Production and specificity of antisera raised against 25-hydroxyvitamin D_3-[C-3]–bovine serum albumin conjugates

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In order to obtain specific antisera for use in the enzyme immunoassay of 25-hydroxyvitamin D_3, three hapten-carrier conjugates having different lengths of bridges at the C-3 position were prepared from 25-hydroxyvitamin D_3 by coupling with bovine serum albumin using the active ester method. The specificity of anti-25-hydroxyvitamin D_3 antisera elicited in rabbits was tested by a cross-reaction study with closely related secosterols and by measuring the plasma levels of 25-hydroxyvitamin D_3 by means of radioimmunoassay using tritium-labeled antigen. The results indicated that the specificity of the antisera obtained is higher than that of vitamin D-binding protein, and that some of these antisera are suitable for enzyme immunoassay. (*Steroids* **57**:488–493, 1992)

Keywords: sterols; 25-hydroxyvitamin D_3; hapten-[C-3]–carrier conjugates; antiserum production; specificity; radioimmunoassay

Introduction

Humans derive their vitamin D [vitamin D_2 (D_2) and vitamin D_3 (D_3)] from two sources. D_3 is mainly biosynthesized in the skin, upon irradiation of provitamin D_3 with ultraviolet (UV) light, whereas D_2 is absorbed solely from the diet. The values of D_2 and its metabolites in biological fluids are usually less than one tenth of those of D_3 and its metabolites. Vitamin D is converted in the liver to 25-hydroxyvitamin D [25-OH-D], which itself is further metabolized in the kidney to 1_{α},25-dihydroxyvitamin D [1,25-$(OH)_2$D] and other compounds. Although about 40 metabolites have been identified in in vivo and/or in vitro experiments, 25-OH-D is the major circulating metabolite and 1,25-$(OH)_2$D is the most potent form of vitamin D in the regulation of calcium and bone resorption.

The levels of 25-OH-D_3 are accepted as a good index of the overall status of vitamin D in the body, and correspond well both with intake of D_3 and exposure to sunlight. The determination of 25-OH-D_3 in serum/plasma is usually accomplished by a competitive protein-binding assay (CPBA) using vitamin D–binding protein (DBP) or high-performance liquid chromatography (HPLC). However, the specificity of these assays is not high enough; thus, some forms of chromatography must be used before assay to remove the other metabolites and accompanying interfering substances.[1] On the other hand, a few antibodies against 25-OH-D_3 have been produced [2,3] and used for radioimmunoassay (RIA), but enzyme immunoassay (EIA) of 25-OH-D_3 has not been reported. Compared with RIA or CPBA, EIA has several advantages, due to its not requiring a radioactive compound; that is, the assay is as sensitive as RIA and can be performed inexpensively, without special facilities and techniques.

To obtain specific antisera for use in EIA of 25-OH-D_3, three hapten-carrier conjugates having different lengths (succinyl, glutaryl, and glutarylglycinyl) of bridges at the C-3 position of 25-OH-D_3 were prepared. Various anti–25-OH-D_3 antisera were then elicited in rabbits against these conjugates, and their specificity was examined by RIA using a tritium-labeled antigen.

Experimental

UV spectra were obtained on a Union Giken SM-401 spectrophotometer (Osaka, Japan). Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded at 400 MHz on a JEOL JNM-GX-400 spectrometer (Tokyo, Japan) by using tetramethylsilane

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as an internal standard. Abbreviations used are s (singlet), d (doublet), m (multiplet), and br (broad). Radioactivities were measured with an Aloka LSC-1000 liquid scintillation spectrometer (Tokyo, Japan). Column chromatography was performed by using Silica gel 60 (70-230 mesh; E. Merck, AG, Darmstadt, Germany). D_3 and 25-OH-D_3 were obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan) and Duphar Co. (Amsterdam, The Netherlands), respectively. 1,25-$(OH)_2D_3$, 24R,25-dihydroxyvitamin D_3 [24,25-$(OH)_2D_3$], and 25-OH-D_2 were supplied by Chugai Pharmaceutical Co. (Tokyo, Japan). [26,27-Methyl-^{3}H]-25-OH-D_3 (180 Ci/mmol) was purchased from Amersham Japan Co. (Tokyo, Japan). Polyvinyl alcohol (average mol wt 2000) was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). Bovine serum albumin (BSA, crystallized) and complete Freund's adjuvant were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and DIFCO Laboratories (Detroit, MI, USA), respectively. Immunobead second antibody (goat anti-rabbit immunoglobulin; DASP beads) was obtained from Bio-Rad Laboratories (Richmond, CA, USA). All other reagents and solvents were of analytical grade.

