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Specificity of the Polyclonal Antibodies Raised against a Novel 25-Hydroxyvitamin D_3–Bovine Serum Albumin Conjugate Linked through the C-11α Position

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To obtain a specific antibody for use in 25-hydroxyvitamin D_3 [25(OH)D_3] immunoassay, a novel hapten–carrier conjugate was prepared by coupling 11α-hemiglutaryloxy-25(OH)D_3 with bovine serum albumin (BSA). Three polyclonal antibodies (Ab_{11}) showing high titer and affinity for 25(OH)D_3 ($K_a = 0.96–2.6 \times 10^9\ M^{-1}$) were elicited in rabbits by repeated immunization with the conjugate. Specificity of the Ab_{11} was investigated by cross-reactivities with 11 related compounds in a radioimmunoassay using a tritium-labeled antigen and compared with that of conventional antibodies (Ab_3) raised against 25(OH)D_3 3-hemiglutarate conjugated with BSA. The Ab_3 could not discriminate the A-ring modified metabolites [1,25$(OH)_2D_3$ (87–290%) and 25(OH)D_3 3-sulfate (S) (130–180%)], although the cross-reactivities with the side chain modified metabolites were satisfactorily low [24,25$(OH)_2D_3$ (2.3–7.4%), 25(OH)D_2 (≤1.1%)]. On the contrary, the Ab_{11} easily discriminated 1,25$(OH)_2D_3$ (0.10–2.4%) and 25(OH)D_3 3S (<0.3%), whereas significant cross-reactivities were found with 24,25$(OH)_2D_3$ (110–120%) and 25,26$(OH)_2D_3$ (66–130%) having a dihydroxylated side chain. These results show that the Ab_{11} are complementary to the A-ring portion of the 25(OH)D_3 molecule which is opposite from the side chain structure recognized by the Ab_3. Thus, the Ab_{11} will compensate for insufficient specificity of the Ab_3 and are expected to be a useful tool for the pretreatment of biological samples in the development of various analyses of vitamin D metabolites including specific 25(OH)D_3 immunoassays using the Ab_3.

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INTRODUCTION

Serum or plasma levels of 25-hydroxyvitamin D_3 [25(OH)D_3], the major circulating metabolite of vitamin D (D), are useful as an index of the overall status of D in the body, and thus their determination is important for clinical or nutritional diagnosis [1, 2]. The 25(OH)D_3 measurements are now usually carried out by competitive protein binding assay (CPBA) employing serum D binding protein (DBP) or high-performance liquid chromatography (HPLC) [2, 3]. However, both methods require tedious and time-consuming pretreatment of biological samples to remove some interfering metabolites.

Immunoassays using specific antibodies are expected to be an alternative methodology which is simpler and more feasible for routine use. Some polyclonal anti-25(OH)D_3 antibodies have already been raised against the haptens linked to carrier proteins through the 3-position of 25(OH)D_3 [4], including those prepared in our laboratory [5]. However, these antibodies lacked specificity toward the A-ring structure of the metabolite as shown by high cross-reactivity with 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$, the active form of vitamin D_3 (D_3)], whereas the side chain structure was adequately recognized.

It is anticipated that the use of the hapten–carrier conjugate exposing both the A-ring and side chain of the metabolite would result in highly specific antibodies that recognize both of these partial structures characterizing the individual D metabolite. From these points of view, we previously synthesized novel haptenic derivatives of 25(OH)D_3 possessing chemical bridges at its 11α-position [6, 7]. In the present study, we produced rabbit polyclonal antibodies using a novel immunogenic conjugate prepared by linking one of the haptens, 11α-hemiglutaryloxy-25(OH)D_3 (11α-HG) with bovine serum albumin (BSA) (Fig. 1). The specificity of the antibodies was then investigated

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in detail by a radioimmunoassay (RIA) using a tritium-labeled antigen and compared with that of conventional antibodies raised against 25(OH)D_3 3-hemiglutarate (3-HG) conjugated with BSA [5].

