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Characterization of urinary metabolites of vitamin D_3 in man under physiological conditions using liquid chromatography-tandem mass spectrometry

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Abstract

The characterization of the urinary metabolites of vitamin D_3 in man under physiological conditions was performed using liquid chromatography-tandem mass spectrometry (LC-MS-MS). The urine specimens obtained from healthy volunteers were treated with β-glucuronidase, purified with disposal solid-phase extraction cartridges, derivatized with a Cookson-type reagent, 4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalyl)ethyl]-1,2,4-triazoline-3,5-dione, and subjected to LC-MS-MS. The derivatization was employed to increase the ionization efficiencies of the vitamin D_3 metabolites, which enabled detection of the metabolites in the picogram range. The identification of the genin parts of the metabolites was done by comparison with authentic samples based on their LC-MS-MS data. The glucuronides of 23*S*,25-dihydroxyvitamin D_3 and 24*R*,25-dihydroxyvitamin D_3 were obtained as the main metabolites from the urine in almost equal amounts. In contrast to the fact that the plasma/serum concentration of the former is much lower than that of the latter, the hydroxylation at the C-23 position was considered to be the important side-chain modification of $25(OH)D_3$ to excrete the excess vitamin D_3 in man. In addition, 23*S*,25-dihydroxy-24-oxo-vitamin D_3 occurred as its glucuronide in most of the urine, which suggested that this metabolite also plays a part in the excretion of vitamin D_3 in man.

Keywords: Vitamin D_3 metabolite; Human urine; Liquid chromatography-tandem mass spectrometry; Derivatization; Cookson-type reagent

1. Introduction

It is well-known that vitamin D_3 is hydroxylated in the liver to 25-hydroxyvitamin D_3 [$25(OH)D_3$], which is further metabolized to the hormonally active form, 1α,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], which has important functions, such as calcium homeostasis and bone formation in man. The bio-conversion of $25(OH)D_3$ to $1,25(OH)_2D_3$ is strictly regulated by serum calcium and inorganic phosphorus levels, and the plasma/serum concentration of $1,25(OH)_2D_3$ is

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about one-thousandth of that of $25(OH)D_3$. Therefore, in the vitamin D-supplemented state, excess $25(OH)D_3$ is considered to be converted to catabolic metabolites to be excreted and then discharged from the human body. Various side-chain oxidized metabolites, such as 24*R*,25-dihydroxyvitamin D_3 [$24,25(OH)_2D_3$], which are formed by a multicatalytic enzyme, $25(OH)D_3$-24-hydroxylase (also called CYP24) [1], have been hitherto considered to be the excretory metabolites because they have a very weak effect on calcium mobilization, but the actual excretory form of vitamin D_3 in man still remains poorly understood.

In a previous paper in this series, the authors reported that large amounts of 23*S*,25-dihydroxyvitamin D_3 [$23,25(OH)_2D_3$] and 23*S*,25-dihydroxy-24-oxovitamin D_3 [$23,25(OH)_2$-24-oxo-D_3] were excreted as their glucuronides into the bile of rats administered $25(OH)D_3$ [2], and hypothesized that hydroxylation at the C-23 position and the following conjugation play an important role in the excretion of vitamin D_3. That is, $23,25(OH)_2D_3$ and $23,25(OH)_2$-24-oxo-D_3 may be produced under physiological conditions to modulate the levels of vitamin D_3 in the body although these compounds have not been hitherto detected as the metabolites of vitamin D_3 in the biological fluids unless animals are consuming excessive amounts of vitamin D_3 metabolites [3].

The authors have developed a highly sensitive method for the determination of vitamin D metabolites using liquid chromatography-tandem mass spectrometry (LC-MS-MS) employing derivatization with a Cookson-type reagent, 4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalyl)ethyl]-1,2,4-triazoline-3,5-dione (DMEQ-TAD) [4] (Fig. 1), which is expected to enable detection of small amounts of metabolites occurring under physiological conditions. In this paper, as part of the studies on the excretion of vitamin D_3 in man, characterization of the urinary metabolites in man under physiological conditions was performed using the derivatization-LC-MS-MS method.

