ELSEVIER

Journal of Chromatography B, 704 (1997) 11–17

JOURNAL OF CHROMATOGRAPHY B

Characteristics of mass spectrometric analyses coupled to gas chromatography and liquid chromatography for 22-oxacalcitriol, a vitamin D_3 analog, and related compounds

Masaki Ishigai*, Yoshihiko Ishitani, Kenji Kumaki

Drug Metabolism and Pharmacokinetics Research Laboratory, Chugai Pharmaceutical Co. Ltd., 3-41-8, Takada, Toshima-ku, Tokyo 171, Japan

Received 15 May 1997; received in revised form 19 August 1997; accepted 21 August 1997

Abstract

The characteristics of the mass spectra of vitamin D_3 related compounds were investigated by GC–MS and LC–MS using 22-oxacalcitriol (OCT), an analog of 1,25-dihydroxyvitamin D_3, and related compounds. Fragmentation during GC–MS (electron impact ionization) of TMS-derivatives of OCT and the postulated metabolites gave useful structural information concerning the vitamin D_3-skeleton and its side-chain, especially with respect to the oxidation positions of metabolites. In contrast, few fragment ions were observed in LC–MS (atmospheric pressure chemical ionization), showing that LC–MS gave poor structural information, except for molecular mass. However, when comparing the signal-to-noise ratio (S/N) observed during GC–MS and LC–MS analysis for OCT in plasma extracts, the S/N in LC–MS was over ten-times greater than in GC–MS, possibly due to the low recovery on derivatization and thermal-isomerization in GC–MS. Furthermore, both the GC–MS and the LC–MS allowed the analysis of many postulated metabolites in a single injection without any prior isolation of target metabolites from biological fluids by LC. These results suggest that GC–MS and LC–MS analysis for vitamin D_3 related compounds such as OCT each have unique and distinct advantages. Therefore, the complementary use of both techniques enables the rapid and detailed characterization of vitamin D_3 related compounds. © 1997 Elsevier Science B.V.

Keywords: Oxacalcitriol; Vitamin D_3

1. Introduction

The characterization of vitamin D_3 derivatives and their analogs in biological fluids has been mainly carried out by gas chromatography–mass spectrometry (GC–MS), and mass fragmentation patterns of many vitamin D_3 derivatives have been assigned [1–4]. GC–MS is a powerful tool to characterize the vitamin D_3 related compounds at the low nanogram level. GC–MS, however, also has some disadvantages in that it is difficult to analyze polar, non-volatile and thermo-labile compounds with sufficient sensitivity because of low recovery from derivatization and the effect of thermal-isomerization [5].

Therefore, considerable interest has been focused on high-performance liquid chromatography–mass spectrometry (LC–MS) using electrospray (ESI) [6] and atmospheric pressure chemical ionization (APCI) [7]. In particular, it was reported that APCI is a suitable ionization technique for the characterization

*Corresponding author.

0378-4347/97/$17.00
PII S0378-4347(97)00423-4

of steroids [8]. LC–MS might overcome some of the disadvantages of GC–MS mentioned above. On the other hand, LC–MS also has disadvantages in that it provides little information on chemical structure.

We compared the sensitivity of detection and the quality of structural information for both GC–MS and LC–MS, which use electron impact ionization (EI) and APCI as ionization mode, respectively, in order to achieve a rapid and exact screening of metabolites from plasma after administration of 22-oxacalcitriol (OCT). This compound is an analog of 1α,25-dihydroxyvitamin D_3, and has been developed as a therapeutic drug for secondary hyperparathyroidism in patients with chronic renal failure [9,10] and psoriasis [11].

In this report we describe the advantages and disadvantages of both GC–MS and LC– MS when we characterize OCT and its postulated metabolites.

