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Determination of 22-oxacalcitriol, a new analog of 1α,25-dihydroxyvitamin D_3, in human serum by liquid chromatography–mass spectrometry

Masaki Ishigai*, Yoshinori Asoh, Kenji Kumaki

Drug Metabolism and Pharmacokinetic Laboratory, Chugai Pharmaceutical, 3-41-8, Takada, Toshima-ku, Tokyo 171, Japan

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Abstract

A sensitive and specific liquid chromatographic–mass spectrometric assay has been developed for the determination of 22-oxacalcitriol (OCT), which is a new analog of 1α,25-dihydroxyvitamin D_3. The analyte was isolated from serum by two solid-phase extraction steps on a C_{18} cartridge and NH_2 cartridge. The recovery of OCT through two extraction steps was more than 90%. A related substance (ED-94), i.e. OCT with the side-chain shortened by one carbon, was used as an internal standard. Extracts were chromatographed on a C_{18} reversed-phase column interfaced to the electrospray ionization source. The mass spectrometer was operated in the positive-ion mode of selected reaction monitoring. The chromatographic run-time for one injection was less than 6 min. The intra- and inter-assay coefficients of variation for the lowest concentration examined (30 pg ml^{-1}) were 9.83 and 10.67, respectively. And the analytical recovery of OCT added to serum was quantitative. Assay linearity was obtained in the range of 20–640 pg ml^{-1}.

Keywords: 22-Oxacalcitriol; 1α,25-Dihydroxyvitamin D_3

1. Introduction

22-Oxacalcitriol (OCT) is an analog of 1α,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) having an oxygen atom at the 22-position in the side chain (Fig. 1), which has a suppressive action on parathyroid hormone (PTH) secretion and exhibits potent differentiation-inducing activity with minimal calcemic activity [1–4]. OCT is now expected to be a unique therapeutic drug for secondary hyperparathyroidism

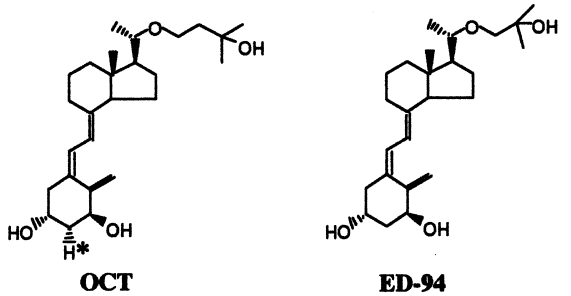

Fig. 1. Chemical structures of OCT and internal standard, ED-94. *: position labeled with ^3H for [^3H]OCT.

*Corresponding author.

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in patients with chronic renal failure and for psoriasis.

To support a clinical study of the pharmacokinetics of OCT, a sensitive method was needed to determine plasma concentrations expected to be in the picogram per ml range because of its low dose (1~3 μg per body). As a method to overcome those problems, radioimmunoassay of OCT has been already developed [5] and has been used for the measurement of a great number of clinical samples. The radioimmunoassay, however, requires several complicated steps which separate OCT from OCT metabolites and endogenous vitamin D_3 metabolites in blood [6–9] in order to achieve a sufficient specificity. We have therefore undertaken the development of a more sensitive and convenient method for determination of OCT. Gas chromatography–mass spectrometry (GC–MS) with selected-ion monitoring (SIM), which is an alternative to bioassay for determination of vitamin D_3 and reference compounds, has been widely accepted as a reliable method. However, it was difficult to achieve sufficient sensitivity for the pharmacokinetic study of these compounds. Komuro et al. have developed higher sensitive GC–MS techniques with high-resolution SIM (HR-SIM) for the determination of a $1,25(OH)_2D_3$ analogue [10]. This method also requires complicated extraction steps including the derivatization of analyte. Moreover, it was considered that high-resolution might be unsuitable for routine analysis of a great number of samples.

We have considered that the combination of high-performance liquid chromatography (HPLC) with electrospray (ESI) mass spectrometry affords a rapid and convenient method for the quantification of drug in biological fluids.