Syntheses of haptenic derivatives

25-Hydroxyvitamin D_3 3-hemisuccinate (HS)[2]. Succinic anhydride (309 mg) was added to a solution of 25-OH-D_3 (25 mg) in pyridine (0.4 ml), and the mixture was stirred at room temperature for 4 days. After the addition of water (~0.05 ml) at 0 C, the mixture was stirred for 1 hour, and then extracted with AcOEt. The organic layer was washed with water, dried over anhydrous Na_2SO_4 and evaporated down. The crude product was subjected to column chromatography [20 × 1 cm internal diameter (ID)], and elution with $CHCl_3$/MeOH (16 : 1) gave the desired compound (HS) as a colorless oily substance (31.9 mg). UV (EtOH) λ_{max} 265 nm, λ_{min} 229 nm; ^{1}H-NMR ($CDCl_3$) δ 0.52 (3H, s, 18-H), 0.93 (3H, d, J = 6.4 Hz, 21-H), 1.20 (6H, s, 26,27-H), 2.61 (4H, s, $COCH_2CH_2CO$), 4.82 (1H, d, J = 2.1 Hz, 19-H), 4.95 (1H, m, 3-H), 5.03 (1H, br s, 19-H), 5.98 (1H, d, J = 11.1 Hz,7-H), 6.19 (1H, d, J = 11.1 Hz, 6-H).

25-Hydroxyvitamin D_3 3-hemiglutarate (HG)[4]. Glutaric anhydride (759 mg) was added to a solution of 25-OH-D_3 (53 mg) in pyridine (0.4 ml), and the mixture was stirred at room temperature for 4 days. After the work-up described earlier, the crude product was subjected to column chromatography (20 × 1 cm ID), and elution with $CHCl_3$/MeOH (20 : 1) gave the desired compound (HG) as a colorless amorphous substance (63.8 mg). UV (EtOH) λ_{max} 265 nm, λ_{min} 229 nm; ^{1}H-NMR ($CDCl_3$) δ 0.54 (3H, s, 18-H), 0.93 (3H, d, J = 6.4 Hz, 21-H), 1.22 (6H, s, 26,27-H), 4.84 (1H, br s, 19-H), 4.96 (1H, m, 3-H), 5.06 (1H, br s, 19-H), 6.03 (1H, d, J = 11.1 Hz, 7-H), 6.21 (1H, d, J = 11.1 Hz, 6-H).

25-Hydroxyvitamin D_3 3-glutarylglycine (GG). N-Hydroxysuccinimide (38 mg) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide · HCl (EDC) (62 mg) were added to a solution of HG (52 mg) in 95% dioxane (0.2 ml), and the mixture was stirred at room temperature for 3 hours. The resulting solution was diluted with AcOEt, washed with water, dried over anhydrous Na_2SO_4, and evaporated down. A portion of the residue containing N-succinimidyl ester of HG (29 mg; determined by UV spectra at 265 nm on the assumption that the molar extinction coefficient is 18,000) was dissolved in pyridine (0.3 ml). To this solution were added glycine (30 mg) and water (0.2 ml), and the mixture was stirred at room temperature for 1 hour. After the usual work-up, the crude product was subjected to column chromatography (20 × 1 cm ID), and elution with $CHCl_3$/MeOH (4 : 1) gave the desired compound (GG) as a colorless amorphous substance (27.4 mg). UV (EtOH) λ_{max} 265 nm, λ_{min} 229 nm; ^{1}H-NMR [$CDCl_3$/CD_3OD(1 : 2)]δ 0.56 (3H, s, 18-H), 0.96 (3H, d, J = 6.4 Hz, 21-H), 1.19 (6H, s, 26,27-H), 3.77 (2H, br s, $NHCH_2CO$), 4.83 (1H, br s, 19-H), 4.94 (1H, m, 3-H), 5.08 (1H, br s, 19-H), 6.04 (1H, d, J = 11.0 Hz, 7-H), 6.22 (1H, d, J = 11.0 Hz, 6-H).