2. Experimental

2.1. Chemical and reagents

OCT, 20*R*(OH)-hexanor-OCT, 20*S*(OH)-hexanor-OCT, 20-oxo-hexanor-OCT, 24*R*(OH)OCT, 24*S*(OH)OCT, (25*R*)-26(OH)OCT, (25*S*)-26(OH)OCT and 24-oxo-OCT (Fig. 1) were synthesized at Chugai Pharmaceutical Research Laboratory [12–14]. All substances were >99% pure as determined by HPLC. Acetonitrile of HPLC-grade, ethyl acetate, isopropanol, hexane, pyridine and tetrahydrofuran of analytical grade were purchased

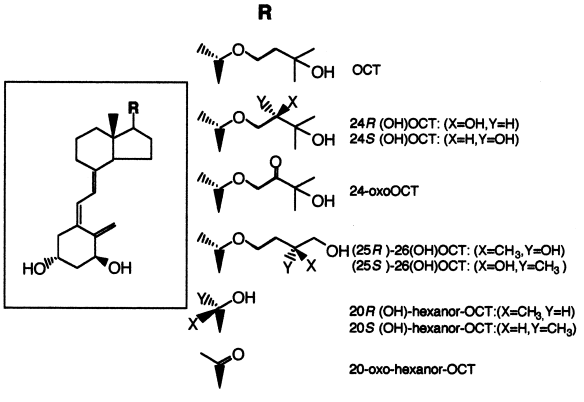


Fig. 1. Chemical structure of OCT and its derivatives.

from Junsei Chemical (Tokyo, Japan). BondElut NH_2 (1 ml/100 mg) and BondElut DEA (1 ml/100 mg) cartridges and Sylon BFT kit (trimethyl silylation reagent) were obtained from Varian (Harbor City, CA, USA) and Supelco (Bellefonte, PA, USA), respectively. The control rat plasma was prepared by blood collected from seven-week old male rats (Jcl:SD, Clea, Tokyo, Japan). All other solvents and reagents used were of the highest grade commercially available.

2.2. Extraction procedure

Three-fold volumes of ethyl acetate were added to plasma sample (1 ml). After extraction, the organic layer was evaporated to dryness under nitrogen gas, and the residue was redissolved in 200 μl of a mixture of isopropanol (IPA)–hexane (4:96, v/v). This solution was applied to a BondElut NH_2 column which had been washed with 2 ml of a mixture of IPA–hexane (4:96, v/v). Elution was performed with 2 ml of IPA–hexane (20:80, v/v). and the eluate was evaporated to dryness under nitrogen gas.

2.3. Trimethylsilyl (TMS) derivatization for GC–MS

The standard of OCT and its analogs or the plasma extracts were dissolved in 80 μl of dry pyridine. To these solutions, 20 μl of each reagent (BFT kit) for TMS derivatization was added, and these mixtures were allowed to react in a hot block bath at 80°C for 1 h. The reaction mixture was evaporated to dryness under nitrogen gas, and the residue was redissolved in 2 ml of hexane. This solution was applied to a BondElut DEA column. The unretained fraction was evaporated to dryness under nitrogen gas, redissolved in tetrahydrofuran, and analyzed by GC–MS.

2.4. GC–MS analysis

GC–MS was carried out using a JEOL JMS DX-300 mass spectrometer (Tokyo, Japan) equipped with a DA-5000 data system. A cross-linked methylsilicon fused-silica capillary column (30 m×0.1 mm I.D.) (J&W Scientific, Folsom, CA, USA) was inserted into the ion source through a heated (280°C)

transfer line. The injector was operated at 300°C in the splitless mode with a flow-rate of helium carrier gas of 1 ml/min. The column oven temperature was successively programmed at a rate of 16°C/min from 200°C to 280°C, at a rate of 4°C/min from 280°C to 320°C, where it was held for 5 min. The ionization energy, ionization current and acceleration voltage were set to 75 eV, 300 μA and 3 kV, respectively. Magnet scanning was operated from m/z 50 to m/z 800 every 1 s.

