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Characterization of vitamin D_3 metabolites using continuous-flow fast atom bombardment tandem mass spectrometry and high-performance liquid chromatography

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ABSTRACT

A mass spectrometric method for the detection of vitamin D_3 metabolites is described. This method involves the derivatization of the metabolites by cycloaddition with 4-phenyl-1,2,4-triazoline-3,5-dione, followed by their characterization by continuous-flow fast atom bombardment (CF-FAB) tandem mass spectrometry (MS–MS) and high-performance liquid chromatography (HPLC). Using HPLC, this derivatization has been shown to increase the UV detectability of 25-hydroxyvitamin D_3 by about 5-fold. The FAB spectra of the adducts are dominated by peaks corresponding to a protonated molecule and a fragment ion derived in part from the loss of the side chain. Multiple reaction monitoring (MRM) of this transition by MS–MS may be utilized for trace level analysis of vitamin D metabolites. Sample introduction by flow injection yields detection limits in the low nanogram to high picogram range, whereas the use of on-line capillary LC has been found to decrease the detection limits to the low picogram level.

INTRODUCTION

Vitamin D_3, a secosteroid involved in the regulation of calcium homeostasis, is synthesized in the skin from 7-dehydrocholesterol on exposure to ultraviolet light. During the past three decades, the metabolism of vitamin D_3 has been extensively investigated [1]. It has been found that vitamin D_3 is first metabolized in the liver to $25(OH)D_3$ [a], followed by metabolism in the kidney to $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$. $1,25(OH)_2D_3$ is the most active metabolite of vitamin D_3, and is now accepted to be the steroid hormone involved in the maintenance of calcium homeostasis [2]. Recently, it has been found that $1,25(OH)_2D_3$ possesses the ability to differentiate several cancer cells [3,4]. *In vitro* studies have shown that $1,25(OH)_2D_3$ can suppress the proliferation of leukemic cells and, at the same time, induce their differentiation towards more mature macrophages. These results indicate that $1,25(OH)_2D_3$ may have a therapeutic potential in the treatment of leukemia. Unfortunately, dosages required to achieve these

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[a] Abbreviations: $25(OH)D_3$ = 25-hydroxyvitamin D_3; $24,25(OH)_2D_3$ = 24,25-dihydroxyvitamin D_3; $1,25(OH)_2D_3$ = 1,25-dihydroxyvitamin D_3.

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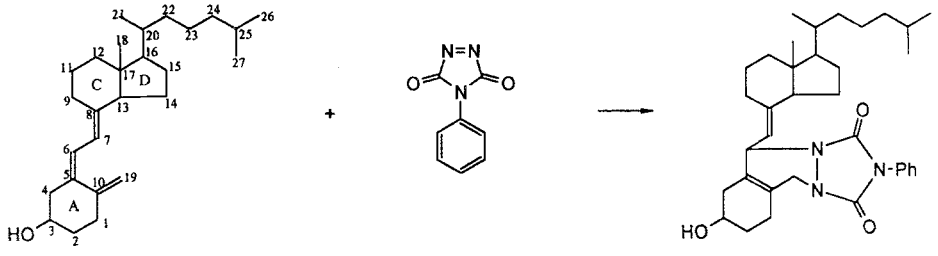
effects were found to cause hypercalcemia. Thus, much interest has arisen in the development of similar analogues as potential anti-cancer agents that are non-calcemic [5].

In the development and testing of new synthetic non-calcemic analogues of $1,25(OH)_2D_3$, their serum levels in the body need to be closely monitored to avoid overdose. Also, routine quantification and identification of natural vitamin D metabolites in the plasma are equally important. However, the detection of these vitamin D metabolites in plasma has proved to be a difficult task, due to their structural and chemical similarities to one another, and the presence of large quantities of other lipids and sterols in samples. Most importantly, the plasma concentrations of these metabolites in a normal human are extremely low. For example, typical levels are [6]: vitamin D_3, 8.1 ± 4.8 ng/ml; $25(OH)D_3$, 27 ± 10 ng/ml; $24,25(OH)_2D_3$, 1.6 ± 0.6 ng/ml; $1,25(OH)_2D_3$, 55 ± 10 pg/ml. At such low levels, the detection of the metabolites requires much sensitivity and specificity.

