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Tandem Immunoaffinity Chromatography for Plasma 1α,25-Dihydroxyvitamin D_3 Utilizing Two Antibodies Having Different Specificities: A Novel and Powerful Pretreatment Tool for 1α,25-Dihydroxyvitamin D_3 Radioreceptor Assays

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We report here a novel and powerful pretreatment method for radioreceptor assays (RRAs) for human plasma 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) based on "Tandem" immunoaffinity chromatography (Tandem IAC). Two antibodies having different specificities were each immobilized on agarose gel with cyanogen bromide to produce immunosorbents which were stable and repeatedly usable. An ethyl ether extract of plasma was applied to the first affinity column, from which $1,25(OH)_2D_3$ could be preferentially eluted and separated from 1α-deoxy type metabolites. The effluent was then submitted to the second column, and the $1,25(OH)_2D_3$ retained was eluted after non- or weakly-adsorbed interfering substances were washed out. This procedure allowed efficient purification without careful handling or strict time-management in the entire operation and enabled avoiding preparative high-performance liquid chromatography (HPLC) from RRA even with a conventional chick intestinal vitamin D receptor. Mean (± SD) plasma $1,25(OH)_2D_3$ values of 56 normal subjects and 10 patients with chronic renal failure, obtained with this Tandem IAC/RRA system, were 36.4 (8.7) and 11.2 (4.0) pg/ml, respectively. The Tandem IAC will also be useful for developing immunoassays or gas chromatography–mass spectrometry of $1,25(OH)_2D_3$.

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INTRODUCTION

1α,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) is the hormonally active form of vitamin D_3 playing an important role in regulating calcium and phosphorus metabolism and bone resorption. The measurement of $1,25(OH)_2D_3$ levels in human serum/plasma is of great value for clinical diagnosis because the biosynthesis of the metabolite can be affected by several diseases influencing vitamin D metabolism, such as decreased renal function and parathyroid disorders [1,2]. The $1,25(OH)_2D_3$ measurement has previously been carried out, mainly by radioreceptor assays (RRAs) based on vitamin D receptors (VDRs) derived from chick intestine [3,4] and some radioimmunoassays (RIAs) using antibodies to $1,25(OH)_2D_3$ have also been attempted [3]. These methods, however, required exhaustive purification steps of serum/plasma samples including preparative high-performance liquid chromatography (HPLC), due to insufficient specificity of the VDRs and antibodies. Consequently, the assays were tedious and time-consuming, and only a limited number samples could be measured at once. In recent years, it has been reported that VDRs prepared from calf thymus are less sensitive to interfering substances in blood [5,6] and permit replacing the preparative HPLC with a single C18/OH cartridge [7-9]. However, developing a novel sample pretreatment

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procedure, which itself has much higher purification efficiency and is feasible for routine use, is worth investigating as an alternative approach for simplifying the $1,25(OH)_2D_3$ assays.

Immunoaffinity chromatography (IAC) seems to be a promising methodology because of its possibility of offering extremely high selectivity based on the antigen–antibody reaction. This method has been applied to the $1,25(OH)_2D_3$ assay using monoclonal antibody against VDR [10]. We describe here an IAC for human plasma $1,25(OH)_2D_3$ in which two kinds of immobilized antibodies having different specificities were employed in an effective combination to provide high selectivity. We called this IAC "Tandem" immunoaffinity chromatography (Tandem IAC). This novel method allowed efficient purification of plasma $1,25(OH)_2D_3$ with a simple operation and enabled valid RRA not requiring any HPLC step (non-HPLC RRA) even with a conventional chick intestinal VDR.

EXPERIMENTAL

Apparatus

Radioactivities were measured with an Aloka (Tokyo, Japan) LSC-700 liquid scintillation spectrometer. HPLC was carried out on a Shimadzu (Kyoto, Japan) LC-6A chromatograph equipped with a Shimadzu SPD-6A UV detector (265 nm). A Develosil 60-5 column (5 μm; 25×0.46 cm i.d.) (Nomura Chemical Co., Seto, Japan) was used under ambient temperature.

Chemicals

Vitamin D_3 and D_2 were purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). 25-Hydroxyvitamin D_3 [$25(OH)D_3$] was obtained from Duphar B.V. Co. (Amsterdam, The Netherlands). 25-Hydroxyvitamin D_3 3-sulfate [$25(OH)D_3$ 3S], 25,26-dihydroxyvitamin D_3 [(25*R*)- and (25*S*)-isomers; $25R,26(OH)_2D_3$ and $25S,26(OH)_2D_3$], 7-dehydro-1α,25-dihydroxycholesterol [pro-$1,25(OH)_2D_3$], and 25-hydroxycholesterol were synthesized in our laboratory by known methods [11]. (23*S*,25*R*)-1α,25-dihydroxyvitamin D_3 26,23-lactone [1,25-lactone] and 1α,25-dihydroxyvitamin D_2 [$1,25(OH)_2D_2$] were donated by Professor S. Takano of Tohoku University (Sendai, Japan) and Maruho Co. (Osaka, Japan), respectively. Other vitamin D derivatives and 1α-hydroxyvitamin D_3 [$1(OH)D_3$] capsules (Alfarol^{R}) were supplied from Chugai Pharmaceutical Co. (Tokyo, Japan). [1α,2α(N)-^{3}H]-Vitamin D_3 (10 Ci/mmol), [26,27-methyl-^{3}H]-$25(OH)D_3$ (172 Ci/mmol), [26,27-methyl-^{3}H]-$1,25(OH)_2D_3$ (172 Ci/mmol) and [23,24(N)-^{3}H]-$24,25(OH)_2D_3$ (83 Ci/mmol) were purchased from Amersham Co. (Tokyo, Japan). For determining the recovery rate through the pretreatment, these labeled compounds were purified by the HPLC prior to use. VDR derived from chick intestine (embryonal duodenum) [4] or calf thymus and dextran-coated charcoal suspension were donated by Yamasa Co. (Choshi, Japan). Clear-sol I liquid scintillation cocktail and cyanogen bromide (CNBr) were obtained from Nacalai Tesque Co. (Kyoto, Japan). Sepharose® 4B and poly(vinyl alcohol) (PVA; average M_W 3000) were purchased from Pharmacia Co. (Uppsala, Sweden) and Polysciences Inc. (Warrington, PA), respectively. Other reagents and solvents were of analytical grade.