Preparation of hapten–BSA conjugates

HS–BSA and HG–BSA. To a solution containing the N-succinimidyl ester of HS or HG (~0.03 mmol) in pyridine (0.77 ml), prepared in the manner described earlier, was added BSA (0.5 μmol) in 0.05 M phosphate buffer (pH 7.4, 0.77 ml). The mixture was stirred overnight at 4 C, and then dialyzed against cold water for 1 day. After addition of acetone and a small amount of NaCl, the resulting suspension was centrifuged at 4 C (1,000 × *g* for 20 minutes), and the supernatant was discarded. The procedure was repeated until free secosterol was removed. The precipitate was dissolved in 20% pyridine (3.0 ml) and dialyzed as described earlier. Lyophilization of the resulting solution afforded the desired conjugate (HS–BSA, HG–BSA) as fluffy powder (each ~30 mg).

GG–BSA. *p*-Nitrophenol (14.4 mg) and EDC (20 mg) were added to a solution of GG (15 mg) in dimethylformamide (0.4 ml), and the resulting solution was stirred at room temperature for 2 hours. The reaction mixture was diluted with AcOEt, washed with water, dried over anhydrous Na_2SO_4, and evaporated down. The obtained crude *p*-nitrophenyl ester was dissolved in pyridine (0.47 ml), and BSA (20 mg) in 0.05 M phosphate buffer (pH 7.4, 0.47 ml) was added to the solution, which was stirred at 4 C overnight. Removal of the unreacted secosterol and lyophilization in the manner described earlier gave the desired conjugate (GG–BSA) as fluffy powder (18 mg).

Determination of the molar ratio of hapten to BSA

UV spectra of each conjugate (~1.2 mg) and BSA (as a control, ~5.5 mg) were measured in 5% sodium lauryl sulfate (4.0 ml). The absorbance due to the hapten molecules at 265 nm was estimated by comparing the spectra of the conjugate with those of BSA. The number of hapten molecules linked to a BSA molecule was calculated on the assumption that the molar extinction coefficient of each hapten is 18,000.

Production of antisera

Four domestic female albino rabbits (3 months old) were used for immunization with each BSA conjugate. The suspension of conjugate (0.2 mg) in sterile isotonic saline (0.5 ml) was emulsified with complete Freund's adjuvant (0.5 ml), and the emulsion was injected subcutaneously at multiple sites along the back. This procedure was repeated biweekly (rabbits 1 and 2) or monthly (rabbits 3 and 4) for 4 months. Then, all the rabbits received five booster injections of the conjugate (1.0 mg) at biweekly intervals. Furthermore, additional immunizations of the same dose were done for HS–BSA and HG–BSA twice and four times, respectively. Blood was collected 7–10 days after the last injection, and allowed to stand at room temperature for 4–6 hours. Centrifugation at 4 C (1,000 × *g* for 15 minutes) afforded the antiserum as supernatant, which was stored at −20 C until use. Each antiserum was abbreviated indicating the hapten (HS–, HG–, GG–) and the number (1–4) of the rabbit from which it was produced.

Assay buffer

The sodium phosphate buffer (0.05 M, pH 7.3) containing 0.1% gelatin, 0.9% NaCl, 0.1% NaN_3, and 1% polyvinyl alcohol was used in the following RIA.

