Characterization of rat and human CYP2J enzymes as vitamin D 25-hydroxylases

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Abstract

Vitamin D is 25-hydroxylated in the liver, before being activated by 1α-hydroxylation in the kidney. Recently, the rat cytochrome P450 2J3 (CYP2J3) has been identified as a principal vitamin D 25-hydroxylase in the rat (Yamasaki *et al*. J. Biol. Chem., 2004). In this study, we examine whether human CYP2J2 that exhibits 73% amino acid homology to rat CYP2J3 has similar catalytic properties. Recombinant human CYP2J2 was overexpressed in *Escherichia coli*, purified, and assayed for vitamin D 25-hydroxylation activity. We found significant 25-hydroxylation activity toward vitamin D_3 (turnover number, 0.087 min^{-1}), vitamin D_2 (0.16 min^{-1}), and 1α-hydroxyvitamin D_3 (2.2 min^{-1}). Interestingly, human CYP2J2 hydroxylated vitamin D_2, an exogenous vitamin D, at a higher rate than it did vitamin D_3, an endogenous vitamin D, whereas rat CYP2J3 hydroxylated vitamin D_3 (1.4 min^{-1}) more efficiently than vitamin D_2 (0.86 min^{-1}). Our study demonstrated that human CYP2J2 exhibits 25-hydroxylation activity as well as rat CYP2J3, although the activity of human CYP2J2 is weaker than rat CYP2J3. CYP2J2 and CYP2J3 exhibit distinct preferences toward vitamin D_3 and D_2.

Key words: Vitamin D_3; Vitamin D_2; 25-Hydroxylase; CYP2J2; CYP2J3

1. Introduction

Two vitamin D compounds, vitamin D_3 and vitamin D_2, are known to be physiologically important for calcium homeostasis [1-5]. Vitamin D_3 can be obtained by the light and heat conversion of 7-dehydrocholesterol in the skin or from the diet, while vitamin D_2 derived from a plant sterol, ergosterol, can only be obtained from the diet. A recent report showed vitamin D_2 is much less effective than vitamin D_3 in humans [6]. Both vitamin D_3 and D_2 are converted into the active forms, $1,25(OH)_2D_3$ and $1,25(OH)_2D_2$, respectively in the body. Vitamin D_2 has an additional active form, $1,24(OH)_2D_2$, which has been reported to be physiologically less active, but less toxic than $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ [7,8]. In both vitamin D compounds, the hydroxylation of the side chain at C-25 or C-24 is a prerequisite for the subsequent 1α-hydroxylation controlled by physiological conditions [3,9,10]. Therefore, characterization of the enzymes involved in the side chain hydroxylation of vitamin D is important for understanding the whole scheme of vitamin D activation.

To date, six vitamin D 25-hydroxylases have been reported: CYP27A1, CYP2R1, CYP3A4, CYP2C11, CYP2D25, and CYP2J3 [11,12]. CYP27A1 is a mitochondrial P450, hydroxylating at C-25 of vitamin D_3 and at C-24 of vitamin D_2, as well as at C-27 of the intermediates of bile acid synthesis [13,14]. The *CYP27A1* gene is highly conserved in all vertebrates. However, knockout mice of the *CYP27A1* gene exhibit no significant changes in vitamin D metabolism. Also, cerebrotendinous xanthomatosis patients, who have serious mutations in the *CYP27A1* gene, exhibit no or

little changes in the serum concentration of 25-hydroxyvitamin D compounds in spite of abnormal cholesterol metabolism [15,16]. Therefore, CYP27A1 is thought to be critical for bile acid synthesis as a sterol 27-hydroxylase.

Recently, CYP2R1 has been identified as a novel microsomal vitamin D 25-hydroxylase by expression cloning using a *CYP27A1*-knockout mouse cDNA library [17]. The *CYP2R1* gene is conserved in all vertebrates, and is dominantly expressed in the liver and testes of mice. The following study of Cheng *et al*. revealed that a patient with low circulating levels of 25-hydroxyvitamin D is homozygous for a mutation of *CYP2R1* gene [18]. This findings have established the CYP2R1 is the essential vitamin D 25-hydroxylase in humans.

CYP3A4 is the most abundant P450 expressed in the adult human liver and intestine, and is involved in the metabolism of about half of the drugs in use. Interestingly, it exhibits 25-hydroxylation activity for vitamin D_2, but not for vitamin D_3 [19]. CYP2C11 and CYP2D25 have been purified as major microsomal vitamin D 25-hydroxylases in male rats and pigs, respectively [20-22]. CYP2C11 is a P450 specific to the male rat. In spite of the significant 25-hydroxylation activity of CYP2D25, its human ortholog, CYP2D6, shows no detectable effect on vitamin D [23]. Thus, CYP2C11, CYP2D25, and CYP3A4 seem to be species-specific vitamin D hydroxylases.

We have recently identified CYP2J3 as a novel vitamin D_3 25-hydroxylase in rats [24]. CYP2J3 is highly expressed in the liver regardless of sex, and exhibits markedly higher (about 10 times) 25-hydroxylation activity toward vitamin D_3 than do

other 25-hydroxylases, such as CYP2C11, CYP2D25, and CYP27A1. Therefore, it is of interest to determine whether human CYP2J2, the sole human CYP2J subfamily member with 73% amino acid homology to CYP2J3, shows 25-hydroxylation activity even though CYP2R1 is the essential 25-hydroxylase in humans. Here, we prepared a recombinant human CYP2J2, and compared its catalytic activity toward vitamin D with that of rat CYP2J3. We found that CYP2J2 shows significant 25-hydroxylation activity, which suggests the contribution of the CYP2J subfamily to vitamin D metabolism.

