Radioimmunoassay Method for the Determination of 22-Oxacalcitriol, a Novel Analog of 1α,25-Dihydroxyvitamin D3 Having Valuable Clinical Potency, in Rat and Human Plasma

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22-Oxacalcitriol is a synthetic analog of 1α,25-dihydroxyvitamin D₃, which is expected to be a novel agent for the treatment of patients with secondary hyperparathyroidism, psoriasis, and/or breast cancer. A sensitive and practical RIA for 22-oxacalcitriol in rat and human plasma has been developed. A rabbit antiserum raised against 22-oxacalcitriol 3-hemiglutarate conjugated with bovine serum albumin was used in combination with a tritium-labeled 22-oxacalcitriol having high specific radioactivity. A plasma sample was extracted with ethyl ether and the extract was successively purified with Bond Elut® C18 and a normal-phase HPLC prior to the RIA to remove interfering substances. Accuracy of the RIA was assessed by various studies including serial dilution and recovery tests. Intra- and inter-assay coefficients of variation were lower than 10%, and the quantification limit was low enough for practical use (33 pg/ml plasma). The present RIA will be useful for the pharmacokinetic studies of 22-oxacalcitriol in both preclinical and clinical stages.

Key words vitamin D₃ analog; 22-oxacalcitriol; RIA; plasma level; pharmacokinetic study

22-Oxacalcitriol (OCT) is an analog of 1α,25-dihydroxyvitamin D₃ [the active form of vitamin D₃; 1,25(OH)₂D₃] having an oxygen atom at the 22-position in the side chain, which was synthesized for separating the cell differentiation activity of 1,25(OH)₂D₃ from its calcemic actions (Chart 1). Although OCT exhibits negligible calcemic activity, its differentiation-inducing activity on myeloid cells is much stronger than that of 1,25(OH)₂D₃. Because of its suppressive action on parathyroid hormone (PTH) secretion with minimal calcemic activity, OCT is now expected to be a unique therapeutic tool in the treatment of secondary hyperparathyroidism in patients with chronic renal failure. Recently, it has also been shown that OCT inhibits the proliferation of psoriatic fibroblasts and estrogen receptor-positive and negative breast cancer cells indicating that the analog has broad and valuable clinical potency.

To study the effectiveness of OCT from a pharmacokinetic aspect, an assay method for determining the concentration of the analog in blood is essential. Because the OCT levels are predicted to be much lower than those of accompanying endogenous vitamin D₃ metabolites in blood, the assay must be highly sensitive and specific.

From these points of view, we have previously produced an anti-OCT antiserum showing high affinity and specificity in an RIA procedure. This paper deals with the development of a sensitive and practical RIA using the antiserum, which is applicable to the determination of OCT in rat and human plasma.

MATERIALS AND METHODS

Reagents Vitamin D₃ and 25-hydroxyvitamin D₃ [25(OH)D₃] were purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan) and Duphar B.V. Co. (Amsterdam, The Netherlands), respectively. OCT, [26-methyl-³H]-OCT (86.5 Ci/mmol), and other related compounds were synthesized in our laboratory (Chugai Pharmaceutical Co.). Bond Elut® C18 (No. 1210—2028) was obtained from Varian Co. (Harbor City, CA, U.S.A.). Clear-sol I liquid scintillation cocktail, dextran (molecular weight 50000—70000), and activated charcoal powder (Norit® EXW) were purchased from Nacalai Tesque (Kyoto, Japan). Calf thymus vitamin D receptor (VDR) was obtained from Yamasa Co. (Choshi, Japan). All the other reagents and solvents were of analytical grade.

Anti-OCT Antiserum The antiserum used in the RIA (corresponds to As-3 in the previous paper) was that elicited in a rabbit by immunization with OCT 3-hemiglutarate conjugated with bovine serum albumin, having a high affinity to OCT (Kᵈ = 1.8 × 10⁻¹⁰ M⁻¹). The cross-reactivities (50% displacement method) of related secosterols in an RIA were as follows: vitamin D₂ < 0.01%; 25(OH)D₃ 0.39%; 1,25(OH)₂D₃ 0.49%; (24R)-

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24,25-dihydroxyvitamin D₃ [24,25(OH)₂D₃] 0.05%; (25R)- and (25S)-25,26-dihydroxyvitamin D₃ 0.02 and 0.06%, respectively; (24,25(Δ2,3)-24-hydroxy-OCT 4.9%; (20S)1α,20-dihydroxy-22,23,24,25,26,27-hexanorvitamin D₃ [1,20(OH)₂hexanor-D₃] 0.02%.  

