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Short communication

Characterization of monoglucuronides of vitamin D_2 and 25-hydroxyvitamin D_2 in rat bile using high-performance liquid chromatography–atmospheric pressure chemical ionization mass spectrometry

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Abstract

The characterization of vitamin D_2 3-glucuronide, 25-hydroxyvitamin D_2 3-glucuronide and 25-hydroxyvitamin D_2 25-glucuronide, biliary metabolites obtained from rats dosed with vitamin D_2 and 25-hydroxyvitamin D_2 per os, was carried out using HPLC–atmospheric pressure chemical ionization (APCI)-MS. The glucuronide obtained from bile specimens was identified by comparison of its chromatographic behaviour with an authentic sample using HPLC–APCI-MS operating in the negative-ion mode. Methylation of the respective fraction with diazomethane gave the methyl ester, which was also confirmed by HPLC–APCI-MS operating in the positive-ion mode. The $(M-H)^-$ and $(M+NH_4)^+$ ions were monitored in the selected-ion monitoring mode.

Keywords: Vitamins; 25-Hydroxyvitamin D_2; Monoglucuronides

1. Introduction

In a previous paper [1] of this series, we reported the separation and characterization of vitamin D_3 (D_3) 3-glucuronide (3G) and 25-hydroxyvitamin D_3 [25(OH)D_3] 3- and 25-glucuronide (25G), biliary metabolites obtained from rats dosed with D_3 and 25(OH)D_3 per os, respectively, by comparison of its chromatographic behaviour with an authentic sample using UV and photodiode array UV detectors. The data obtained from fluorescent derivatization and enzymatic hydrolysis also confirmed its structure [1]. It is interesting that glucuronidation occurred not only at the 3- but also at the highly hindered 25-position of the secosteroid, that is the *tert.*-hydroxy group. Litwiller et al. [2] reported evidence of a monoglucuronide of 1,25(OH)$_2$$D_3$ in rat bile, but its structure has not been clarified. LeVan et al. [3] also reported the isolation and identification of 25(OH)$D_2$25G as a biliary metabolite of D_2 in an avian species, the chick, but its structure was identified by an indirect method using solvolysis due to the absence of an authentic specimen. Recently, we synthesized 25(OH)$D_2$3G and -25G in order to clarify whether the glucuronidation at the 25-position of 25(OH)D_2 also occurs in mammalian bile as in case of 25(OH)D_3 [4].

We report here the characterization of the D_2 and

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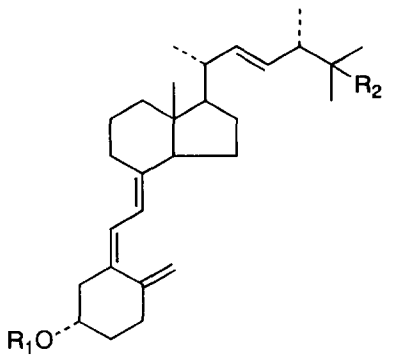

D_2 : R_1=H, R_2=H

$D_2$3G : R_1=G, R_2=H

25(OH)D_2 : R_1=H, R_2=OH

25(OH)$D_2$3G : R_1=G, R_2=OH

25(OH)$D_2$25G : R_1=H, R_2=OG

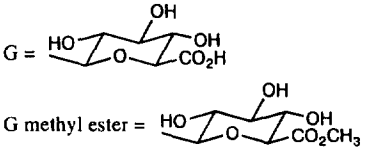

Fig. 1. Structures of D_2, 25(OH)D_2, their glucuronides and methyl esters.

25(OH)D_2 monoglucuronides, biliary metabolites obtained from rats dosed with D_2 and 25(OH)D_2 per os, respectively, using high-performance liquid chromatography (HPLC)–atmospheric pressure chemical ionization (APCI)-MS (Fig. 1).