2. Experimental Procedures

2.1. Materials

Vitamin D_3 was obtained from Katayama Chemical Co. (Osaka, Japan). Vitamin D_2 was obtained from Fluca (Germany). 1α,25$(OH)_2D_3$, 25$(OH)D_3$, 1α$(OH)D_3$, isopropyl-1-thio-β-D-galactoside, and cholic acid were purchased from Wako Pure Chemical Industries (Osaka, Japan). δ-Aminolevulinic acid was obtained from Sigma, and β-NADPH was obtained from Oriental Co. (Tokyo, Japan). Emulgen 911 was supplied by Kao Co. (Tokyo, Japan). A pGro7 expression vector that contained the *GroEL-GroES* gene was obtained from Takara Bio Inc. (Otsu, Japan). Restriction enzymes were obtained from New England Biolabs, Inc. The expression plasmid, pKSN2, was modified from pkk223-3 as previously described by Akiyoshi-Shibata *et al*. [25]. 25$(OH)D_2$ was synthesized as described [26]. Primers for polymerase chain reactions (PCR) were synthesized by Kurabo Industries Ltd. (Osaka, Japan). All other chemicals were of the highest quality commercially available.

2.2. Cloning of CYP2J2 cDNA and construction of N-terminal deletion mutants

CYP2J2 cDNA (GenBank accession no. **U37143**) was cloned from a Cap Site cDNA library of the human liver (Wako Pure Chemical Industries) by PCR using the following primers containing an extra Nde I or Hind III site for ligation into the

expression vector: forward primer (IA-1), 5'-CAT ATG GCT GCA GCA ATG GGC TCT CTG GCG-3' and reverse primer (IA-2), 5'-AAG CTT TCA GAA CAC GTG CCA TGT C-3'. The second codon was changed from wild type Leu (CTC) to Ala (GCT) in the primer IA-1. Two deletion constructs of N-terminal regions (D19 lacking 2-19 amino acid residues, and D34 devoid of 2-34) of CYP2J2 were prepared by PCR using the following forward primers: 5'-CAT ATG GCT CTG TTA TTA GCA ACT GTC GCC-3' for D19 mutant, and 5'-CAT ATG GCT AAA CGT CGT CGC CCA AAG AAC TAC CCG-3' for D34 mutant, together with the IA-2 reverse primer using an isolated CYP2J2 cDNA as the template. Typical PCR parameters were: 95°C for 2 min, 35 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 90 s, and followed by 72°C for 7 min. PCR products were cloned into the pGEM-T Easy vector (Promega), and the sequence integrity was confirmed by sequence analysis with the cloning primers and with two additional primers (5'-GGT TCA CTC TGA CAG CAC TAA GG-3' and 5'-GCA GGT GGT ACC CAG CCA AAG-3'). The sequence of the isolated CYP2J2 cDNA completely agreed with that of the registered CYP2J2 cDNA sequence in GenBank (accession no. **U37143**). The insert cDNAs were excised from the corresponding pGEM-T Easy vectors containing native or modified CYP2J2 cDNAs by digestion with Nde and Hind III. They were then purified, and subsequently ligated into the expression vector pKSN2 at the corresponding restriction sites to construct the expression plasmids (pK2J2, pKD19, and pKD34) encoding 2J2 (wild type), D19, and D34, respectively. *E. coli* JM109 was transformed with pK2J2, pKD19, or pKD34 for expression experiments.

2.3. Overproduction of recombinant CYP2J2 in E. coli

The *E. coli* cells harboring the CYP2J2 expression plasmid were transformed with pGro7 containing the *GroEL-GroES* gene, and selected with chloramphenicol (20 μg/ml). The doubly transformed *E. coli* cells were precultured in LB medium (15 ml) containing ampicillin (50 μg/ml) and chloramphenicol (20 μg/ml) at 37°C overnight, and then 1 ml of the culture was transferred into 40 ml of TB medium containing 5 mg/ml arabinose, and maintained at 37°C. Upon reaching mid-log phase (A_{600} = around 0.6), the culture temperature was reduced to 28°C, and isopropyl 1-thio-β-D-galactopyranoside and δ-aminolevulinic acid were added to the final concentrations of 1 mM and 0.5 mM, respectively. The cultures were maintained for 20 h or 44 h at 28°C. The following operations except the measurement of P450 spectrum were carried out at 4°C or on ice. The cells were harvested by centrifugation, suspended in a glycerol buffer (100 mM potassium phosphate (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol, and 20% glycerol), and treated with 1 mg/ml lysozyme for 1 h. After lysis, cells were disrupted by sonication and centrifuged at 500 x g for 10 min, and the supernatant was recentrifuged at 100,000 x g for 1 h. The supernatant and pellets were separated as cytosol and membrane fractions, respectively. The membrane fraction was suspended in the glycerol buffer and solubilized with 0.5% Emulgen 911 (final concentration 0.5%) for 30 min. After centrifugation at 100,000 x g for 30 min, the supernatant was collected. The cytosol and solubilized membrane fractions were

subjected to the measurement of P450 contents by a reduced CO-difference-spectrum [27].

2.4. Purification of recombinants of CYP2J2 and CYP2J3

All purification procedures were carried out at 4°C. The cells expressing D34 were grown in 600 ml of TB medium for 44 h at 28°C, and the membrane fraction was prepared as described above. After solubilization with sodium cholate (final concentration 0.5%) and centrifugation at 100,000 x g for 30 min, the supernatant was loaded onto an ω-aminohexyl Sepharose column (1.6 x 3.0 cm) equilibrated with buffer A (10 mM potassium phosphate (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol, 20% glycerol, and 0.5% cholate). The column was washed with 2 column volumes of buffer A, and proteins were eluted stepwise with the same buffers containing 0.1% and 0.5% Emulgen 911. The eluted fractions (0.5% Emulgen) containing P450 were pooled, and dialyzed against buffer B (10 mM potassium phosphate (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol, 20% glycerol, and 0.3% cholate) overnight. The dialyzed fractions were loaded onto a hydroxyapatite column (1.6 x 2.0 cm) equilibrated with buffer B, and washed with more than 100 ml of buffer B to remove Emulgen 911 completely. Proteins were eluted stepwise with buffer B containing 100 mM, 150 mM, and 500 mM potassium phosphate (pH 7.4). The fractions (500 mM eluate) containing P450 were dialyzed against buffer B and loaded onto a DEAE-Sepharose column (1.6 x 3.0 cm) equilibrated with buffer B. The column was washed with buffer B and P450 was eluted

stepwise with buffer B containing 100 mM and 150 mM potassium phosphate (pH 7.4). The P450 fraction (150 mM eluate) was dialyzed against buffer C (10 mM potassium phosphate (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol, 20% glycerol, and 0.05% cholate) and stored at -80°C. The N-terminal deletion mutant CYP2J3 (dN2J3) was expressed in *E. coli* and purified as described previously [24]. The final preparations were analyzed for their purity by 10% SDS-polyacrylamide gel electrophoresis according to the method of Laemmli [28]. Protein concentrations were determined by the BCA protein assay kit (Pierce).

