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Notes

Application of 4-(4-Nitrophenyl)-1,2,4-triazoline-3,5-dione to Analysis of 25-Hydroxyvitamin D_3 in Human Plasma by Liquid Chromatography/Electron Capture Atmospheric Pressure Chemical Ionization–Mass Spectrometry

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The utility of 4-(4-nitrophenyl)-1,2,4-triazoline-3,5-dione (NPTAD) as a derivatization reagent in the analysis of 25-hydroxyvitamin D_3 [25(OH)D_3] using liquid chromatography/electron capture atmospheric pressure chemical ionization–mass spectrometry (LC/ECAPCI-MS) was examined. The derivatives of 25(OH)D_3 with NPTAD underwent electron capture in the APCI source in the negative-ion mode and provided 30-fold higher sensitivity compared to an intact compound. This derivatization–LC/ECAPCI-MS method was applied to a plasma 25-hydroxyvitamin D_3 assay, and gave satisfactory results in sensitivity, specificity, measurable range and throughput of the analysis.

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Introduction

Measurements of the concentration of vitamin D (D) metabolites, especially vitamin D_3 metabolites, in biological fluids are widely used for the assessment of the D status and the follow-up of several diseases (parathyroid gland disorders, renal failure, osteoporosis, rickets and sarcoidosis) in humans. Pharmacokinetic studies of synthetic analogues of D are also important to reduce the risk of their side-effects. Liquid chromatography/mass spectrometry (LC/MS) is considered to be a rapid and specific method for the determination of D compounds in biological fluids. However, the ionization efficiencies of D compounds are generally low in various ionization methods, such as electrospray ionization and atmospheric pressure chemical ionization (APCI). Therefore, conventional LC/MS sometimes does not demonstrate the required sensitivity for the trace analysis of D compounds. To overcome this, derivatization suitable for each ionization method has been examined using a Cookson-type reagent (4-substituted 1,2,4-triazoline-3,5-dione), which selectively, rapidly and quantitatively reacts with the *s-cis*-diene of D compounds to form a Diels–Alder adduct.[1]

In positive APCI-MS, the introduction of proton-affinitive atoms, such as oxygen and nitrogen, to the analyte is very effective in increasing the sensitivity of the resulting derivative. Based on this information, we have developed the LC/positive APCI-MS methods for the determination of D compounds employing the derivatization with a proton-affinitive Cookson-type reagent, 4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalyl)ethyl]-1,2,4-triazoline-3,5-dione (DMEQTAD).[2-4] On the other hand, APCI operating in the negative-ion mode is expected to provide greater sensitivity, because background noise is relatively low in this mode. Electron capture APCI (ECAPCI)-MS using a commercial APCI interface in the negative-ion mode, developed by Singh *et al*., is a highly sensitive technique for electron-affinitive compounds, such as those having a pentafluorobenzyl group.[5] Recently, LC/ECAPCI-MS was applied to the analysis of nitroaromatic compounds.[6] We have also found that a nitrophenyl group has an excellent electron-capturing ability in negative APCI, and have synthesized boronic acid and hydrazine derivatives having a nitrophenyl group as derivatization reagents for vicinal diol and carbonyl compounds.[7] In the present paper, the usefulness of 4-(4-nitrophenyl)-1,2,4-triazoline-3,5-dione (NPTAD)[8] as a derivatization reagent for the analysis of 25-hydroxyvitamin D_3 [25(OH)D_3], a major circulating metabolite in humans, in LC/ECAPCI-MS is described.

Experimental

Materials and reagents

25(OH)D_3 and 25-hydroxyvitamin D_4 [25(OH)D_4; internal standard (IS)] were those used in a previous study.[2] All other reagents and solvents were of analytical grade.

Preparation of NPTAD solution

NPTAD was synthesized from 4-nitrobenzoyl chloride in our laboratories by a known method.[8] Because the reagent was unstable in a solid condition, it was stored as a solution in dried AcOEt (*ca*. 2 mg mL^{-1}) under N_2 at –20°C, where the reagent was stable for at least 6 months. An aliquot of this solution was further diluted with AcOEt to the concentration of *ca*. 0.1 mg mL^{-1} and used for derivatization.