**Apparatus** Radioactivity was measured with an Aloka (Tokyo, Japan) LSC-1000 liquid scintillation spectrometer. HPLC was carried out on a Tosoh (Tokyo, Japan) PX-8010 chromatograph equipped with a Tosoh UV-8010 detector (265 nm). A ZorbaX SIL (Du Pont Instruments, Inc., Wilmington, DE, U.S.A.) or a Capcell Pak NH₂ (Shiseido Co., Tokyo, Japan) column (each 5 μm, 25×0.46 cm i.d.) was used under ambient temperature. Injection of samples to the HPLC was done using a Tosoh AS-8000 autosampler. Fractionation of the eluate was performed using a Tosoh FC-8010 fraction collector controlled by a Tosoh FX-8010 fraction collector controller.

**Plasma Samples Used for Assay Validation** In assessment of the accuracy and precision of the RIA, the following plasma samples were used. a) Rat Plasma: Male Sprague Dawley rats (7 weeks old, weighing ca. 300 g) were given an intravenous dose (100 μg/kg body weight) of OCT as a solution in saline. Blood was withdrawn at 30 min and 1.5 h after administration, and plasma (plasma-1 and -2, respectively) was immediately separated.

b) Human Plasma: For the HPLC elution profile study, a pooled plasma from patients with secondary hyperparathyroidism repeatedly given intravenous doses (3 or 5 μg/body; 12 times every other day) of OCT was used. The predose plasma from healthy volunteers, to which standard OCT (ca. 5 or 10 ng) was added (plasma-1 and -2, respectively), was used for the other studies.

**Pretreatment of Plasma Samples** A solution of [³H]OCT (ca. 3000 dpm) in EtOH (10 μl) was added to the plasma (100–400 μl) and the solution was kept at room temperature for 1 h. After the addition of H₂O (1 ml) and Et₂O (5 ml), the mixture was vortex-mixed twice (each for 30 s). The Et₂O layer was separated and evaporated off under an N₂ gas stream at 40°C (bath temperature). The residue was dissolved in a mixture of MeCN–H₂O–0.4 M Na₂HPO₄/NaOH buffer (pH 10.6) (1:2:1; 2 ml) and then applied to the Bond Elut® C18 column which had been serially pre-washed with hexane, CHCl₃, MeOH, and H₂O (each 5 ml). The column was washed with H₂O (5 ml) and then H₂O–MeOH (1:1; 3 ml), and the OCT fraction was eluted with MeCN (5 ml).

After removal of the solvent as described above, the residue was dissolved in a 10% 2-propanol/hexane solution (300 μl). A portion (200 μl) of the solution was injected into the HPLC equipped with a ZorbaX SIL column by the autosampler, which was eluted with the same solvent at a flow rate of 2.7 ml/min. The eluate containing OCT (tᵣ 15—17 min) was automatically collected using a fraction collector, and the solvent was evaporated off as described above. The residue obtained was dissolved in EtOH (100 μl), and the radioactivity of the resulting solution (25 μl) was measured to calculate the recovery rate of OCT. The remaining solution was submitted to the following RIA (if necessary, after being further diluted with EtOH).

**RIA Procedure** RIA was carried out according to the previously described method⁵ with some modification. A 0.05 M sodium phosphate buffer (pH 7.3) containing 0.1% gelatin, 0.9% NaCl, and 0.1% NaN₃ was used in the assay. Standard OCT (0—400 pg) or a plasma sample pretreated as already described, dissolved in EtOH (25 μl), was added to the antiserum diluted with the buffer (1:350000; 500 μl), and the mixture was incubated at 4°C overnight. The [³H]OCT (ca. 16000 dpm) in EtOH (25 μl) was then added to the solution, and the mixture was incubated at 4°C for a further 3 h. After addition of the suspension of the charcoal powder (0.5%) in the buffer containing 0.1% dextran, the mixture was vortex-mixed, left at 0°C for 20 min, and then centrifuged at 4°C (1000 × g for 10 min). The supernatant was transferred to a vial containing scintillation cocktail (10 ml) by decantation to measure its radioactivity.