2.5. Assay of vitamin D 25-hydroxylase activity

Assay methods were described previously [29]. Briefly, the purified P450 (10-150 pmol), 1.0 unit of NADPH-P450 reductase, and a substrate (25 nmol of vitamin D_3, 25 nmol of vitamin D_2, or 5 nmol of 1α(OH)D_3) were mixed in 0.49 ml of reaction buffer containing 100 mM Tris-HCl (pH 7.7 at 37°C) and 1 mM EDTA. Reaction was started by adding 10 μl of 50 mM β-NADPH. Under these conditions, the reaction products linearly increased with amount of P450 in range of 10-150 pmol and up to 10 min. After a 5 or 10 min incubation at 37°C, reaction products were extracted with 4-ml of benzene and subjected to HPLC analysis. The HPLC system was composed of a PU-980 pump, a UV975 detector, and a Fine Pack SIL-5 column (4.6 x 250 mm) (JASCO Co. Ltd. Tokyo, Japan). 25(OH)D_2 and 25(OH)D_3 were eluted by Solvent A

(hexane:isopropanol:methanol = 93:5:2) at a constant flow rate of 1.2 ml/min. 1α,25(OH)$_2$D$_3$ was eluted by solvent B (hexane:isopropanol:methanol = 86:7:7) at a constant flow rate of 1.4 ml/min. The 25-hydroxylated products were identified by comparison of retention times with authentic 25-hydroxylated compounds and quantified from the peak heights on the normal phase HPLC. The separated products were subjected to further identification using reverse phase HPLC with a Fine Pack SIL C18T-5 column (4.6 x 250 mm). Samples were eluted by 95% methanol at a flow rate of 1.0 ml/min for 25(OH)D$_2$ and 25(OH)D$_3$. Kinetic parameters (K_m and V_{max}) were determined using Lineweaver-Burke plots with the substrate concentrations of 0.625, 1.25, 2.5, and 5 μM.

2.6. Mass spectrometric analysis of the metabolites

Metabolites were purified by the normal and reverse phase HPLC. The isolated products were dissolved in ethanol (50 μl) and infused via a syringe pump (Holliston, Ma, USA) at 10 μl/min into the ionspray source of an Applied Biosystems/QSTAR XL (Concord, Ontario, Canada) triple quadrupole instrument. The collision gas was nitrogen and the instrument was optimized for maximum response at unit mass resolution. The data were averaged over 100 scans collected in duplicate.

3. Results

3.1. Heterologous expression of human CYP2J2 in E. coli

We have prepared three CYP2J2 expression vectors, for a full-length protein (2J2) as well as for two N-terminal deletion mutant proteins (D19 and D34), as shown in Fig. 1, since substitutions and/or deletions of the N-terminal hydrophobic region (membrane anchor region) of microsomal P450 improve expression in *E. coli* [30]. 2J2 has one Leu (CTC) to Ala (GCT) substitution at the second codon, as is also the case for CYP17 [31]. D19 lacks half of a putative membrane anchor region, and the N-terminal sequence is modified similarly to that of a CYP3A4 mutant [32]. D34 was completely devoid of the N-terminal hydrophobic region, with the new N-terminal region modified like that of the CYP2J3 mutant [24]. In spite of these modifications, none of the constructs expressed P450 when analyzed by CO-difference spectra. Since CYP2J3 expression was markedly improved by the co-expression of *E. coli* chaperone proteins [24], we applied the same strategy to CYP2J2 expression experiments. Co-expression of GroEL-GroES dramatically increased the expression levels of the P450 (Fig. 2 and Table 1). The P450 expression level of *E. coli* cells containing pKD34/pGro7 was more than 100 times higher than of those containing pKD19/pGro7. D34 was distributed both in cytosol and membrane fractions equally, whereas D19 predominantly localized in the membrane fraction. Long culture times (44 h) did not increase the P450 level (Table 1).

3.2. Purification of D34

D34 was purified from the membrane fraction because of its higher P450 content therein relative to the cytosol fraction (Table 1). The membrane fraction was solubilized and applied to hydrophobic and subsequent hydroxyapatite column chromatographies. In the hydroxyapatite column step, Emulgen 911 was completely removed by extensive washing (while monitoring its UV absorption at 275 nm), since it significantly inhibits the vitamin D hydroxylation activity of CYP2J2 (data not shown). Finally, samples were subjected to a DEAE-Sepharose column for purification and concentration, and dialyzed against a buffer (containing 0.05% cholate) that did not inhibit vitamin D 25-hydroxylase activity. SDS-polyacrylamide gel electrophoresis of the final preparations showed single major bands with the predicted molecular weights (Fig. 3). The P450 contents of D34 and dN2J3 (recombinant CYP2J3) were more than 10 nmol P450/mg proteins.

3.3. Analysis of 25-hydroxylation activities of CYP2J enzymes

Vitamin D 25-hydroxylation assays were carried out using a reconstituted system consisting of NADPH-P450 reductase. The products were analyzed by comparison of their retention times with authentic 25-hydroxylated compounds, as described in Experimental Procedures. Fig. 4 shows the typical results of the HPLC

analysis for 1α(OH)D_3, vitamin D_3, and vitamin D_2 on the normal phase HPLC. The major peaks were identified as 25-hydroxylation products. Several minor peaks of unidentified products were also observed. The 25-hydroxylation products were collected and their identities confirmed by reverse phase HPLC (data not shown). The product identified as 25(OH)D_2 by HPLC was subjected to mass spectrometric analysis. As shown in Fig. 5, the major molecular ion (m/z 413, M + H^+) clearly indicates the metabolite was the monohydroxylated product of vitamin D_2. The amounts of the products were calculated from their peak heights, and kinetic parameters were determined as described in Experimental Procedures (Table 2). Interestingly, human CYP2J2 exhibited higher activity toward vitamin D_2 than D_3 (0.16 and 0.087 nmol/min/nmol P450, respectively), whereas rat CYP2J3 showed higher activity toward vitamin D_3 than D_2 (1.4 and 0.86 nmol/min/nmol P450, respectively) (Table 3). CYP2J2 showed no 1α-, 24-, and 26-hydroxylation activity toward 25(OH)D_3, and no 2α- and 16α-hydroxylation activity toward testosterone, indicating similar enzymatic characteristics to CYP2J3 (data not shown). Ebastin, a histamine H1-receptor antagonist, is a well-characterized drug that is metabolized to pharmacologically active metabolite via the hydroxylation catalyzed by CYP2J2 or CYP2J3 in the intestine [33]. The activity of CYP2J2 and CYP2J3 toward vitamin D was lower compared to that toward ebastin (Table 3).