Buffers

Buffer A, 0.05 M NaH_2PO_4–Na_2HPO_4 (pH 7.3); buffer B, buffer A containing 0.9% NaCl and 0.1% NaN_3; buffer C, buffer B containing 0.1% gelatin and 0.5% PVA (a solubilizing agent for hydrophobic vitamin D derivatives [12, 13]); buffer D, 0.1M $NaHCO_3$–Na_2CO_3 (pH 9.0); buffer E, 0.4M K_2HPO_4–KOH (pH 10.6); and buffer F, 0.05M KH_2PO_4–K_2HPO_4 (pH 7.4) containing 0.3M KCl and 5 mM dithiothreitol.

Antibodies and their characterization

The Ab-3 or -11 used for preparing affinity columns is the IgG fraction of rabbit antiserum against the conjugate of bovine serum albumin with $25(OH)D_3$ 3-hemisuccinate or that of 11α-hemiglutaryloxy-$25(OH)D_3$ (each correspond to HS-1 [14] and As_{11}-3 [13], respectively) produced in our laboratory. The binding properties of these antibodies were tested by an RIA system employing the [^{3}H]$1,25(OH)_2D_3$ (for the Ab-3)[13] or [^{3}H]$25(OH)D_3$ (for the Ab-11) [11] as a labeled antigen.

Clinical samples

All the plasma samples were prepared using heparin as an anticoagulant and were stored at -20°C prior to use. The 56 plasma samples (27 men and 29 women) obtained from healthy adult subjects (ages 20–41 y) were donated by Dr K. Mawatari of the School of Allied Medical Profession, Kanazawa University (Kanazawa, Japan). The 10 plasma specimens from patients with chronic renal failure (pre-dialysis phase; 6 men and 4 women) were given by Dr S. Kamachi of Chugai Pharmaceutical Co. Plasma samples after $1(OH)D_3$ load were prepared from two healthy male volunteers (36 and 51 y) at 6, 10 and 31 h after administration of an oral dose (4 μg) of this agent as Alfarol^{R} capsules.

Preparation of immunosorbent

To a stirred suspension of Sepharose^{R} 4B (50 ml; swelled gel volume) in water (100 ml), CNBr (5 g) was added portionwise at 0°C at a controlled pH (10.5–11.0; adjusted with 2 M NaOH). After stirring for 20 min at 0°C, the activated gel was washed twice with buffer D (each 500 ml) and then suspended in buffer D (50 ml). A solution of the Ab-3 or -11

(containing 50 mg of IgG; determined by absorption at 280 nm with an E-value of 14) in buffer D (approx. 20 ml) was added to an aliquot of the activated gel (25 ml; swelled gel volume). The mixture was shaken continuously at 4°C for 1 h and then left at 4°C overnight. After centrifugation at 4°C (35*g* for 10 min), the precipitate was resuspended in 0.1 M Tris–HCl buffer (pH 8.0; 50 ml) and shaken at 4°C for 1 h. The suspension was centrifuged as above and the precipitated gel was washed 5 times with buffer A (approx. 50 ml). The immunosorbent thus obtained was stored in buffer B at 4°C prior to use.

Preparation and treatment of affinity column

The proper volume of the immunosorbent immobilizing the Ab-3 or -11 was packed into a disposable polypropylene column (4.4 × 0.9 cm i.d.) (Sepacol·mini·PP; Seikagaku Kogyo Co., Tokyo, Japan). These affinity columns (column-3 and -11, respectively) were serially washed with 95% methanol (10 ml), water (10 ml) and buffer B (5 ml): column-11 was further washed with buffer C–ethanol (10:1, v/v; 5 ml) prior to use. After use, the columns were washed with aqueous methanol, water and then buffer B as above. Then the immunosorbent was recovered from the column to a bottle, suspended in buffer B and stored at 4°C until next use.

Ethyl ether extraction of plasma

A ethanolic solution of [^{3}H]1,25$(OH)_2D_3$ (3000 dpm; 25 μl) was mixed with a plasma sample (0.75–2.0 ml) and left at room temperature for 1 h. The solution was diluted with buffer E (4.0 ml) and then vortex-mixed with ethyl ether (12 ml) twice each for 30 s. After separation of the organic layer, ethyl ether (12 ml) was again added to the aqueous layer and vortex-mixed as above. The organic layer was combined with the former one, and the solvent was evaporated with a nitrogen gas stream.

Tandem IAC procedure

The following chromatographic operation was done at room temperature and atmospheric pressure. The plasma extract prepared as above was dissolved in buffer C–ethanol (10:1, v/v; 3.2 ml) and applied to the column-11 (immunosorbent, 2 ml). The column was washed with water (10 ml) and then eluted with 25% acetonitrile (15 ml). The effluent was mixed with water (10 ml) and submitted to the column-3 (immunosorbent, 1 ml). After serial washing of the column with water and 20% acetone (each 5 ml), the adsorbed substances were eluted with 95% methanol (10 ml). The solvent was evaporated with a nitrogen gas stream at 45°C (bath temperature) and the residue was dissolved in methylene chloride (3.0 ml) which was then washed once with water (1.5 ml). Finally, the solvent was evaporated again and the residue including 1,25$(OH)_2D_3$ (representing the 1,25$(OH)_2D_3$ fraction) was dissolved in the proper volume of ethanol. The radioactivity of its aliquot (50–150 μl) was measured using Clear-sol I (10 ml) to estimate the recovery rate, and the remaining solution was submitted to the RRA.

