Separation and Characterization of Monoglucuronides of Vitamin D₃ and 25-Hydroxyvitamin D₃ in Rat Bile by High-Performance Liquid Chromatography

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Received November 27, 1995; accepted December 25, 1995

The separation and characterization of vitamin D₃- and 25-hydroxyvitamin D₃-monoglucuronides, biliary metabolites obtained from rats dosed with D₃ and 25-hydroxyvitamin D₃ per os, respectively, were carried out by HPLC. The glucuronide fractions were obtained from bile specimens by the combined use of a Bond Elut C18 cartridge, for solid phase extraction, and a lipophilic gel (piperidinohydroxypropyl Sephadex LH-20), for ion-exchange chromatography. Each glucuronide was identified by comparison with an authentic sample in three ways: its chromatographic behavior, that of its fluorescent labeled derivative using 4-[4-(6-methoxy-2-benzoxazolyl)phenyl]-1,2,4-triazoline-3,5-dione and data obtained following enzymatic hydrolysis using β-glucuronidase.

Key words monoglucuronide; vitamin D₃; 25-hydroxyvitamin D₃; rat; biliary metabolite; identification

In general, conjugation is one of the major metabolic pathways for endo- and xenobiotics. Over the past few decades, interest in the metabolism and physiological action of vitamin D (D) has increased exponentially.¹ Despite recent intensive investigation of D metabolism, conjugates of D metabolites still remain poorly understood. Axelsson² and we³,⁴,⁵ have reported that 25-hydroxyvitamin D₃ [25(OH)D₃] 3-sulfate is a major circulating form of D₃ in man. Some investigators have shown the presence of glucuronides (G) of D₃,¹ 1,25(OH)₂D₃,⁶ and 25(OH)D₂,⁷ in mammalian or chick bile, but their structures were not always identified due to the absence of authentic specimens or inconsistencies with authentic compounds. In order to identify the structures of these metabolites, we synthesized the positionally isomeric G of 25(OH)D₃ and 25(OH)D₂ and reported the substrate specificities for these positional isomers found following enzymatic hydrolysis using β-glucuronidase from different sources.⁸

This paper deals with the separation and characterization of D₃- and 25(OH)D₃-monoglucuronides, biliary metabolites obtained from rats dosed with D₃ and 25(OH)D₃ per os, respectively, by comparison with an authentic sample based on its chromatographic behavior using UV and photodiode array UV detectors (Fig. 1). Data obtained from fluorescent derivatization and enzymatic hydrolysis also confirmed these structures.

MATERIALS AND METHODS

Materials and Reagents D₃ was obtained from Tokyo Kasei (Tokyo) and 25(OH)D₃ was generously donated by Teikoku Hormone Mfg. (Tokyo). β-Glucuronidase from Escherichia coli (E. coli, Type IX-A) was obtained from Sigma (St. Louis, MO, U.S.A.). D₃G,⁵,⁹ 25(OH)D₃G,⁸ and the fluorescent derivatization reagent, 4-[4-(6-methoxy-2-benzoxazolyl)phenyl]-1,2,4-triazoline-3,5-dione (MBOTAD)⁶ were synthesized in our own laboratories.

![Diagram of D₃, 25(OH)D₃, and its G](image)

Fig. 1. Structures of D₃, 25(OH)D₃, Its G and MBOTAD Adducts

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Bond Elut C18 cartridges (500 mg; Varian, Harbor, CA, U.S.A.) were obtained from Uniflex (Tokyo) and piperidinoxypropyl Sephadex LH-20 (PHP-LH-20) was prepared in our own laboratories.11

HPLC  HPLC was performed on a Shimadzu LC-6A chromatograph (Kyoto) equipped with a Shimadzu SPD-6AV UV (265 nm), -6A6 photodiode array UV or a Hitachi F-1050 fluorescence (FL: \( \lambda_{ex} \approx 320 \text{ nm}, \lambda_{em} \approx 380 \text{ nm} \)) (Tokyo) detector at a flow rate of 1 ml/min under ambient conditions. The following reversed-phase columns were used: J'sphere ODS-M80 (4 \( \mu \text{m} \)), YMC-GEL C8 (5 \( \mu \text{m} \)) (YMC, Kyoto), TSKgel ODS-80TM (5 \( \mu \text{m} \)) (Tosoh, Tokyo) (each 15 \( \times \) 0.46 cm i.d.). The pH of the mobile phase containing AcONH4 or NaClO4 was adjusted with AcOH or HClO4, respectively.

Bile Samples from Rats  Three and two male Wistar rats weighing ca. 200 g were used for the administration of D3 and 25(OH)D3, respectively. The rats were anesthetized with diethyl ether and the bile duct cannulated with a polyethylene tube (SP 31) (Natsume, Tokyo) for the collection of bile. All animals were starved overnight prior to administration of D3 or 25(OH)D3. A suspension of D3 or 25(OH)D3 (1 ng) in dimethylsulfoxide (0.1 ml) with saline (0.7 ml) and Tween 80 (0.2 ml) was given orally to each rat, and bile was collected over a period of 24 h following administration.