2.5. LC–MS analysis

LC–MS was carried out using a Finnigan TSQ-700 triple stage quadrupole mass spectrometer (San Jose, CA, USA) equipped with Shiseido Nanospace SI-1 (Tokyo, Japan). HPLC was performed using a 250×4.5 mm column packed with Capcell Pak C_{18} SG120, 5 μm from Shiseido. The mobile phase, consisting of acetonitrile–water (40:60, v/v), was maintained at a flow-rate of 1 ml/min. MS detection was performed in positive ion mode by APCI with a nebulizer probe temperature setting of 500°C. The nebulizing gas (nitrogen) pressure and auxiliary flow were set at 70 p.s.i. and 20 p.s.i., respectively (1 p.s.i.=6894.76 Pa). Gas-phase chemical ionization was effected by a corona discharge needle (5 μA) and positive ions were sampled into the quadrupole mass analyzer. The voltages for capillary, skimmer and octapole were set 30 V, 100 V and −10 V, respectively. The ions produced were scanned from m/z 100 to m/z 500 every 2 s.

2.6. Sensitivity

The quality control samples were prepared by addition of OCT (5, 50, 500 ng) in control rat plasma (1 ml). The sample was extracted as described in Section 2.2. For GC–MS analysis, the final extract was dissolved in dry pyridine for TMS derivatization. After derivatization, the TMS-derivative was dissolved in 25 μl of tetrahydrofuran, and 5 μl was then injected into the GC system. For LC–MS analysis, the final extract was dissolved in 50 μl of LC mobile phase, and 20 μl was then injected into the HPLC system. The $[M]^+$ ions in EI spectra (GC–MS) and the $[M+NH_4]^+$ ion in APCI spectra (LC–MS) were used for calculation of each S/N ratio.

3. Results and discussion

3.1. GC–MS analysis

Fig. 2 shows the GC–MS total ion chromatogram and the spectrum of the Tri-TMS derivative of OCT (Tri-TMS-OCT). Two main peaks are apparent in the total ion chromatogram. The spectra for I and II gave identical molecular ion peaks and similar fragment patterns. In general, it is known that the TMS derivatives of the related compounds of vitamin D_3 thermally isomerize when analyzed by GC, yielding several isomers such as the pyro form and the isopyro form [5]. Furthermore it has been reported that the pyro form and the isopyro form are formed at a constant ratio (pyro/isopyro=2~3) [15]. Fig. 3 shows the derivatization reaction and proposed thermally induced isomerization for OCT.

The retention times (t_Rs) of TMS derivatives of OCT and the postulated metabolites in GC are listed in Table 1. Some differences in t_R values between the side-chain cleavage derivatives (SCCDs) and the side-chain oxidation derivatives (SCODs) were observed. SCODs such as 24*R*(OH)OCT, 24*S*(OH)OCT, 24-oxo-OCT, (25*R*)-26(OH)OCT and (25*S*)-26(OH)OCT gave two peaks corresponding to the pyro form and the isopyro form similar to OCT isomerization. SCCDs such as 20-oxo-hexanor-OCT, 20*R*(OH)-hexanor-OCT, 20*S*(OH)-hexanor-OCT, gave another broad peak as well as the two peaks corresponding to the pyro form and the isopyro form, that appeared between the pyro form and the isopyro form; the third peak, and gave almost the same mass spectrum. The fragmentations of TMS derivatives of OCT and the postulated metabolites in GC–MS are summarized in Table 2. All compounds showed fragmentation of the vitamin D_3 skeleton (cleavages A, B, C and G in Table 2). Thus, these fragmentations are diagnostic for modification (such as oxidation) of the A, B and D rings. In addition, fragments D, E and F (involving cleavage of the C_{20-22} bond) were observed in the specific of SCCDs. Furthermore, unique fragment ions derived from the side-chain in the spectra of SCCDs were observed. These