Recovery rate of vitamin D derivatives through the pretreatment

An ethanolic solution containing one of the following labeled compounds, [^{3}H]1,25$(OH)_2D_3$ (3000 dpm; 25 μl), [^{3}H]vitamin D_3, [^{3}H]25$(OH)D_3$ or [^{3}H]24,25$(OH)_2D_3$ (each 60,000 dpm; 100 μl) was mixed with an aliquot of pooled plasma (2.0 ml) from healthy subjects and left at room temperature for 1 h. Each solution was extracted with ethyl ether, submitted to the IAC, and then the radioactivity of the 1,25$(OH)_2D_3$ fraction obtained was measured as above. As for the other metabolites, a solution of a standard compound (10 or 100 ng) in buffer C–ethanol (10:1, v/v; 2.0 ml) was pretreated in place of the plasma specimen. The amount of metabolite recovered in the 1,25$(OH)_2D_3$ fraction was measured by an RIA system using the corresponding standard compound for calibration, in combination with the following antibody and labeled antigen: for measuring 1,25$(OH)_2D_2$, 1,25-lactone and 1,24,25$(OH)_3D_3$, "Ab-2" and [^{3}H]1,25$(OH)_2D_3$ [13]; for 25$(OH)D_3$ 3S, "HG-2" and [^{3}H]25$(OH)D_3$ [14]; for 25*R*,26$(OH)_2D_3$ and 25*S*,26$(OH)_2D_3$, "Ab_{11}-2" and [^{3}H]25$(OH)D_3$ [11].

RRA procedure

An ethanolic solution of standard 1,25$(OH)_2D_3$ (0–20 pg) or the 1,25$(OH)_2D_3$ fraction of plasma (50 μl; in duplicate) was mixed with the VDR solution (500 μl) in buffer F, which was prepared to bind about 40% of added total radioactivity. After incubation at room temperature for 1 h, an ethanolic solution of the [^{3}H]1,25$(OH)_2D_3$ (10,000 dpm; 25 μl) was added to the mixture, which was further incubated at 4°C for 16 h. The dextran-coated charcoal suspension (200 μl) was added to the solution at 0°C, and the resulting suspension was vortex-mixed and then left at 4°C for 30 min. After centrifugation at 4°C (1000 ***g*** for 10 min), a portion of the supernatant (500 μl) was transferred into a vial containing the tT 21 scintillation cocktail [15] (10 ml), and the radioactivity was measured. The plasma 1,25$(OH)_2D_3$ values were corrected using the recovery rate through the pretreatment calculated as above.

Serial dilution and precision studies

Plasma from healthy volunteers (2.0 ml) was pretreated as above, and the 1,25$(OH)_2D_3$ fraction was dissolved in ethanol (200 or 350 μl) to prepare the 1/4 or 1/7 sample. The 1/4 (or 1/7) sample was (serially) diluted with ethanol to produce the 1/7 (or 1/14 and 1/28) sample. The 1,25$(OH)_2D_3$ levels of

Table 1. Binding properties of antibodies and VDRs

	Cross-reactivity* (%)			
	Antibody		VDR	
	Ab-3	Ab-11	Intestinal	Thymus
$1,25(OH)_2D_3$	100	0.10	100	100
$1,25(OH)_2D_2$	<0.05	—	81	78
Vitamin D_3	0.08	3.6	<0.001	<0.001
Vitamin D_2	<0.1	—	<0.001	<0.001
$25(OH)D_3$	21	100	0.39	0.61
$25(OH)D_33S$	51	<0.3	0.001	0.002
$24,25(OH)_2D_3$	6.8	110	0.07	0.07
$25R,26(OH)_2D_3$	0.48	71	0.01	0.02
$25S,26(OH)_2D_3$	3.2	100	0.01	0.03
$1,24,25(OH)_3D_3$	1.8	<1	25	27
1,25-Lactone	0.08	<0.02	0.34	1.1
$1,24(OH)_2D_3$	<0.1	—	64	87
Pro-$1,25(OH)_2D_3$	<0.05	—	0.01	0.003
25-Hydroxycholesterol	—	<0.03	—	—
Titer†	1:88000	1:59000	—	—
K_a‡ ($M^{-1} \times 10^{-9}$)	2.8	2.2	—	—

*The relative amounts required to reduce the initial binding of the [^{3}H]$1,25(OH)_2D_3$ (Ab-3 and VDRs) or [^{3}H]$25(OH)D_3$(Ab-11) by half, where the mass of the corresponding unlabeled compound was arbitrarily taken as 100% [16].

†The final dilution of antibodies required to bind 40% of added [^{3}H]$1,25(OH)_2D_3$(Ab-3) or [^{3}H]$25(OH)D_3$(Ab-11).

‡Affinity constant obtained by Scatchard analysis (more than seven points, n=1) [17].

these samples were simultaneously determined by the intestinal VDR-based RRA.

Analytical recovery studies

An ethanolic solution of standard $1,25(OH)_2D_3$ (0, 50, 100 or 200 pg; 100 μl) was mixed with aliquots of pooled plasma from a healthy volunteer (2.0 ml), left at room temperature for 1 h, and was pretreated as above. The $1,25(OH)_2D_3$ fraction was dissolved in the proper amount of ethanol and submitted to the intestinal VDR-based RRA.

Comparison with a reference method involving preparative HPLC

The plasma specimen (2.0 ml) was pretreated as above, and the $1,25(OH)_2D_3$ fraction was dissolved in ethanol (600 μl). The solvent of this portion (300 μl) was evaporated, and the residue was applied to the HPLC which was eluted with 2-propanol–hexane (3:17, v/v) at a flow rate of 2.5 ml/min. Typical t_R of some endogenous vitamin D_3 derivatives were as follows: vitamin D_3, 1.5 min; $25(OH)D_3$, 2.0 min; $24,25(OH)_2D_3$, 3.2 min; $1,25(OH)_2D_3$, 7.8 min; 1,25-lactone, 13.0 min; $1,24,25(OH)_3D_3$, 15.8 min. The effluent containing $1,25(OH)_2D_3$ (t_R 7.5–10.5 min) was collected, the solvent was evaporated, and the residue was dissolved in ethanol (250 μl). These samples (before and after the HPLC) were simultaneously submitted to the intestinal VDR-based RRA (Tandem IAC/RRA and Tandem IAC/HPLC/RRA). The recovery rates of $1,25(OH)_2D_3$ in both samples, with which the assay values were corrected, were calculated as above.