EXPERIMENTAL

Materials

D_3 and 25(OH)D_3 were purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan) and the Duphar B.V. Co. (Amsterdam, The Netherlands), respectively. 1,25$(OH)_2D_3$, (24R)-24,25$(OH)_2D_3$, (24R)-1α,24,25-trihydroxyvitamin D_3 [1,24,25$(OH)_3D_3$] and 25-hydroxyvitamin D_2 [25$(OH)D_2$] were supplied by Chugai Pharmaceutical Co. (Tokyo, Japan). (23S,25R)-1α,25-dihydroxyvitamin D_3 26,23-lactone (1,25-lactone) was donated by Professor S. Takano of Tohoku University (Sendai, Japan). 25,26-Dihydroxyvitamin D_3 [both (25R)- and (25S)-isomers; 25*R*,26$(OH)_2D_3$ and 25*S*,26$(OH)_2D_3$] [8], vitamin D_3 sulfate (D_3 S) [9], 25-hydroxyvitamin D_3 3-sulfate [25(OH)D_3 3S] [9] and 25-hydroxycholesterol [6, 7] were synthesized in our laboratory by known methods. The haptenic derivative, 11α-HG, was that reported in previous papers [6, 7]. Polyvinyl alcohol (PVA; average M_w 2000) was obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). BSA and complete Freund's adjuvant were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.) and DIFCO Labs (Detroit, MI, U.S.A.), respectively. [26,27-Methyl-^{3}H]25(OH)D_3 (173.5 Ci/mmol) and the protein A column kit (Ampure™ PA Kit) were obtained from the Amersham Japan Co. (Tokyo, Japan). Three kinds of antisera, **As_3-1**, 2 and 4 (corresponding to HG-1, 2 and 4 in the previous paper [5]) were prepared in our laboratory using 3-HG as a hapten. Dextran (M_w 50,000–70,000) and activated charcoal powder (Norit® EXW) were purchased from Nacalai Tesque Co. (Kyoto, Japan). All other reagents and solvents were of analytical grade.

Apparatus

UV spectra were obtained on a Union Giken (Osaka, Japan) SM-401 spectrophotometer. Radioactivities were measured with an Aloka (Tokyo, Japan) LSC-700 liquid scintillation spectrometer.

Preparation of hapten–BSA conjugate

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide·HCl (EDC) (44 mg, 0.23 mmol) and *N*-hydroxysuccinimide (26 mg, 0.23 mmol) were added to the hapten 11α-HG (14.8 mg, 27.9 μmol) dissolved in dioxane–H_2O (19:1, v/v) (0.2 ml), and the mixture was stirred at room temperature for 5 h. The resulting solution was diluted with AcOEt, washed with water, and dried over anhydrous Na_2SO_4. Removal of the organic solvent gave crude *N*-succinimidyl ester (11.2 mg, 17.8 μmol; determined by UV spectra at 265 nm on the assumption that the molar extinction coefficient is 17,700 [10]), which was then dissolved in pyridine (0.7 ml). A solution of BSA (30.0 mg, 0.455 μmol) in 0.05 M sodium phosphate buffer (pH 7.4) (0.7 ml) was added to the solution of the ester. The resulting 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 (1000 *g* for 20 min) and the supernatant was discarded. The procedure was repeated until free secosterols were removed. The precipitate was dissolved in 20% pyridine (5 ml) and dialyzed again as described above. Lyophilization of the resulting solution gave the desired conjugate as a fluffy powder (23.6 mg). The number of hapten molecules linked to a BSA molecule was calculated from the UV absorption [5] on the assumption that the molar extinction coefficient of the hapten is 17,700 [10].

Production of antisera and their IgG fractions

Three domestic albino rabbits (female, 3 months old) were used for immunization. The suspension of the hapten–BSA conjugate (3.0 mg) in isotonic saline (1.5 ml) was emulsified with complete Freund's adjuvant (1.5 ml), and a portion of the emulsion (approx. 1.0 ml) was injected into each rabbit subcutaneously at multiple sites along the back. This procedure was repeated biweekly for 8 months using the conjugate (1.5 mg). Blood was collected 10 days after the last immunization and allowed to stand at room temperature for 4 h. Centrifugation at 4°C (1000 *g* for 20 min) afforded the desired antisera (**As_{11}-1**, 2 and 3). A portion of each **As_{11}** or **As_3** (see *Materials*) (200 μl) was applied to the protein A column, and the immunoglobulin G (IgG) fractions obtained (expressed as **Ab_{11}-1**, 2, 3 and **Ab_3-1**, 2, 4; the recoveries of IgG were not determined) were diluted with 0.05 M sodium phosphate buffer (pH 7.3) containing NaN_3 (0.02%) (8 ml; corresponding to 1:40), and stored at −20°C prior to use.