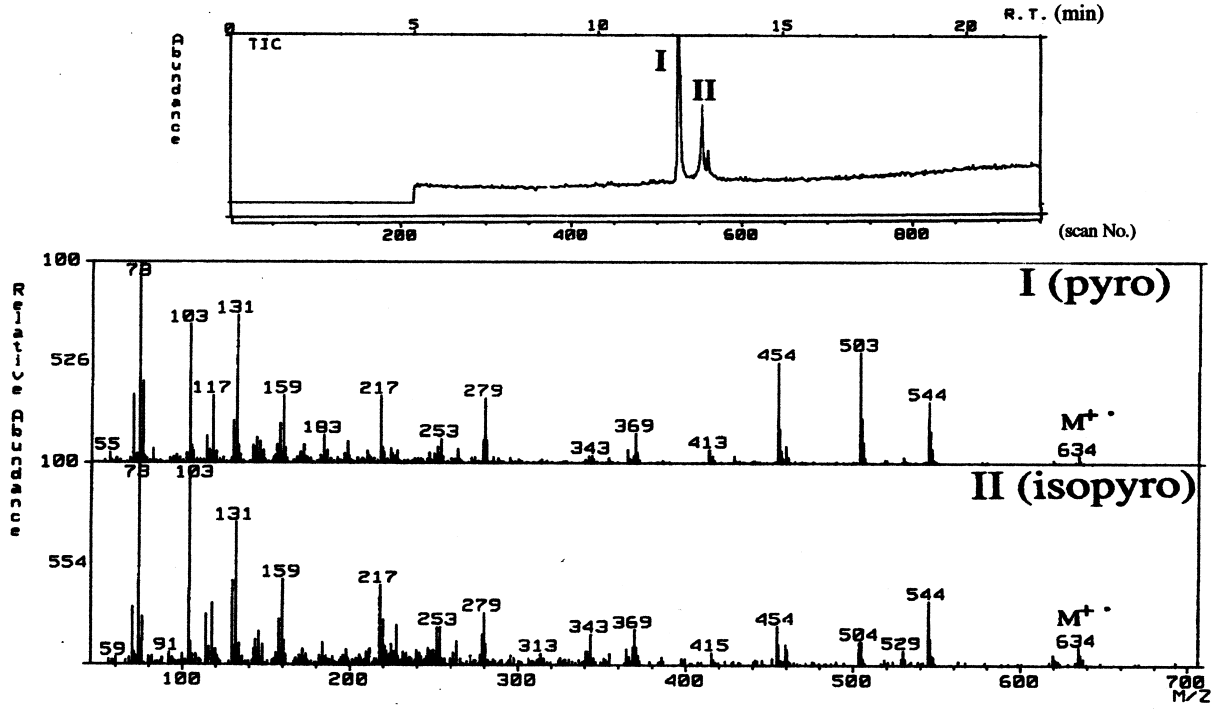


Fig. 2. GC–MS chromatogram and spectra of Tri-TMS-OCT.

fragment ions also make it possible to determine the oxidation positions of the side-chain.

3.2. LC–MS analysis

The spectrum of OCT obtained by LC–MS (APCI) is shown in Fig. 4. OCT gave a single peak at 25.8 min in the total ion chromatogram (data not shown). The spectrum shows prominent fragments in addition to ions reflecting the intact analyte. The ions at m/z 436, 419 and 401 corresponded to $[M+NH_4]^+$, $[M+H]^+$ and $[M+H-H_2O]^+$. The fragment ion at m/z 315 suggested cleavage at the 20–22 position, and other main ions at m/z 297 and 279 indicated the loss of one or two water molecules from the ions at m/z 315. Table 3 summarizes the fragmentations and t_R values of OCT and the postulated metabolites recorded by LC–MS. Most com-

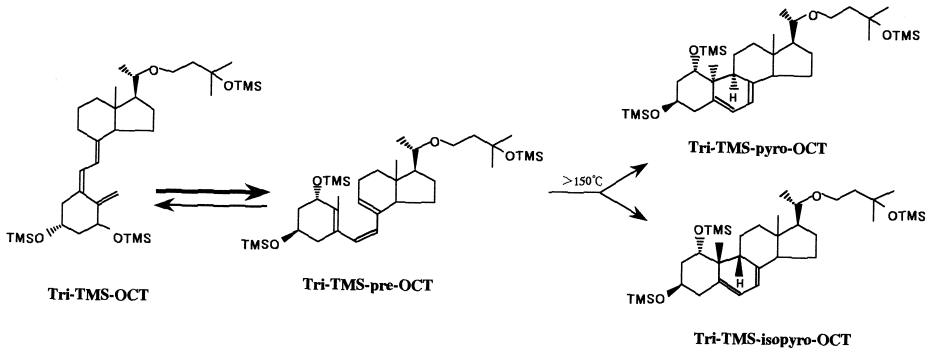


Fig. 3. Postulated mechanism for the thermal-isomerization of Tri-TMS-OCT.