Current methods that are most frequently used for characterizing these metabolites include saturation analysis, high-performance liquid chromatography (HPLC) and gas chromatography–mass spectrometry (GC–MS) measurements. HPLC with UV detection is only sensitive enough for measuring metabolites such as vitamin D_3 and $25(OH)D_3$ [7] which occur at higher concentration levels. GC–MS with isotope dilution has been applied to the detection of vitamin D_3 metabolites as well. However, the metabolites are not suitable for GC analysis, and some form of pre-column derivatization must be carried out in order to make them compatible with the high temperature in the column. Typical derivatization employed includes their conversion into trimethylsilyl and *tert.*-butyldimethylsilyl ethers [8]. While the electron impact (EI) spectra of these derivatives are often structurally informative, the compounds still suffer from thermal lability. Moreover, the ion peak intensity at the high mass is relatively low and molecular weight information may be obscured. Saturation analysis includes the use of antibodies, plasma binding proteins or receptor proteins. These methods in general have sufficient sensitivity for quantifying the metabolites; however, they lack the necessary specificity [9], and cross reactions often occur. Consequently, each metabolite needs to be completely isolated before reactions with antibodies or proteins can be carried out, thus posing more stringent requirements on the preliminary HPLC separations.

Mass spectrometry (MS) is generally recognized as one of the most definitive analytical techniques currently available. When used in conjunction with a GC or LC system, MS detection can provide excellent sensitivity and specificity. Besides GC–MS, however, there has not been much advancement in such combined techniques in vitamin D research. Yergey *et al.* [10] described a quantitative technique for metabolic assay using thermospray LC–MS with isotope dilution. Although it was successful for many drug metabolites, it did not have a detection limit that is physiologically realistic for $1,25(OH)_2D_3$. It is therefore deemed important



I II III

Scheme 1

to develop a combined LC–MS method that is capable of detecting vitamin D metabolites at biological levels.

In this paper, we describe a MS technique that allows for sensitive detection of vitamin D metabolites by capillary LC–MS. A simple pre-column derivatization step is employed, using the Cookson reagent 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD, **II**) [11]. PTAD is one of the strongest dienophiles known; it is capable of attacking the 5,7-diene bonds in the vitamin D structure and forms an adduct via a Diels–Alder reaction (Scheme 1). Due to the high UV response of the phenyl ring associated with the PTAD structure, this reaction is found to be promising for the selective labelling of diene compounds for HPLC separation, where large amounts of interferences might be present [12]. PTAD and its methyl analogue, 4-methyl-1,2,4-triazoline-3,5-dione (MTAD), have previously been used as derivatizing reagents for the determination of compounds containing a conjugated diene moiety in an aliphatic chain [13,14]. It has also been examined as a protecting agent for the diene bonds in vitamin D_3 as an approach for A ring derivatization [15]. It is thus quite clear that PTAD is an ideal candidate for the selective labelling of vitamin D metabolites. In our laboratory, we have been examining the utility of these vitamin D_3–PTAD adducts for analysis by MS [16] and HPLC [16,17]. Since the adducts formed are too polar to be ionized by the traditional EI or chemical ionization techniques, fast atom bombardment (FAB) MS operating in the continuous-flow mode has been employed, as reported here.

EXPERIMENTAL SECTION

Chemicals

Vitamin D_3 and PTAD were purchased from Aldrich (Milwaukee, WI, USA). Other metabolites were synthesized by Dr. M.R. Uskokovic of Hoffmann LaRoche (Nutley, NJ, USA). [26,27-^{3}H]-25(OH)D_3 was obtained from Amersham (Arlington Heights, IL, USA) with a specific activity of 20 Ci/mmol. All solvents used were of the highest qualities available.

HPLC conditions

A Waters 600E LC system equipped with a Model 990 photodiode array detector (Waters, Milford, MA, USA) was used. Wavelengths monitored were 226 nm for the adduct, and 265 nm for the metabolite. The column used was a 15 cm × 4.6 mm C_8 column (Rainin, Woburn, MA, USA) with 5 μm particle size. Mobile phase was made up of water–acetonitrile (50:50, v/v) (both HPLC grade and filtered). Flow-rate was maintained at 2.0 ml/min. Fractions were collected using a Retriever II fraction collector. ^{3}H counting was done by a scintillation counter (TM Analytic, Bensenville, IL, USA) using Scintilene (Fisher Scientific, Pittsburgh, PA, USA) as the reagent.