2. Experimental

2.1. Materials and reagents

$25(OH)D_3$, $23,25(OH)_2D_3$, $24,25(OH)_2D_3$, 3-epi-$24,25(OH)_2D_3$ and 25*S*,26-dihydroxyvitamin D_3 [$25,26(OH)_2D_3$] were those used in a previous study [5]. $23,25(OH)_2$-24-oxo-D_3 was donated by Kureha Chemical Co. (Tokyo, Japan). [26,26,26,27,27,27-$^2H_6$]-$24,25(OH)_2D_3$ [d_6-$24,25(OH)_2D_3$] was synthesized in our laboratories according to known methods [6,7] using C^2H_3I instead of CH_3I. DMEQTAD [8] was obtained from Wako Pure Chemical Co. (Osaka, Japan). β-Glucuronidase originating from *E. coli* (Type IX-A) was obtained from Sigma (St. Louis, MO, USA). OASIS HLB cartridges (60 mg; Waters Assoc., Milford, MA, USA) were successively washed with AcOEt (2 ml), MeOH (2 ml) and H_2O (2 ml) prior to use. Bond Elut Si cartridges (500 mg; Varian, Harbor, CA, USA) were successively washed with $CHCl_3$–MeOH (30:1, v/v, 3 ml) and $CHCl_3$ (3 ml) prior to use. All other reagents and solvents were of analytical grade.

2.2. Urine samples

Urine samples were obtained from healthy volunteers (six males and four females, age range: 21–58 years) known not to have received vitamin D supplements.

2.3. LC-MS-MS

LC-MS-MS was performed using a ThermoQuest LCQ (San Jose, CA) liquid chromatograph-ion trap-mass spectrometer connected to a JASCO PU-980 (Tokyo) chromatograph, and atmospheric pressure chemical ionization (APCI) was used in the positive-ion mode. A J'sphere ODS H-80 (4 μm, 150 × 4.6 mm i.d.: YMC, Kyoto, Japan) column was used at a flow rate of 1 ml/min at 40 °C. MeOH–H_2O (13:7, v/v) or MeCN–H_2O (2:3, v/v) was used as the mobile phase. Helium was used as the collision gas for the MS-MS. The heated capillary temperature and the vaporizer temperature were set at 200 and 500 °C, respectively. The source current and the

sheath gas flow rate were 5 μA and 80 U, respectively. The capillary voltage and the tube lens offset voltage were 3 and 15 V, respectively. The LC eluent from 0 to 4 or 5 min after injection did not enter the mass spectrometer using a diversion valve.

2.4. Separation of dihydroxyvitamin D_3 metabolites from urine

A urine sample (2 ml) was incubated with β-glucuronidase (1000 Fishman U/ml) in AcONa–AcOH buffer (pH 5.0; 0.1 M) (2 ml) at 37 °C for 2 h. The reaction mixture was added with the solution of d_6-24,25$(OH)_2D_3$ (200 pg in 20 μl of EtOH) and MeCN (2 ml), then subjected to centrifugation (1500 × *g*, 10 min). The supernatant was passed through an OASIS HLB cartridge. After washing with H_2O (2 ml), 70% MeOH (2 ml) and hexane (1 ml), the metabolites were eluted with AcOEt (1 ml), which was evaporated under a N_2 gas stream. The residue was dissolved in $CHCl_3$ (0.2 ml × 2) and applied to a Bond Elut Si cartridge. After washing with $CHCl_3$ (3 ml), the

