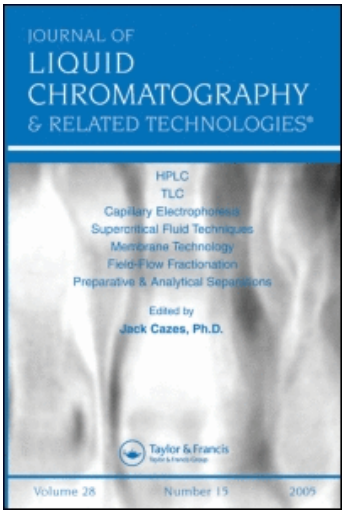

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF VITAMIN D_3 3-FATTY ACID ESTERS AND THEIR LIQUID CHROMATOGRAPHY/MASS SPECTROMETRY

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF VITAMIN D_3 3-FATTY ACID ESTERS AND THEIR LIQUID CHROMATOGRAPHY/MASS SPECTROMETRY

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ABSTRACT

The separation of authentic vitamin D_3 3-stearate, -palmitate, -oleate, and -linoleate, possible metabolites of vitamin D_3, was carried out using reversed-phase high performance liquid chromatography. Liquid chromatography/atmospheric pressure chemical ionization - mass spectrometry (LC/APCI-MS) of these esters was also examined, and a deesterified peak was detected as a base peak. On the contrary, the fatty acid ester derivatized with a Cookson-type reagent, 4-phenyl-1,2,4-triazoline-3,5-dione, showed a quasi-molecular ion as an intense peak. The LC/APCI-MS data on the adducts of another Cookson-type reagent having an electron-capture substituent were also reported.

INTRODUCTION

In general, conjugation (glucuronidation, sulfation, *N*-acetylglucosaminidation, fatty acid esterification, etc.) 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 (usually D_3 and D_2) has increased exponentially.[1]

Despite recent intensive investigation of D metabolism, the conjugates of D metabolites still remain poorly understood. Axelson[2] and we have reported that 25-hydroxyvitamin D_3 [25(OH)D_3] 3-sulfate is a major circulating form of D_3 in human.[3,4]

We also reported the characterization of 25(OH)D 25-glucuronide together with 3-glucuronide as biliary metabolites obtained from rats dosed with 25(OH)D *per os*,[5,6] which was also observed in an *in vitro* experiment using a rat liver microsomal fraction as an enzyme source.[7]

On the other hand, Zagalak et al. reported that endogenous D_3 is present in urine as a genin, sulfate, and glucuronide but mainly as the non-polar esterified form, which is also present in serum.[8] However, these structures were proposed on the basis of gas chromatography (GC)/mass (MS) fragmentographic assay of D_3 or D_2 obtained from hydrolysis of the conjugates, so ambiguity has still remained regarding its conjugation form.

In this report, the separation of authentic D_3 3-stearate, -palmitate, -oleate and -linoleate (possible metabolites of D_3) was carried out using reversed-phase high performance liquid chromatography (HPLC). Liquid chromatography-atmospheric pressure chemical ionization - MS spectrometry (LC/APCI-MS) of these esters and their derivatives with a Cookson-type reagent[9] were also reported (Figure 1).

EXPERIMENTAL

Instrumentation

HPLC was carried out using a Hitachi L-7110 (Tokyo, Japan) chromatograph equipped with a Shimadzu SPD-10A UV detector (265 nm) (Kyoto, Japan) at a flow rate of 1 mL min^{-1}. A reversed-phase column [YMC-Pack ODS-AM (5 μm), YMC-Pack C8 (5 μm) and J'sphere ODS-L80 (4 μm) (150 x 4.6 mm i.d.; YMC, Kyoto)] was used at 40°C.