RIA procedure

A sodium phosphate buffer (0.05 M, pH 7.3) containing 0.1% gelatin, 0.9% NaCl, 0.1% NaN_3 and 1% PVA was used in the RIA. A series of standard 25(OH)D_3 (0–2000 pg) and [^{3}H]25(OH)D_3 (approx. 15,000 dpm), each dissolved in EtOH (each 25 μl), were added to the antibody (IgG fraction) diluted with the buffer (500 μl), and the mixture was incubated at 4°C for 4 h. After addition of a charcoal (2%) suspension in the buffer containing dextran (0.05%) but no PVA (500 μl), the mixture was vortex-mixed, allowed to stand at 0°C for 20 min, and then centrifuged at 4°C (1000 *g* for 10 min). The supernatant was transferred by decantation into a vial containing the tT 21 scintillation cocktail (10 ml) [11], and the radioactivity was measured.

Cross-reaction study

The cross-reactions of the antibodies were tested with 11 kinds of compounds having structures closely related to 25(OH)D_3. The relative amounts required

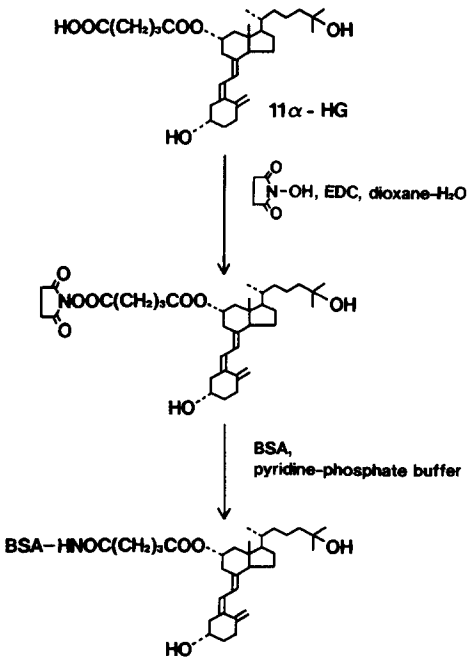

Fig. 1. Preparation of BSA conjugate of the hapten 11α-HG.

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 standard curves [12].

RESULTS

Preparation of hapten–BSA conjugate

The hapten–BSA conjugate was prepared by reacting 40-fold excess *N*-succinimidyl ester of 11α-HG with BSA in a mixture of pyridine and a sodium phosphate buffer at 4°C, as outlined in Fig. 1 [13]. The hapten–BSA molar ratio of the obtained conjugate was determined to be 11, and the value was judged to be sufficient for its use as an immunogen.

Production of polyclonal antibodies

Three kinds of antisera ($\mathbf{As_{11}}$-1, 2 and 3) against the hapten 11α-HG were elicited by the repeated immunization of three rabbits with the conjugate prepared above. The IgG fractions of the $\mathbf{As_{11}}$ and $\mathbf{As_3}$ were then prepared using the protein A column kit. The titer of these polyclonal antibodies (the $\mathbf{Ab_{11}}$ and $\mathbf{Ab_3}$) in the RIA system was determined as the final dilution that

Table 1. *Titer and affinity constant of antibodies*

Antibody	Final dilution	K_a ($M^{-1} \times 10^{-9}$)
Ab_{11}-1	1:44,000	0.96
Ab_{11}-2	1:51,000	2.6
Ab_{11}-3	1:59,000	2.2
Ab_3-1	1:50,000	2.2
Ab_3-2	1:17,000	1.3
Ab_3-4	1:75,000	1.2

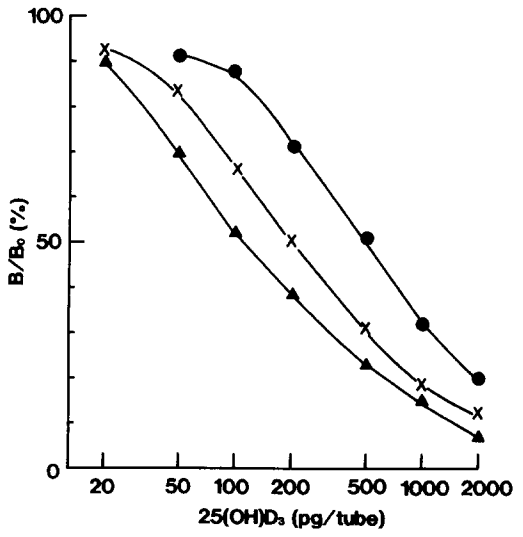

Fig. 2. Dose–response curves for 25(OH)D_3 RIA using the Ab_{11}. ●——●; Ab_{11}-1, ▲——▲; Ab_{11}-2, × —— ×; Ab_{11}-3.