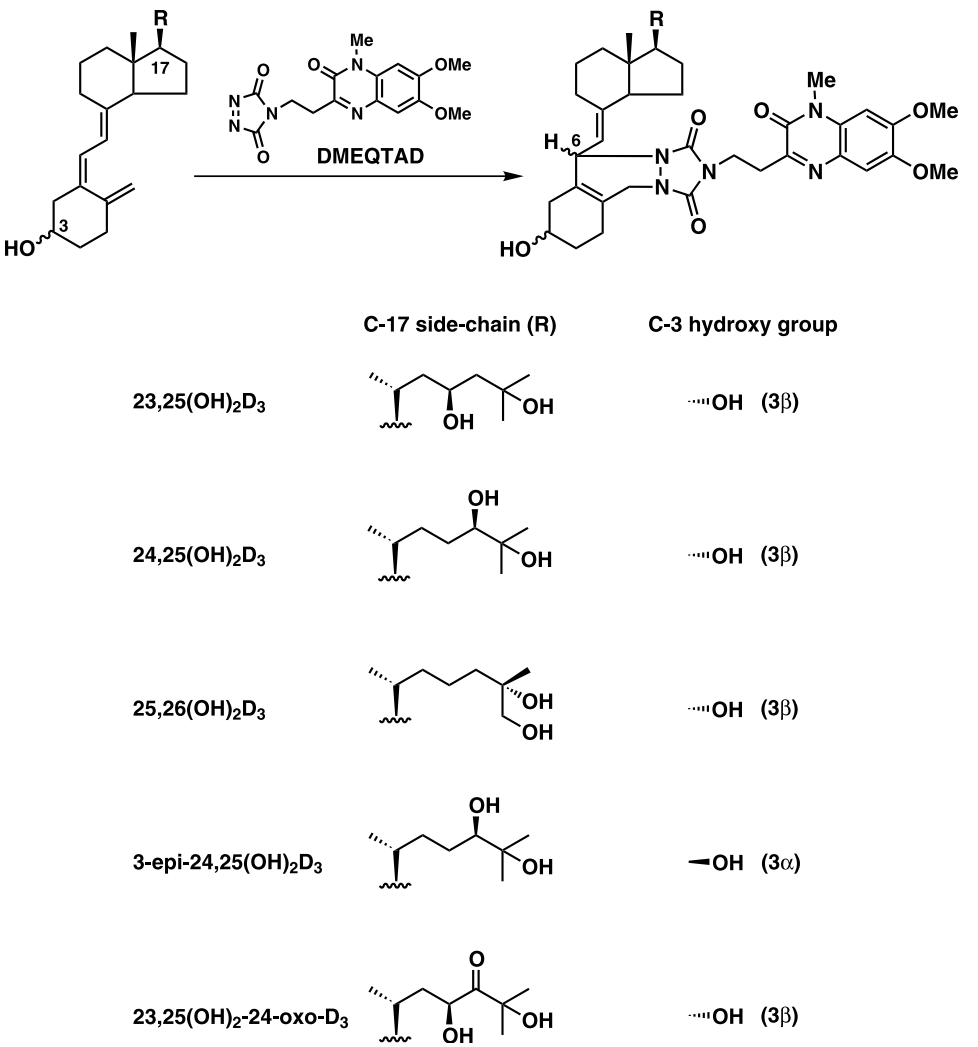

Fig. 1. Derivatization of vitamin D_3 metabolites with DMEQTAD.

metabolites were eluted with $CHCl_3$–MeOH (30:1, v/v, 3 ml). After evaporation, the residue was subjected to derivatization.

2.5. Separation of 23,25(OH)$_2$-24-oxo-D$_3$ from urine

A urine sample (6 ml) was incubated with β-glucuronidase (1000 Fishman U/ml) in AcONa–AcOH buffer (pH 5.0; 0.1 M) (6 ml) at 37 °C for 2 h. The reaction mixture was mixed with MeCN (6 ml) and subjected to centrifugation (1500 × *g*, 10 min). The supernatant was passed through an OASIS HLB cartridge and treated as described above. The eluent was evaporated under a N_2 gas stream and the residue was dissolved in $CHCl_3$ (0.2 ml × 2), which was applied to a Bond Elut Si cartridge. After washing with $CHCl_3$ (3 ml) and $CHCl_3$–MeOH (30:1, v/v, 0.5 ml), the desired compound was eluted with $CHCl_3$–MeOH (30:1, v/v, 2.5 ml). After evaporation, the residue was subjected to derivatization.

