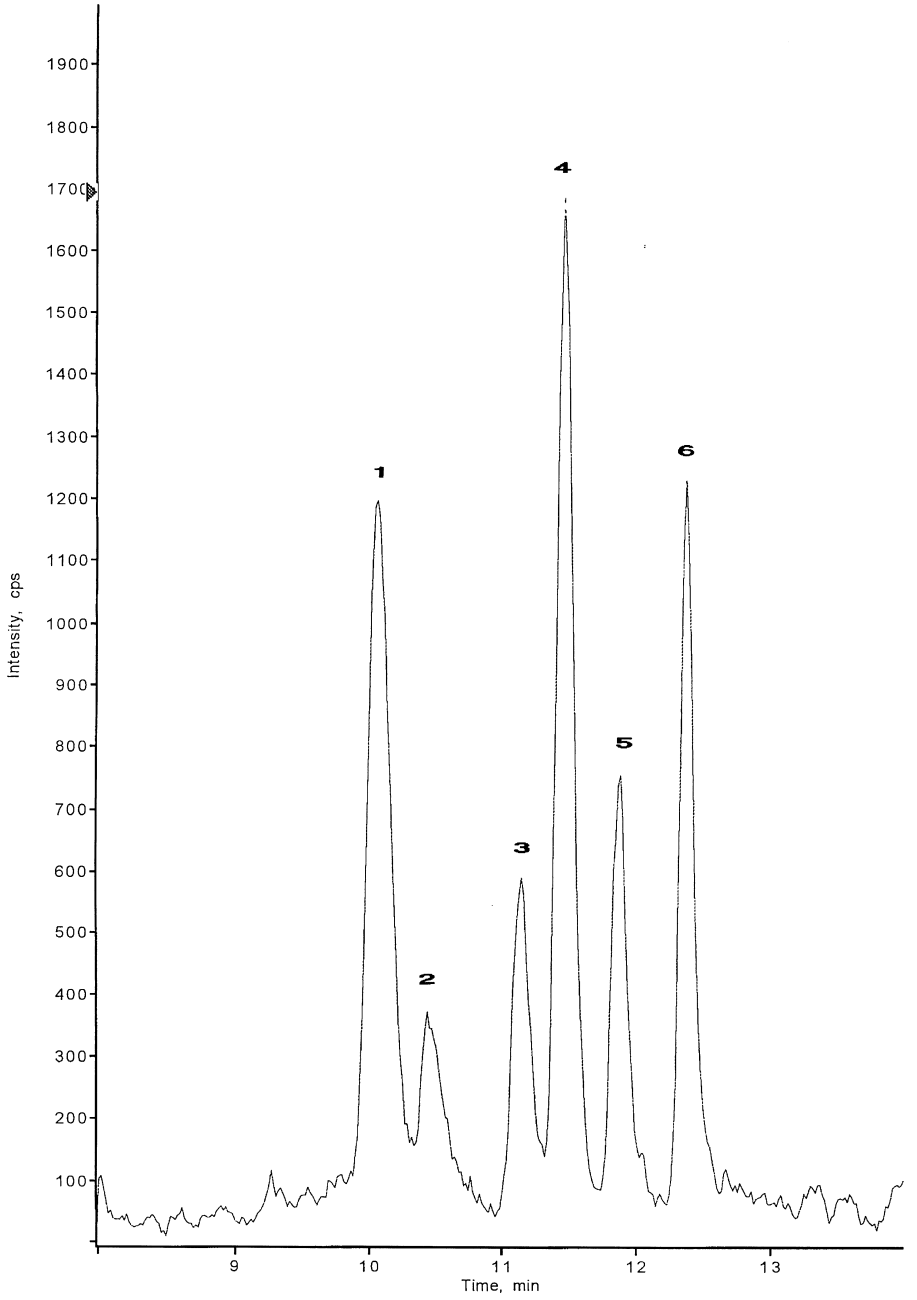

Fig. 4. Selected reaction monitoring (SRM) ion chromatogram (transition: m/z 488.4→453.4) of 26-hydroxy and 26a-hydroxy derivatives of seocalcitol: 26a(I)-OH seocalcitol (1); (25*R*),26*R*-OH seocalcitol (2); (25*R*),26*S*-OH seocalcitol (3); 26a(II)-OH seocalcitol (4); (25*S*),26*S*-OH seocalcitol (5) and (25*S*),26*R*-OH seocalcitol in the relative concentrations: 0.5:1:1:0.4:1:1.

the mean height of a spiked sample versus the mean height of a blank sample spiked with analytes after extraction [including correction for the dilution (0.86) done in connection with the extraction]. The mean ($N=6$) recoveries are stated in Table 4. The data suggest the recoveries of 1α,25$(OH)_2D_3$ to be

Table 1
Calibration curve equations using 1/concentration weighing of 1α,25(OH)$_2$D$_3$ in 5% BSA

Run	Slope	Intercept	Correlation coefficient (r)
1	0.00111	0.00639	0.9998
2	0.00105	0.00335	0.9994
3	0.00112	0.00038	0.9999

Table 2
Percentage residuals of the calibration curve of 1α,25(OH)$_2$D$_3$ in 5% BSA

Nominal concentration (pg ml^{-1})	Run no. (%)		
	1	2	3
20	1.1	−1.2	0.5
40	Ex[a]	0.8	−0.3
60	−1.9	2.5	−0.6
80	−0.1	−2.4	−0.4
100	1.1	0.4	0.8

[a] Ex=Excluded to optimise linear regression.

independent on the concentration in the tested ranges.