Procedure for Separation and Characterization of Monoglucone in Rat Bile  A bile specimen (12 ml) was diluted with 0.5 M sodium phosphate buffer (pH 7.0) (480 ml), one-fifth of which was passed through a Bond Elut C18 cartridge. After washing with H2O (10 ml), steroids were eluted with MeOH (5 ml), and H2O (0.56 ml) was added to the eluate. The whole was applied to a column (2 \( \times \) 0.6 cm i.d.) of PHP-LH-20. After washing with 90% MeOH (5 ml) and 0.1 M AcOH in 90% MeOH (5 ml), G was eluted with 0.2 M HCOOH in 90% MeOH (5 ml), which was evaporated in vacuo. The residue obtained was subjected to preparative (prep.) HPLC [TSKgel ODS-80TM, D3:G; MeOH–2% NaClO4 (pH 3.0) (12:1), \( t_{R} \approx 10.5-11.5 \text{ min} \); 25(OH)D3:25G: MeCN–2% NaClO4 (pH 3.0) (1:1), \( t_{R} \approx 14-16 \text{ min} \); 25(OH)D3:3G: MeCN–2% NaClO4 (pH 3.0) (1:1), \( t_{R} \approx 17-19 \text{ min} \); UV]. After neutralization with 5% NaHCO3 and diluted with H2O, part of the corresponding fraction thus obtained was applied to a Bond Elut C18 cartridge in the manner described above to remove inorganic salts. The MeOH eluate was evaporated in vacuo and the residue was subjected to the following HPLC analysis in comparison with an authentic sample [J’sphere ODS-M80, D3:G; MeOH–2% NaClO4 (pH 3.0) (14:1), \( t_{R} \approx 8.9 \text{ min} \); 25(OH)D3:25G: MeOH–2% NaClO4 (pH 3.0) (4:1), \( t_{R} \approx 14.7 \text{ min} \); MeCN–2% NaClO4 (pH 3.0) (9:10), \( t_{R} \approx 16.4 \text{ min} \); 25(OH)D3:3G: MeOH–2% NaClO4 (pH 3.0) (4:1), \( t_{R} \approx 15.6 \text{ min} \); MeCN–2% NaClO4 (pH 3.0) (1:1), \( t_{R} \approx 14.2 \text{ min} \); UV and photodiode array UV].

Derivatization Reaction with MBOTAD3,10. The above-described residue obtained from prep. HPLC was dissolved in AcOEt (0.1 ml) containing MBOTAD (ca. 60 \( \mu \text{g} \)) in a cuvette, kept at room temperature for 1 h, and then MeOH (3 drops) was added to decompose excess reagent. The mixture was evaporated under N2 gas, and the residue redissolved in 90% MeOH (1 ml) was transferred to a column (2 \( \times \) 0.6 cm i.d.) of PHP-LH-20. After washing with 90% MeOH (10 ml) and 0.1 M AcOH in 90% MeOH (15 ml), derivatized G was eluted with 0.1 M AcONH4 in 90% MeOH (ca. 4 ml). An aliquot of the eluate was subjected to the following HPLC analysis in comparison with an authentic sample [YMC-GEL C8, D3:G; MBOTAD: MeOH–0.5% AcONH4 (pH 5.0) (5:1), \( t_{R} \approx 11.4, 13.5 \text{ (min) \text{ min}} \); MeCN–0.5% AcONH4 (pH 5.0) (8:5), \( t_{R} \approx 9.6 \text{ min} \); 25(OH)D3:MBOTAD: MeOH–0.5% AcONH4 (pH 5.0) (3:1), \( t_{R} \approx 10.0, 12.6 \text{ (min) min} \); MeCN–0.5% AcONH4 (pH 5.0) (7:1), \( t_{R} \approx 15.4, 18.6 \text{ (min) min} \); 25(OH)D3:3G:MBOTAD: MeOH–0.5% AcONH4 (pH 5.0) (3:1), \( t_{R} \approx 12, 14.5 \text{ (min) min} \); MeCN–0.5% AcONH4 (pH 5.0) (7:10), \( t_{R} \approx 17.6 \text{ min} \); FL].

Enzymatic Hydrolysis of Monoglucone3b  The G-containing fractions obtained from prep. HPLC [each contained ca. 0.25 mmol of G in EtOH (20 \( \mu \text{l} \)] and \( \beta \)-glucuronidase (from E. coli; 500 Fishman units), each of which was dissolved in acetate buffer (0.1 M AcONa–AcOH (pH 5.0) (0.2 and 0.8 ml, respectively), were separately pre-incubated at 37 °C for 15 min. The two solutions were then mixed and incubated at 37 °C for 2 h. The reaction mixture was extracted with AcOEt and the organic layer was evaporated in vacuo. The residue was submitted to the following HPLC analysis and the compounds were identified by comparison with an authentic sample [J’sphere ODS-M80, MeOH–2% NaClO4 (pH 3.0) (14:1), \( t_{R} \approx 10.2 \text{ min} \); 25(OH)D2:MeCN–H2O (29:1), \( t_{R} \approx 20.6 \text{ min} \); MeOH–0.5% AcONH4 (pH 5.0) (5:1), \( t_{R} \approx 7.3 \text{ min} \); 25(OH)D3:3G, \( t_{R} \approx 6.5 \text{ min} \); 25(OH)D3:25G, \( t_{R} \approx 20.8 \text{ min} \); 25(OH)D3: MeCN–H2O (8:3), \( t_{R} \approx 17.0 \text{ min} \); 25(OH)D3: UV].