was capable of binding 40% of the labeled antigen (Table 1). Affinity constants obtained by the Scatchard analysis [14] are also listed in Table 1.

Dose–response curve

All the antibodies afforded the dose–response curves whose measurable ranges were 20 ($\mathbf{Ab_{11}}$-2, 3 and $\mathbf{Ab_3}$) or 50 ($\mathbf{Ab_{11}}$-1) pg to 2000 pg per tube. Typical standard curves obtained with the $\mathbf{Ab_{11}}$ and $\mathbf{Ab_3}$ are shown in Figs 2 and 3, respectively.

Cross-reaction study

The cross-reactions of the $\mathbf{Ab_{11}}$ and $\mathbf{Ab_3}$ with 11 compounds having closely related structures are listed in Table 2. Three antibodies derived from the same hapten showed a similar cross-reactivity pattern, although some degree of variation, which might be ascribed to the individual differences among rabbits for immune response, was observed.

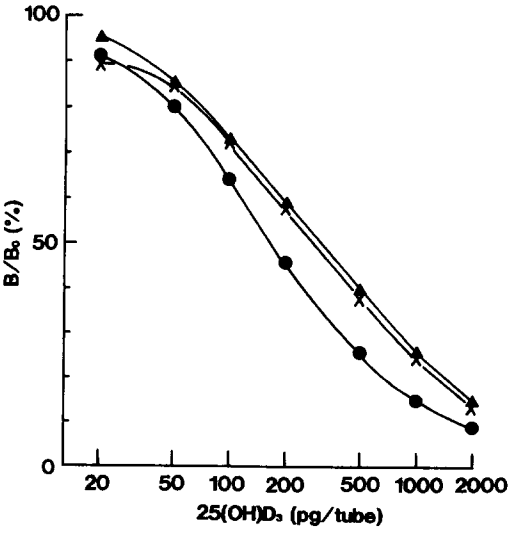

Fig. 3. Dose–response curves for 25(OH)D_3 RIA using the Ab_3. ●——●; Ab_3-1, ▲——▲; Ab_3-2, × —— ×; Ab_3-4.

Table 2. Percent cross-reaction of antibodies with related compounds

	Ab_{11}			Ab_3		
Compound	1	2	3	1	2	4
$25(OH)D_3$	100	100	100	100	100	100
D_3	1.1	1.0	3.6	<0.04	0.60	<0.05
$1,25(OH)_2D_3$	0.20	2.4	0.10	290	87	92
$24,25(OH)_2D_3$	110	120	110	7.4	3.8	2.3
$25R,26(OH)_2D_3$	66	96	71	1.4	3.1	<1
$25S,26(OH)_2D_3$	76	130	100	9.5	19	27
$1,24,25(OH)_3D_3$	<1	<3	<1	<2	12	<2
1,25-Lactone	<0.06	1.4	<0.02	1.4	4.5	<0.05
D_3 S	<0.06	<0.02	<0.02	<0.03	0.20	<0.07
$25(OH)D_3$ 3S	<0.1	<0.1	<0.3	130	180	170
$25(OH)D_2$	3.1	15	24	0.60	1.1	<1
25-Hydroxycholesterol	<0.08	<0.02	<0.03	0.40	<0.05	<0.05

The cross-reactivities of the Ab_3 with some of these compounds had been examined by an RIA based on double-antibody solid phase method [5] and an EIA employing β-galactosidase-labeled antigen [15]. Present results were compatible with the previous data. Namely, the Ab_3 showed acceptably low cross-reactivites with the following side chain modified metabolites: D_3 ($\leqslant$0.60%), $24,25(OH)_2D_3$ (2.3–7.4%), $25R,26(OH)_2D_3$ ($\leqslant$3.1%) and $25(OH)D_2$ ($\leqslant$1.1%) except that somewhat higher cross-reactivities were observed with $25S,26(OH)_2D_3$ (9.5–27%). However, nearly equal or larger reactivities compared with that of $25(OH)D_3$ were found with $1,25(OH)_2D_3$ (87–290%) and $25(OH)D_3$ 3S (130–180%) which have structural modification of the A-ring.