RESULTS

Characterization of antibodies

Table 1 lists some binding properties [16,17] of the antibodies used for preparing the affinity columns in the RIA procedure. The Ab-3 and -11 showed high titer and affinity for $1,25(OH)_2D_3$ and for $25(OH)D_3$, respectively. The Ab-3 exhibited higher reactivity to $1,25(OH)_2D_3$ than $25(OH)D_3$, despite the fact that the hapten used $25(OH)D_3$ 3-hemisuccinate lacks the 1α-hydroxy group. This property has previously been observed in an RIA system using the [^{3}H]$25(OH)D_3$ as a labeled antigen, and the reason for this has been suggested [14]. The Ab-3 reasonably discriminated side chain-modified metabolites [$1,25(OH)_2D_2$, vitamin D_3 and D_2, $24,25(OH)_2D_3$, $25,26(OH)_2D_3$, $1,24,25(OH)_3D_3$, 1,25-lactone and $1,24(OH)_2D_3$], though high cross-reactivity was observed with $25(OH)D_3$ 3S modified on the A-ring. On the other hand, the Ab-11 cross-reacted equally with $25(OH)D_3$, $24,25(OH)_2D_3$ and $25,26(OH)_2D_3$ while the metabolites having a 1α-hydroxy or a 3-sulfonate group [$1,25(OH)_2D_3$, $1,24,25(OH)_3D_3$, 1,25-lactone and $25(OH)D_3$ 3S] were satisfactorily discriminated [11]. Both the antibodies were specific to the vitamin D skeleton as demonstrated from the negligible cross-reactivity with pro-$1,25(OH)_2D_3$ (the Ab-3) or 25-hydroxycholesterol (the Ab-11).

Table 2. Typical retention behavior of $1,25(OH)_2D_3$ and $25(OH)D_3$ in IAC: effect of solvent composition used as effluent or washing solution of the affinity column

Organic solvent	Concentration* (%, by vol)	Recovery (%) IAC-11† $1,25(OH)_2D_3$	IAC-11† $25(OH)D_3$	IAC-3‡ $1,25(OH)_2D_3$	IAC-3‡ $25(OH)D_3$
Methanol	30	—	—	96	95
	35	—	—	82	91
	45	80	4.3	—	—
	50	85	33	—	—
Acetonitrile	20	62	7.9	80	88
	25	86	11	82	85
	30	94	47	77	80
Acetone	20	—	—	96	84
	30	76	24	90	75
	40	85	78	15	67

*Concentration of the organic solvent in water is given.

†Standard $1,25(OH)_2D_3$ (50 pg) or $25(OH)D_3$ (25 ng) and the corresponding tritium-labeled compound (5000 dpm) dissolved in the buffer C–ethanol (10:1, v/v; 2.1 ml) were applied to the column-11 (immunosorbent, 1 ml). After washing with water (10 ml), the column was eluted with the aqueous organic solvent (10 ml) shown above, and the radioactivity of the effluent was measured to estimate the recovery rate of each compound.

‡Standard $1,25(OH)_2D_3$ (100 pg) or $25(OH)D_3$ (40 ng) and the corresponding tritium-labeled compound (5000 dpm) dissolved in 25% acetonitrile (5 ml) were applied to the column-3 (immunosorbent, 1 ml). After washing with water (5 ml) and the aqueous organic solvent (5 ml) shown above, the column was eluted with 95% methanol (5 ml), and the radioactivity of the effluent was measured to estimate the recovery rate.

Preparation of immunosorbents

Two kinds of immunosorbents were prepared by immobilizing the Ab-3 or -11 on a Sepharose® 4B using CNBr [18, 19]. The mean (± SD) coupling yields of the added antibody, which were calculated from the absorption at 280 nm due to unreacted antibody in repeated experiments, were 98 (1.0)% for the Ab-3 (n=3) and 95 (4.5)% for the Ab-11 (n=5), respectively. To estimate the binding capacity of the resulting immunosorbents, increasing amounts of $1,25(OH)_2D_3$ were added to the column-3. After washing with water, the adsorbed $1,25(OH)_2D_3$ on the column was eluted with the aqueous methanol, and the recovery rate was calculated using the $[^3H]1,25(OH)_2D_3$ as a tracer. The recovery was over 90% even when 100 ng of the metabolite was applied, showing that the immunosorbent has sufficient capacity for practical use (data not shown).

Optimization of IAC procedure

The Tandem IAC procedure was set up as follows. Standard $1,25(OH)_2D_3$ or $25(OH)D_3$ was added to each column, and their retention behavior was examined using the corresponding tritium-labeled compound as a tracer (in detail, see legend of Table 2). The latter compound is one of the most abundant and interfering endogenous metabolites which should be removed as much as possible at the pretreatment stage: thus its recovery rate is available as an index for obtaining higher selectivity. Considering the cross-reactivity of the Ab-11 with $1,25(OH)_2D_3$, we expected that the metabolite could be quantitatively recovered from the column-11 in the non-adsorbed (passed-through) fraction, leaving most of the $25(OH)D_3$ retained on this column. However, the column-11 also adsorbed $1,25(OH)_2D_3$ under the condition that $25(OH)D_3$ was sufficiently retained. This discrepancy can be explained in relation to the heterogeneity of this polyclonal antibody: the Ab-11 may contain a large amount of an antibody subpopulation which cross-reacts with $1,25(OH)_2D_3$ with relatively low affinity. Thus, we next attempted to elute the retained $1,25(OH)_2D_3$ selectively from the column with an aqueous organic solvent. Among various solvent systems tested, 25% acetonitrile gave the best result (Table 2). It may be worth pointing out that the retention behavior was obviously affected by the kind of organic solvent: e.g., aqueous acetone provided only a poor selectivity under conditions where $1,25(OH)_2D_3$ was satisfactorily recovered. On the other hand, column-3 should quantitatively retain $1,25(OH)_2D_3$ contained in the effluent from column-11. After dilution with water to reduce the acetonitrile concentration, we applied the effluent to column-3. The column was then washed with various solvents and the recoveries in the adsorbed fraction eluted with the aqueous methanol were compared (Table 2). Although only slight selectivity was observed in all the tested solvents, which was again somewhat inconsistent with the cross-reactivity of the Ab-3 with $25(OH)D_3$, 20% acetone was found to be superior to the others. The volume of immunosorbent in each column (column-11, 2 ml; column-3, 1 ml) was determined on the basis that endogenous $1,25(OH)_2D_3$ contained in 2 ml of plasma could be recovered at a rate comparable to that in the above