**Serial Dilution Test** The rat plasma or the human plasma (each 100 μl) was pretreated as above, and the residue was dissolved in EtOH (8.0 ml and 200 μl, respectively). The resulting solution (represented as 1:1) was serially diluted twice with EtOH to produce the samples (1:2 and 1:4), and each of them was submitted to the RIA.

**Recovery Test** Solutions of standard OCT (0, 120, 240, 480, 600, 760, and 960 pg) in EtOH (10 μl) were added to a predose of plasma from a rat or human (100 μl). Each of the resulting solutions was pretreated and the residue was dissolved in EtOH (100 μl), which was then submitted to the RIA.

**Time Course of Plasma OCT Concentration in Human Volunteers** An intravenous dose (6 μg) of OCT was given with saline to each of two healthy male volunteers (volunteer-1 and -2). Blood was taken at 5, 10, 20, 30, 60, 120, and 240 min after administration. The plasma specimens obtained were pretreated as previously mentioned and submitted to the RIA. The OCT levels of these samples were also measured by the radioreceptor assay (RRA) described below, and the results were compared with those obtained by the RIA.

**Pretreatment for RRA** A solution of [³H]OCT (ca. 3000 dpm) in EtOH (25 μl) was added to the plasma (1.0 ml) and the solution was kept at room temperature for 30 min. After the addition of 1 ml HCl (1 ml), the mixture was applied to a Sep-Pak t₁₈ Plus column (Waters Assoc., Milford, MA, U.S.A.) which had been serially pre-washed with MeCN and 4% AcOH (each 5 ml). The column was washed with 4% AcOH and then 70% MeOH (each 5 ml), and the OCT fraction was eluted with MeCN (6 ml). The eluate was mixed with 14% NH₄OH (50 μl) and then the solvent was evaporated off. The residue was dissolved in MeOH–2-propanol–hexane (2:6:92; 200 μl) and submitted to HPLC using the Capcell Pak NH₂ column which was eluted with the same solvent at a flow rate of 2.0 ml/min. The OCT recovered from the eluate (tᵣ 14—16 min) was dissolved in EtOH (200 μl), and the radioactivity of an aliquot (50 μl) was measured to calculate the recovery rate of OCT. The remaining solution was used as a sample for the following RRA.

**RRA Procedure** A 0.05 M potassium phosphate buffer (pH 7.4) containing 0.3 M KCl was used in the RRA. An EtOH solution of standard OCT (0—250 pg) or a plasma sample pretreated as above (50 μl) was mixed with VDR
solution [prepared by the addition of the buffer (20 ml) to a vial of the VDR] (1.0 ml). After incubation at room temperature for 1 h, the [3H]OCT (ca. 12000 dpm) dissolved in EtOH (30 μl) was added to the mixture at 0°C, which was then incubated at room temperature for an additional 1 h. After the addition of a dextran-coated charcoal suspension (supplied from the manufacturer of VDR) (200 μl) at 0°C, the mixture was vortex-mixed, left at 0°C for 30 min, and then centrifuged at 4°C (1000 × g for 20 min). The supernatant (1.0 ml) was transferred to a vial containing the scintillation cocktail (5 ml) by decantation to measure its radioactivity.

RESULTS

Dose–Response Curves To measure serum/plasma OCT levels with high sensitivity, an RRA using calf thymus VDR39 seemed to also be applicable because it had been determined that the VDR has significant binding activity to OCT. Figure 1 shows the dose–response curve of the RIA together with that of the RRA, representing the mean from 9 (RIA) and 5 (RRA) assays with duplicate determination of OCT standards. The S.D. values of each point (represented as vertical bars) indicated that the RIA afforded a highly reproducible dose–response curve compared with the RRA. The detection limit of the RIA and the RRA, defined as the amount of OCT required to give a B/Bo of 2 S.D. below was 2.5 and 7.5 pg/tube, respectively. Although the RRA showed a steeper response in the high dose area, this result indicated that the RIA has slightly higher sensitivity than that of the RRA.