2.6. Derivatization with DMEQTAD

The samples were extensively dried and then dissolved in AcOEt (25 μl) containing DMEQ-TAD (2.5 μg). The mixture was kept at room temperature for 30 min, then an additional reagent (2.5 μg/25 μl of AcOEt) was added and the entire mixture was further kept at room temperature for 1 h. After the addition of EtOH (40 μl) to decompose the excess reagent, the solvent was evaporated and the residue was dissolved in the mobile phase (40 μl), 15 μl of which was subjected to LC-MS-MS.

2.7. Preparation of authentic samples

The authentic 25(OH)D_3, 23,25(OH)$_2D_3$, 24,25(OH)$_2D_3$, 3-epi-24,25(OH)$_2D_3$, 25,26(OH)$_2$ D_3 and 23,25(OH)$_2$-24-oxo-D_3 (each 5 ng) were derivatized with DMEQTAD (5 μg in 50 μl of AcOEt) as described above and then dissolved in the mobile phase (100 μl), which were used as authentic samples.

3. Results

3.1. Identification of dihydroxyvitamin D$_3$ metabolites in urine

The behavior of the DMEQTAD derivatives of the dihydroxyvitamin D_3 metabolites in LC-APCI-MS was first examined. The reaction products of a vitamin D compound with DMEQTAD consisted of 6*R* (minor) and 6*S* (main) isomers (Fig. 1), because the reagent attacked at the s-*cis*-diene of the compound from the α- and β-sides, respectively [8]. Therefore, each derivative usually gives characteristic twin peaks on its chromatogram run using a conventional C_{18} column, which is advantageous in identifying metabolites; that is, identification of the metabolites is doubly confirmed by the retention times and the ratio of the twin peaks in one analysis.

All examined derivatives gave very intense protonated molecular ions at *m*/*z* 762 as base ions together with their dehydrated ions with very weak intensities in the positive-APCI-MS. The authors have reported that the MS–MS mode using a low collision energy (relative energy 15%), in which the protonated molecular ion is used as the precursor ion and the same residual ion is used as monitoring ion, is very effective in reducing the background noise [4]. This technique was also used in the present studies.

The retention times of the derivatized metabolites for the two mobile phase systems are summarized in Table 1. When MeOH–H_2O (13:7, v/v) was used as the mobile phase, the 23,25(OH)$_2D_3$ and 24,25(OH)$_2D_3$ derivatives were satisfactorily separated. However, the 6*S* isomer of the 25,26(OH)$_2D_3$ derivative migrated close to those of the above two derivatives and the separation of these three compounds was incomplete. On the other hand, the mobile phase using MeCN as the organic modifier separated the 25,26(OH)$_2D_3$ derivative from the 23,25(OH)$_2D_3$ and 24,25(OH)$_2D_3$ derivatives but failed in separation of the latter two. Thus a mobile phase system that was able to separate all the derivatives clearly could not be obtained; therefore, identification of the dihydroxyvitamin D_3 metabolites was carried out by the complementary use of the above two

Table 1
Retention times and isomer ratios of DMEQTAD derivatives of vitamin D_3 metabolites

Compound (as derivative)	Retention time (min)				Ratio (6*R*/6*S*)
	MeOH–H_2O (13:7, v/v)		MeCN–H_2O (2:3, v/v)		
	6*R*	6*S*	6*R*	6*S*	
23,25$(OH)_2D_3$	6.5	11.0	6.4	10.3	1/3.7
24,25$(OH)_2D_3$	7.0	12.0	6.6	10.1	1/4.4
25,26$(OH)_2D_3$	7.5	11.5	6.2	9.1	1/3.5
3-epi-24,25$(OH)_2D_3$[a]	9.1		8.2		–
23,25$(OH)_2$-24-oxo-D_3	4.8	6.9	5.8	8.8	1/4.1

[a] The isomers were not separated, therefore, the ratio was not determined.

mobile phase systems. Incidentally, the 3-epi-24,25$(OH)_2D_3$ derivative was completely separated from the above three derivatives (Table 1).