RIA procedure

Diluted antiserum (0.1 ml) and DASP beads suspension (4.0 mg/ml, 0.75 ml) prepared with the assay buffer were incubated at 4 C for 2 hours. After addition of the buffer (1.0 ml), the mixture was centrifuged at 4 C (500 × *g* for 10 minutes), and the supernatant was aspirated off. The precipitate was washed once and resuspended with 1.0 and 0.5 ml of the buffer, respectively. To these suspensions were added a series of 25-OH-D_3 standards (0–2,000 pg) or plasma extracts, and [^{3}H]-25-OH-D_3 (~15,000 disintegrations per minute (dpm)), which were dissolved in EtOH (each 25 μl). After being incubated at 4 C for 2 hours followed by addition of the buffer (0.35 ml), the mixtures were centrifuged at 4 C (500 × *g* for 10 minutes). The aliquot (0.5 ml) of supernatant was transferred into a scintillation vial containing the tT-21 scintillation cocktail (4.0 ml),[5] and the radioactivity was measured.

Cross-reaction study

The cross-reactivities of antisera were tested with four kinds of secosterols having structures related to 25-OH-D_3. The relative amounts required to reduce the initial binding of [^{3}H]-25-OH-D_3 by half, where the mass of unlabeled 25-OH-D_3 was arbitrarily taken as 100%, were calculated from the standard curves.[6]

Extraction of 25-hydroxyvitamin D_3 from plasma

Plasma specimens were obtained from three healthy volunteers in July, and extracted according to the reported method,[7] which was modified as described later. To the plasma (0.2 ml) was added [^{3}H]-25-OH-D_3 (~6,000 dpm) in EtOH (10 μl), and the solution was left at room temperature for 30 minutes. Then, $CHCl_3$ (0.25 ml) and MeOH (0.5 ml) were added, vortex-mixed, and the mixture was allowed to stand at 4 C for 1 hour. After addition of $CHCl_3$ (0.25 ml) and water (0.2 ml), the mixture was centrifuged at 4 C (1,000 × *g* for 15 minutes). The $CHCl_3$ layer was washed once with water (0.15 ml), and evaporated down under an N_2 gas stream. The residue was dissolved in EtOH (1.6 ml), an aliquot of which was used for radioactivity counting to estimate the recovery rate. The remaining solution was submitted for assay.

Results

Preparation of hapten–BSA conjugates

Three kinds of 25-OH-D_3–BSA conjugates were prepared as follows. Haptens HS and HG were obtained by acylation of the hydroxy group at the 3 position of 25-OH-D_3 with a large excess (~50 Eq) of succinic and glutaric anhydrides, respectively. Hapten GG was synthesized by condensing HG with glycine via its N-succinimidyl ester. All the reactions used here were performed at room temperature with satisfactory yield. The structures of these haptens were confirmed by UV absorption (265 nm; D triene structure) and ^{1}H NMR spectra.

The conjugates, HS–BSA and HG–BSA, were prepared by the N-succinimidyl ester method.[8] On the other hand, hapten GG was linked with BSA via its *p*-nitrophenyl ester to give GG–BSA,[9] because the conversion of GG into its N-succinimidyl ester was unsuccessful due to the formation of several by-products. The hapten/BSA molar ratio of these conjugates was determined to be 18, 24, and 7 for HS–BSA, HG–BSA, and GG–BSA, respectively. These values were judged to be sufficient for their use as immunogens.

Production of antisera

Because of the possibility that the rabbits might suffer from vitamin D intoxication, immunization was started with two different schedules (biweekly and monthly intervals) using a relatively small dose of the conjugate (0.2 mg/body). However, such an undesirable symptom was not observed for 4 months. Therefore, all of the rabbits were further immunized with an increased dose at biweekly intervals. After 5–9 months from the primary immunization, the blood was drawn and the antisera (HS–, HG–, and GG–,1~4) were separated.

The titer of the antiserum in RIA was determined as the final dilution that was capable of binding 50% of the labeled antigen. Nine antisera of 12 gave satisfactory results (titer >1 : 5,000) that warranted further consideration (Table 1).