LC/MS(/MS)

LC/MS(/MS) was performed using a ThermoQuest LCQ (San Jose, CA, USA) liquid chromatograph/ion trap-mass spectrometer connected to a JASCO PU-980 (Tokyo, Japan) chromatograph, and APCI was used in the negative-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^{-1} at 40°C. MeOH–H_2O (7:1, v/v) was used as the mobile phase. For an

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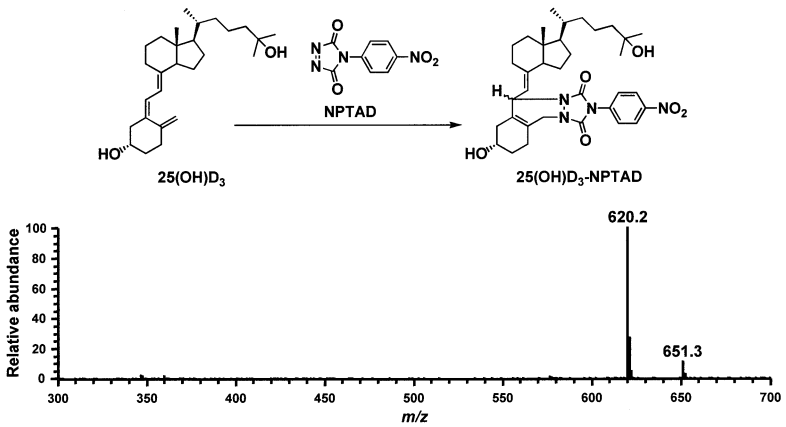

Fig. 1 Derivatization reaction of 25(OH)D_3 with NPTAD and ECAPCI mass spectrum of the resulting derivative.

Table 1 Limit of detection of 25(OH)D_3 and its derivatives[a]

25(OH)D_3 derivative	MS mode	Monitoring ions (*m*/*z*)	t_R (min)	LOD (per injection)
Intact	Positive APCI-MS	Sum of 401.0 $[M + H]^+$ (24),[b] 383.0 $[401 - H_2O]^+$ (100) and 365.0 $[401 - 2H_2O]^+$ (21)	5.0	300 fmol[c]
NPTAD	ECAPCI-MS	620.2 $[M]^-$ (100)	4.7	10 fmol
DMEQTAD	Positive APCI-MS/MS	746.0 $[M + H]^+$ (100)	4.6	12.5 fmol[c]

a. The t_R and LOD values of the derivatives are those of the 6*S*-isomer. b. The values in parentheses are relative intensities. c. Data from the previous study.[2]

MS/MS experiment, helium was used as the collision gas and the relative collision energy was set at 15%. The ECAPCI-MS conditions for the NPTAD derivative is as follows: sheath gas flow rate, 60 units; vaporizer temperature, 375˚C; capillary temperature, 200˚C; capillary voltage, –10 V; tube lens offset voltage, –15 V. The analysis of the DMEQTAD derivative of 25(OH)D_3 was performed according to the previously reported method.[2]

Pretreatment of plasma sample

Plasma samples were obtained from healthy male volunteers and stored at –20˚C prior to use. A plasma sample was pretreated as previously described.[2] Briefly, a plasma sample (20 μL) was added with IS and extracted with MeCN. The extract was dissolved in AcOEt, washed with H_2O and then subjected to derivatization.

Derivatization with NPTAD

A sample was dried and then dissolved in AcOEt (25 μL) containing NPTAD (*ca*. 2.5 μg). After the mixture was kept at room temperature for 30 min, an additional reagent (*ca*. 2.5 μg in 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 any excess reagent, the solvent was evaporated and, unless otherwise indicated, the residue was dissolved in the mobile phase (40 μL), 15 μL of which was subjected to LC/MS.

Effect of derivatization in enhancing the sensitivity

The effect of derivatization with NPTAD in enhancing the sensitivity was evaluated by the limit of detection [LOD; the amount of the derivative per injection giving a signal to noise ratio (*S*/*N*) of 3]. Two hundred picograms (500 fmol) of 25(OH)D_3 were derivatized with NPTAD, as described above, dissolved in the mobile phase (200 μL) to prepare solutions of 2.5 fmol μL^{-1} and subjected to LC/MS. By decreasing the injection volume of the resulting solution stepwise, the amount of derivative giving an *S*/*N* of 3 was determined.

Results and Discussion

LC/ECAPCI-MS(/MS) of NPTAD derivative of 25(OH)D_3

An adduct of 25(OH)D_3 with NPTAD consisted of 6*S* and 6*R* isomers (6*S*:6*R* = *ca*. 7:1) (Fig. 1), because the reagent attacked at the *s*-*cis*-diene of the compound from the α- and β-sides.[1] The derivative gave the intense molecular anion at *m*/*z* 620, but not the deprotonated ion, as shown in its mass spectrum. This demonstrated that the derivative with NPTAD underwent non-dissociative electron capture in the APCI source. Incidentally, the ion at *m*/*z* 651 was inferred to be the $[M - H + O_2]^-$ species.

The MS/MS experiment was also performed, where the molecular anion was used as the precursor ion, but the mode gave only a low intense deprotonated molecular anion (*m*/*z* 619.2) and was not advantageous in sensitivity. Considering these results, the mass chromatography mode of monitoring the molecular anion was used in the following studies.