The urine specimens were pretreated, derivatized and then subjected first to LC-MS-MS using MeOH–H_2O (13:7, v/v) as the mobile phase (Fig. 2(a)). The peaks corresponding to the derivatives of 23,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$ were observed. In order to clarify whether 25,26$(OH)_2D_3$ occurred in the sample, the same sample was analyzed with the mobile phase using MeCN, but peaks derived from 25,26$(OH)_2D_3$ were not found. The peaks corresponding to the 3-epi-24,25$(OH)_2D_3$ derivative were not detected in both mobile phases. All the examined urine samples obtained from healthy volunteers ($n = 10$) showed similar results; that is, the peaks corresponding to the derivatives of 23,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$ appeared, but those of 25,26$(OH)_2D_3$ and 3-epi-24,25$(OH)_2D_3$ were not found. Furthermore, the peak area ratios of the 6*R* and 6*S* isomers of the derivatized 23,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$ in the urine samples were almost identical with those of the authentic samples. In addition, no peak corresponding to the derivatized metabolite was observed in both mobile phase systems without enzymic hydrolysis. These data suggested that 23,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$ were constantly excreted in the urine as their glucuronides in man.

To identify the genins of the urinary metabolites more reliably, MS–MS analyses with higher collision energy (25%) were performed, in which the protonated molecular ions were also used as the precursor ions. Both product ion mass spectra of the authentic 23,25$(OH)_2D_3$ (Fig. 2(b)) and 24,25$(OH)_2D_3$ derivatives (Fig. 2(c)) demonstrated product ions at m/z 744, 726 and 468. The former two ions were formed by the loss of one or two water molecules from the precursor ions, and the ion at m/z 468 was assigned as the A-ring fragment ions derived from the cleavage of the C-6–7 bond of vitamin D. The 23,25$(OH)_2D_3$ derivative gave an another product ion at m/z 688 inferred to be formed by the cleavage of the C-23–24 bond on the side chain (Fig. 2(b)), which is characteristic of the C-23 hydroxylated compounds and not observed in the 24,25$(OH)_2D_3$ and 25,26$(OH)_2D_3$ derivatives. The product ion mass spectra of the derivatized putative 23,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$ obtained from urine were identical with those of authentic samples (Fig. 2(d) and (e), respectively). These results led to the conclusion that the genins of the metabolites occurring in human urine were certainly 23,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$.

3.2. Identification of 23,25$(OH)_2$-24-oxo-D_3 in urine

The authentic 23,25$(OH)_2$-24-oxo-D_3 almost only gave a protonated molecular ion (m/z 776) in APCI-MS by derivatization with DMEQTAD, just as the above metabolites; therefore, a similar MS–MS technique, in which this ion was used as a precursor and monitoring ion, was employed for