4. Discussion

We have analyzed the vitamin D 25-hydroxylation activity of human CYP2J2 and compared it with that of the rat's orthologous P450, CYP2J3, since CYP2J3 shows markedly high 25-hydroxylation activity toward vitamin D_3, and appears to play an important role as a vitamin D 25-hydroxylase in the rat's liver [24]. Here, we have clearly demonstrated that human CYP2J2 also exhibits significant 25-hydroxylation activity for vitamin D_3, even though human CYP2J2 and rat CYP2J3 show a 27% amino acid sequence difference. Interestingly, human CYP2J2 exhibited twofold higher hydroxylation activity for vitamin D_2 than for vitamin D_3. This preference was opposite to that of the rat CYP2J3 (Table 3). Therefore, human and rat CYP2J proteins exhibit distinct preferences for vitamin D compounds.

CYP2J2 has a high catalytic ability for drugs and xenobiotics such as ebastin [33], in keeping with the present observation that CYP2J2 prefers vitamin D_2 (exogenous) to vitamin D_3 (endogenous). However, when the same amount of vitamin D_2 and D_3 is orally administered, rats exhibits a higher blood-concentration of 25(OH)D_2, whereas humans show a higher concentration of 25(OH)D_3 [6,34,35]. These results are opposite to those one would predict from the 25-hydroxylation preferences of CYP2J2 and CYP2J3. Therefore, the serum 25(OH)D status is likely to be determined by several factors, such as the substrate specificity of other 25-hydroxylases, affinity for vitamin D binding protein, clearance rates, and absorption efficiencies in the intestine.

Humans express two microsomal P450's (CYP2R1 [17] and CYP3A4 [19]) and a mitochondrial P450 (CYP27A1 [13]) that exhibit vitamin D 25-hydroxylation activity. CYP2R1 is mainly expressed in the liver and testis, and hydroxylates both vitamin D_3 and vitamin D_2 at C-25 with equal efficiency. It is the most important enzyme for 25(OH)D_3 synthesis in humans, since the patient carrying a mutation in the *CYP2R1* gene shows very low 25(OH)D level in the blood [18]. Cheng *et al*. confirmed that the single point mutation of CYP2R1 protein abolishes 25-hydroxylation activity toward vitamin D_3 and strongly diminishes 25-hydroxylation activity toward vitamin D_2 on the expression experiments of CYP2R1 cDNA using HEK 293 cells [18].

CYP3A4, a major P450 involved in xenobiotics metabolism in the liver and intestine, shows 25-hydroxylation activity for vitamin D_2 but not for vitamin D_3 [19]. CYP27A1 is expressed ubiquitously [36,37], and exhibits 25-hydroxylation activity toward vitamin D_3 and 24-hydroxylation activity toward vitamin D_2 [13]. Our results have demonstrated that CYP2J2 exhibits a relatively higher 25-hydroxylation activity for vitamin D_2 than for vitamin D_3. It localizes in the intestine, liver, and heart [38]. Therefore, it is likely that, similar to xenobiotics, vitamin D_2 undergoes a first-pass activation in the intestine by CYP2J2 or CYP3A4 [39], because vitamin D_2 is supplied only through the diet. The physiological importance of CYP2J2 as a vitamin D_3 25-hydroxylase *in vivo* is unclear since its turnover numbers are low than that for vitamin D_2 and its apparent K_m values appear to be higher than the concentration of circulating vitamin D_3 (ca 2.5-70 nM for humans). Taken together, humans have at least 4 vitamin D 25-hydroxylases that show distinct features, *i.e*. substrate specificity,

position selectivity, and tissue distribution.

Although CYP2J2 expressed in baculovirus and yeast systems has been used to analyze its catalytic properties [33,38], no expression experiment of CYP2J2 in *E. coli* has been reported. Our *E. coli* expression system has achieved a very high expression level of CYP2J2 by combining the deletion of a putative membrane anchor region in the N-terminal with the co-expression of the *E. coli* chaperone protein GroEL-GroES (Table 1). Therefore, the modification applied here is useful for the preparation of CYP2J2 protein. It should be noted that the truncations of the N-terminal hydrophobic region do not severely affect the catalytic activity and substrate specificity of P450 [24,40]. The deletion of an N-terminal hydrophobic region may account for the equal distribution of D34 in cytosol and membrane fractions (Table 1).

Recently, Shinkyo *et al*. analyzed the 25-hydroxylation activity of CYP2J2 toward vitamin D using baculovirus-infected insect cells [41]. The reported turnover numbers for 1α(OH)D_3, vitaminD_2, and 1α(OH)D_2 were 0.060 ± 0.006 min^{-1}, 0.020 ± 0.003 min^{-1}, and 0.860 ± 0.003 min^{-1}, respectively. Their values for vitamin D_2 and 1α(OH)D_3 are much lower than found here (about 8 times and 35 times respectively), and moreover, no activity toward vitamin D_3 was found in their study. However, the preference of vitamin D_2 structure to that of D_3 can be seen in both their results (1α(OH)D_3 and 1α(OH)D_2) and our results (vitamin D_3 and D_2). The differences between the two studies is likely to be due to the differences in the samples, *i.e*. crude CYP2J2 in the microsomes of insect cells vs. purified CYP2J2 protein. Another reason may be the polymorphisms of the *CYP2J2* gene, of which there are five variants,

according to King *et al*. [42]. These CYP2J2 variants showed different hydroxylation activity toward arachidonic acid [42].