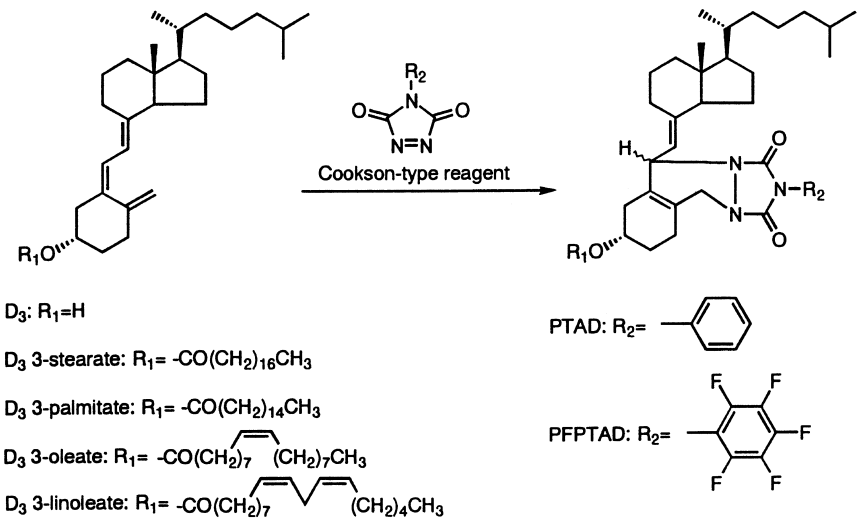

Figure 1. Structures of D_3 3-fatty acid esters and their adducts with Cookson-type reagents.

LC/APCI-MS was performed using a Hitachi M-1000H MS spectrometer connected to a Hitachi L-6200 chromatograph. The temperatures of the vaporizer and the de-solvator were set at 300°C and 399°C, respectively. The drift, multiplier, and focus voltages were set at +/-20 V, 2.5 kV, and +/-110 V, respectively.

Proton nuclear magnetic resonance (^{1}H-NMR) spectra were measured with a JEOL JNM-EX 270 (270.06 MHz) spectrometer (Tokyo) using $CDCl_3$ and tetramethylsilane as a solvent and internal standard, respectively. The following abbreviations are used; s=singlet, d=doublet, q=quartet, m=multiplet, br=broad. Electron-impact MS (EI-MS) was measured with a JEOL JMS-S 102A MS spectrometer. UV spectra were measured with a Hitachi U-2000 spectrophotometer.

Materials and Reagents

D_3 was obtained from Tokyo Kasei (Tokyo). 4-Phenyl-1,2,4-triazoline-3,5-dione (PTAD) was synthesized from 4-phenylurazole (Nacalai Tesque, Kyoto) and purified by sublimation prior to use.[9] 4-Pentafluorophenyl-1,2,4-triazoline-3,5-dione (PFPTAD),[10] D_3 3-stearate, -palmitate[11] were synthesized in these laboratories by known methods. Thin layer chromatography (TLC) and

preparative (prep.) TLC were conducted with 0.25 and 0.5 mm pre-coated Silica gel $60F_{254}$ (Merck, Darmstadt, Germany), respectively. Silica gel 60 (70-230 mesh, Merck) was used for column chromatography. The other reagents and solvents were of analytical grade.

Preparation of D_3 3-Oleate and -Linoleate

D_3 (50 mg), fatty acid (oleic acid or linoleic acid; 50 mg), dimethylaminopyridine (15.6 mg) and dicyclohexylcarbodiimide (26.8 mg) were dissolved in abs. toluene (2 mL), and the whole was kept at room temperature under an N_2 gas stream for 12 h. After the formed urea was filtered off, the filtrate was diluted with benzene which was washed with 5% HCl, 5% $NaHCO_3$, brine, and then dried over Na_2SO_4. The organic solvent was evaporated off and the residue was subjected to column chromatography (200 x 11 mm i.d.). The desired compounds were obtained from hexane-AcOEt (50:1) fraction as oily substances. D_3 3-oleate; ^{1}H-NMR δ: 0.53 (3H, s, H-18), 0.84-0.93 [12H, m, H-21, 26, 27, C*H*$_3$(CH$_2$)$_7$CH=], 4.82 and 5.05 (1H each, br s, H-19), 4.89 (1H, m, H-3α), 5.32-5.34 (2H, m, fatty acid CH=CH), 6.02 and 6.20 (total 2H, AB q, *J*=10.9 Hz, H-6, 7). EI-MS *m/z*: 648 $[M]^+$. UV λ_{max} (EtOH) nm: 265. D_3 3-linoleate; ^{1}H-NMR δ: 0.54 (3H, s, H-18), 0.85-0.92 [12H, m, H-21, 26, 27, C*H*$_3$(CH$_2$)$_4$CH=], 4.84 and 5.05 (1H each, br s, H-19), 4.95 (1H, m, H-3α), 5.28-5.41 [4H, m, fatty acid (CH=CH) x 2], 6.03 and 6.21 (total 2H, AB q, *J*=11.2 Hz, H-6, 7). EI-MS *m/z*: 646 $[M]^+$. UV λ_{max} (EtOH) nm: 262.