Dose–response curve

Every antiserum gave a dose–response curve showing the useful range of 20–2,000 pg/tube. The typical example obtained with antiserum HG-2 is shown in Figure 1. The amounts of 25-OH-D_3 required to displace 50% of labeled antigen (midpoint) in each standard curve are also shown in Table 1.

Cross-reaction study

The cross-reactions of antisera with four closely related secosterols are listed in Table 1. Every antiserum showed a similar pattern of cross-reactivity, although some degree of variation was observed, which might be ascribed to the individual differences among rabbits for immune response. Little effect of the bridge length on the specificity was observed. The values obtained with 24,25-$(OH)_2D_3$ (8–27%) and 25-OH-D_2 (<1–3.8%) were significantly low. On the contrary, DBP used in CPBA cross-reacts equally with both metabolites,[10,11] although avian DBP discriminates the latter compound.[12] In addition, only negligible values were obtained with D_3 by our antisera. On the other hand, higher reactivity with 1,25-$(OH)_2D_3$ than that with 25-OH-D_3 was found in almost all the antisera.

The four antisera (HS-3 and HG-1,-2,-4) were evaluated to be superior to the others on the basis that the cross-reactivity with 24,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ were below 20% and 200%, respectively.

Measurement of plasma 25-hydroxyvitamin D_3 levels

The 25-OH-D_3 levels of three plasma specimens obtained from healthy volunteers were measured by RIA using the selected antisera as described earlier (Table 2). The extraction using chloroform and methanol[7] was used as a pretreatment method. Observed values were corrected on the basis of the recovery rate of [^{3}H]-25-OH-D_3 added to each plasma sample. The recovery rate of tracer was 92.9 ± 8.7%, which was estimated

Table 1 Percent cross-reaction of antisera

Compound	Antiserum (titer[a])								
	HS-1 (1 : 22,000)	HS-2 (1 : 49,500)	HS-3 (1 : 27,500)	HS-4 (1 : 22,000)	HG-1 (1 : 16,500)	HG-2 (1 : 19,250)	HG-4 (1 : 55,000)	GG-2 (1 : 8,250)	GG-4 (1 : 12,650)
25-OH-D_3	100	100	100	100	100	100	100	100	100
D_3	<1	<1	<1	<1	<1	<1	<1	<1	<1
24,25-$(OH)_2D_3$	26	18	19	27	14	8.0	10	22	23
1,25-$(OH)_2D_3$	230	230	160	210	190	80	130	190	190
25-OH-D_2	<1	1.7	<1	<1	1.1	3.8	<1	<1	<1
Midpoint[b]	500	340	400	310	340	300	400	380	580

[a] The final dilution of antisera needed to bind 50% of labeled antigen.
[b] The amount of 25-OH-D_3 required to displace 50% of labeled antigen (pg/tube).

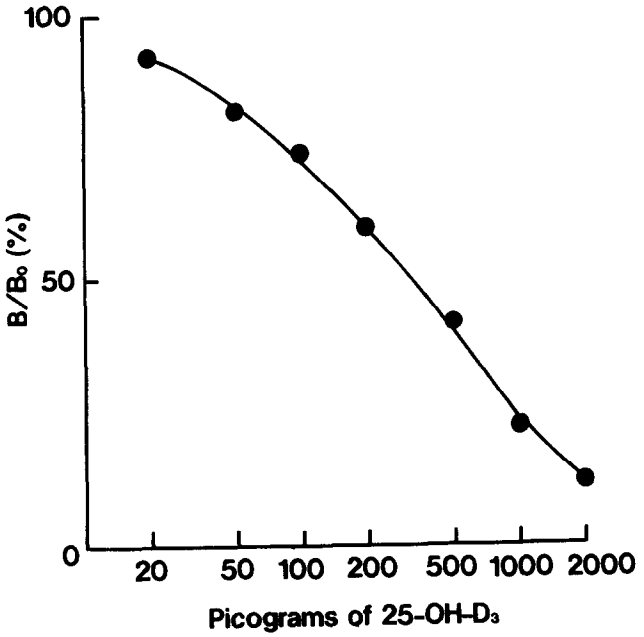

Figure 1 Dose–response curve for 25-OH-D_3 radioimmunoassay using antiserum HG-2.