In conclusion, CYP2J2 is the forth P450 exhibiting vitamin D_3 25-hydroxylation activity in humans. It catalyzes the 25-hydroxylation of vitamin D_3 and D_2, ($D_3 < D_2$), although CYP2J2 appears not to be a principal vitamin D 25-hydroxylase in humans compared to CYP2J3 in rats. Future research will be directed toward the pharmacological function of CYP2J2 for vitamin D_2 activation in the intestine, and the relationship between its polymorphisms and activity.

References

1. Brown AJ, Dusso A, Slatopolsky E. Vitamin D. Am J Physiol 1999;277(2 Pt 2):F157-75.

2. DeLuca HF. The vitamin D story: a collaborative effort of basic science and clinical medicine. Faseb J 1988;2(3):224-36.

3. DeLuca HF. Overview of general physiologic features and functions of vitamin D. Am J Clin Nutr 2004;80(6 Suppl):1689S-96S.

4. Jones G, Strugnell SA, DeLuca HF. Current understanding of the molecular actions of vitamin D. Physiol Rev 1998;78(4):1193-231.

5. Omdahl JL, Morris HA, May BK. Hydroxylase enzymes of the vitamin D pathway: expression, function, and regulation. Annu Rev Nutr 2002;22:139-66.

6. Armas LA, Hollis BW, Heaney RP. Vitamin D_2 is much less effective than vitamin D_3 in humans. J Clin Endocrinol Metab 2004;89(11):5387-91.

7. Knutson JC, LeVan LW, Valliere CR, Bishop CW. Pharmacokinetics and systemic effect on calcium homeostasis of 1α,24-dihydroxyvitamin D_2 in rats. Comparison with 1α,25-dihydroxyvitamin D_2, calcitriol, and calcipotriol. Biochem Pharmacol 1997;53(6):829-37.

8. Brown AJ, Ritter CS, Holliday LS, Knutson JC, Strugnell SA. Tissue distribution and activity studies of 1,24-dihydroxyvitamin D_2, a metabolite of vitamin D_2 with low calcemic activity in vivo. Biochem Pharmacol 2004;68(7):1289-96.

9. Mawer EB, Jones G, Davies M, Still PE, Byford V, Schroeder NJ, Makin HL, Bishop CW, Knutson JC. Unique 24-hydroxylated metabolites represent a significant pathway of metabolism of vitamin D_2 in humans: 24-hydroxyvitamin D_2 and

1,24-dihydroxyvitamin D_2 detectable in human serum. J Clin Endocrinol Metab 1998;83(6):2156-66.

10. Horst RL, Koszewski NJ, Reinhardt TA. 1α-Hydroxylation of 24-hydroxyvitamin D_2 represents a minor physiological pathway for the activation of vitamin D_2 in mammals. Biochemistry 1990;29(2):578-82.

11. Ohyama Y, Yamasaki T. Eight cytochrome P450s catalyze vitamin D metabolism. Front Biosci 2004;9:3007-18.

12. Prosser DE, Jones G. Enzymes involved in the activation and inactivation of vitamin D. Trends Biochem Sci 2004;29(12):664-73.

13. Guo YD, Strugnell S, Back DW, Jones G. Transfected human liver cytochrome P-450 hydroxylates vitamin D analogs at different side-chain positions. Proc Natl Acad Sci U S A 1993;90(18):8668-72.

14. Usui E, Noshiro M, Ohyama Y, Okuda K. Unique property of liver mitochondrial P450 to catalyze the two physiologically important reactions involved in both cholesterol catabolism and vitamin D activation. FEBS Lett 1990;274(1-2):175-7.

15. Rosen H, Reshef A, Maeda N, Lippoldt A, Shpizen S, Triger L, Eggertsen G, Björkhem I, Leitersdorf E. Markedly reduced bile acid synthesis but maintained levels of cholesterol and vitamin D metabolites in mice with disrupted sterol 27-hydroxylase gene. J Biol Chem 1998;273(24):14805-12.

16. Moghadasian MH. Cerebrotendinous xanthomatosis: clinical course, genotypes and metabolic backgrounds. Clin Invest Med 2004;27(1):42-50.

17. Cheng JB, Motola DL, Mangelsdorf DJ, Russell DW. De-orphanization of cytochrome P450 2R1: a microsomal vitamin D 25-hydroxylase. J Biol Chem 2003;278(39):38084-93.

18. Cheng JB, Levine MA, Bell NH, Mangelsdorf DJ, Russell DW. Genetic evidence that the human CYP2R1 enzyme is a key vitamin D 25-hydroxylase. Proc Natl Acad Sci U S A 2004;101(20):7711-5.

19. Gupta RP, Hollis BW, Patel SB, Patrick KS, Bell NH. CYP3A4 is a human microsomal vitamin D 25-hydroxylase. J Bone Miner Res 2004;19(4):680-8.

20. Andersson S, Jornvall H. Sex differences in cytochrome P-450-dependent 25-hydroxylation of C27-steroids and vitamin D_3 in rat liver microsomes. J Biol Chem 1986;261(36):16932-6.

21. Hayashi S, Noshiro M, Okuda K. Isolation of a cytochrome P-450 that catalyzes the 25-hydroxylation of vitamin D_3 from rat liver microsomes. J Biochem (Tokyo) 1986;99(6):1753-63.

22. Axen E, Bergman T, Wikvall K. Purification and characterization of a vitamin D_3 25-hydroxylase from pig liver microsomes. Biochem J 1992;287 (Pt 3):725-31.

23. Hosseinpour F, Wikvall K. Porcine microsomal vitamin D_3 25-hydroxylase (CYP2D25). Catalytic properties, tissue distribution, and comparison with human CYP2D6. J Biol Chem 2000;275(44):34650-5.

24. Yamasaki T, Izumi S, Ide H, Ohyama Y. Identification of a novel rat microsomal vitamin D_3 25-hydroxylase. J Biol Chem 2004;279(22):22848-56.

25. Akiyoshi-Shibata M, Sakaki T, Ohyama Y, Noshiro M, Okuda K, Yabusaki Y. Further oxidation of hydroxycalcidiol by calcidiol 24-hydroxylase. A study with the mature enzyme expressed in *Escherichia coli*. Eur J Biochem 1994;224(2):335-43.