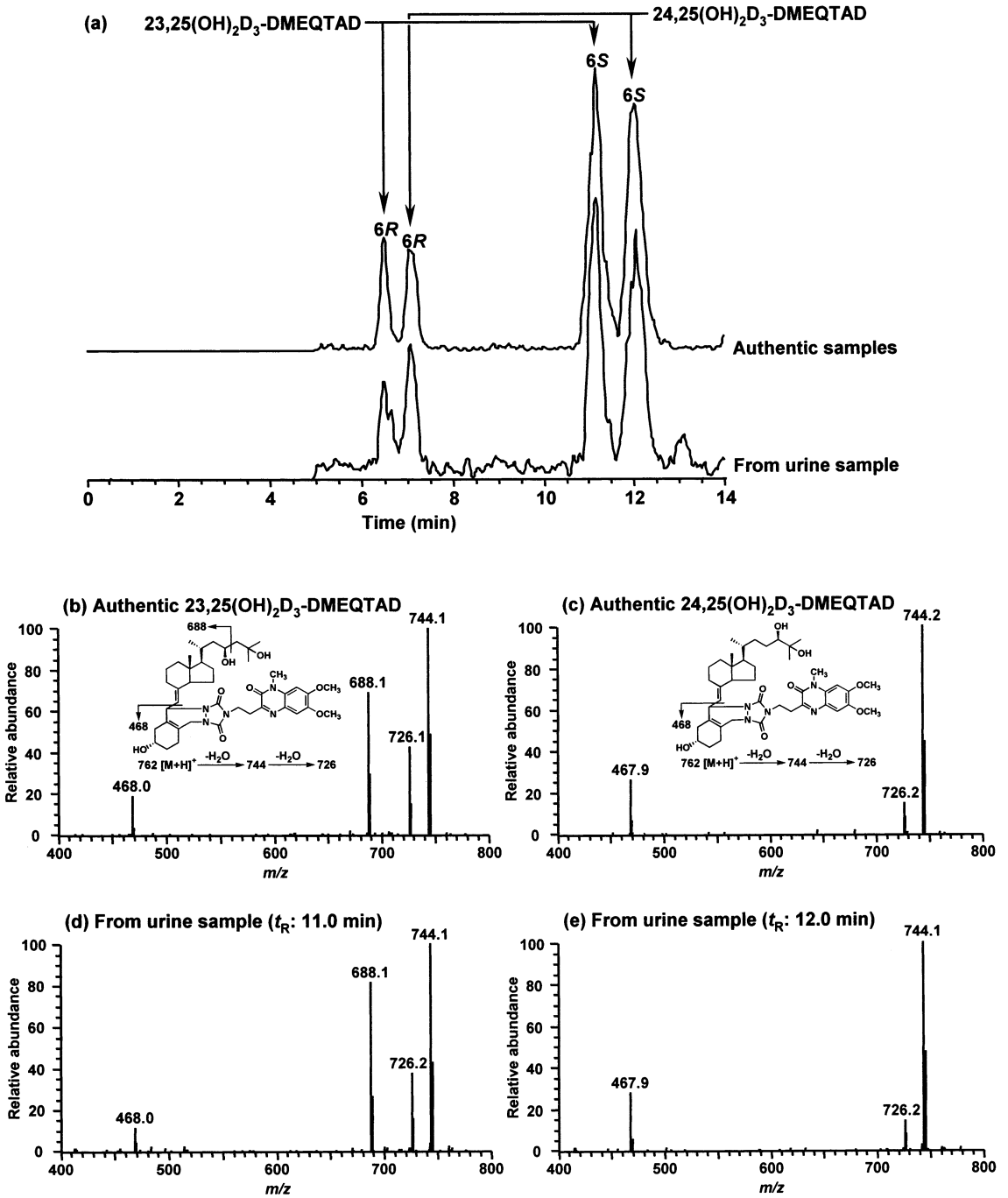

Fig. 2. LC-MS-MS data of derivatized dihydroxyvitamin D_3 metabolites using MeOH–H_2O (13:7, v/v) as the mobile phase. (a) Mass chromatograms: relative collision energy, 15%; precursor ion, m/z 762; monitoring ion, m/z 762. (b–d) Product ion mass spectra: relative collision energy, 25%; precursor ion, m/z 762; monitoring ion, m/z 400–800.

search of the metabolite in human urine. Anticipating that the amount of the metabolites in the urine was very small, 6 ml of urine was used for this purpose.

When the pretreated and derivatized samples were subjected to LC-MS-MS, the peaks corresponding to the 23,25(OH)$_2$-24-oxo-D$_3$ derivative were detected in eight of ten samples in the two mobile phase systems, and as an example, the chromatogram using MeOH–H$_2$O (13:7, v/v) as the mobile phase is shown in Fig. 3(a). For some samples containing relatively large amounts of the metabolite, an MS–MS analysis with a 25% relative collision energy was performed to confirm the structure. As shown in Fig. 3(b) and (c), the product ion mass spectrum obtained from the urine sample completely agreed with that of the authentic sample. These data indicated that 23,25(OH)$_2$-24-oxo-D$_3$ occurred in most normal human urine as its glucuronide.