Table 3. Recovery rates of various vitamin D derivatives through the Tandem IAC-based pretreatment

Compound	Added (ng)	Recovery (%)*	*n*
$1,25(OH)_2D_3$	—	66.4 (5.0)	55
Vitamin D_3	—	0.6 (0.4)	4
$25(OH)D_3$	—	2.6 (0.2)	4
$24,25(OH)_2D_3$	—	1.1 (0.3)	4
$1,25(OH)_2D_2$	100	3.2	2
$25(OH)D_33S$	10	15	2
	100	12	2
$25R,26(OH)_2D_3$	10	<1	2
	100	<0.1	2
$25S,26(OH)_2D_3$	10	<1	2
	100	<0.1	2
$1,24,25(OH)_3D_3$	10	24	2
	100	20	2
1,25-Lactone	100	4.2	2

*The recovery rates of the corresponding tritium-labeled compounds from a pooled plasma [mean (±SD)] or the recovery rates of standard compounds added to the plasma, which are measured by RIA (upper or lower column relative to the dotted line).

experiment (data not shown). The final step of the procedure, that is washing the $1,25(OH)_2D_3$ fraction dissolved in methylene chloride with water, was intended to remove possible free antibodies leaked out from the affinity columns [20, 21] which, if any, will interfere with the following RRA.

Selectivity of the pretreatment based on Tandem IAC

The recovery rates of $1,25(OH)_2D_3$ and $25(OH)D_3$ through ethyl ether extraction were about 90 and 35%, respectively. This step was necessary to obtain a satisfactory recovery rate in subsequent Tandem IAC. As shown in Table 3, the recovery rate of $1,25(OH)_2D_3$ through the present pretreatment procedure, that is ethyl ether extraction followed by Tandem IAC, was comparable to those in the methods using the C18/OH cartridge (64.4–68.9%) [8] and was reproducible. On the contrary, the recoveries of related endogenous vitamin D derivatives were lower than 5%, except for $25(OH)D_3$ 3S and $1,24,25(OH)_3D_3$ which showed somewhat higher values. Thus, the present method efficiently removed $25(OH)D_3$ and $24,25(OH)_2D_3$, which are the major metabolites interfering with $1,25(OH)_2D_3$ assays. It was also shown that $1,25(OH)_2D_2$ was substantially separated: this is a unique feature of this method utilizing an antibody specific to the 25-monohydroxylated vitamin D_3 side chain (the Ab-3).

Sensitivity and specificity of RRA

The present RRAs, using chick intestinal VDR and calf thymus VDR gave similar dose–response curves (Fig. 1). The detection limit, defined as the amount of $1,25(OH)_2D_3$ required to give a B/B_0 of 2 SD below, was 0.5 and 0.3 pg per tube, with the intestinal

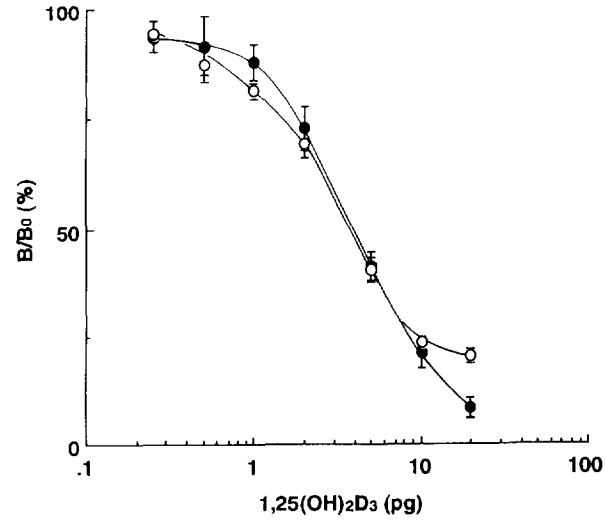

Fig. 1. Dose–response curves of the $1,25(OH)_2D_3$ RRAs using the chick intestinal VDR (●) or calf thymus VDR (○). Bars represent mean±SD for quadruplicate determinations.

and thymus VDR, respectively. The cross-reactivities of the VDRs examined in our laboratory were also similar to each other (Table 1) and were compatible with those in previous reports [3]. Thus, high cross-reactivities were observed with $1,25(OH)_2D_2$, $1,24,25(OH)_3D_3$ and $1,24(OH)_2D_2$, which is a synthetic analog of $1,25(OH)_2D_3$ having potent calcemic activity [22]. On the other hand, the other 9 derivatives showed fairly low values, although the cross-reactivity with $25(OH)D_3$ could not be ignored taking into account the fact that its serum/plasma levels are about 1000-fold higher than those of $1,25(OH)_2D_3$ [1–3].