Table 1
The retention times in GC–MS of TMS derivatives of OCT and the postulated metabolites

Compound	Retention time (min)		
	1st Peak (pyro)		2nd Peak (isopyro)
20-oxo-Hexanor-OCT	8.67	(8.93)[a]	9.27
20*R*-(OH)-Hexanor-OCT	8.98	(9.63)[a]	9.83
20*s*(OH)-Hexanor-OCT	9.57	(9.75)[a]	9.98
OCT	12.28	–	12.95
24*R*(OH)OCT	13.17	–	13.88
24*S*(OH)OCT	13.30	–	14.05
24-oxo-OCT	13.30	–	14.05
(25*R*)-26(OH)OCT	13.82	–	14.58
(25*S*)-26(OH)OCT	13.92	–	14.58

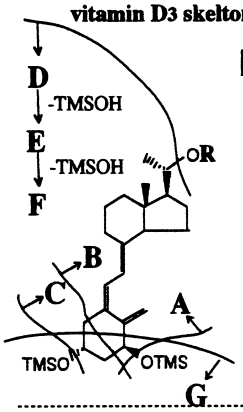
[a] Broad peak.

pounds show good separation in the LC condition used, though the t_R values of some compounds indicated incomplete separation for isomers. In the MS spectrum of each compound, a difference was observed in fragmentations between SCODs and SCCDs: the main ion species of SCODs was $[M+NH_4]^+$. In contrast, $[M+NH_4]^+$ ions were not detected in MS spectra of SCCDs but $[M+H-H_2O]^+$ was observed as the predominant ion peak. The fragmentation of vitamin D_3-related compounds by LC–MS (APCI) was relatively simple, making it possible to readily assign molecular ions. However, there were no unique ions to elucidate the chemical structure of the side-chain and vitamin D_3 skeleton in the LC–MS spectra.

3.3. Comparison between GC–MS and LC–MS

The *S*/*N* ratio for OCT in several different concentrations of quality control samples observed during GC–MS and LC–MS analysis was compared. The detection of OCT was carried out after the

Table 2
The possible fragmentations in GC–MS of TMS-derivatives of OCT and the postulated metabolites

Ion species		OCT	24*R*(OH)OCT 24*S*(OH)OCT	(25*R*)-26(OH)OCT (25*S*)-26(OH)OCT	24-oxoOCT	20*S*(OH)-hexanor -OCT 20*R*(OH)-hexanor -OCT	20-oxo-hexanor -OCT
vitamin D3 skelton				m/z			
	[M]$^{+\cdot}$	634	722	722	648	548	474
	A	544	632	632	558	458	384
	B	503	591	591	517	417	343
	C	454	542	542	468	368	294
	D	459	459	459	459	—	—
	E	369	369	369	369	—	—
	F	279	279	279	279	—	—
	G	217	217	217	217	217	217
side chain		159	157	219 157 145 129		117	
the other ions		131 103 73	131 103 73	— 103 73	131 103 73	131 103 73	131 103 73

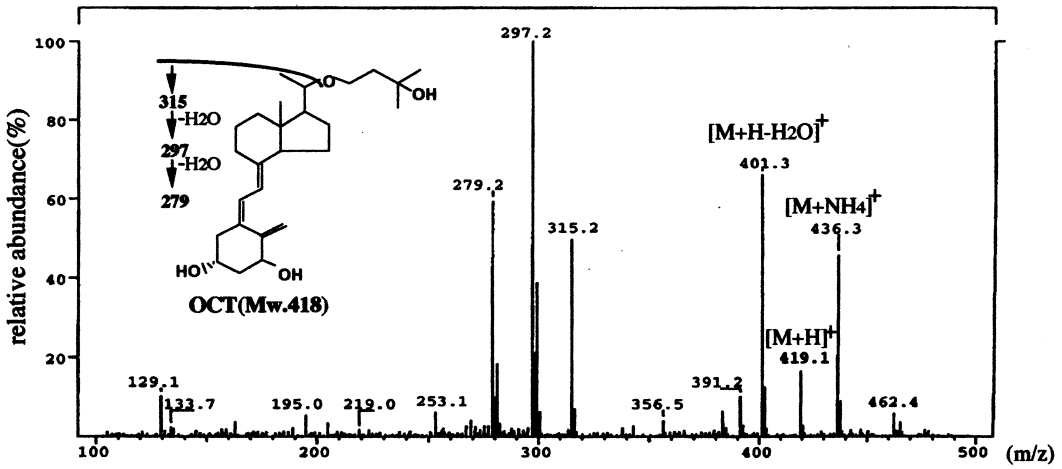


Fig. 4. LC–MS (APCI) spectrum of OCT.