4. Discussion

It has been accepted that vitamin D$_3$ is excreted after successive oxidation of the side-chain, which leads to the formation of polar compounds, the C-23 carboxylic acid [1,9], but to our knowledge, the formation of the carboxylic acid has been demonstrated only in in vitro experiments. On the contrary, the authors have reported that the hydroxylation at the C-23 position and following glucuronidation is one of the major excretory routes of vitamin D$_3$ in rats [2]. However, the actual excretory form of vitamin D$_3$ in man under physiological conditions still remains poorly understood.

In this respect, the urinary metabolites of vitamin D$_3$ were studied using the LC-MS-MS method the authors had developed [4]. In the method, the metabolites were derivatized to the DMEQTAD adducts, by which the metabolites in the picogram range could be detected.

23,25(OH)$_2$D$_3$ and 24,25(OH)$_2$D$_3$ were detected as major metabolites in the urine samples treated with β-glucuronidase. Although a precise quantitative determination was not performed, the approximate amounts of 23,25(OH)$_2$D$_3$ and 24,25(OH)$_2$D$_3$ obtained from 1 ml of urine were almost equal [23,25(OH)$_2$D$_3$/24,25(OH)$_2$D$_3$ ratio = 0.81 ± 0.19 (mean ± standard deviation

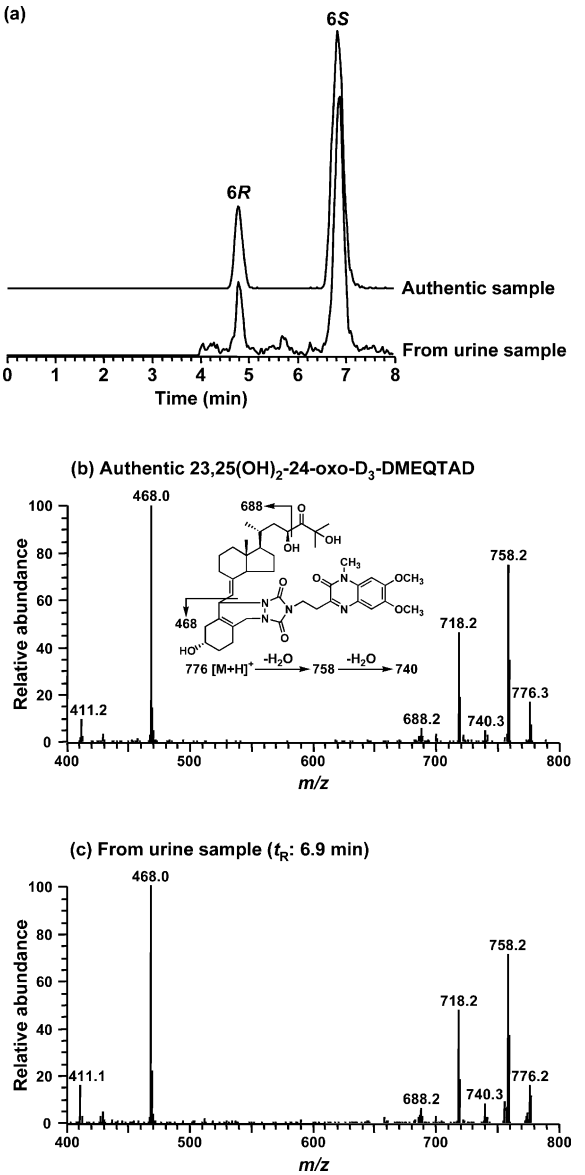

Fig. 3. LC-MS-MS data of derivatized 23,25(OH)$_2$-24-oxo-D$_3$ using MeOH–H$_2$O (13:7, v/v) as the mobile phase. (a) Mass chromatograms: relative collision energy, 15%; precursor ion, m/z 776; monitoring ion, m/z 776. (b) and (c) Product ion mass spectra: relative collision energy, 25%; precursor ion, m/z 776; monitoring ion, m/z 400–800. The assignments of the ions at m/z 411 and 718 have not been done.