This paper is the first report which describes the determination of vitamin D_3 derivatives using LC–ESI-MS–MS.

2. Experimental

2.1. Chemicals

OCT and ED-94 were synthesized at the Chugai Pharmaceutical Research Laboratories (Tokyo, Japan) [11,12]. Both substances were >99% pure as determined by HPLC. [^{3}H]OCT (Fig. 1) also was synthesized at the Chugai Pharmaceutical Research Laboratories. The specific activity was 814 MBq mg^{-1} and the radiochemical purity was more than 98% as judged by HPLC with a radioactivity detector. Methanol of HPLC grade, isopropanol, hexane, hydrochloric acid and ammonium acetate of analytical grade were purchased from Junsei Chemical (Tokyo, Japan). BondElut C_{18} (3 cc/200 mg) and BondElut NH_2 (1 cc/100 mg) cartridges were obtained from Varian (Harbor City, CA, USA). Control human serum was obtained from Cosmo Bio (Tokyo, Japan). All other solvents and reagents were of the highest commercial grade available.

2.2. Standard and sample preparation

Stock solutions of OCT and ED-94 were prepared as 1 μg ml^{-1} solutions in ethanol. Subsequent dilutions were carried out in the range of 1 ng ml^{-1} to 32 ng ml^{-1} for OCT and 16 ng ml^{-1} for ED-94 with ethanol. Standard and quality-control samples were prepared by the addition of known amounts of each standard solution (20 μl) to 1 ml of control human serum. The concentrations of OCT in standard serum samples were 20, 40, 80, 160, 320 and 640 pg ml^{-1}. Quality-control samples were prepared at 30, 150 and 600 pg OCT per ml of serum.

2.3. Extraction procedure

A serum sample (1 ml) was spiked with 20 μl of the internal standard solution (ED-94), and was mixed after the addition of 1 *M* hydrochloric acid (0.2 ml). Two steps of solid-phase extraction were applied. In the first step, the mixture was applied to a BondElut C_{18} cartridge, that had been prewashed and conditioned successively with methanol–water (80:20, v/v, 3 ml), distilled water (2 ml) and 1 *M* hydrochloric acid (2 ml). Following sample application, the cartridge was rinsed successively with distilled water (2 ml) and methanol–water (50:50, v/v, 3 ml). OCT was then eluted with methanol–water (80:20, v/v, 4 ml). The eluate was evaporated to dryness under a stream of nitrogen at 60°C. The residue was dissolved in isopropanol (IPA)–hexane

(HEX) (10:90, v/v, 0.1 ml). In the second step, the solution was applied to a BondElut NH_2 cartridge that had been prewashed and conditioned successively with IPA–HEX (30:70, v/v, 1 ml) and IPA–HEX (10:90, v/v, 1 ml). Following sample application, the cartridge was rinsed with IPA–HEX (10:90, v/v, 1 ml). Finally, OCT was eluted with IPA–HEX (30:70, v/v, 2 ml). After evaporation the eluate was dissolved in methanol–10 m*M* ammonium acetate (90:10, v/v, 50 μl), which was then subjected to HPLC.

Extraction efficiency was determined using [^{3}H]OCT as follows. A known concentration of [^{3}H]OCT (approximately 814 Bq ng^{-1}) was added to the control serum. The extraction was carried out in the same way as described above, and the radioactivity in the final extract was counted using a LSC-900 liquid scintillation counter (Aloka, Japan).

2.4. LC–MS–MS analysis

The HPLC system consisted of Shiseido Nanospace SI-1 (Tokyo, Japan) equipped with autoinjector.