26. Yamada S, Shiraishi M, Ohmori M, Takayama H. Facile and stereoselective synthesis of 25-hydroxyvitamin D_2. Tetrahedron Lett 1984;25:3347-3350.

27. Omura T, Sato R. The Carbon Monoxide-Binding Pigment of Liver Microsomes. II.

Solubilization, Purification, and Properties. J Biol Chem 1964;239:2379-85.

28. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227(5259):680-5.

29. Ohyama Y, Hayashi S, Usui E, Noshiro M, Okuda K. Assay of vitamin D derivatives and purification of vitamin D hydroxylases. Methods Enzymol 1997;282:186-99.

30. Gonzalez FJ, Korzekwa KR. Cytochromes P450 expression systems. Annu Rev Pharmacol Toxicol 1995;35:369-90.

31. Barnes HJ, Arlotto MP, Waterman MR. Expression and enzymatic activity of recombinant cytochrome P450 17α-hydroxylase in *Escherichia coli*. Proc Natl Acad Sci U S A 1991;88(13):5597-601.

32. Iwata H, Fujita K, Kushida H, Suzuki A, Konno Y, Nakamura K, Fujino A, Kamataki T. High catalytic activity of human cytochrome P450 co-expressed with human NADPH-cytochrome P450 reductase in *Escherichia coli*. Biochem Pharmacol 1998;55(8):1315-25.

33. Hashizume T, Imaoka S, Mise M, Terauchi Y, Fujii T, Miyazaki H, Kamataki T, Funae Y. Involvement of CYP2J2 and CYP4F12 in the metabolism of ebastine in human intestinal microsomes. J Pharmacol Exp Ther 2002;300(1):298-304.

34. Trang HM, Cole DE, Rubin LA, Pierratos A, Siu S, Vieth R. Evidence that vitamin D_3 increases serum 25-hydroxyvitamin D more efficiently than does vitamin D_2. Am J Clin Nutr 1998;68(4):854-8.

35. Horst RL, Napoli JL, Littledike ET. Discrimination in the metabolism of orally dosed ergocalciferol and cholecalciferol by the pig, rat and chick. Biochem J 1982;204(1):185-9.

36. Andersson S, Davis DL, Dahlback H, Jornvall H, Russell DW. Cloning, structure,

and expression of the mitochondrial cytochrome P-450 sterol 26-hydroxylase, a bile acid biosynthetic enzyme. J Biol Chem 1989;264(14):8222-9.

37. Reiss AB, Martin KO, Rojer DE, Iyer S, Grossi EA, Galloway AC, Javitt NB. Sterol 27-hydroxylase: expression in human arterial endothelium. J Lipid Res 1997;38(6):1254-60.

38. Wu S, Moomaw CR, Tomer KB, Falck JR, Zeldin DC. Molecular cloning and expression of CYP2J2, a human cytochrome P450 arachidonic acid epoxygenase highly expressed in heart. J Biol Chem 1996;271(7):3460-8.

39. Matsumoto S, Hirama T, Matsubara T, Nagata K, Yamazoe Y. Involvement of CYP2J2 on the intestinal first-pass metabolism of antihistamine drug, astemizole. Drug Metab Dispos 2002;30(11):1240-5.

40. Larson JR, Coon MJ, Porter TD. Purification and properties of a shortened form of cytochrome P-450 2E1: deletion of the NH_2-terminal membrane-insertion signal peptide does not alter the catalytic activities. Proc Natl Acad Sci U S A 1991;88(20):9141-5.

41. Shinkyo R, Sakaki T, Kamakura M, Ohta M, Inouye K. Metabolism of vitamin D by human microsomal CYP2R1. Biochem Biophys Res Commun 2004;324(1):451-7.

42. King LM, Ma J, Srettabunjong S, Graves J, Bradbury JA, Li L, Spiecker M, Liao JK, Mohrenweiser H, Zeldin DC. Cloning of CYP2J2 gene and identification of functional polymorphisms. Mol Pharmacol 2002;61(4):840-52.

Footnotes

[1] The abbreviations used are: 25(OH)D_3, 25-hydroxyvitaimin D_3; 1,25 $(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 1α(OH)D_3, 1α-hydroxyvitamin D_3; CYP, cytochrome P450; HPLC, high performance liquid chromatography; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.

Table 1.
Comparison of expression levels of CYP2J2 mutants and their distribution in *E. coli*

Expression vector	Amount of P450 expression (nmol/ liter of culture)			
	20 h		44 h	
	cytosol	membrane	cytosol	membrane
pk2J2/pGro7	– [a)]	–	–	–
pkD19/pGro7	–	0.4	–	1.0
pkD34/pGro7	145	182	128	138

[a)] not detected.

Table 2
Kinetic parameters of CYP2J2 (D34) for vitamin D compounds

Substrates	Hydroxylation position	Turnover number (nmol/min/nmol P450)	K_m (μM)
Vitamin D_3	C-25	0.087 ± 0.013 [a)]	7.7 ± 1.2 [a)]
Vitamin D_2	C-25	0.16 ± 0.03	2.0 ± 0.3
1α(OH)D_3	C-25	2.2 ± 1.0	4.4 ± 0.7
25(OH)D_3	C-24 or 1α	n.d. [b)]	n.d.

[a)] The results are means ± SE of at least three separate experiments.
[b)] n.d., not detected.

Table 3
Comparison of catalytic activities of CYP2J2 and CYP2J3

Substrate	Turnover number (nmol/min/nmol P450)	
	CYP2J2	CYP2J3
Vitamin D_3	0.087	1.4
Vitamin D_2	0.16	0.86
$1\alpha(OH)D_3$	2.2	22
Ebastin[a)]	40.6[b)]	6.4[b)]

[a)] a potent and selective histamine H_1-recepter antagonist *in vivo*
[b)] Hashizume T., et al. (33)

FIGURE LEGENDS

Figure 1. N-Terminal region of deletion mutants of CYP2J2. 2J2 indicates the wild type with a modification at the second amino acid residue (Lue to Ala). D19 and D34 represent deletion mutants of the anchor region lacking 2-19 and 2-34 residues, respectively. A putative membrane anchor region is underlined in 2J2. Substitution of amino acids and the corresponding codons are as follows: for D19, 19R(CGC) to M(ATG), 20T(ACT) to A(GCT), 21L(CTC) to L(CTG), 22L(CTA) to L(TTA), 23L(CTG) to L(TTA), and 24G(GGC) to A(GCA), and for D34, 34F(TTT) to M(ATG), 35L(CTC) to A(GCT), 37R(AGA) to R(CGT), and 38R(CGG) to R(CGT). The substituted amino acids are indicated in italics in D19 and D34.