(S.D.), $n = 10$)], and most were on the order of several dozens picograms, which were determined according to the peak area ratio with d_6-24,25$(OH)_2D_3$ (100 pg/ml urine) added after the enzymic hydrolysis. Incidentally, the recovery rates involving the derivatization rates of 23,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$ (each 100 pg/ml urine) from the urine samples after the enzymic hydrolysis were 87.2 ± 2.8 and $83.6 \pm 4.8\%$, respectively (mean $\pm$ S.D., $n = 3$). It should be noted that almost equal amounts of 23,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$ were produced from the urine, in contrast to the fact that the plasma/serum concentration of 24,25$(OH)_2D_3$ is much higher than that of 23,25$(OH)_2D_3$ [3]. Based only on their plasma/serum concentration, it seems that the C-23 hydroxylation is much less efficient than the C-24 hydroxylation in the 25$(OH)D_3$ metabolism, though the actual efficiencies of these hydroxylations have not been clarified. Furthermore, Napoli et al. [10] commented that 23,25$(OH)_2D_3$ was rapidly metabolized to 25-hydroxyvitamin D_3-26,23-lactone, which was the major reason that the blood concentration of the former is very low. Although there is no denying this possibility, our results strongly demonstrate that 23,25$(OH)_2D_3$ is more easily conjugated with glucuronic acid and more promptly excreted than the other vitamin D_3 metabolites.

25$(OH)D_3$ is also hydroxylated at the C-26 position to 25,26$(OH)_2D_3$, whose serum concentration is on the order of several hundred picograms per milliliter [3]. Although the biological importance of the metabolite in man has not been clearly demonstrated, the present study revealed that the metabolite is hardly involved with the vitamin D_3 excretion. In addition, 25$(OH)D_3$, the most abundant metabolite circulating in the blood, including its glucuronide, was also not found in the urine samples.

3-Epi-24,25$(OH)_2D_3$ has been isolated from rat plasma and bile as a major metabolite in the free [5,11] and glucuronidated forms [2], respectively, when the rats were dosed with a large amount of 25$(OH)D_3$ or 24,25$(OH)_2D_3$, but the metabolite was not detected in human urine. It was recently reported that C-3 epimerization plays an important role in modulating the concentration and biological activity of vitamin D_3 metabolites in target tissues [12]. On the contrary, the present results suggest that the contribution of the C-3 epimerization in the excretion of vitamin D_3 is minor under physiological conditions and more predominant during hypervitaminosis D_3. In other words, very little production of the C-3 epimerized metabolites would be expected in man under physiological conditions.

23,25$(OH)_2$-24-oxo-D_3 was also isolated and identified from most of the urine samples treated with β-glucuronidase, which is the first reported instance of its occurrence in human biological fluids. Its approximate yield from 1 ml of urine was mostly less than that of 23,25$(OH)_2D_3$ or 24,25$(OH)_2D_3$ and several picograms, though a few samples yielded several dozens of picograms of the metabolite, which were estimated by comparison with a known amount of the authentic sample. In contrast the metabolite was not detected in two samples. No clear correlation was observed between the amounts of 23,25$(OH)_2$-24-oxo-D_3 and those of its precursor, 24,25$(OH)_2D_3$. In any case, the present results indicate that 23,25$(OH)_2$-24-oxo-D_3 also plays a part in the excretion of vitamin D_3 in man. The conjugation position of these metabolites will be clarified in future work.

In conclusion, the authors demonstrated that 23,25$(OH)_2D_3$, 24,25$(OH)_2D_3$ and 23,25$(OH)_2$-24-oxo-D_3 occurred in the urine of man under physiological conditions as their glucuronides. It was also suggested that the C-23 hydroxylation was an important side-chain modification of 25$(OH)D_3$ to excrete the excess vitamin D_3 in man.

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