3.6. Stability

The stability of 1α,25(OH)$_2$D$_3$ under various conditions was investigated. A stock solution of 100 μg ml^{-1} of 1α,25(OH)$_2$D$_3$ in 2-propanol was demonstrated to be stable for at least 1 year when stored at −18°C. A working solution of 40 ng ml^{-1} of 1α,25(OH)$_2$D$_3$ in methanol was found to be stable for at least 1 month at −18°C. The stability of 1α,25(OH)$_2$D$_3$ was also investigated in rat and pig serum. After three freeze–thaw cycles, the concentrations were within 85–115% range of the initial concentration (Table 5). The extended stability of 1α,25(OH)$_2$D$_3$ in serum stored at −18°C and −80°C is on-going. Data are not yet available. Finally, stability of 1α,25(OH)$_2$D$_3$ in extracted and reconstituted 5% BSA or rat serum samples stored in the autosampler at 8°C for 24 h was demonstrated, as the

Table 3
Intra- and inter-run precision and accuracy (N=6) of 1α,25(OH)$_2$D$_3$ in 5% BSA and authentic serum from rat and pig

		Nominal concentration in 5% BSA		
		20 pg ml^{-1}	50 pg ml^{-1}	100 pg ml^{-1}
Intra	Precision RSD (%)	12.5	8.4	2.1
	Accuracy (%)	98	88	108
Inter	Precision RSD (%)	5.7	9.1	10.7
	Accuracy (%)	108	92	96
		Authentic serum samples		
		Rat	Pig	
Intra	Precision RSD (%)	7.1	7.6	
	Mean (pg ml^{-1})	87.5	80.0	
Inter	Precision RSD (%)	8.1	8.8	
	Mean (pg ml^{-1})	74.1	83.8	

Table 4
Percentage recovery of 1α,25(OH)$_2$D$_3$ and [^{2}H$_6$]1α,25(OH)$_2$D$_3$ from 5% BSA[a]

Recovery (%)			
1α,25(OH)$_2$D$_3$			[^{2}H$_6$]1α,25(OH)$_2$D$_3$
20 pg ml^{-1}	50 pg ml^{-1}	100 pg ml^{-1}	1000 pg ml^{-1}
52 (10/4.6)	59 (6.2/3.3)	59 (5.2/2.1)	57 (4.9/3.7)

[a] The numbers in brackets are RSDs (%) of the peak height found in spiked and extracted samples and in blank samples spiked after the extraction procedure, respectively.

Table 5
Freeze–thaw stability of $1\alpha,25(OH)_2D_3$ in rat and pig serum

Species	Percentage of initial concentration after three freeze–thaw cycles
Rat	102
Pig	89

concentrations compared between 0 and 24 h of storage were within the above ranges of acceptance (Table 6).

3.7. Application of the assay

After validation the assay has successfully been used to simultaneously determine the level of endogenous $1\alpha,25(OH)_2D_3$ and seocalcitol after repeated administration of seocalcitol to rats and pigs [38]. The chromatograms in Fig. 3 originate from a toxicokinetic study in pigs demonstrating that repeated administration of toxicological doses of seocalcitol completely suppresses the endogene presence of $1\alpha,25(OH)_2D_3$ in serum. The validation of $1\alpha,25(OH)_2D_3$ in human serum has not yet been fully completed, as the LC–MS–MS assay needs to be cross-validated against the presently most-used assays, e.g., the receptor binding assay and the radioimmunoassay [39–41]. In addition, due to the lower levels of $1\alpha,25(OH)_2D_3$ in human serum (at least from Scandinavians) compared to rats and minipigs it is necessary to use an aliquot of 2 ml of serum in order to obtain a sufficient sensitivity (LLOQ$\leq$10 pg ml^{-1}).

The assay has also been utilised for other purposes. For example, the assay is used to determine the level of $1\alpha,25(OH)_2D_3$ and $24,25(OH)_2D_3$ in different cell cultures [42]. $24,25(OH)_2D_3$ is another metabolite of vitamin D_3 showing the same optimal MRM setting as $1\alpha,25(OH)_2D_3$ but with a shorter retention time. In addition, as to cell culture samples, the sample preparation procedure can be modified by using a liquid-phase extraction procedure instead of the solid-phase extraction procedure.

Table 6
Twenty-four hour autosampler stability of $1\alpha,25(OH)_2D_3$ in 5% BSA and rat serum in reconstituted samples

Percentage of nominal concentration at 24 h in autosampler			
25 pg ml^{-1}	50 pg ml^{-1}	100 pg ml^{-1}	Rat serum
97	99	97	89

4. Conclusion

A liquid chromatography–tandem mass spectrometry assay for the determination of $1\alpha,25(OH)_2D_3$ has been developed and validated with a lower limit of quantitation of 20 pg ml^{-1} using 5% BSA as the biological matrix for calibration. The assay is specific, sensitive and has a capacity of more than 100 samples per day making it suitable for routine analysis. The assay can be used for the analysis of $1\alpha,25(OH)_2D_3$ separately or simultaneously with seocalcitol or other vitamin D analogs. Except for the MRM settings the present assay is also considered to be applicable for many other vitamin D analogs.

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