MS conditions

All data were obtained on a Fisons/VG Quattro triple quadrupole mass spectrometer (Fisons/VG Biotech, UK). For CF-FAB, the matrix solution was made up as follows: 1% tetramethylene sulfone, 5% acetonitrile and 19% ethanol in deionized water. This solution was delivered at a flow-rate of 5 μl/min using an Isco μLC-500 pump (Isco, Lincoln, NE, USA) to the dynamic probe tip through a 50 μm I.D. fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA). A stainless-steel wire mesh [5 μm pore size, 0.003 in. thick (1 in. = 2.54 cm) $\approx$ 2 mm in diameter] was placed at the tip to stabilize the solution flow. Samples were introduced by flow injection via a Rheodyne injector (No. 7520) equipped with a 0.5-μl internal sample loop (Rheodyne, Cotati, CA, USA). Source temperature was maintained at 35°C in the MS. The collision induced dissociation (CID) (static probe) and multiple reaction monitoring (MRM) data were obtained with a collision energy set at 70 eV. Peak intensity was attenuated by 75% using argon gas.

Capillary LC and LC–MS conditions

A Fisons/Carlo Erba Phoenix 30 capillary LC system (Fisons, Danvers, MA, USA) was used in the isocratic mode at 5 μl/min with the following solvents: (1) Vitamin D_3– and 25(OH)D_3–PTAD: acetonitrile–H_2O–thioglycerol (85:14:1, v/v/v); (2) 24,25$(OH)_2D_3$– and 1,25$(OH)_2D_3$–

PTAD: acetonitrile–H_2O–thioglycerol (60:39:1, v/v/v). A C_{18} Fusica column of 320 μm I.D. (LC Packings, Switzerland) was used in all work. The LC eluent was delivered to the CF-FAB probe via a 50 μm I.D. fused-silica capillary.

Reaction of metabolites with PTAD

Samples of metabolites were dissolved and stored in ethanol. Solid PTAD was dissolved in acetonitrile in concentrations in the order of several hundred μg/ml. The PTAD solution was added directly to each metabolite, until the red color persisted for a few seconds. The reaction mixtures were vortexed for a few minutes, and then left at room temperature for at least 30 min, during which time the color would gradually change to orange or straw-like. For HPLC studies, the samples were first dried down by nitrogen gas and resuspended in the mobile phase before injections. For MS analyses, samples were examined directly in their original solvent mixture.

Tritium counting experiment

For the study of the effect of PTAD derivatization on HPLC–UV sensitivity, 20 μg of 25(OH)D_3 was spiked with 1 μCi of ^{3}H-labelled 25(OH)D_3. A part (3/4) of this solution was reserved for reaction with PTAD, following the conditions as outlined above and using a 1000 molar excess of PTAD to ensure complete conversion. The remaining sample was injected into the HPLC under isocratic conditions. Fractions were collected from 14 to 21 min and peak areas were summed, which contained the eluted 25(OH)D_3 at 17 min. Of each fraction one third was kept for ^{3}H counting, whereas the rest was pooled and reinjected into the HPLC. This procedure was repeated three more times. Similar experiment was carried out with the [^{3}H]-25(OH)D_3–PTAD adduct, with fractions collected between 3 to 8 min. Four sets of readings were also taken of the ^{3}H counts and of the peak areas for the adduct.

RESULTS AND DISCUSSION

Several aspects of the pre-column derivatization with PTAD were evaluated. These included reaction requirements, potential usefulness of the derivatization for HPLC–UV analysis and detectability of the adducts by MS. Results of these studies are outlined below.

Evaluation of the derivatization reaction

Vitamin D_3 solutions of various concentrations ranging from 10^{-3} to 10^{-8} M were allowed to react with different amounts of PTAD. Each reaction product was examined by static FAB-MS, with close attention paid to the protonated molecular ion regions of the vitamin D_3–PTAD adduct (m/z 560) and the unreacted vitamin D_3 (m/z 385). It was found that at a concentration of 10^{-3} M, a 2-fold molar excess of PTAD was