2. Experimental

2.1. Materials and reagents

D_2 was obtained from Tokyo Kasei (Tokyo, Japan), while $D_2$3G, 25(OH)D_2 [5] and its monoglucuronide [4] were synthesized in our own laboratories. Bond Elut C_{18} cartridges (500 mg, Varian, Harbor, CA, USA) were obtained from UNIFLEX (Tokyo, Japan) and piperidinohydroxypropyl Sephadex LH-20 (PHP-LH-20) was prepared in our own laboratories [6].

2.2. Instrumentation

HPLC was performed on a Shimadzu LC-6A chromatograph (Kyoto, Japan) equipped with a Shimadzu SPD-6AV UV (265 nm) detector at a flow-rate of 1 ml/min under ambient conditions. The following reversed-phase columns were used: TSKgel ODS-80TM (5 μm) (Tosoh, Tokyo, Japan), J'sphere ODS-M80 (4 μm) (YMC, Kyoto, Japan), (each 15×0.46 cm I.D.). The pH of the mobile phase containing $AcONH_4$ or $NaClO_4$ was adjusted with AcOH or $HClO_4$, respectively.

HPLC–MS was performed on a Hitachi M-1000H APCI-MS system (Tokyo, Japan) connected to a Hitachi L-6200 chromatograph at a flow-rate of 1 ml/min. The desolvator temperature and the multiplier voltage were set at 399°C and 2.5 kV, respectively. The focus voltage was set at 110 V (positive-ion mode) or −110 V (negative-ion mode). The chromatography was performed on a J'sphere ODS-H80 column (4 μm, 15×0.46 cm I.D.; YMC) and the column temperature was maintained at 40°C.

2.3. Bile samples from rats

Two male Wistar rats weighing ca. 200 g were separately used for the administration of D_2 and 25(OH)D_2. The rats were anaesthetized with diethyl ether and the bile duct cannulated with a polyethylene tube (SP 31) (Natsume, Tokyo, Japan) for the collection of bile. All animals were starved overnight prior to the administration of D_2 or 25(OH)D_2. A suspension of D_2 (1 mg) or 25(OH)D_2 (0.5 mg) in dimethylsulphoxide (0.1 ml) with saline (0.7 ml) and Tween 80 (0.2 ml) was orally given to each rat, and bile was collected over a period of 24 h following the administration.

2.4. Procedure for separation and characterization of monoglucuronide 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 C_{18} cartridge. After washing with H_2O (10 ml), steroids were eluted with MeOH (5 ml), and H_2O (0.56 ml) was added to the eluate. The entire sample was applied to a column (2×0.6 cm I.D.) of PHP-LH-20.