RESULTS  The separation and characterization of monogluconides of D3 and 25(OH)D3, biliary metabolites obtained from rats dosed with D3 or 25(OH)D3 per os, respectively, were carried out according to the procedure shown in Fig. 2.

Separation of Biliary Metabolites and Characterization by HPLC  Bile from rats following oral administration of D3 or 25(OH)D3 (1 mg) was diluted with sodium...
phosphate buffer and the desired compound was extracted using a Bond Elut C18 cartridge and then chromatographed on a PHP-LH-20 column to remove coexisting substances. The desired fraction was further purified by prep. HPLC using a reversed-phase column to give D$_3$G, 25(OH)D$_3$-3G or -25G as a single peak, which was confirmed by co-chromatography with an authentic sample using UV detection. Photodiode array UV detection of these metabolites showed the characteristic UV absorbance ($\lambda_{\text{min}}$, ca. 230 nm, $\lambda_{\text{max}}$, ca. 266 nm) of the D structure (Fig. 3). Although precise quantitative determination was not performed, the following amounts of monoglucuronide appeared to be present in rat bile: D$_3$G (92 ng/ml, mean, $n = 3$), 25(OH)D$_3$-3G (137 ng/ml, mean, $n = 2$), 25(OH)D$_3$-25G (54 ng/ml, mean, $n = 2$).

**Characterization by Fluorescent Derivatization** Recently, we described the fluorescent derivatization reagent, MBOTAD, which specifically reacts with the s-cis-diene-structure of D and gives an adduct highly responsive to FL detection.$^{3,10}$ We then used the reagent for the identification of these metabolites. The fraction obtained from prep. HPLC was derivatized with MBOTAD, followed by application to the PHP-LH-20 column. The corresponding peak was clearly detected by FL. The MBOTAD adduct of D produces twin compounds corresponding to the z- and $\beta$-side-linked adducts, with the latter being the main component (Fig. 1).$^{10}$ The adducts of the examined metabolites showed single or twin peaks in their chromatograms depending on the chromatographic conditions, which was also confirmed by comparison with an authentic sample (Fig. 4).

**Characterization by Enzymatic Hydrolysis** In a previous paper, we showed that $\beta$-glucuronidase from *E. coli* hydrolyzed both positional isomers of 25(OH)DG.$^{8}$ Based on these data, the G-containing fraction was subjected to enzymatic hydrolysis using this enzyme, and the obtained residue showed a peak corresponding to D$_3$.
or 25(OH)D₃ on the reversed-phase column with two other solvent systems, which was confirmed by co-chromatography with an authentic sample (Fig. 5).

It is obvious from these data that D₃G and 25(OH)D₃G (3- and 25-G) are excreted in rat bile after oral administration of D₃ and 25(OH)D₃, respectively.

DISCUSSION

Bell and Kodicek reported the presence of the glucuronide conjugate of D₃ in rat bile, but the metabolite was not identical with synthetic D₃G. Litwiler et al. also reported the presence of the monoglucuronide of 1,25(OH)₂D₃, but the site of conjugation was not been determined. Although LeVan et al. suggested the existence of 25(OH)D₃-25G as a biliary metabolite of D₃ in the chick, its structure was determined from hydrolysis data due to the absence of authentic specimens. We have now shown that D₃-3G and 25(OH)D₃-3G, -25G are excreted in rat bile after oral administration of D₃ and 25(OH)D₃, and this was confirmed by comparison with an authentic sample based on its chromatographic behavior, that of its MBOTAD labeled derivative and data obtained from enzymatic hydrolysis. Recently, we reported that the Koenigs-Knorr reaction of 25-hydroxyprovitamin D₃ [25(OH)proD₃] gave 3-monoglucuronide acetate methyl ester (3G') and 25G' in a ratio of 4 to 1; these data are compatible with the reactivity of the hydroxyl groups, sec- and tert-hydroxyl groups, respectively. It should be noted that not only 25(OH)D₃ but also 25(OH)D₂25G was excreted in bile, as suggested by LeVan et al., and the formation ratio (5 to 2) of these metabolites was similar to that (4 to 1) of the synthetic ones using 25(OH)proD₃ as a substrate.

Further studies on the determination of D metabolites in biological fluids are now under investigation in our laboratories.

Acknowledgements The authors thank Teikoku Hormone Mfg. for providing 25(OH)D₃. This work was supported by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan.

REFERENCES