General Procedure for Preparation of the Authentic Adducts with Cookson-Type Reagents

A solution of a Cookson-Type reagent in AcOEt was added to a solution of each D_3 3-stearate or -palmitate in AcOEt until the color of excess reagent persisted in the reaction mixture and kept at room temperature for 1h. The reaction was stopped with the addition of MeOH, and the solvent was evaporated off under an N_2 gas stream. The residue obtained was subjected to prep. TLC, and the corresponding zone was extracted with AcOEt to give the desired adducts.

D_3 3-stearate-PTAD adduct (6*S* and 6*R* isomers)

Both 6*S* and 6*R* isomers were obtained as colorless oily substances, whose ratio was *ca.* 6:1.[12, 13] 6*S*; ^{1}H-NMR δ: 0.50 (3H, s, H-18), 0.85-0.91 [12H, m, H-21, 26, 27, C*H*$_3$(CH$_2$)$_{16}$-], 3.89 and 4.20 (total 2H, AB q, *J*=15.5 Hz, H-19), 4.76 and 4.96 (total 2H, AB q, *J*=9.5 Hz, H-6, 7), 5.17 (1H, m, H-3α), 7.35-7.46 (5H, m, Ph-H). 6*R*; ^{1}H-NMR δ: 0.50 (3H, s, H-18), 0.85-0.91 [12H, m, H-21, 26, 27,

C*H*$_3$(CH$_2$)$_{16}$-], 2.32 (1H, d, *J*=7.2 Hz, H-4α), 3.89 and 4.20 (total 2H, AB q, *J*=15.5 Hz, H-19), 4.79 and 4.98 (total 2H, AB q, *J*=9.5 Hz, H-6, 7), 5.07 (1H, m, H-3α), 7.41-7.50 (5H, m, Ph-H).

D_3 3-stearate-PFPTAD adduct (6*S* and 6*R* isomers)

Both 6*S* and 6*R* isomers were obtained as colorless, amorphous, and oily substances, respectively, whose ratio was *ca.* 6:1. 6*S*; ^{1}H-NMR δ: 0.45 (3H, s, H-18), 0.85-0.91 [12H, m, H-21, 26, 27, C*H*$_3$(CH$_2$)$_{16}$-], 3.98 and 4.21 (total 2H, AB q, *J*=15.5 Hz, H-19), 4.76 and 4.96 (total 2H, AB q, *J*=9.8 Hz, H-6, 7), 5.16 (1H, m, H-3α). 6*R*; ^{1}H-NMR δ: 0.54 (3H, s, H-18), 0.85-0.95 [12H, m, H-21, 26, 27, C*H*$_3$(CH$_2$)$_{16}$-], 2.43 (1H, d, *J*=7.5 Hz, H-4α), 3.89 and 4.20 (total 2H, AB q, *J*=15.5 Hz, H-19), 4.79 and 4.98 (total 2H, AB q, *J*=9.8 Hz, H-6, 7), 5.07 (1H, m, H-3α).

D_3 3-palmitate-PTAD adduct (6*S* isomer)

Colorless, oily substances. APCI-MS *m/z*: 798.8 $[M+H]^+$. The corresponding *6R* isomer has not been obtained because of the shortage of the sample.

D_3 3-palmitate-PFPTAD adduct (6*S* isomer)

Colorless, oily substances. APCI-MS *m/z*: 886.6 $[M-1]^+$. The corresponding *6R* isomer has not been obtained because of the shortage of the sample.

LC/APCI-MS of D_3 3-Fatty Acid Esters and Their Adducts with Cookson-Type Reagents

LC/APCI-MS of D_3 3-stearate, -palmitate and their adducts (6*S* isomers) with PTAD or PFPTAD were examined with the flow injection mode. The mobile phase, MeOH containing AcONH$_4$ (10 mM), was maintained at a flow rate of 1 mL min^{-1}.