extraction of OCT from the quality control samples in control rat plasma, following the extraction procedure as described in the Section 2.2. As shown in Table 4, the S/N ratio in LC–MS was ten-times greater than in GC–MS. The practical detection limits, for $S/N>3$, for OCT in 1 ml of plasma were 5 ng for LC–MS and 50 ng for GC–MS. One of the reasons for the great differences between detection limits was that the relative intensity of $[M]^+$ in EI spectra was much lower than that of $[M+NH_4]^+$ in APCI spectra. However, there are other fundamental problems in GC–MS such as low recovery on derivatization and thermal-isomerization that lead to lower detection limits. LC–MS (APCI), as compared with GC–MS (EI), is a more convenient and sensitive technique for determining of the molecular masses of polar, non-volatile and thermo-labile vitamin D_3-related compounds without any derivatization being required. Therefore, LC–MS should be more suitable for a preliminary identification with synthetic postulated metabolites. On the other hand, GC–MS is a superior technique to identify and

Table 3
The possible fragmentations and retention times of OCT and the postulated metabolites in LC–MS

Ion species	OCT	24*R*(OH)OCT	24*S*(OH)OCT	(25*R*)-26(OH)OCT (25*S*)-26(OH)OCT	24-oxo-OCT	20*S*(OH)-hexanor-OCT 20*R*(OH)-hexanor-OCT	20-oxo-hexanor-OCT
	(*m*/*z*)						
$[M+NH_4]^+$	436 (base)[a]	452 (base)	452 (base)	452 (base)	450 (base)	N.D.	N.D.
$[M+H]^+$	419	435	435	435	433	N.D.	N.D.
$[M+H-H_2O]^+$	401	417	417	417	415	315 (base)	313 (base)
$[M+H-2H_2O]^+$	383	399	399	399	397	297	295
$[M+H-3H_2O]^+$	N.D.[b]	N.D.	N.D.	N.D.	N.D.	279	N.D.
$[F]^+$[c]	315	315	315	315	315	–	–
$[F-H_2O]^+$	297	297	297	297	297	–	–
$[F-2H_2O]^+$	279	279	279	279	279	–	–
Retention time (min)	25.83	9.00	9.33	8.07	11.53	6.83	10.93

[a] Base ion peak.
[b] N.D.=Not detected.
[c] [F]: The fragment ion cleaved at the 20–22 position.

Table 4
Comparison of S/N ratios observed during GC–MS and LC–MS analysis for OCT in extracts of plasma

Method	Ionization	Signal-to-noise (S/N) ratio			
		Amounts spiked in plasma (1 ml)			
		5 ng	10 ng	50 ng	500 ng
GC–MS	EI	N.M.[a]	N.D.[b]	<4	20
LC–MS	APCI	13	N.M.	40	N.M.

Each value was calculated from the mass chromatograms of $[M]^+$ for GC–MS or $[M+NH_4]^+$ for LC–MS. The value for GC–MS was calculated from the peak of pyro-isomer.
[a] N.M.=Not measured.
[b] N.D.=Not detected.

characterize these metabolites. In particular, GC–MS is the more powerful technique to elucidate in detail the chemical structure of small amounts of unknown compounds. The interpretation of spectra for the products which were generated after chemical reactions such as a periodate oxidation and $NaBH_4$ reduction of an analyte, as well as the interpretation of spectrum of an intact analyte itself would allow us to elucidate chemical structures in even more detail. Furthermore, both GC–MS and LC–MS methods established in this paper allowed us to analyze many postulated metabolites in a single injection without isolation of target metabolites from biological fluids by LC before GC analyses, in contrast to methods described previously [16–20]. The extraction procedure and chromatographic conditions used in this work could be applied to rapid analysis of other vitamin D_3-related compounds such as OCT and have unique and distinct advantages. Therefore, the complementary use of both techniques enables a rapid and detailed characterization of vitamin D_3-related compounds.