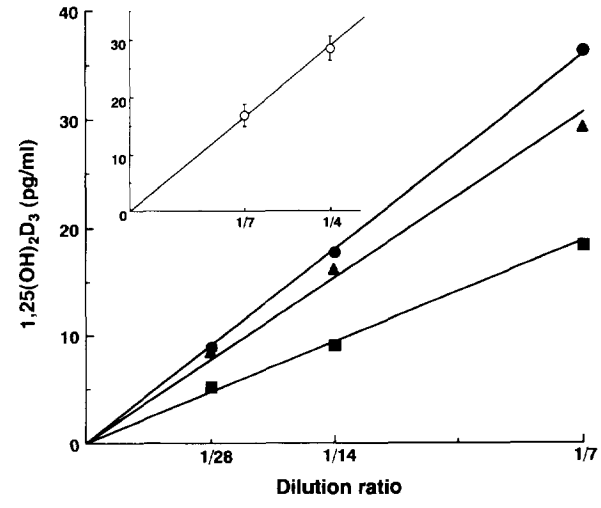

Fig. 2. Serial dilution study in the RRA using the intestinal VDR with the Tandem IAC-based pretreatment. Plasma $1,25(OH)_2D_3$ levels of normal subjects (n=3; ●, ▲ and ■) obtained with the 1/14 and 1/28 samples are represented without correction by the dilution factor against the 1/7 samples. In the inset, the level of a 1/7 sample is shown as above against the corresponding 1/4 sample (○): the bars express mean±SD for the within-assay variance (n=3).

Table 4. Analytical recovery of 1,25(OH)$_2$D$_3$ from pooled human plasma in the RRA using the intestinal VDR with the Tandem IAC-based pretreatment

1,25(OH)$_2$D$_3$ pg/ml			
Added	Expected	Observed	Recovery (%)
0	–	26.3	–
25	51.3	49.1 ± 1.5*	95.7 ± 2.9
50	76.3	74.7 ± 2.4*	97.9 ± 3.1
100	126.3	118.6 ± 3.7*	93.9 ± 2.9

*Mean ± SD for the within-assay variance (n=6).

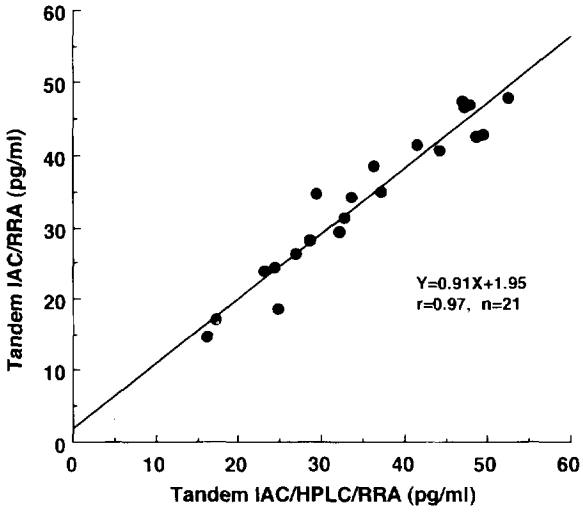

Fig. 3. Correlation between plasma 1,25(OH)$_2$D$_3$ levels of normal subjects (n=21) obtained by the Tandem IAC/RRA (y) and the Tandem IAC/HPLC/RRA (x).

Applicability of Tandem IAC to RRA using chick intestinal VDR

Good paralellism was observed between the 1,25(OH)$_2$D$_3$ assay values and the dilution ratio (1/7, 1/14 and 1/28) of the pretreated samples. A linear relationship between a further lower dilution (1/4) and that of 1/7 was also confirmed for measuring fairly low 1,25(OH)$_2$D$_3$ levels in the patients with chronic renal failure (Fig. 2). As shown in Table 4, satisfactory mean recovery rates (93.9–97.9%) were

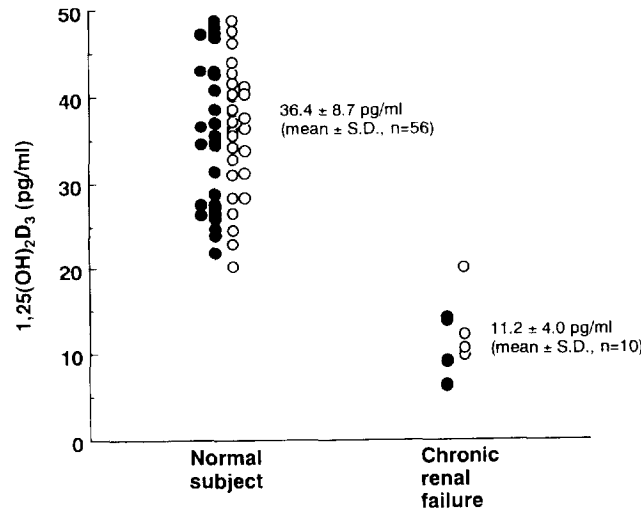

Fig. 4. Plasma 1,25(OH)$_2$D$_3$ levels of normal subjects and patients with chronic renal failure obtained by the RRA using the intestinal VDR with the Tandem IAC-based pretreatment. The plasma specimens were collected from both men (○) and women (●).

obtained. The plasma 1,25(OH)$_2$D$_3$ levels of healthy subjects determined by Tandem IAC/RRA (the present method) and those by Tandem IAC/HPLC/RRA (the reference method) were quite similar (Fig. 3). As for the measurement of 1(OH)D$_3$-administered volunteers, a slight overestimation (maximum 1.2-fold; e.g. 31h after administration, 57.8 vs 48.3 pg/ml) was observed with the present method. However, these assay values were compatible with those by the reference method and acceptable for routine clinical studies. Within- and between-assay coefficients of variation for the measurement of healthy volunteers were below 10% (Table 5).

The assay linearity was obtained in 0.75, 1.0, 1.5 and 2.0 ml of plasma. Using the maximum applicable volume (2.0 ml), 1.5 pg/ml of 1,25(OH)$_2$D$_3$ can be measured with the sample dilution of 1/4, regarding the mean recovery rate of the metabolite through the pretreatment and the detection limit of the RRA (see above). Thus, the practical sensitivity of the assay system was comparable to or higher than that of previous RRA systems [3, 6–9] and was high enough to measure the plasma levels of the patients with chronic

Table 5. Precision of the RRA using the intestinal VDR with the Tandem IAC-based pretreatment

	Within-assay (n=8)*			Between-assay (n=6)†	
Plasma	Mean 1,25(OH)$_2$D$_3$ (pg/ml)	CV (%)	Plasma	Mean 1,25(OH)$_2$D$_3$ (pg/ml)	CV (%)
A (1/7)	14.2	9.9	B (1/7)	25.5	8.6
(1/14)	14.7	7.5	(1/14)	26.9	9.3

*A pooled plasma from healthy volunteers (A; 2.0 ml) was pretreated in octuplicate. The 1,25(OH)$_2$D$_3$ fractions obtained were each diluted with ethanol to produce 1/7 and 1/14 samples, which were simultaneously submitted to the RRA.