Figure 2. Reduced CO-difference spectrum of recombinant D34 expressed in *E. coli*. D34 was co-expressed with GroES-GroEL and purified from a membrane fraction. The analysis was performed with a membrane fraction (2.4 mg) solubilized with 0.5% Emulgen 911 in 100 mM potassium phosphate (pH 7.4), 1 mM EDTA, and 20% glycerol.

Figure 3. SDS-PAGE analysis of the purified CYP2J2 (D34) and CYP2J3 (dN2J3) proteins used for activity analysis.

Lane 1, molecular weight markers; lane 2, CYP2J2 (300 ng); lane 3, CYP2J3 (300 ng). Gel were visualized by CBB staining. Calculated molecular weights from amino acid sequences are 54202 and 54373 for D34 and d2J3, respectively.

Figure 4. Normal phase HPLC analysis of the metabolites of 1α(OH)D_3, vitamin D_3, and vitamin D_2 by purified D34. *A*, 1α(OH)D_3; *B*, vitamin D_3; *C*, vitamin D_2. Elution positions of authentic 1,25-$(OH)_2D_3$, 25(OH)D_3, and 25(OH)D_2 are indicated by arrows. Detailed HPLC conditions are described under "EXPERIMENTAL PROCEDURES".

Figure 5. Mass spectrum of the 25-hydroxylated metabolite of vitamin D_2 in Fig. 4C. The purified metabolite was analyzed by mass spectrometry as described under "EXPERIMENTAL PROCEDURES". The peak of m/z 413 agrees with the molecular ion of 25(OH)D_2 + H^+.

Fig. 1 Aiba et al.

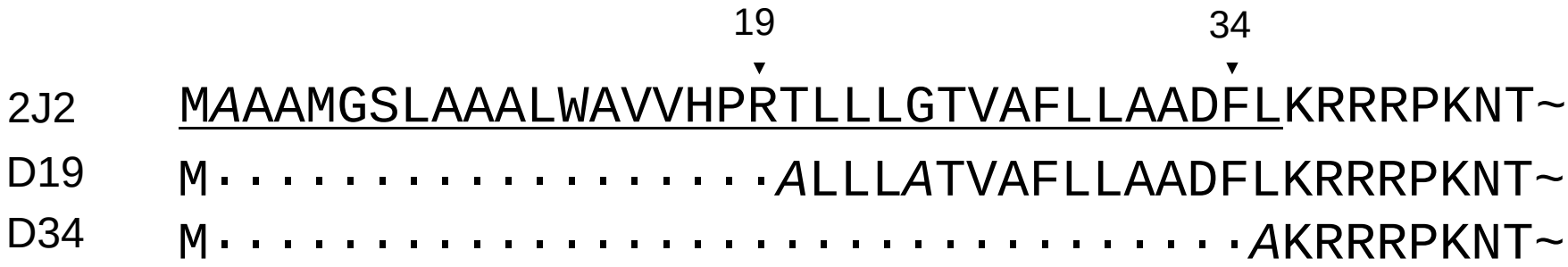

Fig. 2 Aiba et al.

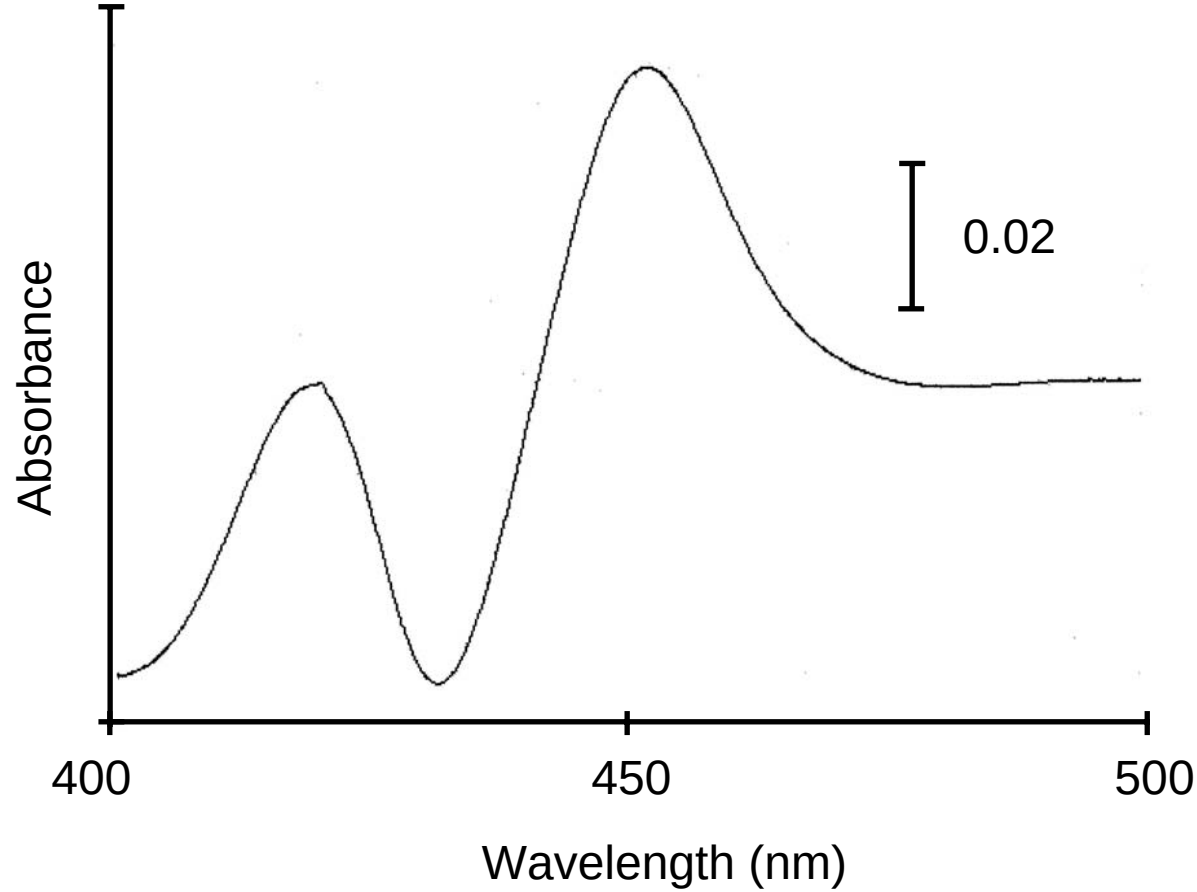

Fig. 3 Aiba et al.