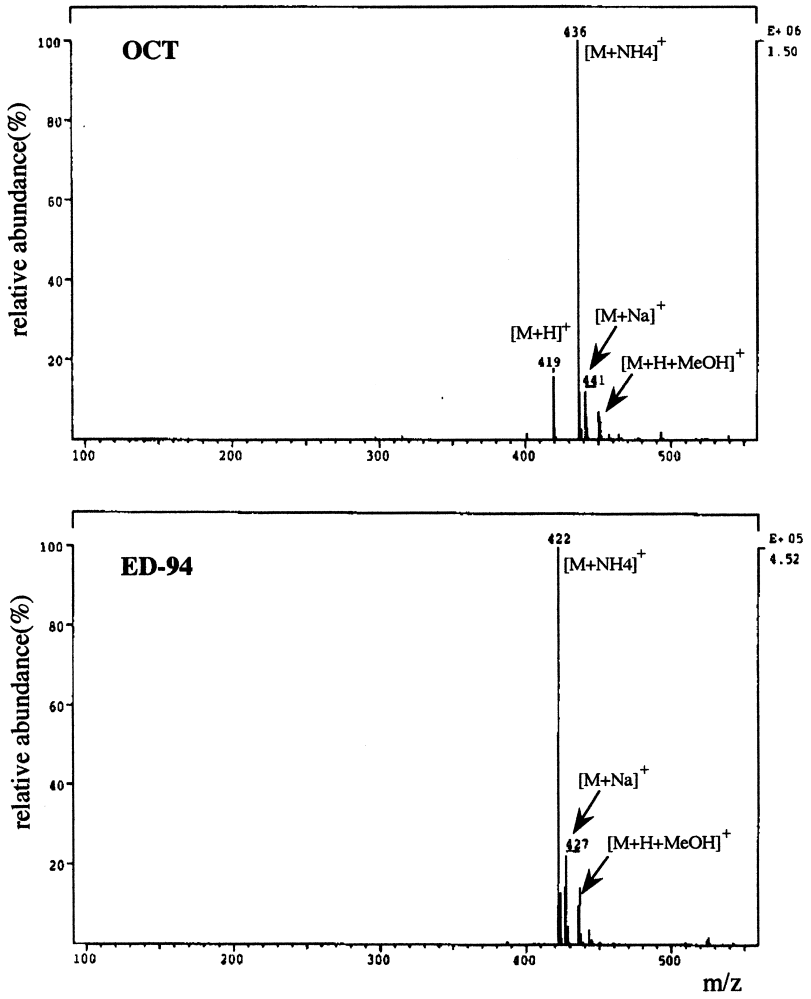

Fig. 2. Electrospray ionization normal-scan spectra of OCT (top panel) and internal standard, ED-94 (bottom panel); 10 ng of each substance was analyzed.

HPLC was performed using a 2×250 mm column packed with Capcell Pak C18 UG120, 5 μm from Shiseido and 20-μl aliquots of the extract were taken for analysis. The mobile phase was methanol–10 m*M* ammonium acetate (90:10, v/v) at a flow-rate of 0.2 ml min^{-1}.

Mass spectrometric detection was carried out using a Finnigan TSQ-700 triple-quadrupole instrument (San Jose, CA, USA), using electrospray in positive-ion mode. The electrospray voltage was set at 4.5 kV and the tube lens voltage was set at 120 V. OCT and ED-94 were detected by selected reaction monitoring (SRM: *m*/*z* 436→297 and *m*/*z* 422→297, respectively). For collision-activated dissociation (CAD), argon was used at a pressure of approximately 2.0 mTorr. The collision energies were set at −20 eV for OCT and −15 eV for ED-94. Scans for respective compounds were switched every second. The area ratios for calibration curves and quantitative analysis were calculated using ISIS-QUAN (ver.2.1). and Lotus 1-2-3 (ver.R2.3J).

The calibration curves were constructed using a nonweighted linear least-squares regression of the serum concentrations and the measured area ratios.