On the contrary, the Ab_{11} exhibited only negligible cross-reactivities with $1,25(OH)_2D_3$ (0.10–2.4%) and $25(OH)D_3$ 3S (<0.3%). As for side chain analogs, acceptably low cross-reactivities were obtained with D_3 (1.0–3.6%), indicating that the Ab_{11} recognize the hydroxy group at the 25-position. However, surprisingly high cross-reactivities were found with the metabolites having a dihydroxylated side chain: $24,25(OH)_2D_3$ (110–120%), $25R,26(OH)_2D_3$ (66–96%) and $25S,26(OH)_2D_3$ (76–130%). Significant cross-reactions were also observed with $25(OH)D_2$ (3.1–24%).

The metabolites modified both on the side chain and A-ring [D_3 S, $1,24,25(OH)_3D_3$ and 1,25-lactone] were easily discriminated by both the Ab_{11} and Ab_3 except that the Ab_3-2 showed slight cross-reactivities. Negligibly low cross-reactivities with 25-hydroxycholesterol demonstrated the high ability of all the antibodies to recognize the D skeleton.

DISCUSSION

It is well known that the specificity of antibodies for small molecules, such as D metabolites, is significantly influenced by the position on the compound used for conjugation to carrier proteins. Generally, the antigen-binding site of antibodies is complementary to the hapten portion remote from the position used for attachment to carrier proteins. Therefore, hapten–carrier conjugates linked through a position on the C-ring, especially via the 11α-position, seems promising for the production of antibodies showing higher specificity, because both A-ring and side chain of the sterol are expected to be well exposed. However, no attempt has been made so far, though some D derivatives functionalized at C-11 have recently been synthesized [16–18]. In the present study, the polyclonal antibodies (Ab_{11}) were produced against the novel $25(OH)D_3$–BSA conjugate linked through the C-11α position, and their specificity in the RIA system was investigated in detail from comparative view with those of the conventional antibodies (Ab_3) previously prepared in our laboratory [5].

The RIA procedure employed here was a simple liquid phase method using [^{3}H]$25(OH)D_3$ as a labeled antigen. PVA was added to the assay buffer to solubilize hydrophobic D derivatives and make it possible to estimate their cross-reactivities correctly [19]. The Ab_{11} and Ab_3 were the IgG fractions of rabbit antisera (the As_{11} and As_3) prepared by a protein A column: this partial purification was intended to avoid the interference by DBP in the antisera with characterization of the antibodies.

All 6 antibodies (Ab_{11}-1, 2, 3 and Ab_3-1, 2, 4) exhibited satisfactorily high titers and affinity constants (Table 1). The dose–response curves of the RIA using these antibodies were sensitive enough to measure serum/plasma $25(OH)D_3$ levels, and the quantification limit (20 or 50 pg per tube) was lower than that of CPBA (Figs. 2 and 3).

The Ab_3 easily discriminated the side chain modified analogs of $25(OH)D_3$, as expected, taking into account the hapten structure used in immunization (3-HG). Exceptionally, the (25S)-isomer of $25,26(OH)_2D_3$ cross-reacted to a somewhat higher extent (especially, the Ab_3-2 and 4), but the (25R)-isomer was satisfactorily discriminated. On the other hand, the Ab_3 possessed equal or higher affinity for $1,25(OH)_2D_3$ or $25(OH)D_3$ 3S compared with $25(OH)D_3$: thus the modification at the 1- or 3-position was scarcely recognized by these antibodies. These extraordinarily high cross-reactivities with the A-ring modified analogs might be the result of the carbonyl group of the bridge

structure (glutaryl moiety) contained in the hapten–carrier conjugate mimicking the 1α-hydroxy group of 1,25$(OH)_2D_3$ [5, 20] or the sulfonyl group of 25(OH)D_3 3S at the immunization, rather than a result based on masking of the 1- or 3-position by the carrier protein. However, the highly cross-reactive property of the **Ab_3** with 25(OH)D_3 3S would be useful to develop the immunoassays of this important metabolite which had been suggested to be one of the major circulating D metabolites in human plasma [21]: its separation from the accompanying 25(OH)D_3 in serum/plasma will be easily performed by simple extraction with organic solvents or immunoaffinity chromatography (IAC) immobilizing the **Ab_{11}** as discussed below. To discriminate D_3 S nearly completely will be an additionally favorable feature of the **Ab_3** for this purpose, since its existence in mammalian body fluids had also been suggested [22].