Table 2 Plasma 25-hydroxyvitamin D_3 levels obtained by radioimmunoassay using various antisera (ng/ml)

Plasma sample[a]	Antisera			
	HS-3	HG-1	HG-2	HG-4
I	28.3	29.7	28.3	40.4
II	23.9	21.8	29.6	28.2
III	20.4	21.2	26.9	34.2

[a] Extracted with $CHCl_3$/MeOH.

separately by using many specimens (n = 28). The antisera HS-3, HG-1, and HG-2 gave similar values for each sample, which were in good agreement with those reported as normal 25-OH-D_3 levels.[13] On the other hand, HG-4 afforded values somewhat higher, suggesting that the specificity was lower than that of the other antisera.

Discussion

To establish a sensitive and specific EIA for a small molecule, including vitamin D metabolites, attention should be paid to designing the structure of the haptenic derivative for antibody production. It is well known that the specificity of antibodies is significantly influenced by the position on the hapten molecule used for the conjugation with a carrier protein. On the other hand, bridge heterologous combination between enzyme-labeled antigen and hapten-carrier conjugate is often required to gain satisfactory sensitivity. Hosoda et al.[14,15] reported that, in steroid EIA, the use of an enzyme-labeled antigen having a chemical bridge shorter than that used for antibody production (i.e., for linkage of hapten to carrier) is advantageous in increasing the assay sensitivity.

Taking these factors into account, the site for the linkage to the carrier should not only be remote from the functional groups characterizing the structure of the analyte, but also should be able to react with bridges of various length. The C-3 position of 25-OH-D_3 satisfies these requirements. Therefore, we synthesized three haptens (HS, HG, and GG) having different lengths of bridges and prepared their conjugates with BSA. Then, nine anti-25-OH-D_3 antisera having satisfactory titer were obtained by immunization of rabbits with these conjugates.

It is reported that antiserum exhibiting a lack of specificity in RIA using tritium as a tracer is also not specific in EIA.[16] This provides a good criterion for selecting a suitable antibody for EIA. Therefore, we examined the specificity of obtained antisera by using [^{3}H]-25-OH-D_3 as a labeled antigen. It should be mentioned that the RIA procedure was set up to avoid the interference by DBP in antiserum. Therefore, the competition reaction between labeled and unlabeled antigens was performed after separation of immunoglobulin from the other serum components by using DASP beads. In CPBA and RIA, some kinds of protein or detergent were added to the assay buffer to solubilize 25-OH-D_3 sufficiently, and to reduce its nonspecific adsorption onto the glass walls of assay tubes.[17] We examined several solubilizers with respect to their effect on nonspecific adsorption and antigen–antibody

reaction. Polyvinyl alcohol that had been reported to be excellent in CPBA[18] gave most favorable results in our RIA system, the details of which will be reported elsewhere.

Every antiserum afforded a dose–response curve with a sensitivity that was slightly higher than that of CPBA, and was high enough to measure plasma 25-OH-D_3 levels. The cross-reaction study indicated that all the antisera recognized the structure of the 25-OH-D_3 side-chain adequately, as expected from the hapten structure used. It is especially noteworthy that the cross-reactivity with 24,25-$(OH)_2D_3$, one of the major interfering metabolites of 25-OH-D_3 assays, was markedly lower than the cross-reactivity of DBP. The property of discriminating against 25-OH-D_2 is also remarkable. These results indicate that the antisera could be used in an immunoassay that can measure 25-OH-D_3 selectively without complicated pretreatments (including HPLC), which are nearly inevitable in CPBA to avoid the overestimation due to these metabolites. However, we must pay considerable attention to evaluating the assay values in assessing the vitamin D status of the patients when administering D_2 as a supplement. The assay might be of little clinical usefulness for these patients. On the other hand, unexpectedly high cross-reactivities were observed with 1,25-$(OH)_2D_3$ in all the antisera. Yamamoto and Matsuura[4] have reported a similar result: a monoclonal antibody specific to 1,25-$(OH)_2D_3$ rather than 25-OH-D_3 has been produced by using 25-OH-D_3 3-hemiglutarate (i.e., HG in this report) as hapten. They have also deduced that the ability of their antibody to recognize the 1 α-hydroxy group might be based on the conformational approach of the carbonyl group on the glutaryl bridge to the 1 α position in the hapten-carrier conjugate. This interpretation is interesting, whereas the explanation based on masking the 1α position, which is located relatively near the bridge, by carrier protein may also be possible.