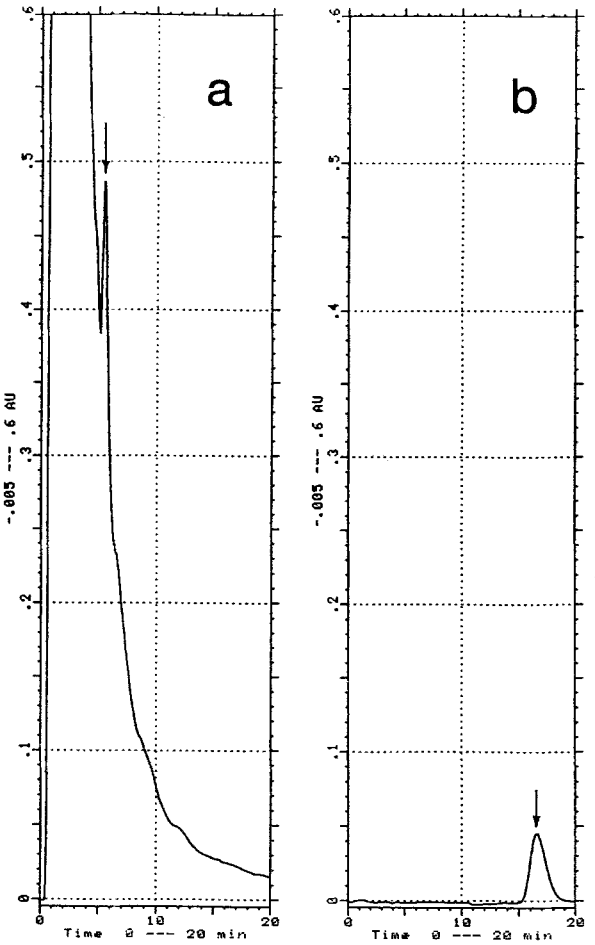


Fig. 1. HPLC chromatograms, shown in the same scale, of (a) ^{3}H-labelled 25(OH)D_3–PTAD, 5 μg; (b) ^{3}H-labelled 25(OH)D_3, 5 μg.

sufficient to convert all the vitamin D_3 into the adduct, as no peak was visible at m/z 385. At a concentration as low as 10^{-8} M, it appeared that the reaction was complete using a 4000-fold molar excess of PTAD. The 10^{-8} M concentration would be equivalent to about 3 ng/ml of vitamin D_3 in plasma, which is a physiologically realistic level.

After conversion to the PTAD adducts, it was expected that the phenyl ring in the adduct structure would increase the UV sensitivity of the metabolites. In order to examine this potential increase quantitatively, we used [^{3}H]-25(OH)D_3 as the starting substrate and compared the UV absorbance generated by the [^{3}H]-25(OH)D_3 and its PTAD adduct by HPLC, as outlined in the Experimental section. The advantage of this method is that peak areas from HPLC could be directly related to and quantified by post-column ^{3}H counting, thereby eliminating the need to account for the recovery of sample after HPLC. The chromatograms of labelled 25(OH)D_3 and 25(OH)D_3–PTAD are shown in Fig. 1. A plot of ^{3}H counts *vs.* peak areas for both compounds indicates that the PTAD adduct of 25(OH)D_3 is about 5 times more UV sensitive than the unreacted metabolite in terms of peak area per ^{3}H count. (Slope $= 9.0 \times 10^{-7}$ for 25(OH)D_3 with $r = 0.9745$; slope $= 5.2 \times 10^{-6}$ for 25(OH)D_3–PTAD with $r = 0.9741$). This result further establishes the advantage of pre-column derivatization of the vitamin D_3 metabolites with PTAD for UV detection purposes.

Detectability by FAB-MS

Fig. 2 shows the normal full scan positive ion FAB spectra of PTAD adducts of the four most common vitamin D_3 metabolites. Since the adducts contain nitrogens, protonation by FAB is facilitated and this is advantageous for increasing the detectability of the analytes by MS. In each mass spectrum, the protonated molecules (MH^+) can be seen clearly, along with the $[MH - H_2O]^+$ peaks. Typical of FAB spectra, these spectra are quite simple and contain few fragment ion peaks. Prominent in the spectra of the adducts of vitamin D_3, 25(OH)D_3 and 24,25(OH)$_2D_3$ is the fragment ion of m/z 298. For the A ring hydroxylated metabolite