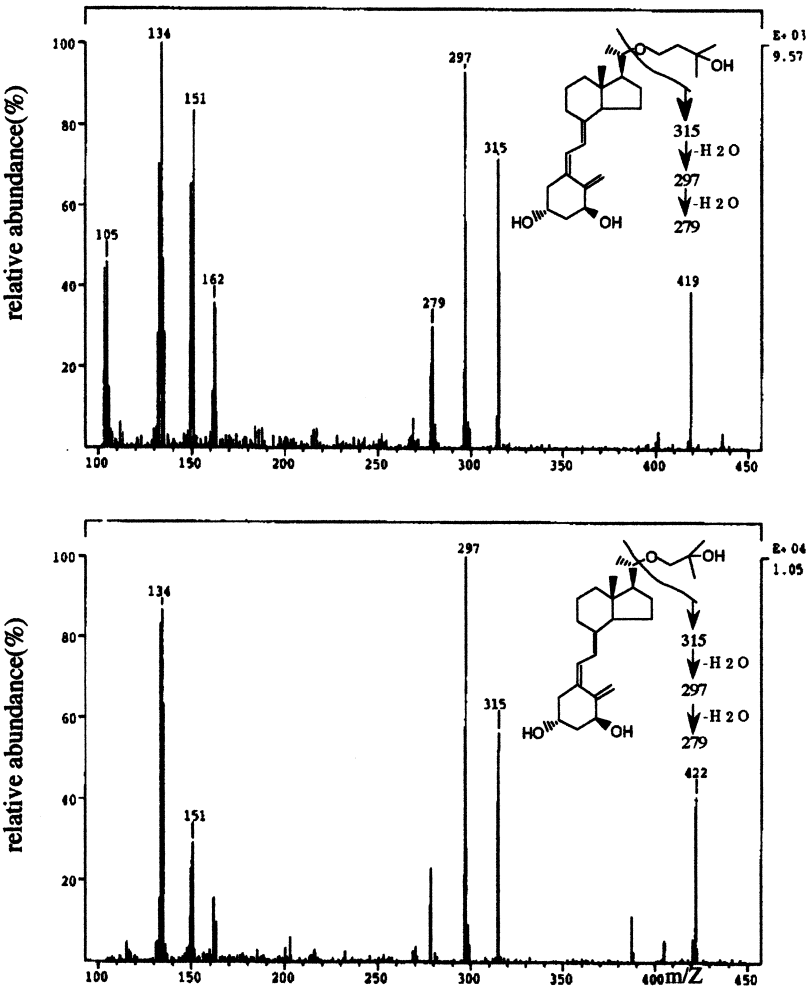

Fig. 3. Product-ion mass spectra of OCT (*m*/*z* 436, top panel) and internal standard, ED-94 (*m*/*z* 422, bottom panel); 10 ng of each substance was analyzed.