Pretreatment of Plasma Samples In competitive binding assays of vitamin D metabolites, some types of lipid10 and serum protein, which is incompletely separated during the initial extraction procedure,11 are suggested to be interfering substances in addition to the cross-reactive secosterols. To remove such substances, the ethyl ether extract of the plasma was further purified with a reversed-phase cartridge having an octadecylsilyl group (Bond Elut® C18) which seemed to be suitable for this purpose.9a,12 It should be mentioned that the recovery rate of 25(OH)D3 through the ethyl ether extraction was lower than 20% while that of OCT was over 85% (determined using tritium-labeled compounds). Thus, the extraction step was helpful to remove a large portion of 25(OH)D3, which is a major vitamin D3 metabolite and is predicted to interfere with the OCT assay. To determine whether

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Fig. 1. Dose–Response Curves for the RIA and the RRA of OCT
Each point was calculated from result (mean ± S.D.) of 9 and 5 experiments for RIA (○) and RRA (○), respectively.

Fig. 2. HPLC Elution Profile of a Rat Plasma Extract Determined as Immunoactivity (A), and of Some Standard Compounds Related to OCT (B)
HPLC was carried out using the Zorbax SIL column, according to the conditions described in Materials and Methods. (A) The rat plasma-1 (100 μl) was extracted with Et2O and then purified with Bond Elut® C18. The purified plasma extract containing OCT was applied to the HPLC. The eluate was fractionated every minute, and a portion (1/200) of each obtained fraction was submitted to the RIA. Immunoreactivity detected was represented as plasma concentration of OCT equivalent. (B) 1. Vitamin D2; 2. 25(OH)D2; 3. 24,25(OH)2D2; 4. 1,25(OH)2D3; 5. OCT; 6. pro-OCT (7-dehydro-1α,25-dihydroxy-22-oxacholesterol); 7. 1,25(OH)2- hexanor-D2; 8. pre-OCT ((6αZ)-(15,3αR)-22-oxa-9,10-secocholesta-5(10), 6,8-triene-1,3,25-triol).
or not a further purification is needed, the OCT fraction of a postdose of rat plasma, prepared by the above-mentioned procedure, was further submitted to a normal-phase HPLC to examine the elution profile of immunoreactivity (Fig. 2A). A typical chromatogram of several compounds related to vitamin D₃ or OCT in this HPLC condition is also shown in Fig. 2B. 1,20(OH)₂Hexanor-D₃ is a major metabolite of OCT, which has been identified in rat postdose plasma. It was shown that all the related compounds tested here were satisfactorily separated from OCT. Although its extent was much lower than that of the OCT fraction (15—17 min), we found an immunoreactive fraction (4 min) which might be ascribable to an unidentified less-polar OCT metabolite. It is conceivable that the ratio of these immunoreactivities may vary widely with OCT dose, or between animals and/or patients. Furthermore, this variation may, in some cases, result in significant overestimation of the plasma OCT concentrations. Thus, we employed a purification step by this HPLC after the treatment with a Bond Elut® C18 cartridge, to obtain reliable assay values for any plasma samples. The recovery rate of OCT through the whole pretreatment procedure was 74.8±5.9% (mean ± S.D.; n=20), which was satisfactory including its reproducibility.

Accuracy of the RIA  a) Serial Dilution test and HPLC Elution Profile of Immunoreactivity: The OCT levels of a postdose of rat plasma and OCT-added human plasma were determined after the above-mentioned pretreatment followed by serial dilution (Fig. 3). A good parallel between the assay values and the sample dilution was observed for both specimens. Thus, it was suggested that, for measurement of rat plasma levels, the pretreatment procedure successfully removed both endogenous and OCT-derived interfering substances. However, this result can not exclude the possibility that the RIA may overestimate the human plasma levels by OCT metabolites, which are characteristic of human, and co-migrate with OCT by the chromatographies in the pretreatment because the human plasma used in this test does not contain any OCT metabolites. Thus, plasma specimens of OCT-administered patients with secondary hyperparathyroidism were collected and pretreated with the established procedure. The OCT-fraction thus obtained was submitted to an additional HPLC based on a different separation condition using the Capcell Pak NH₂ column (Pretreatment for RRA in Materials and Methods), and the elution profile of immunoreactivity was examined in a manner similar to that described above. No immunoreactivity except that eluted at the t₂ of OCT (ca. 14—15 min) was found (data not shown), indicating that the assay values of human plasma are free from the above-mentioned interference and are as reliable as those of rat plasma.