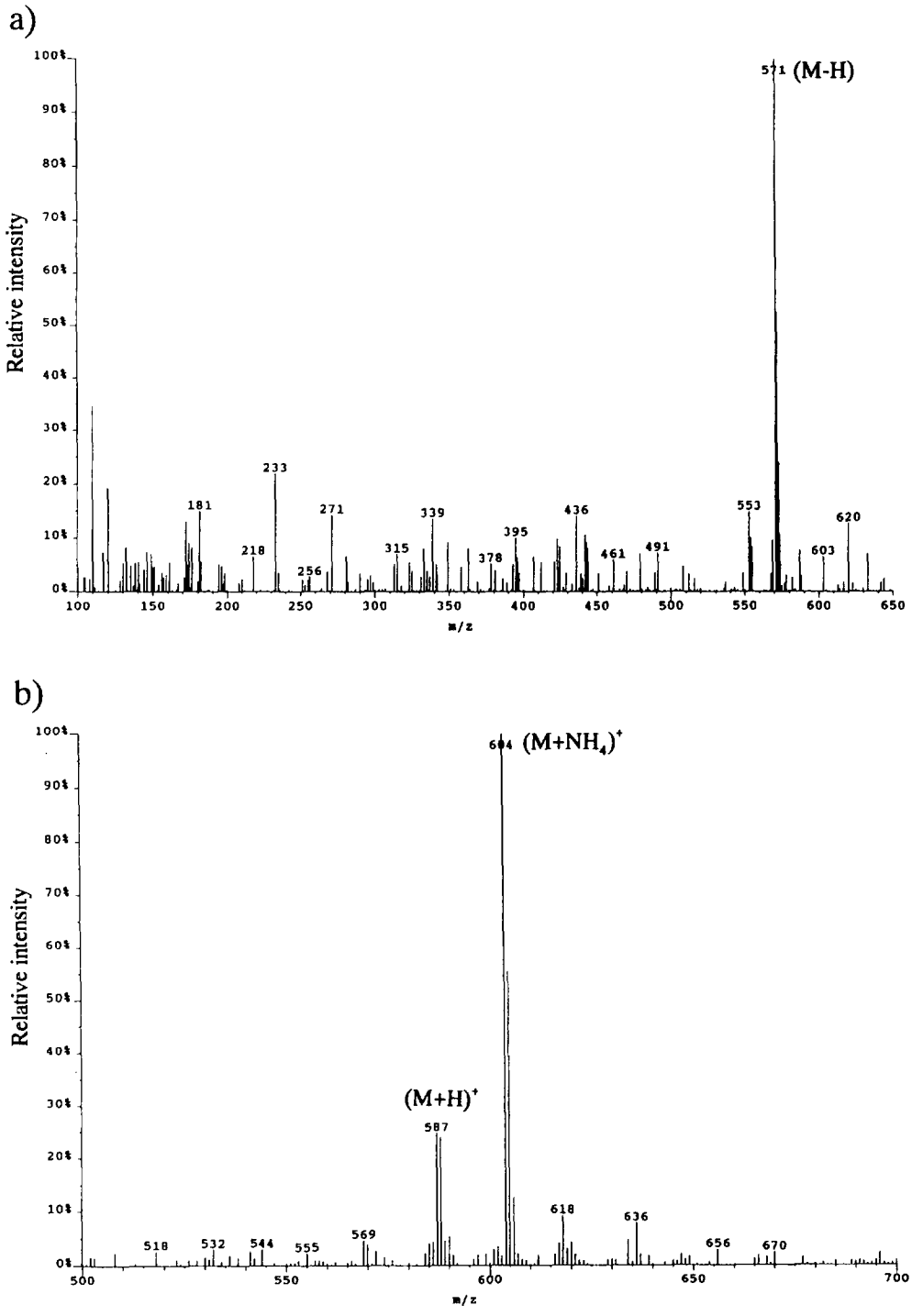

Fig. 2. APCI mass spectra of $D_2$3G and its methyl ester. (a) $D_2$3G, (b) $D_2$3G methyl ester. Conditions: flow injection analysis; mobile phase, (a) MeOH–H_2O (19:1) containing $AcONH_4$ (65 m*M*), (b) MeOH–H_2O (97:3) containing $AcONH_4$ (65 m*M*); MS, drift voltage, (a) −80 V, (b) 15 V; vaporizer temperature, (a) 170°C, (b) 180°C.