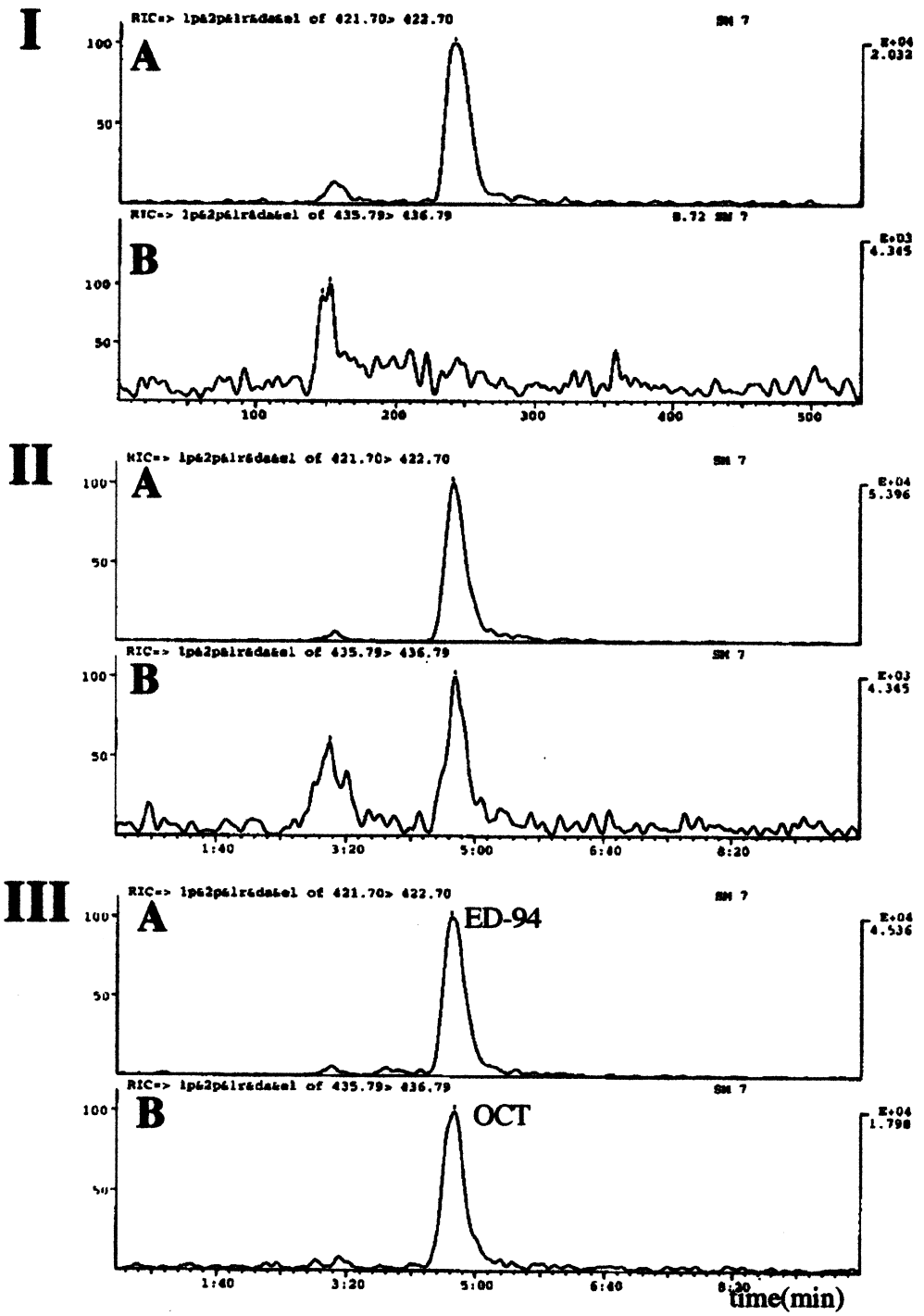

Fig. 4. Chromatograms obtained by selected reaction monitoring of extracts of human serum; channel A: m/z 422→297 for ED-94 (internal standard); channel B: m/z 436→297 for OCT; I=control serum; II=spiked at the conc. of 20 pg/ml OCT in control serum; III=spiked at the conc. of 160 pg/ml OCT in control serum. All samples contain internal standard (320 pg/ml).

3. Results and discussion

3.1. Full scan and product scan mass spectra

The ESI normal scan mass spectra of OCT and ED-94 standards are shown in Fig. 2. The base peaks of OCT and ED-94 were m/z 436 and m/z 422 respectively which correspond to $[M+NH_4]^+$ ion species. Both spectra showed little fragmentation. Fig. 3 shows the product ion spectra of the $[M+NH_4]^+$ ions under CAD conditions. In each case, the same fragmentation pattern of the $[M+NH_4]^+$ ions to some product ions at m/z 315, 297, 279 and so on were demonstrated. These fragment ions were assigned to cleavage of the carbon-20 and oxygen-22 bond with subsequent losses of water (Fig. 3).

In each compound, the fragment ion with the greatest intensity was the ion at m/z 297. This ion was utilized in all subsequent determinations by MS–MS.

3.2. Extraction efficiency

Table 1 shows the extraction efficiency determined by [^{3}H]OCT. A high extraction efficiency of more than 90% in each step was observed.

3.3. Chromatograms

No peak except a solvent peak in void volume was observed in chromatograms of some different control sera without both OCT and ED-94 which were monitored in two channels (m/z 436→297 for OCT, m/z 422→297 for ED-94). Fig. 4 shows typical LC–SRM chromatograms for OCT spiked and control sera without OCT.