Acknowledgements

The authors would like to thank Drs. N. Kubodera and H. Watanabe of the Exploratory Research Laboratory, Chugai Pharmaceutical Co. Ltd., for providing all the synthetic compounds for postulated metabolites of OCT.

References

[1] M.F. Holick and H.F. Deluca, in D.E.M. Lowson (Editor), Vitamin D, Academic Press, London, 1978, p. 51.
[2] C.E. Porteous, R.D. Coldwell, D.J.H. Trafford, H.L.J. Makin, J. Steroid Biochem. 28 (1988) 785.
[3] R.D. Coldwell, D.J.H. Trafford, M.J. Varley, D.N. Kirk, H.L.J. Makin, Clin. Chim. Acta 180 (1989) 157.
[4] R.D. Coldwell, D.J.H. Trafford, M.J. Varley, D.N. Kirk, H.L.J. Makin, Steroids 55 (1990) 418.
[5] P.P. Nair, C. Bucana, S. De Leon, D.A. Turner, Anal. Chem. 37 (1965) 631.
[6] M. Yamashita, J.B. Fenn, J. Phys. Chem. 88 (1984) 4451.
[7] M. Sakanishi, H. Kambara, Anal. Chem. 61 (1989) 1159.
[8] Y. Kobayashi, K. Saiki, F. Watanabe, Biol. Pharm. Bull. 16 (1993) 1175.
[9] E. Murayama, K. Miyamoto, N. Kubodera, T. Mori, I. Matsunaga, Chem. Pharm. Bull. 34 (1986) 4410.
[10] A.J. Brown, C.R. Ritter, J.L. Finch, J. Morrissey, K.J. Martin, E. Murayama, Y. Nishii, E. Slatopolsky, J. Clin. Invest. 84 (1989) 728.
[11] Y.Nishii, J. Abe, K. Sato, T. Kobayashi, T. Okano, N. Tsugawa, Slatopolsky, A.J. Brown, A. Dusso and L.G. Raisz, in A.W. Norman, R. Bouillon and M. Thomasset (Editors), Vitamin D: Gene Regulation, Structure–Function Analysis and Clinical Application, Walter de Gruyter, Berlin and New York, 1991, p. 289.
[12] E. Murayama, K. Miyamoto, N. Kubodera, T. Mori, I. Matsunaga, Chem. Pharm. Bull. 34 (1986) 4410.
[13] N. Kubodera, H. Watanabe, T. Kawanishi, M. Matsumoto, Chem. Pharm. Bull. 40 (1992) 1494.
[14] N. Kubodera, H. Watanabe, S. Hatakeyama, K. Tazumi, S. Takano, Bioorg. Med. Chem. Lett. 4 (1994) 753.
[15] L.V. Avioli, S.W. Lee, Anal. Biochem. 16 (1966) 193.
[16] M.F. Holick, H.K. Schnoes, H.F. Deluca, R.W. Gray, I.T. Boyle, T. Suda, Biochemistry 11 (1972) 4251.
[17] T.A. Reinhardt, J.L. Napoli, B. Praminik, E.T. Littledike, D.C. Beitz, J.J. Partridge, M.R. Uskokovic, R.D. Horst, Biochemistry 20 (1981) 6230.
[18] S. Ishizuka, S. Ishimoto, A.W. Norman, Biochemistry 23 (1984) 1473.
[19] F. Qaw, M.J. Calverley, N.J. Schroeder, D.J.H. Trafford, H.L.J. Makin, G. Jones, J. Biol. Chem. 268 (1993) 282.
[20] S. Masuda, V. Byford, R. Kremer, H.L.J. Makin, N. Kubodera, Y. Nishii, A. Okazaki, T. Okano, T. Kobayashi, G. Jones, J. Biol. Chem. 271 (1996) 8700.