After washing with 90% MeOH (5 ml) and 0.1 *M* AcOH in 90% MeOH (5 ml), monoglucuronide was eluted with 0.2 *M* HCOOH in 90% MeOH (10 ml), which was evaporated in vacuo. The obtained residue was subjected to preparative (prep.) HPLC [TSKgel ODS-80Tm, $D_2$3G: MeOH-163 m*M* $NaClO_4$ (pH 3.0) (12:1), t_R 10.0–11.5 min; 25(OH)$D_2$25G: MeCN–163 m*M* $NaClO_4$ (pH 3.0) (10:9), t_R 12.5–14.0 min; 25(OH)$D_2$3G: MeCN–163 m*M* $NaClO_4$ (pH 3.0) (10:9), t_R 15.5–16.5 min]. After neutralization with 5% $NaHCO_3$ and dilution with H_2O, part of the corresponding fraction thus obtained was applied to a Bond Elut C_{18} cartridge in the manner described above to remove any inorganic salts. The MeOH eluate was evaporated in vacuo and part of the residue was subjected to the following HPLC analysis for comparison with an authentic sample [J'sphere ODS-M80, $D_2$3G: MeOH–163 m*M* $NaClO_4$ (pH 3.0) (12:1), t_R 11.5 min; MeOH–65 m*M* $AcONH_4$ (pH 5.0) (12:1), t_R 8.5 min; 25(OH)$D_2$25G: MeOH–163 m*M* $NaClO_4$ (pH 3.0) (4:1), t_R 17.2 min; MeCN–65 m*M* $AcONH_4$ (pH 5.0) (2:3), t_R 9.7 min; 25(OH)$D_3$3G: MeOH–163 m*M* $NaClO_4$ (pH 3.0) (4:1), t_R 18.9 min; MeCN–65 m*M* $AcONH_4$ (pH 5.0) (2:3), t_R 14.9 min].

Part of the above residue was also subjected to the following HPLC–APCI-MS operating in the negative-ion mode for comparison with an authentic sample [$D_2$3G: MeOH–H_2O (19:1) containing $AcONH_4$ (65 m*M*), t_R 4.9 min; 25(OH)$D_2$3G: MeCN–H_2O (2:3) containing $AcONH_4$ (65 m*M*), t_R 13.8 min; 25(OH)$D_2$25G: MeCN–H_2O (2:3) containing $AcONH_4$ (65 m*M*), t_R 7.7 min]. The drift voltage was set at −80 V. The vaporizer temperature was set at 170°C ($D_2$3G) or 210°C [25(OH)D_2 monoglucuronide]. The $[M-H]^-$ ion [$D_2$3G: *m/z* 571; 25(OH)D_2 monoglucuronide: *m/z* 587] was monitored in the selected-ion monitoring (SIM) mode.

Part of the above residue dissolved in MeOH (0.1 ml) was treated with diazomethane in diethyl ether at room temperature for 1 h. After evaporation of the solvent, the obtained residue was subjected to the following HPLC–APCI-MS operating in the positive-ion mode for comparison with an authentic sample [$D_2$3G methyl ester: MeOH–H_2O (97:3) containing $AcONH_4$ (65 m*M*), t_R 6.3 min; 25(OH)$D_2$3G methyl ester: MeOH–H_2O (17:3) containing $AcONH_4$ (65 m*M*), t_R 6.6 min; 25(OH)$D_2$25G methyl ester: MeOH–H_2O (17:3) containing $AcONH_4$ (65 m*M*), t_R 6.4 min]. The drift voltage and the vaporizer temperature were set at 15 V and 180°C, respectively. The $[M+NH_4]^+$ ion [$D_2$3G methyl ester: *m/z* 604, 25(OH)D_2 monoglucuronide methyl ester: *m/z* 620] was monitored in the SIM mode.

3. Results and discussion

3.1. Detection of vitamin D glucuronide using HPLC–APCIMS

The mass spectra of $D_2$3G and 25(OH)D_2 monoglucuronide were scanned using flow injection analysis. The glucuronides produced strong $[M-H]^-$ ions in the negative-ion mode (Fig. 2a), and these ions [$D_2$3G: *m/z* 571; 25(OH)D_2 monoglucuronide: *m/z* 587] were selected for SIM. The mobile phase, drift voltage and vaporizer temperature were set as described in the experimental section in order to obtain a high sensitivity [7,8]. In contrast, the peak corresponding to a molecular ion or quasi-molecular ion has not been observed in the positive-ion mode. These data prompted us to derivatize the glucuronide with diazomethane to give the methyl ester. The obtained $D_2$3G methyl ester and 25(OH)D_2 monoglucuronide methyl ester showed no significant ions in their mass fragmentograms using the MeOH–H_2O or MeCN–H_2O system as the mobile phase of HPLC. However, the $[M+NH_4]^+$ ions were clearly detected along with a weak $[M+H]^+$ ion in the

