Substrate Specificity for Enzymic Glucuronidation of 24R,25-Dihydroxyvitamin D₃ and Related Compounds

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Keywords Substrate specificity, enzymic glucuronidation, 24R,25-dihydroxyvitamin D₃, pro-form, 24S-isomer, high-performance liquid chromatography

24R,25-Dihydroxyvitamin D₃ [24R,25(OH)₂D₃] is one of the major metabolites of vitamin D₃, which is being examined as an anti-osteoporosis medicine¹, and much interest is focused on the metabolism of 24R,25(OH)₂D₃. In a previous paper of this series, we reported the *in vitro* glucuronidation of 24R,25(OH)₂D₃ using rat liver microsome as an enzyme source, in which its 3- and 24-glucuronides (G) were formed as the main products together with a small amount of the corresponding 25G.²

On the other hand, we also reported that substrate specificity was observed between the *in vitro* glucuronidation of 25-hydroxyvitamin D₃ [25(OH)D₃] and that of its pro-form.³ The former and the latter gave 3G and 25G as the main product, respectively, which means that glucuronidation at the sterically hindered *tert*-hydroxy group occurred mainly on the pro-form.

In the present work, we examined whether this specificity was also observed on 24R,25(OH)₂D₃ and its pro-form [24R,25(OH)₂proD₃]. Furthermore, the *in vitro* glucuronidation of the stereoisomer, 24S,25(OH)₂D₃, was compared with that of 24R,25(OH)₂D₃ (Fig. 1).

Experimental

Materials and reagents

24R,25(OH)₂D₃ was obtained from Duphar (Amsterdam, The Netherlands) and 24R,25(OH)₂proD₃ was synthesized in our laboratories by a known method.⁴ 24S,25(OH)₂D₃ was supplied by Kureha Chemical Co. (Tokyo, Japan). 24R,25(OH)₂D₃ G² and 24R,25(OH)₂proD₃ G (-3G, -24G and -25G) were synthesized in our laboratories; the syntheses of the latter are described below. 4-Phenyl-1,2,4-triazoline-3,5-dione (PTAD) was synthesized from 4-phenylurazole (Nacalai Tesque, Kyoto, Japan) and purified by sublimation prior to use.⁵ Isolute C18 (EC) cartridges (500 mg; International Sorbent Tech., Hengoed, UK) were obtained from Uniflex (Tokyo), and piperidinohydroxy-

propyl Sephadex LH-20 (PHP-LH-20)⁶ was prepared in our laboratories. The other reagents and solvents were commercially available and of analytical grade.

Instruments

High-performance liquid chromatography (HPLC) was performed on a Shimadzu LC-10AT chromatograph (Kyoto) equipped with a Shimadzu SPD-10A UV (265 nm) or a Shimadzu SPD-M6A photodiode array UV (200–340 nm) detector. J'sphere ODS-H80 (4 μm, 150×4.6 mm i.d.) and J'sphere ODS-M80 (4 μm, 150×4.6 mm i.d.) (YMC, Kyoto) columns were used at a flow rate of 1 ml/min at 30°C. The pH of the mobile phase containing AcONH₄ or NaClO₄ was adjusted to the desired values with AcOH or HClO₄, respectively.

Liquid chromatography/tandem mass spectrometry (LC/MSⁿ) was performed using a Finnigan MAT LCQ (San Jose, CA, USA) connected to a JASCO PU-980 (Tokyo) chromatograph and electrospray ionization (ESI) was used. The following conditions were used for the analysis. The spray needle voltage was 5 kV; the heated capillary temperature, the sheath gas flow rate and the auxiliary gas were set at 200°C, 70 units and 20 units, respectively. The capillary voltage was 1 or -1 V and the tube lens offset was 10 or -10 V in the positive- or the negative-ion mode, respectively. A YMC-Pack Pro C18 (5 μm, 150×3.0 mm i.d.) column was used at a flow rate of 0.4 ml/min at 30°C. For a MSⁿ analysis, helium was used as the collision gas and the relative collision energy was set at 20%. Fast-atom bombardment (FAB) mass spectra were determined with a Jeol JMS-DX-303 spectrometer (Tokyo).