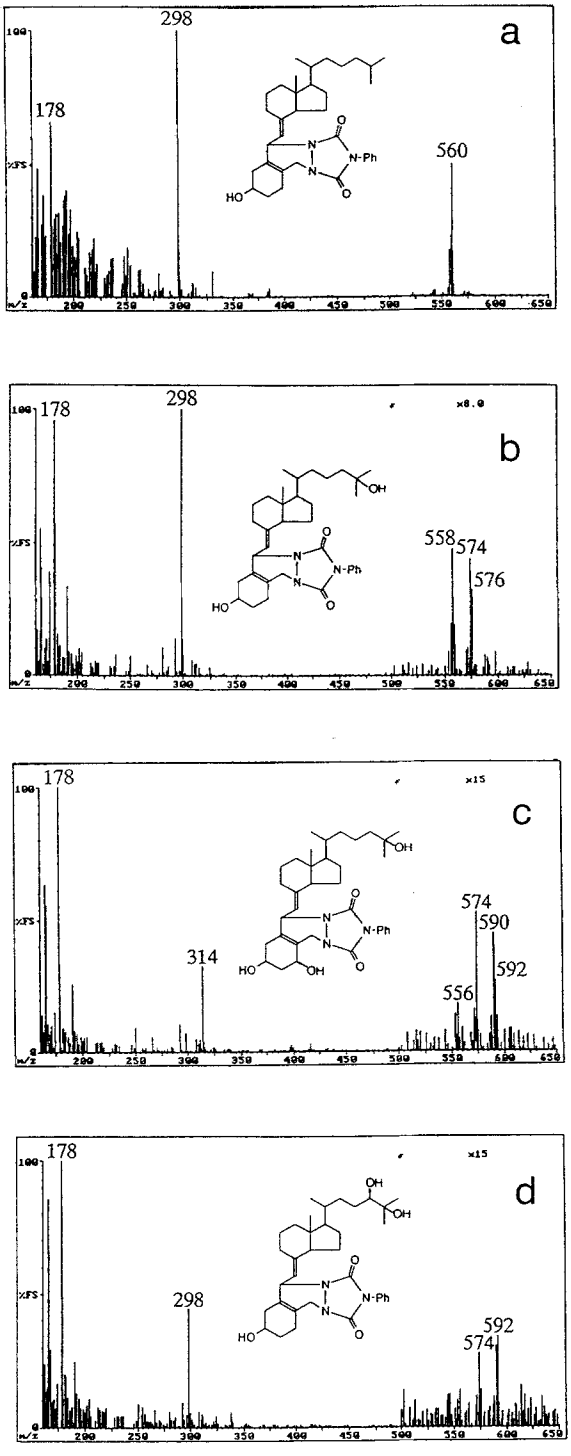
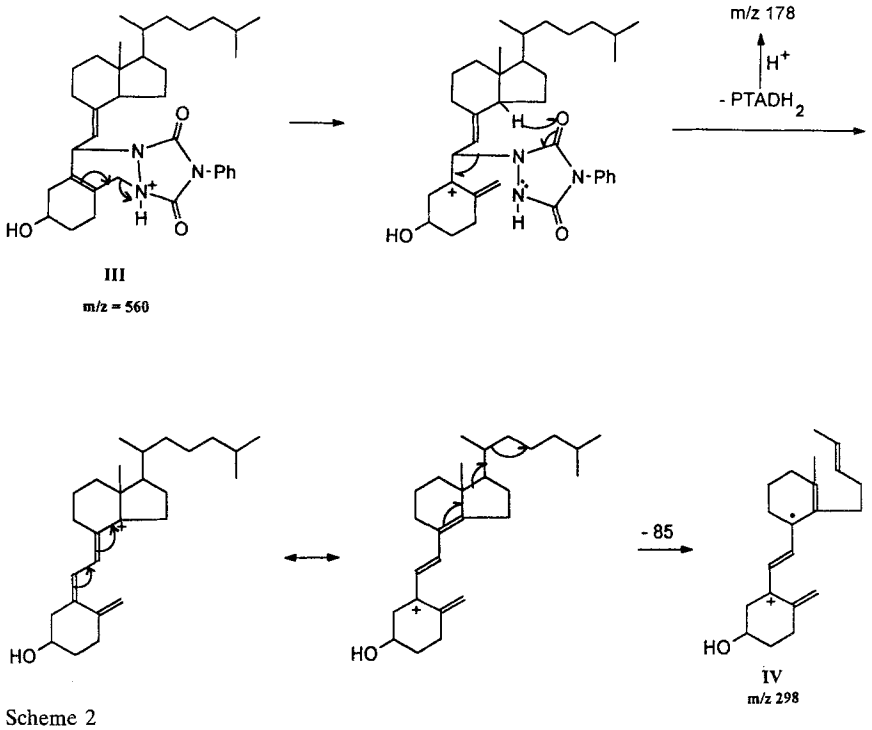