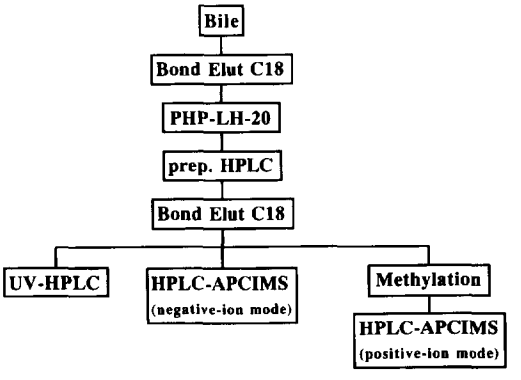

Fig. 3. Procedure for the separation and characterization of $D_2$3G and 25(OH)D_2G in rat bile.

positive-ion mode using the mobile phase containing $AcONH_4$ and a relatively low drift voltage (15 V) (Fig. 2b). The corresponding $[M+NH_4]^+$ ions [$D_2$3G methyl ester: *m/z* 604, 25(OH)D_2 monoglucuronide methyl ester: *m/z* 620] were then selected for SIM.

3.2. Separation and characterization of biliary metabolites

The separation and characterization of monoglucuronides of the D_2 and 25(OH)D_2, biliary metabolites obtained from rats dosed with D_2 or 25(OH)D_2 per os, respectively, were carried out according to the procedure shown in Fig. 3. A bile specimen from rats following oral administration of D_2 (1 mg) or 25(OH)D_2 (0.5 mg) was diluted with sodium phosphate buffer and the desired compound was extracted using a Bond Elut C_{18} 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_2$3G, 25(OH)D_2-3G or -25G as a single peak, which was confirmed by co-chro-