Syntheses of monoglucuronides of 24R,25(OH)₂proD₃

A solution of 0.1 M KOH-MeOH (*ca.* 0.2 ml) was added to a solution of methyl{[(24R)-24-acetoxycholesta-5,7-dien-25-ol-3β-yl]-2,3,4-tri-*O*-acetyl-β-D-glucopyranosid}uronate, methyl{[(24R)-3β-acetoxycholesta-5,7-dien-25-ol-24-yl]-2,3,4-tri-*O*-acetyl-β-D-glucopyranosid}uronate or methyl{[(24R)-3β-acetoxycholesta-5,7-dien-24-ol-25-yl]-2,3,4-tri-*O*-acetyl-β-D-

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Fig. 1 Structures of substrates.

glucopyranosid}uronate² (*ca.* 2 mg) in MeOH/CH₂Cl₂ (5:2, v/v, *ca.* 0.3 ml), and the mixture was stirred at room temperature for 5 h. The mixture was diluted with H₂O and neutralized with 5% HCl under ice-cooling. After the addition of NaCl, the mixture was extracted with THF. The organic layer was washed (brine) and then dried over anhydrous Na₂SO₄. After removal of the solvent under reduced pressure, the crude product thus obtained was purified by prep. TLC [5×20 cm, CHCl₃/MeOH/H₂O (70:30:4, v/v/v) (*R_f* *ca.* 0.4)] to yield 24R,25(OH)₂proD₃-3G, -24G or -25G as a colorless semi-solid (*ca.* 0.8 mg), respectively. FAB-MS: 24R,25(OH)₂proD₃ 3G *m/z* 591 [M-H]⁻ (8.7%), 153 (100%), 24R,25(OH)₂proD₃ 24G *m/z* 591 [M-H]⁻ (8.1%), 153 (100%), 24R,25(OH)₂proD₃ 25G *m/z* 591 [M-H]⁻ (2.3%), 153 (100%).

Derivatization

PTAD adduct: the glucuronide was dissolved in AcOEt (50 μl) containing PTAD (5 μg) and kept at room temperature for 1.5 h. MeOH (50 μl) was added in order to decompose the excess reagent, and the solvent was evaporated under a N₂ stream. The residue was redissolved in EtOH.

Acetylation: the glucuronide was dissolved in pyridine-Ac₂O (2:1, v/v, 50 μl) and stored at room temperature for 2.5 h. MeOH (50 μl) was added to the mixture, and the solvent was evaporated under a N₂ stream.

Incubation procedure

The liver microsomal fraction from female Wistar strain rats (*ca.* 140 g, 7 w, Japan S. L. C., Hamamatsu, Japan) was prepared as previously reported.³ The assay medium contained 24R,25(OH)₂D₃, 24R,25(OH)₂proD₃ or 24S,25(OH)₂D₃ [10 μg in EtOH (10 μl)], uridine-5'-diphosphoglucuronic acid [4 μM in 0.1 M Tris-HCl buffer (Tris buffer; pH 7.2; 0.2 ml)], MgCl₂ [10 μmol in Tris buffer (50 μl)], Triton X-100 [0.5 μl in Tris buffer (50 μl)], ethylenediaminetetraacetic acid-2Na [20 nmol in Tris buffer (50 μl)], microsomal fraction [2 mg protein in Tris buffer containing 0.25 M sucrose (60 μl)] and Tris buffer in a total volume of 1.0 ml. The mixture was incubated in air at 37°C for 6 h. The reaction was stopped by cooling on ice.