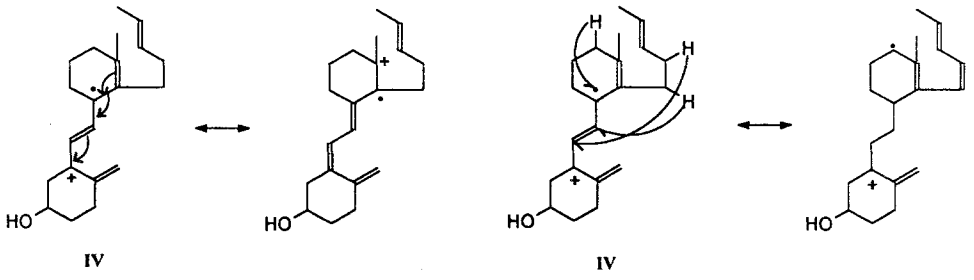
Fig. 2. Full scan FAB spectra of the four adducts studied: (a) vitamin D_3–PTAD, 40.6 ng; (b) 25(OH)D_3–PTAD, 119 ng; (c) 1,25(OH)$_2D_3$–PTAD, 61 ng; (d) 24,25(OH)$_2D_3$–PTAD, 50 ng.



Scheme 2

$1,25(OH)_2D_3$, this peak is shifted by 16 mass units to m/z 314. It may be postulated that this fragment is formed via the process depicted in Scheme 2, starting with a hydrogen transfer which leads to the losses of a reduced PTAD molecule and part of the side chain. Presumably, these ions can undergo a series of rearrangements, resulting in the localization of a charge in the D ring (Scheme 3). The ions of m/z 298 and m/z 314 have been subjected to CID and the data (Fig. 3) are consistent with the indicated structural assignments (**IV**).

The CID data of the MH^+ ion of all the adducts show high relative abundance for the fragment 298/314 ions. In view of the high abundance of the latter in the normal MS spectra, MRM utilizing these ions appeared to be an attractive detection scheme for the metabolites. In MRM, the transition under collision conditions is monitored in a fashion similar to single-



Scheme 3

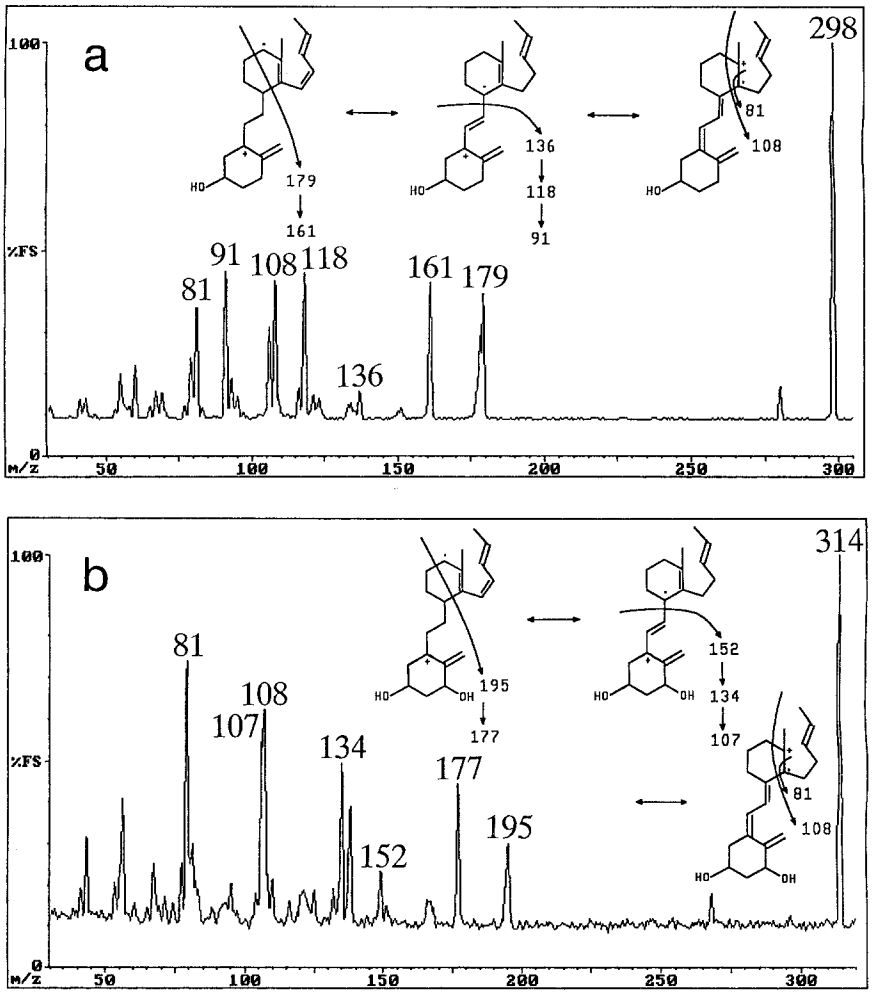


Fig. 3. CID spectra of: (a) m/z 298 and (b) m/z 314. Fragmentation schemes are shown in the inserts.