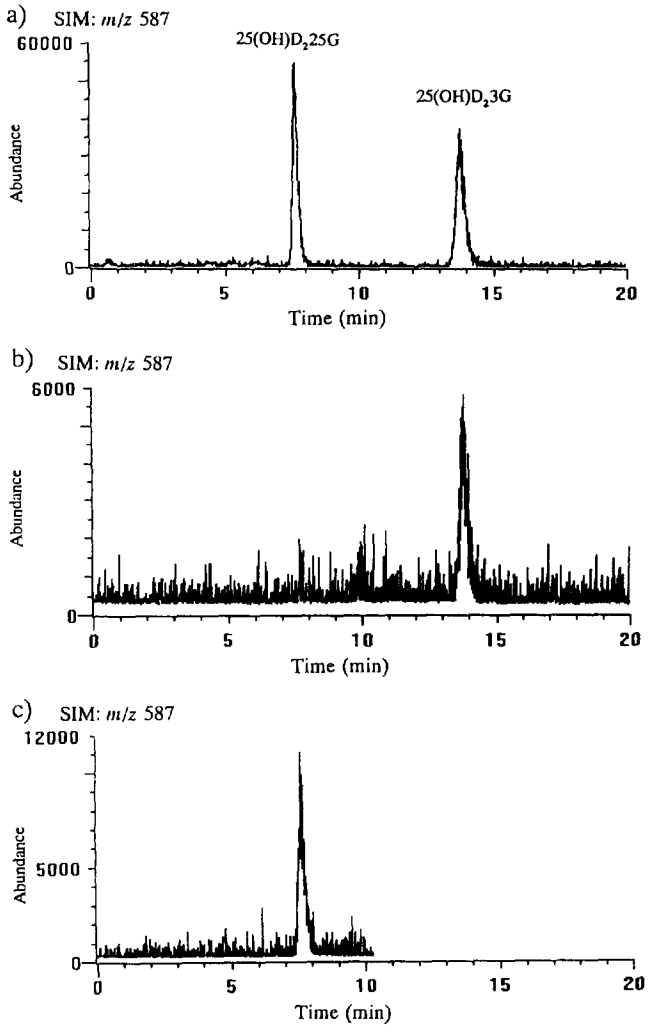

Fig. 4. Mass fragmentograms of 25(OH)D_2G. (a) Authentic sample [25(OH)$D_2$25G, 515 ng: 25(OH)$D_2$3G, 308 ng], (b) 25(OH)$D_2$3G from rat bile, (c) 25(OH)$D_2$25G from rat bile. Conditions: column, J'sphere ODS-H80; mobile phase, MeCN–H_2O (2:3) containing $AcONH_4$ (65 m*M*); MS, drift voltage, −80 V; vaporizer temperature, 210°C.

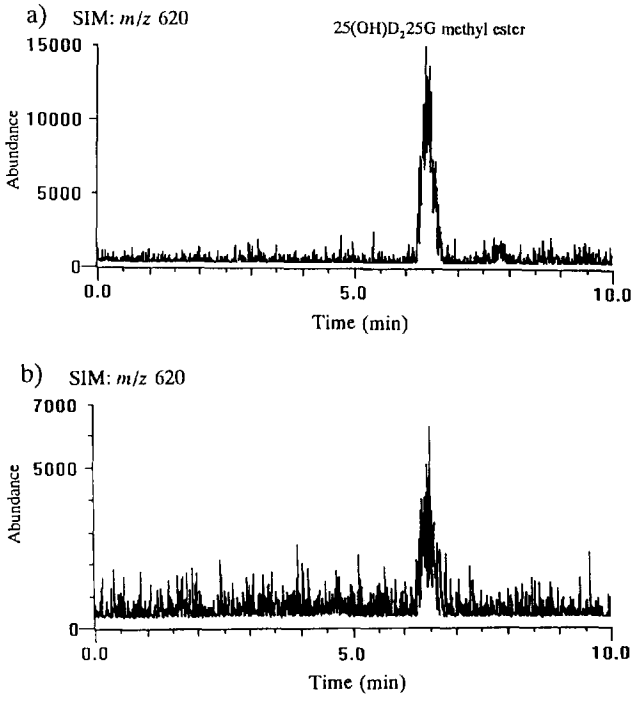

Fig. 5. Mass fragmentograms of 25(OH)$D_2$25G methyl ester. (a) Authentic sample (300 ng), (b) from rat bile. Conditions: column, J'sphere ODS-H80; mobile phase, MeOH–H_2O (17:3) containing $AcONH_4$ (65 m*M*); MS, drift voltage, 15 V; vaporizer temperature, 180°C.

matography with an authentic sample using UV detection. Although precise quantitative determination was not performed, the following amounts of the monoglucuronide appeared to be present in the rat bile: $D_2$3G (70.3 ng/ml, mean, $n=2$), 25(OH)$D_2$3G (156.5 ng/ml, mean, $n=2$), 25(OH)$D_2$25G (143.1 ng/ml, mean, $n=2$).

The structures of these compounds were also confirmed by the above described HPLC–APCI-MS. Each glucuronide was identified by comparison of its chromatographic behaviour with an authentic sample using HPLC–APCI-MS operating in the negative-ion mode of SIM as shown in Fig. 4. Methylation of the respective fraction with diazomethane gave the methyl ester, which was also confirmed by HPLC–APCI-MS operating in the positive-ion mode of SIM as shown in Fig. 5.

This study showed that $D_2$3G and 25(OH)D_2 monoglucuronide are excreted into rat bile after oral administration of D_2 and 25(OH)D_2, respectively. It should be noted that not only 25(OH)$D_2$3G but also 25(OH)$D_2$25G was excreted into the bile, as suggested by LeVan et al. [3], and in the case of 25(OH)D_3. Since we used a complicated sample preparation including prep. HPLC step prior to HPLC–MS to confirm the structure of these glucuronides, a more simplified sample preparation is necessary to further develop the determination method of these compounds in biological fluids using HPLC–MS. Such studies are now in progress in our laboratories.

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