On the contrary, the **Ab_{11}** exhibited rather unexpected specificity: the reactivities with 24,25$(OH)_2D_3$, 25*R*,26$(OH)_2D_3$ and 25*S*,26$(OH)_2D_3$ were similar to that with 25(OH)D_3, while 1,25$(OH)_2D_3$ and 25(OH)D_3 3S, which cross-reacted significantly with the **Ab_3**, were satisfactorily discriminated. Thus, the antibodies have little ability to recognize the additional hydroxylation on the 25-hydroxylated side chain in spite of the fact that the 11α-position used for the linkage with the carrier protein is located substantially apart from this partial structure. One possible explanation for this result is that the rabbits might have no B-lymphocyte clones equipping the surface receptors which are capable of recognizing both the A-ring and the side chain structures on the hapten moiety of the present [C-11α]–BSA conjugate. It is also conceivable that the flexible nature of the bridge structure in the conjugate (glutaryl group) might result in the masking of the side chain structure of the hapten moiety by BSA, and thus the immunogen failed to stimulate the above-mentioned B-lymphocytes. Therefore, immunization of another animal species (e.g. mouse, rat or chicken) and/or the use of other [C-11α]-conjugates linked through chemical bridges containing bulkier and more rigid structures (e.g. phenyl group) might afford antibodies having the expected specificity. The production of monoclonal antibodies by the hybridoma technology [23] would be another approach to the desired antibody.

However, the **Ab_{11}** obtained in this study are widely useful as a tool for pretreatment of biological specimens in various analytical methods of D metabolites including the 25(OH)D_3 immunoassays. Recently, we first introduced an IAC as a pretreatment for plasma specimens in a 25(OH)D_3-EIA [15] and concluded that the methodology must have great potential for simplifying the conventional D analyses. The IAC using an **Ab_{11}**-immobilized column will be an effective pretreatment method in the 25(OH)D_3 immunoassays using the **Ab_3**, because the **Ab_{11}** have a recognition profile that compensates for the insufficient specificity of the **Ab_3**. The IAC with the **Ab_{11}** will also allow us to develop a profile analysis of 25(OH)D_3, 24,25$(OH)_2D_3$ and 25,26$(OH)_2D_3$ by various chromatographies [i.e. HPLC, GLC, or gas chromatography–mass spectrometry (GC–MS)], because the antibodies show similar reactivity to each of these compounds. Furthermore, the IAC must be an effective tool for the removal of these 1α-deoxylated metabolites from serum/plasma specimens: this application is especially valuable in the determination of 1,25$(OH)_2D_3$ or 1,25-lactone, which circulate at lower concentrations than these metabolites in serum/plasma. In addition, the IAC would enable us to develop an immunoassay for 25(OH)D_3 3S using the **Ab_3**, because it would easily separate this metabolite and 25(OH)D_3 as mentioned above.

In conclusion, the **Ab_{11}** prepared using the novel hapten–[C-11α]–BSA conjugate did not show sufficient specificity for the 25(OH)D_3 side chain, although the A-ring structure was well recognized. However, their unique recognition profile which compensates for the insufficient specificity of the **Ab_3**, and in other words, is group-specific for 25(OH)D_3, 24,25$(OH)_2D_3$ and 25,26$(OH)_2D_3$ will provide us with effective pretreatment tools for various analyses of D metabolites.

The synthesis of novel [C-11α]-haptens of 1,25$(OH)_2D_3$ [24] and 24,25$(OH)_2D_3$ [25] and antibody production using them has recently been accomplished in our laboratory. Development of highly selective IAC of plasma 1,25$(OH)_2D_3$ utilizing the **Ab_{11}** in combination with an **Ab_3**-like antibody [raised against 25(OH)D_3 3-hemisuccinate] is also in progress as a pretreatment for its radioreceptor assays or immunoassays. Details will be reported elsewhere in the future.

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