Separation of the formed monoglucuronides from incubation specimens

A pretreatment of the incubation specimen was carried out as previously reported.² Briefly, the incubation specimen was deproteinized with EtOH, and the supernatant was passed through an Isolute C18 cartridge. After washing with H₂O, the fraction containing steroids was eluted with EtOH and then subjected to PHP-LH-20 column chromatography to remove unconjugated compounds. The glucuronides were eluted with 0.1 M AcONH₄ in 90% EtOH and applied to an Isolute C18 cartridge again to remove AcONH₄. The desired compounds were eluted with EtOH, which was evaporated under a N₂ stream. The residue was redissolved in EtOH and stored at -20°C until use.

Results and Discussion

Characterization of monoglucuronides formed in incubation specimen

In a previous study, the glucuronides formed from 24R,25(OH)₂D₃ in the incubation specimen were identified in comparison with standard samples based on their chromatographic behavior during HPLC and the data obtained from LC/ESI-MS.² In the present study, the identification of the glucuronides formed from the pro-form [24R,25(OH)₂proD₃] and the stereoisomer [24S,25(OH)₂D₃] was carried out with HPLC and LC/ESI-MS using the derivatization method.⁷

First, the incubation specimen using 24R,25(OH)₂proD₃ as a substrate was pretreated and subjected to UV-HPLC [J'sphere ODS-M80, MeCN/0.5% AcONH₄ (pH 5.0)(4:9, v/v)]. A peak coinciding with 24R,25(OH)₂proD₃ 24G (*t_R* 25.7 min) was clearly observed, but the corresponding 3G and 25G could not be detected even when a large amount of incubation specimen was used. The formed 24,25(OH)₂proD₃ 24G was identified by another solvent system [J'sphere ODS-M80, MeCN/2% NaClO₄ (pH 3.0)(9:16, v/v), *t_R* 27.0 min]. Furthermore, photodiode array UV detection of this metabolite showed the same UV absorbance as the authentic sample (*λ_{max}* 283 nm).

Second, the pretreated incubation specimen with 24S,25(OH)₂D₃ was subjected to UV-HPLC [J'sphere

Fig. 2 Chromatograms of monoglucuronides formed from 24*R*,25(OH)₂D₃ (a) and 24*S*,25(OH)₂D₃ (b) in incubation specimen. Conditions: column, J'sphere ODS-H80; mobile phase, MeCN/2% NaClO₄ (pH 3.0)(2:3, v/v).

ODS-H80, MeCN/2% NaClO₄ (pH 3.0)(2:3, v/v)]. A large peak at *t_R* 9.5 min and a small peak at *t_R* 14.3 min were newly observed [Fig. 2 (b)]. Although authentic samples of 24*S*,25(OH)₂D₃ G were not available, based on the chromatographic behavior of 24*R*-isomers, the peak at 9.5 min was inferred to be the 3G form. In order to clarify this point, the metabolite was purified with prep. HPLC (the above system), followed by an Isolute C18 cartridge to remove NaClO₄ as described in the experimental section, and subjected to LC/ESI-MS. We have reported that the glucuronides of vitamin D metabolites give characteristic product ions due to the loss of water and sugar moieties by MS/MS in the positive-ion mode.² Furthermore, we have proved that the PTAD adducts of the conjugated vitamin D metabolites were cleaved at the C6-7 bond of the vitamin D skeleton and provided a product ion indicating the conjugation position by MS/MS/MS.⁷ Based on these data, the intact metabolite and its PTAD derivative were separately subjected to LC/ESI-MS [MeOH/10 mM AcONH₄ (3:1, v/v)]. The former gave product ions similar to those observed for 24*R*,25(OH)₂D₃ 3G by MS/MS using *m/z* 610 [M+NH₄]⁺ as a precursor ion: *m/z* 593 [M+H]⁺, 575 [593-H₂O]⁺, 557 [593-2H₂O]⁺, 539 [593-3H₂O]⁺, 417 [genin+H]⁺, 399 [417-H₂O]⁺, 381 [417-2H₂O]⁺ and 363 [417-3H₂O]⁺. The PTAD derivative gave a product ion at *m/z* 474, which indicated that the glucuronic acid was at the 3-position.⁷ These results showed that this metabolite was 24*S*,25(OH)₂D₃ 3G.