The signals for OCT and ED-94 both appeared at around 4.5 min. The chromatogram for control serum revealed that there was no interference from endogenous serum components in the determination of OCT or ED-94. A signal-to-noise value of 3 was obtained for a sample of OCT concentration 20 pg/ml, which therefore represents the detection of quantification.

Table 1
Solid-phase extraction efficiency of [^{3}H]OCT from human serum

Step no.	Recovery (mean±S.D., n=3) (%)
1 (BondElut C_{18})	99.92±3.66
2 (BondElut NH_2)	94.13±6.84
Total (1+2)	91.39±2.16

3.4. Calibration curve

Fig. 5 showed combined data from six standard curves analyzed on six separate days. The equation of the line was $y=0.0029x+0.0230$, with a correlation coefficient (r^2) of 0.999. The data demonstrated a good fit to the nonweighted regression lines and good linearity in the range of 20–640 pg ml^{-1}.

3.5. Method validation

The data obtained from the quality-control samples assayed for validation of the method are shown in Table 2. The calibration curves routinely yielded correlation coefficients (r^2) of 0.999. Though the concentration determined in the intra-day replicate analyses of the 30 pg ml^{-1} sample was somewhat greater (113.35%) than theoretical, differences between the value obtained and the theoretical one for other concentration levels indicated discrepancies of less than 5%. The coefficient of variation in three concentrations examined were less than 10%. In the

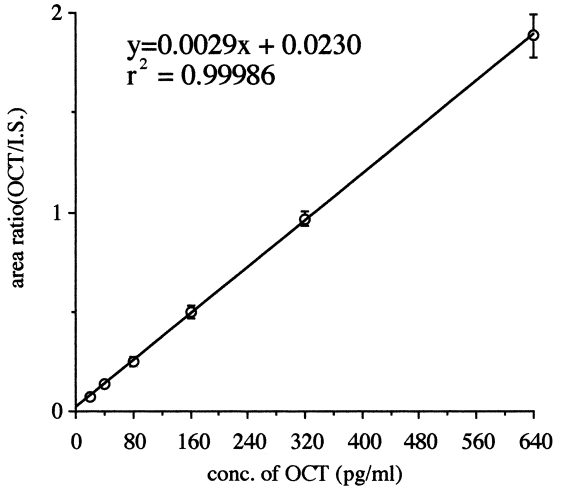

Fig. 5. Typical calibration curve of detection of OCT in human serum. The curve is a composite of six calibration curves analyzed on six separate days.

Table 2
Intra- and inter-day accuracy and precision for the determination of OCT by LC–MS–MS

Method	Actual concentration (pg/ml)	Number of replicates	Observed concentration (mean±S.D., pg/ml)	Accuracy (% of found/added)	Precision (% C.V.)
Intra-day	30	6	34.01±3.34	113.35	9.83
	150	6	143.92±10.43	95.95	7.25
	600	6	598.82±21.38	99.80	3.57
Inter-day	30	3	30.56±3.26	101.87	10.67
	150	3	149.14±7.63	99.43	5.12
	600	3	600.46±18.47	100.08	3.08

inter-day replicate analysis, differences between the expected and found values were less than 2%. The average coefficient of variation at 30 pg ml^{-1} was 10.67%, which is considered to be an acceptable value for clinical sample analysis. The precision of serum concentrations higher than 30 pg ml^{-1} were within 6%. These validation data indicated high reproducibility for this determination method.

4. Conclusions

The validated LC–ESI-MS–MS method has been shown to be specific for the determination of OCT in human serum. The method achieved high specificity with simple extraction of OCT from serum, and it was possible to obtain higher sensitivity in comparison with radioimmunoassay. In addition, the method is rapid, with a sample analysis time of 6 min from one injection to the next injection. The short chromatographic run-time made it possible to analyze 100 samples per 10 h.

In conclusion, a simple, selective, and sensitive method with MS–MS detection has been developed for determination of OCT in human serum. We believe it will be suitable for routine assays of clinical samples after administration of OCT.

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