b) Recovery Test: The predose plasma specimens to which known amounts of OCT had been added were pretreated and submitted to the RIA. The recovery rates ranged from 98 to 113% (rat plasma) and from 93 to 104% (human plasma), both of which were evaluated to be satisfactory (Table I). It should be noted that no OCT was detectable in the predose plasma itself.

Precision of the RIA Intra-assay coefficients of variation determined in rat (n=10) and human (n=8) plasma samples, both of which contain low or high levels of OCT, were 4.1—6.9%. Excellent inter-assay coefficients of variation (n=5; 4.8—9.0%) were also obtained (Table II). Thus, the present RIA proved to be satisfactorily precise.

Independence of Plasma Volume and Practical Quantification Limit As shown in Fig. 4, the RIA gave an assay value which seemed to be independent of plasma volume

| Table I. Recovery of OCT Added to Predose Plasma |
|---|---|---|---|
| Added (pg/tube) | Rat plasma | Human plasma | |
| | Found (%) | Recovery (%) | Found (%) | Recovery (%) |
| 0 | 0 | — | 0 | — |
| 30 | — | — | 30 | 100 |
| 60 | 59 | 98 | 56 | 93 |
| 120 | 117 | 98 | 125 | 104 |
| 150 | 162 | 108 | — | — |
| 190 | 215 | 113 | — | — |
| 240 | 267 | 111 | 229 | 95 |
| Mean ± S.D. | 105.6±6.4 | 98.0±4.3 |

Table II. Intra- and Inter-Assay Coefficients of Variation (CV) of OCT Measurements by the RIA

<table>
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<th>Plasma sample</th>
<th>OCT (ng/ml)</th>
<th>CV (%)</th>
<th>n</th>
<th>OCT (ng/ml)</th>
<th>CV (%)</th>
<th>n</th>
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</table>

Fig. 3. Effect of Serial Dilution of Plasma Samples on Assay Values of OCT Obtained by the RIA

Rat plasma (●) and human plasma (○) were used. Assay values obtained at the dilution of 1:2 and 1:4 were represented without correction by the dilution factor.
plasma OCT thus obtained was reasonable and reliable. On the other hand, the RRA afforded somewhat irregular and unreasonable plasma concentration time curves (especially for volunteer-1), although we have no detailed account of the cause.

DISCUSSION

We have succeeded in developing a RIA procedure for measuring plasma OCT levels which was found to be more suitable than the RRA for this purpose. This assay exhibited enough sensitivity for practical use and reliable assay values for both rat and human plasma specimens. The satisfactory result is mainly ascribable to the high affinity and specificity of the anti-OCT antiserum used here, which had been generated using a well-designed haptenic derivative (OCT 3-hemigluturate), and the use of a tritium-labeled OCT having high specific radioactivity.

Because OCT shows only negligible binding to a serum vitamin D binding protein (DBP), the antiserum itself was used in the RIA system without any purification to remove DBP. It should also be mentioned that OCT has a minimal tendency to be adsorbed on the glass walls, probably due to its higher polarity than that of 1,25(OH)₂D₃; about 95% of the added OCT could be recovered from the RIA incubation mixture (data not shown). Therefore, no special solubilizing agent (such as polyvinyl alcohol) was necessary to be added to the buffer for the RIA, in contrast with the immunoassays of 25(OH)D₃ and 1,25(OH)₂D₃.

Although the assay procedure involves a preparative HPLC step in sample pretreatment, the use of an autosampler coupled with an automatic fraction collector permitted the handling of many plasma specimens without the constant attendance of a technician.

The present RIA will be useful for the pharmacokinetic studies of OCT in preclinical and clinical stages, promoting the development of this analog as a novel agent for the treatment of patients with secondary hyperparathyroidism, psoriasis, and/or breast cancer.

REFERENCES