ion monitoring, and the results are represented as chromatographic profiles. Fig. 4 shows the profiles of the MRM transitions of $MH^+ \rightarrow$ 298(314) for vitamin D_3– and $1,25(OH)_2D_3$–PTAD near their detection limits by flow injection. The data of Fig. 4 indicate that detection of low nanogram quantites is readily achievable using this derivative and the appropriate MRM transition.

Given the encouraging results obtained via the flow injection mode, we next considered the analysis of vitamin D–PTAD derivatives by on-line capillary LC–CF-FAB-MS–MS. The focus thus far has been on the further improvement of the detection limits in view of the concentration effects associated with the use of a chromatographic inlet. Indeed, a significant enhancement in detection limit is indicated for both vitamin D_3– and $1,25(OH)_2D_3$–PTAD as shown in Fig. 5. Triplicate injections are shown at 5 pg (13 fmol) for vitamin D_3–PTAD, indicating a relative standard deviation of 25% in terms of area counts and a S/N ratio of 3.6. As expected, an improvement in signal reproducibility (relative standard deviation = 16%) was observed for analysis at the 50 pg level. For $1,25(OH)_2D_3$–PTAD, injections of 150 pg (360 fmol) give a S/N of 2.3 and relative standard deviation of

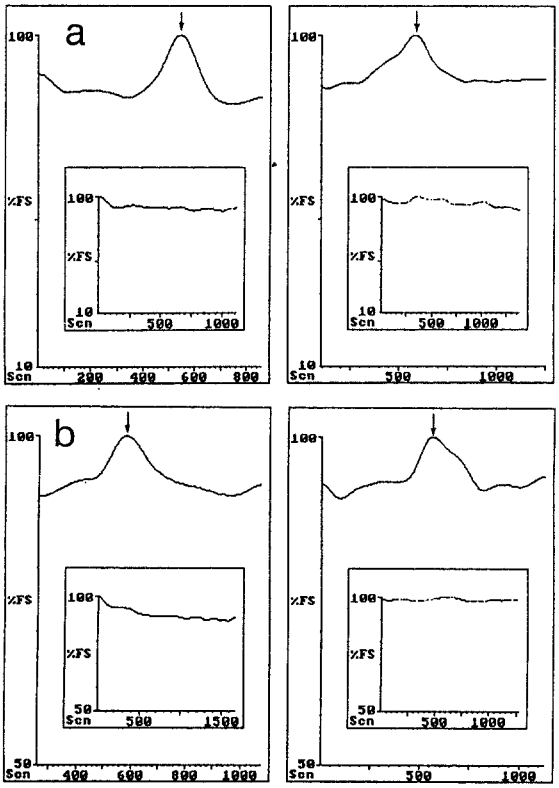


Fig. 4. Detection limits using MRM, by flow injection (in duplicates) of: (a) vitamin D_3–PTAD, 740 pg; (b) $1,25(OH)_2D_3$–PTAD, 3 ng. Blanks are shown in the inserts.

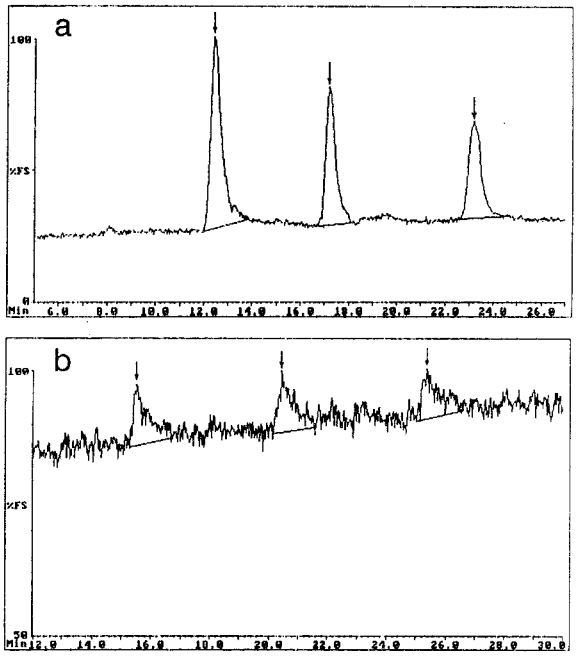


Fig. 5. LC–CF-FAB-MS–MS data, from three injections of: (a) vitamin D_3–PTAD, MRM of m/z 560→298, 5 pg (13 fmol); (b) $1,25(OH)_2D_3$–PTAD, MRM of m/z 592→314, 150 pg (360 fmol).