RESULTS AND DISCUSSION

Separation of D_3 3-Fatty Acid Esters

In order to separate and characterize D_3 3-fatty acid esters in biological fluids, four authentic samples having a common fatty acid (stearic, palmitic, oleic, or linoleic acid) in human plasma have been synthesized and their

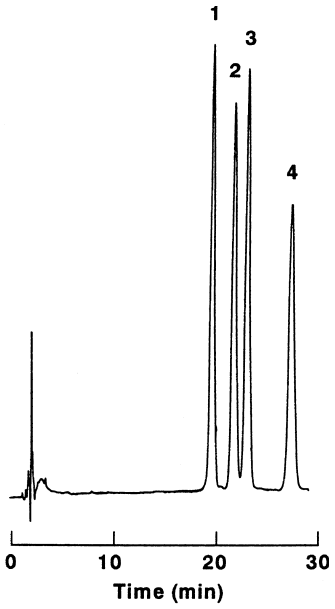

Figure 2. Separation of D_3 3-fatty acid esters. 1) D_3 3-linoleate, 2) D_3 3-palmitate, 3) D_3 3-oleate, 4) D_3 3-stearate. HPLC conditions: column, YMC-Pack C8; mobile phase, MeOH-H_2O (97:3); flow rate, 1 mL min^{-1}; temperature, 40°C; detection, UV 265 nm.

structures were confirmed by ^{1}H-NMR and MS spectra data. The separation of these fatty acid esters using HPLC is a pre-requisite for identifying the compound in biological fluids. Initially, efforts were directed at the separation of four D_3 3-fatty acid esters during reversed-phase HPLC using a conventional octadecylsilyl column (YMC-Pack ODS-AM).

Although two mobile phases (MeOH, MeCN) were examined, the complete separation of D_3 3-oleate and -palmitate has not been done, and some compounds were not eluted within 1 h. These data prompted us to use a less hydrophobic column, J'sphere ODS-L80 or YMC-Pack C8, the former has a low-carbon-loaded phase and the latter is an octylsilyl column. Both columns gave short retention time; however, D_3 3-oleate and -palmitate were not separated using a J'sphere ODS-L80 column.

Satisfactory separation of the four D_3 3-fatty acid esters was achieved within 30 min using a YMC-Pack C8 column and MeOH-H_2O (97:3) as the mobile phase. A typical chromatogram is shown in Figure 2.

LC/APCI-MS of D_3 3-Fatty Acid Esters and Their Adducts with Cookson-Type Reagents

As described in the introduction section, Zagalak *et al.* reported that endogenous D_3 is mainly present in urine and serum as the non-polar esterified form.[8] However, these structures were proposed on the basis of GC/MS fragmentographic assay of D_3 obtained from hydrolysis of the conjugates, so ambiguity has still remained regarding its conjugation form. In order to clarify this ambiguity, it is necessary to obtain the information on the intact form of these esters, and LC/MS is the most promising analytical method for the identification of these non-volatile compounds.[6]

This data prompted us to examine the LC/APCI-MS of D_3 3-fatty acid esters using D_3 3-stearate and -palmitate as the model compounds. The APCI-MS of these esters showed their deesterified ion (*m/z*: 367) as a base peak in the positive ion mode, but a quasi-molecular ion $[QM]^+$ (*m/z*: 651 and 623, respectively) was detected with weak intensity (<12 %). In the negative ion mode, these esters did not show any characteristic ion (Table 1).

It is well-known that a Cookson-type reagent selectively reacts with a compound having a diene structure such as D metabolites under mild conditions to give the stable Diels-Alder adducts,[9] and PTAD is usually used as a protecting group for syntheses of D metabolites or related compounds (Figure 1).[14] The adducts of D metabolites usually consist of 6*S* and 6*R* isomers, and the former is the main product.[12,13] Recently, a Cookson-type reagent having a fluorophore at the 4-position has been developed and used for the determination of D metabolites in biological fluids.[12,15] Yeung *et al.* reported the use of PTAD for the determination of the D analog using LC/electrospray ionization-MS/MS.[16] These data prompted us to examine the LC/APCI-MS of adducts (6*S* isomer) of D_3 3-fatty acid esters with PTAD.