†The pooled plasma (2.0ml) was singly pretreated and measured by the RRA as above, and this procedure was repeated five more times.

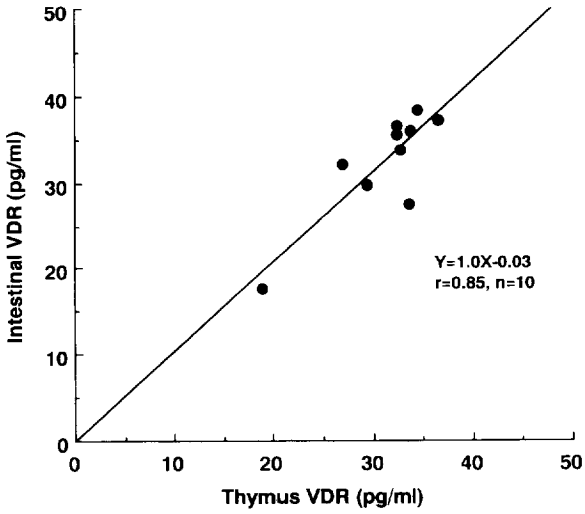

Fig. 5. Correalation between plasma $1,25(OH)_2D_3$ levels of normal subjects (n=10) obtained by the Tandem IAC/RRA using the intestinal VDR (y) and the thymus VDR (x).

renal failure. The normal value of plasma $1,25(OH)_2D_3$, obtained by 56 samples from apparently healthy blood donors (27 men and 29 women), was 36.4 ± 8.7 (mean ± SD) pg/ml and no sex related difference was observed. As for 10 patients with chronic renal failure (6 men and 4 women), apparently lower $1,25(OH)_2D_3$ levels (11.2 ± 4.0 pg/ml) were obtained (Fig. 4).

Applicability of Tandem IAC to RRA using calf thymus VDR

The plasma $1,25(OH)_2D_3$ levels of healthy subjects were determined by two RRA procedures using the intestinal VDR or the thymus VDR after the present pretreatment (Fig. 5). The assay values obtained with the thymus VDR correlated well with those measured with the intestinal VDR.

DISCUSSION

The main subject in developing an improved $1,25(OH)_2D_3$ assay has been to omit the preparative HPLC step, which is tedious and time-consuming but was essential in the conventional methods (intestinal VDR-based RRAs and the RIAs) to remove accompanying cross-reactive secosterols and non-specific interfering substances such as lipids. Although a few non-HPLC assays (e.g. RRAs [23, 24] and an RIA [25]) had been reported, they seemed to leave much room for further validation by measuring samples from vitamin D-supplemented volunteers or patients and/or comparison with a reasonable reference method (validity of some previous non-HPLC RRAs has been discussed in ref. [6]). On the other hand, calf thymus VDRs were found to be less sensitive to lipid interference than those from the chick intestinal VDRs and useful for developing non-HPLC RRAs [5,6]. These assays are now carried out in combination with sample purification based on phase-switching elution on C18/OH cartridges [7–9]. This pretreatment method itself has, however, somewhat limited efficiency for purification, as clearly shown by the fact that it afforded extraordinarily high assay values in an RRA using a chick intestinal VDR [9]. It is also presumed that the use of strictly selected cartridges containing octadecylated and non-endo-capped silanol groups in a suitable ratio should be crucial for performing reproducible purification with a defined solvent system for elution: this was suggested in the first paper reporting a prototype of these assays using a C18 cartridge in place of the C18/OH cartridge [7].

A pretreatment procedure based on IAC should be a valuable and attractive tool in developing simplified $1,25(OH)_2D_3$ assays, because it should provide custom-made selectivity by immobilizing an antibody having the desired specificity which itself would be producible by immunization with a well-designed haptenic derivative. Thus, the method is expected to offer higher efficiency, as well as reproducibility due to the high stability of the immobilized antibodies, for sample purification so as to facilitate the RRAs even with intestinal VDR. Indeed, we have recently developed the first enzyme immunoassay for human plasma $25(OH)D_3$ by employing an IAC as a pretreatment [26]. This is also the first report dealing with the application of IAC in vitamin D analysis. However, when we undertook this study, such a highly specific antibody, which permits practically selective extraction of $1,25(OH)_2D_3$ from blood, had not been generated due to difficulty in synthesizing suitable haptenic derivatives. Thus, we devised the Tandem IAC, utilizing two affinity columns (column-3 and -11) each containing an immobilized antibody (Ab-3 and -11) having different specificity. Although each of these antibodies alone has only limited utility for this purpose, tandem use of them allowed efficient purification of $1,25(OH)_2D_3$ from plasma by overlapping and focusing their specificity on this metabolite. Namely, a plasma extract was first applied to column-11 immobilizing the Ab-11 which binds more strongly with $25(OH)D_3$, $24,25(OH)_2D_3$ and $25,26(OH)_2D_3$, than with $1,25(OH)_2D_3$. Thus, a large part of these interfering 1α-deoxy-type metabolites should be adsorbed on the column, while the compounds reacting only weakly with the Ab-11, involving $1,25(OH)_2D_3$, can be preferentially eluted. The effluent containing $1,25(OH)_2D_3$ was then submitted to column-3. Because the Ab-3 immobilized therein is specific to vitamin D_3 derivatives, having a 25-monohydroxylated side chain, the metabolites with a different side chain (e.g. with no hydroxy group, dihydroxylated, lactonized or that of vitamin D_2 derivatives) should be efficiently washed out of the column, together with interfering substances of vitamin D-unrelated structure (e.g. lipids and incompletely removed proteins).