However, the cross-reactivity with 1,25-$(OH)_2D_3$ will be negligible for practical use, because the serum/plasma levels of this metabolite are extremely low compared with those of 25-OH-D_3 (usually 1/100~1/1,000). Therefore, the specificity of four antisera (HS-3 and HG-1,-2,-4) that gave relatively favorable results were further investigated from the viewpoint of applicability to measurement of plasma levels. Plasma specimens were obtained from healthy volunteers and the Bligh-Dyer extraction[7] was used as a pretreatment method with a slight modification. On the basis that the assay values obtained with them were in agreement with those reported so far,[13] the antisera HS-3, HG-1, and HG-2 were judged to be satisfactorily specific for practical use. On the other hand, the antiserum HG-4 gave somewhat higher values. We interpreted the result that the antiserum HG-4 cross-reacts with some metabolites that were not tested in this study, and judged it to be less specific compared with the others.

In conclusion, we have succeeded in producing anti-25-OH-D_3 antisera reasonably specific in [^{3}H]-RIA, using the hapten–BSA conjugates linked through different lengths of bridges at the C-3 position of 25-OH-D_3. These antisera would be useful for developing a practical EIA of 25-OH-D_3. The antisera HG-1 and HG-2 in particular are expected to provide highly sensitive bridge heterologous EIA systems by the combination with an enzyme-labeled antigen prepared with hapten HS. Studies on the development of an EIA are in progress in our laboratories.[19]

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Appendix

Haptens identical with HS and HG in this report have been already used for the production of anti-25-OH-D_3[2] and anti 1,25-$(OH)_2D_3$[4] antibodies, respectively. However, the details about the synthesis and/or characterization of the compound were not given in this report. The hapten GG is a new derivative of 25-OH-D_3.

The following trivial names of secosterols were used in this report: vitamin D_2 = (5Z,7E,22E)-(3S)-9,10-secoergosta-5,7,10(19),22-tetraen-3-ol; vitamin D_3 = (5Z,7E)-(3S)-9,10-secocholesta-5,7,10(19)-trien-3-ol; 25-hydroxyvitamin D_2 = (5Z,7E,22E)-(3S)-9,10-secoergosta-5,7,10(19),22-tetraene-3,25-diol; 25-hydroxyvitamin D_3 = (5Z,7E)-(3S)-9,10-secocholesta-5,7,10(19)-triene-3,25-diol; 1α,25-dihydroxyvitamin D_3 = (5Z,7E)-(1S,3R)-9,10-secocholesta-5,7,10(19)-triene-1,3,25-triol; 24R,25-dihydroxyvitamin D_3 = (5Z,7E)-(3S,24R)-9,10-secocholesta-5,7,10(19)-triene-3,24,25-triol; 25-hydroxyvitamin D_3 3-hemisuccinate = (5Z,7E)-(3S)-25-hydroxy-9,10-secocholesta-5,7,10(19)-trien-3-yl hemisuccinate; 25-hydroxyvitamin D_3 3-hemiglutarate = (5Z,7E)-(3S)-25-hydroxy-9,10-secocholesta-5,7,10(19)-trien-3-yl hemiglutarate; 25-hydroxyvitamin D_3 3-glutarylglycine = (5Z,7E)-(3S)-25-hydroxy-9,10-secocholesta-5,7,10(19)-trien-3-yl 4-[N-(carboxymethyl)carbamoyl]butanoate.