On the other hand, the peak at 14.3 min in Fig. 2 (b) was supposed to be the 24G or 25G form. The com-

Table 1 Formation ratio in enzymic glucuronidation

Substrate	Formation ratio (mean, <i>n</i> =3) ^a		
	3G	24G	25G
24 <i>R</i> ,25(OH) ₂ D ₃	100	92	5
24 <i>R</i> ,25(OH) ₂ proD ₃	N.D. ^b	100	N.D.
24 <i>S</i> ,25(OH) ₂ D ₃	100	N.D.	<7

a. The main product was taken as 100.

b. Not detected.

pound was easily acetylated, and the acetate gave an adduct ion ([M+NH₄]⁺) at *m/z* 820 in the positive LC/ESI-MS. The increased molecular weight was 210, which meant that the metabolite has five reactive *sec*-hydroxy groups. Furthermore, the product ion mass spectra from this ion showed the following characteristic ions: *m/z* 803 [M+H]⁺, 743 [803-AcOH]⁺, 501 [genin moiety+H]⁺, 483 [803-sugar moiety]⁺, 441 [501-AcOH]⁺ and 381 [501-2AcOH]⁺. Although further analysis could not be carried out due to the shortage of the sample, these data indicated that the peak contained 24*S*,25(OH)₂D₃ 25G at any rate. However, the existence of the corresponding 24G was not clarified.

Substrate specificity

The formation ratios in enzymic glucuronidation of 24*R*,25(OH)₂D₃ and related compounds are summarized in Table 1. As mentioned above, we have reported that 24*R*,25(OH)₂D₃ gave 3G and 24G as the main products in almost equal amounts, but only a small amount of the corresponding 25G was obtained in the *in vitro* glucuronidation [Fig. 2 (a)].² In the present study, we examined the enzymic glucuronidation of 24*R*,25(OH)₂proD₃ and 24*S*,25(OH)₂D₃, and obtained the following new evidence.

In the case of 24*R*,25(OH)₂proD₃, its 24G was the only product. On the basis of these data, it was suggested that the 3-hydroxy group of 24*R*,25(OH)₂D₃ is rather flexible because the B-ring is open; therefore, it is much more easily conjugated than that of its pro-form. In an *in vitro* study using 25(OH)proD₃ as a substrate, the glucuronidation occurred at the 3- and 25-positions with a ratio of *ca.* 1:5.² These results demonstrated that in the absence of a *sec*-hydroxy group on the side chain, the glucuronidation occurs at the *tert*-one on the side chain in a large proportion, and although the 3-hydroxy group is a *sec*-one, it is much more inactive than the 25-hydroxy group in the pro-form.

On the contrary, the stereoisomer, 24*S*,25(OH)₂D₃, mainly provided its 3G, and the corresponding 24G could not be detected. The 24*S*-position was supposed to be rather flexible and expected to be conjugated as much as the 3-position. One of the causes for the unexpected result may be that 24*S*,25(OH)₂D₃ is a synthetic analogue, but not a natural metabolite in mammals.

This is supported by the report that 24*S*,25(OH)₂D₃ was hardly metabolized by 25(OH)D₃ 1 α -hydroxylase in comparison with the 24*R*-isomer.⁸

These data showed that LC/ESI-MS using derivatization is useful for the characterization of these glucuronides, and remarkable substrate specificity was observed on enzymic glucuronidation of 24*R*,25(OH)₂D₃ and related compounds.

A part of this work was supported by a grant from the Ministry of Education, Science, Sport and Culture of Japan. We thank Kureha Chemical Co. for supplying 24*S*,25(OH)₂D₃.

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(Received February 19, 1999)

(Accepted April 19, 1999)