The adducts with PTAD showed $[QM]^+$ in relatively strong intensity (>53%) together with the corresponding $[M-261]^+$ ion as a base peak in a positive ion mode. It may be postulated that these fragment ions were formed by cleavage at the C_{6-7} bond of the D skeleton and loss of the CD-ring with a side chain, which is helpful to identify the fatty acid moiety of these esters (Figure 3). In the negative ion mode, PTAD adducts showed $[QM]^-$ or a cluster ion, which was postulated as the addition of a Cl^- ion derived from $CHCl_3$ used as an injection solvent. Although the peak corresponding to *m/z*: 319 has been observed as a base peak of the PTAD adduct of D_3 3-stearate, its identification has not been done (Table 1).

Wang *et al.* applied a Cookson-type reagent having an electron-capture substituent at the 4-position to the determination of the D analog in plasma with LC/particle-beam negative ionization MS and reported that the derivatization

Table 1

LC/APCI-MS Data on D_3 3-Fatty Acid Esters and Their Adducts with Cookson-Type Reagents

Compound	MW	Observed Ion (*m/z*) Positive Ion Mode	Negative Ion Mode
D_3 3-Stearate	650	367.4 $[M+H-C_{18}H_{36}O_2]^+$ (100)[a] 651.7 $[M+H]^+$ (10)	N.D.[b]
PTAD[c]	825	564.5 $[M-261]^+$ (100) 826.7 $[M+H]^+$ (93)	319.1 (100) 860.5 $[M+35]^-$ (76)
PFPTAD	915	370.2 (100) 654.5 $[M-261]^+$ (74)	319.1 (100) 951.1 $[M+36]^-$ (14)
D_3 3-Palmitate	622	367.4 $[M+H-C_{16}H_{32}O_2]^+$ (100) 623.6 $[M+H]^+$ (12)	N.D.
PTAD	797	536.4 $[N-261]^+$ (100) 798.8 $[M+H]^+$ (53)	796.9 $[M-H]^-$ (20) 832.6 $[M+35]^-$ (100)
PFPTAD	887	370.1 (81) 626.2 $[M-261]^+$ (100) 886.6 $[M-1]^+$ (61)[e]	----[d]

[a] Relative intensity (%).
[b] Not detected; [c] Adduct; [d] Not determined.
[e] Ambiguity still remains in its identification.

with the reagent significantly increased the sensitivity.[17] These data prompted us to use PFPTAD having an electron-capture substituent, and the adducts (6*S* isomer) with PFPTAD also showed LC/APCI-MS spectra similar to those of the adducts of PTAD, but a couple of unidentified peaks (*m/z*: 370 and 319) have been observed as a base peak or a strong intense peak (81%)(Table 1).

The preparation and purification of PTAD is much easier than those of PFPTAD, because the precursor (4-phenylurazole) of PTAD is commercially available and PTAD is easily purified by sublimation.[9] All of these data gave us the conclusion that the adduct of PTAD is suitable for the identification of D_3 3-fatty acid ester in biological fluids using LC/APCI-MS in the positive ion mode.

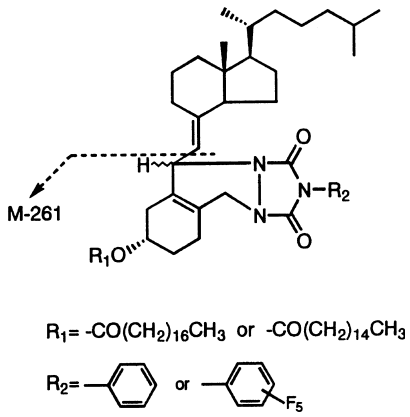

Figure 3. Fragmentation of adducts of D_3 3-fatty acid esters with Cookson-type reagents.

CONCLUSIONS

The separation of authentic D_3 3-stearate, -palmitate, -oleate and -linoleate, possible metabolites of D_3, was carried out using reversed-phase HPLC. Satisfactory separation was obtained using a YMC-Pack C8 column. These data are pre-requisites for characterizing D_3 3-fatty acid esters in biological fluids. The adduct of D_3 3-stearate and -palmitate with a Cookson-type reagent, PTAD, gave an intense quasi-molecular ion together with the base peak consisting of the fatty acid residue in LC/APCI-MS in a positive ion mode, which is useful for the identification of D_3 3-fatty acid esters in biological fluids.

The occurrence of D_3 3-fatty acid esters and the quantitative determination of these esters in biological fluids using the above-described method are now in progress in our laboratories, and the details will be reported in the near future.

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