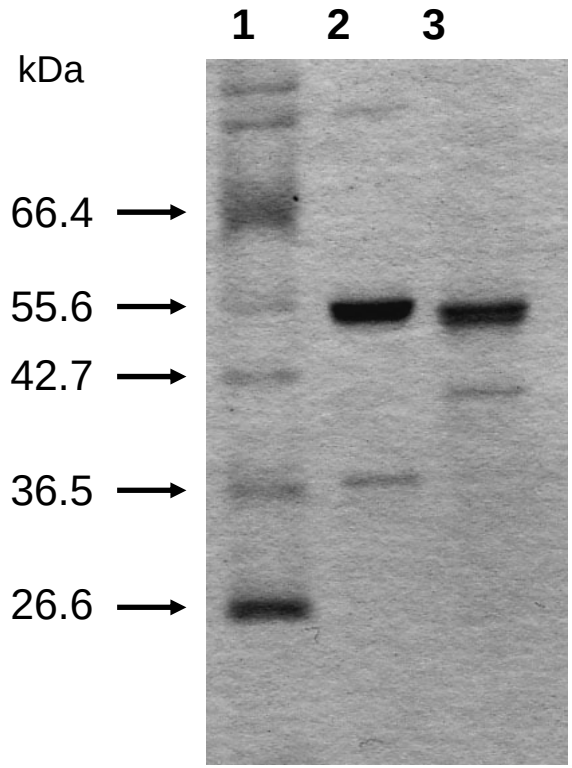

Fig. 4 Aiba et al.

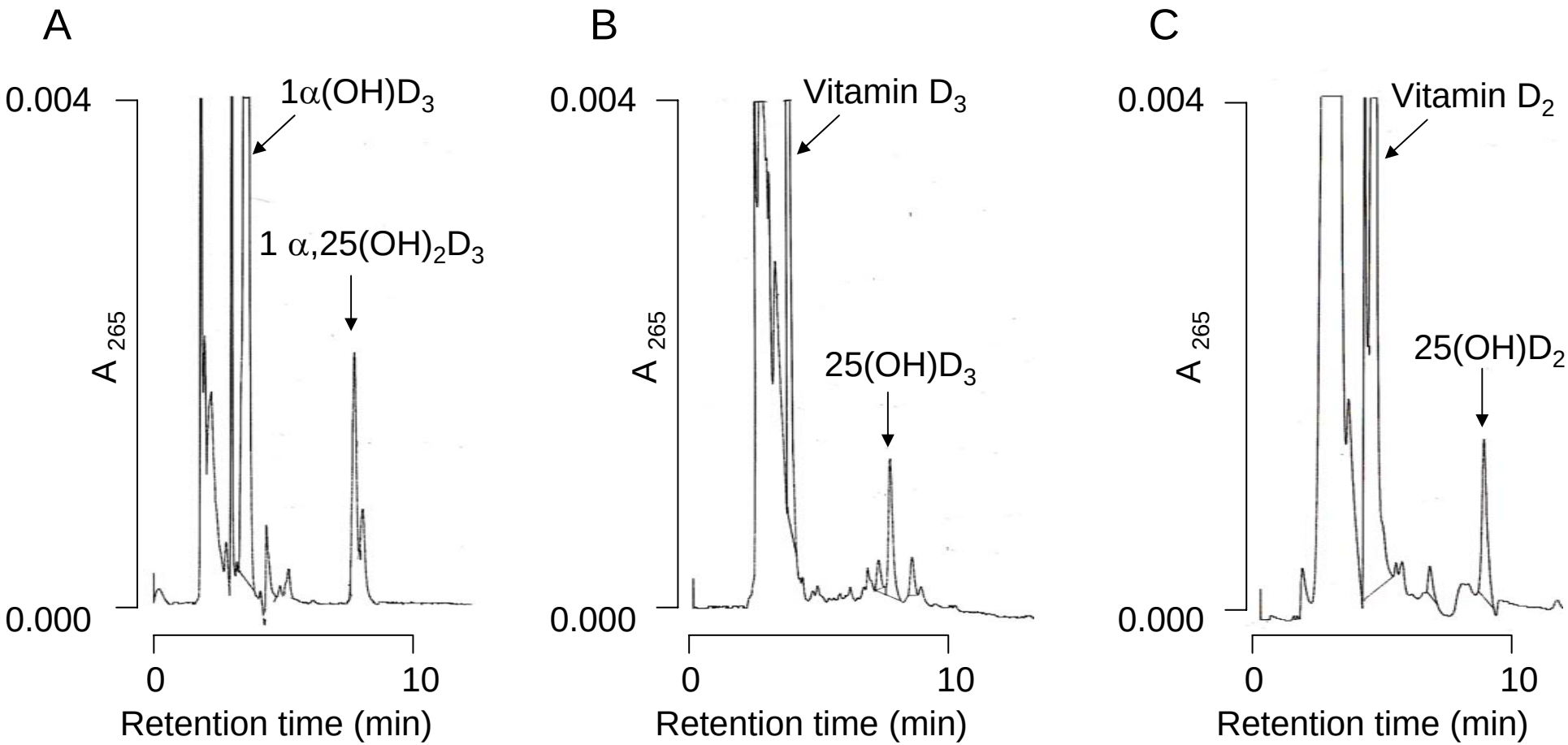

Fig. 5 Aiba et al.