In fact, the Tandem IAC-based pretreatment satisfactorily removed interfering vitamin D derivatives, as shown in Table 3. Only $25(OH)D_3$ 3S and $1,24,25(OH)_3D_3$ showed somewhat high recovery rates. It is reasonable that the sulfate co-migrates with $1,25(OH)_2D_3$ through the Tandem IAC, considering the specificity of the antibodies. Thus the partial selectivity observed here will come from the ethyl ether extraction step. However, this should not induce serious overestimation in the RRAs, in spite of the relatively high concentration of the sulfate in blood [27], because the metabolite is nearly completely discriminated by the VDRs (Table 1). On the other hand, the recovery of $1,24,25(OH)_3D_3$ was rather discrepant with the cross-reaction of Ab-3, and this might also be ascribable to the antibody heterogeneity as described above (see Results). The contamination of $1,24,25(OH)_3D_3$ with the observed ratio, however, did not seem to affect the assay values at least for the normal subjects, because of its fairly lower plasma levels than those of $1,25(OH)_2D_3$ [28]. The result that IAC mostly removed $1,25(OH)_2D_2$ should also be discussed. Although this feature provides an assay specific to the D_3 form of the active metabolite without chromatographic separation, it might restrict application to patients treated with a large amount of vitamin D_2 or its analogs.

To evaluate the purification efficiency of the Tandem IAC-based pretreatment in comparison with previous methods, we examined its applicability to an RRA using a chick intestinal VDR. Serial dilution and analytical recovery studies and comparison with a reference method involving preparative HPLC (Tandem IAC/HPLC/RRA) demonstrated that this Tandem IAC/RRA system is satisfactorily accurate for the measurement of normal subjects. The accuracy of the method was, however, further evaluated by measuring the plasma levels of $1(OH)D_3$-supplemented volunteers. This compound is a synthetic analog of $1,25(OH)_2D_3$, which is often prescribed in the treatment of various diseases accompanying a disorder in vitamin D metabolism and osteoporosis. It has been pointed out that oral administration of vitamin D_3 or its analogs, such as this agent, significantly increases the plasma levels of some metabolites cross-reactive with VDRs [8] (i.e. $1,24,25(OH)_3D_3$ and 1α,25,26-trihydroxyvitamin D_3 are conceivable [29]), and thus especially careful pretreatment of the specimens is required to obtain the correct $1,25(OH)_2D_3$ concentration. The Tandem IAC/RRA system gave slightly higher values compared with the reference method, but practically acceptable assay values. A little overestimation observed here would be mainly ascribable to insufficiency of the pretreatment in removing $1,24,25(OH)_3D_3$ as referred to above.

The precision of this assay system was certified by within- and between-assay variance. The assay permitted measuring 1.5 pg/ml of plasma $1,25(OH)_2D_3$, and was sensitive enough to determine its levels in patients with chronic renal failure. The mean plasma $1,25(OH)_2D_3$ levels of healthy subjects and the patients with chronic renal failure obtained by the present method were in good agreement with those reported so far [3]. Although the present determination of normal subjects was done using 0.75–2.0 ml of plasma specimens at a 1/7 or larger dilution ratio, the volume can be reduced to 0.5 ml by measuring at the 1/4 dilution.

Thus, the Tandem IAC developed here enabled the first valid non-HPLC RRA using chick intestinal VDR. We have not yet obtained any extraordinary assay value with the present pretreatment method. It should be mentioned that the VDR used here is the same commercially available product as that affording an unsatisfactory result with the C18/OH cartridge [9] (referred to above). This pretreatment method was also shown to be applicable to the thymus VDR-based RRA. These results clearly indicated that the Tandem IAC has a higher efficiency for purification of $1,25(OH)_2D_3$ from plasma, compared with various mini-columns and cartridges (including the C18/OH cartridge) previously used for the pretreatment of this metabolite. This encourages us to apply the IAC-based pretreatment in developing the analyses of other minor vitamin D metabolites, such as $1,25(OH)_2D_2$, $1,24,25(OH)_3D_3$ and 1,25-lactone. If an antibody specific enough to the target analyte is difficult to produce, tandem use of two (or more) antibodies, each of which recognizes a different partial structure and is easier to obtain, as in this study, will provide a satisfactory pretreatment system. In some cases, a successful result could be obtained even with a Tandem IAC where an antibody (an immunosorbent) is repeatedly used.

By the use of the CNBr method [18, 19], immunosorbents having a high binding capacity could be prepared with excellent reproducibility and a much higher coupling yield of antibody than achieved in our previous study, where an oligosaccharide moiety-based immobilization was employed [26]. They could be stored for at least 6 months without appreciable decrease in the binding activity and were repeatedly usable more than ten times. The Tandem IAC allowed simultaneous processing of about 20 specimens within 4–5 h without careful handling or strict time-management of the entire operation. It will be possible to shorten the time by carrying out the chromatographic procedure under reduced pressure utilizing a commercially available vacuum rack. Recently, a non-HPLC RRA system, which seems to be well validated, has been reported where a VDR prepared from porcine intestine was employed in combination with a bound/free separation method based on a monoclonal anti-VDR antibody [10]. A recent paper on a non-HPLC RRA utilizing a VDR from bovine mammary gland should also be noted [30].

In conclusion, the Tandem IAC has proved to be a powerful pretreatment tool for $1,25(OH)_2D_3$ in human plasma. This method will also be useful for developing immunoassay or gas chromatography–mass spectrometry of the metabolite as well as RRA. If an antibody having higher specificity recognizing both the A-ring and side chain structures of $1,25(OH)_2D_3$ is available, more feasible IAC based on a single affinity column will be established. We are now developing a new "Simplified" IAC utilizing highly specific antibodies which have recently been generated against a new haptenic derivative [31]. Details of these results will be reported subsequently.

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