6.9% for triplicate injections. In general, the use of a PTAD derivative and analysis by LC–CF-FAB-MS–MS has been found to give detection limits in the range of 100–300 pg for other vitamin D_3 compounds examined thus far.

CONCLUSIONS

Our work in the derivatization of vitamin D_3 metabolites with PTAD has demonstrated the following: (1) The reaction is simple and goes to completion with all the vitamin D_3 metabolites tested in this study, even in very dilute solutions; (2) The $25(OH)D_3$–PTAD adduct shows 5 times more UV response than the underivatized metabolite; (3) From the FAB spectra we can distinguish the A ring hydroxylated metabolites from side chain hydroxylated isomeric metabolites, *e.g.* $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$; (4) The transition from the protonated molecule to m/z 298/314 can be monitored by MS–MS for trace level detection of specific vitamin D metabolites; (5) Use of capillary LC–CF-FAB-MS–MS has been shown to give detection limits in the mid to low picogram range (mid to high femtomole) for the vitamin D metabolites investigated. These detection limits appear to be in line with the physiologically encountered levels of most commonly occurring metabolites in plasma except for $1,25(OH)_2D_3$. Further work to improve on the detectability of this and other vitamin D_3 compounds is currently in progress.

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REFERENCES

1 J.L. Napoli and R.L. Horst, in R. Kumar (Editor), *Vitamin D: Basic Clinical Aspects*, Martinus Nijhoff Publishing, Boston, MA, 1984, p. 91.
2 A.W. Norman, J. Roth and L. Orci, *Endocr. Rev.*, 3 (1982) 331–366.
3 E. Abe, C. Miyaura, H. Sakagami, M. Takeda, K. Konno, T. Yamazaki, S. Yoshiki and T. Suda, *Proc. Natl. Acad. Sci. U.S.A.*, 78 (1981) 4990.
4 T. Suda, T. Shinki and N. Takahashi, *Annu. Rev. Nutr.*, 10 (1990) 195.
5 Y.J. Zhou, A.W. Norman, M. Lübbert, E.D. Collins, M.R. Uskokovic and H.P. Koeffler, *Blood*, 74 (1989) 82.
6 R.D. Coldwell, D.J.H. Trafford, M.J. Varley, D.N. Kirk and H.L.J. Makin, *Steroids*, 55 (1990) 418.
7 R.D. Coldwell, C.E. Porteous, D.J.H. Trafford and H.L.J. Makin, *Steroids*, 49 (1987) 155.
8 G. Jones, D.A. Seamark, D.J.H. Trafford and H.L.J. Makin, in A.P. De Leenheer, W.E. Lambert and M.G.M. De Ruyter (Editors), *Modern Chromatographic Analysis of the Vitamins*, Marcel Dekker, New York, 1985, p.73.
9 R.M. Bauwens, J.A. Kint, M.P. Devos, K.A. Van Brussel and A.P. De Leenheer, *Clin. Chim. Acta*, 170 (1987) 37.
10 A.L. Yergey, N.V. Esteban and D.J. Liberato, *Biomed. Environ. Mass Spectrom.*, 14 (1987) 623.
11 R.C. Cookson, S.S. Gutpe, I.D.R. Stevens and C.T. Watts, *Org. Synth.*, 51 (1971) 121.
12 K. Shimada, T. Oe and T. Mizuguchi, *Analyst*, 116 (1991) 1393.
13 D.C. Young, P. Vouros, B. Decosta and M.F. Holick, *Anal. Chem.*, 59 (1987) 1954.
14 D.C. Young, P. Vouros and M.F. Holick, *J. Chromatogr.*, 522 (1990) 295.
15 D.J. Aberhart and A.C.T. Hsu, *J. Org. Chem.*, 41 (1976) 2098.
16 B. Yeung, G.S. Reddy and P. Vouros, *Proceedings of the 40th ASMS Conference on Mass Spectrometry and Allied Topics, May 31–June 5, 1992, Washington, D.C*, p. 1089.
17 B. Yeung, G.S. Reddy and P. Vouros, *J. Bone Miner. Res.*, 7 (1982) S 171.