Effect of derivatization in enhancing sensitivity

The 6*S*-isomer (main product) was used for the determination of the LOD (*S*/*N* = 3) (Table 1). LC/MS conditions of the derivative were optimized, as described in the experimental section, and MeOH–H_2O (7:1, v/v) was used as the mobile phase, because the ionization efficiency of the derivative in the mobile phase using MeOH was about twice that using MeCN. The LOD of the NPTAD derivative was 10 fmol, equivalent to *ca*. 4 pg of intact 25(OH)D_3, which was 30-times that obtained without derivatization. The LOD value was slightly smaller

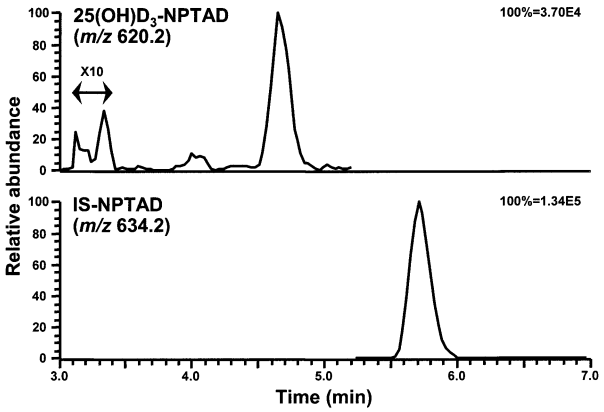

Fig. 2 Typical mass chromatograms of derivatized 25(OH)D_3 and IS in human plasma. The ion abundance (*m*/*z* 620.2) between 3.1 and 3.4 min was magnified 10 times.

than that of the DMEQTAD derivative using positive APCI-MS/MS (12.5 fmol, equivalent to 5 pg).[2]

Applicability of derivatization method with NPTAD to plasma 25(OH)D_3 assay

The applicability of the derivatization method with NPTAD to the biological sample analysis was evaluated in a plasma 25(OH)D_3 assay, an assessment of the D status in humans. As well as the previous study,[2] 25(OH)D_4, a non-endogenous D compound, was used as an IS. Its NPTAD derivative showed behavior in ECAPCI-MS similar to that of 25(OH)D_3; the derivative of IS gave the intense molecular anion (*m*/*z* 634.2) as the base ion. When MeOH–H_2O (7:1, v/v) was used as the mobile phase, the retention times (t_R s) of the respective NPTAD derivatives were as follows; 25(OH)D_3, 4.0 (6*R*) and 4.7 min (6*S*), and IS, 4.5 (6*R*) and 5.7 min (6*S*). Because only the 6*S*-isomers were used for a quantitative analysis of 25(OH)D_3 in the plasma, the LC eluent entered the mass spectrometer from 3 to 7 min after injection by a diversion valve, and a single LC/MS analysis was divided into two segments and the following mass chromatographic method was used: the first segment (3 – 5.2 min) for 25(OH)D_3 derivative: scan range, *m*/*z* 615 – 625; monitoring ion, *m*/*z* 620.2; the second segment (5.2 – 7 min) for IS derivative: scan range, *m*/*z* 630 – 640; monitoring ions, *m*/*z* 634.2.

The calibration curve was constructed by plotting peak area ratios [25(OH)D_3/IS] against the amounts of 25(OH)D_3 per tube [0.05, 0.1, 0.2, 0.5 and 1 ng of 25(OH)D_3 and 1 ng of IS per tube]. The regression line obtained from the combination of three standard curves was y (peak area ratio) = 0.7811x (amount, ng) +0.0197 with the squared correlation coefficient of 0.998. The coefficient of variation of the y values at 0.05 ng of 25(OH)D_3 per tube (lower limit of quantitation) was 8.2% (n = 5). Generally, deuterium labeled analyte is advisable for an IS in LC/MS analysis, but the present method using 25(OH)D_4, an analogue of 25(OH)D_3, as an IS demonstrated good linearity in the range of 0.05 – 1 ng of 25(OH)D_3 per tube with satisfactory precision.

25(OH)D_3 was extracted from plasma by a previously developed method,[2] derivatized and subjected to LC/MS. Due to the use of a large excess of the reagent, the derivatization efficiency was almost quantitative. The absolute recovery rate of 25(OH)D_3 through the extraction procedure was determined to be 83% in the previous study.[2] As shown in Fig. 2, the peaks with satisfactory shapes corresponding to the 25(OH)D_3 and IS derivatives were clearly observed, where the 25(OH)D_3 concentration was 16.8 ng mL^{-1}. The peak height of the main isomer was about 30-times as the background noise (3.1 – 3.4 min; magnified 10 times). Based on these data, *ca*. 3 ng mL^{-1} of 25(OH)D_3 in plasma is measurable when only a 20-μL sample is used, which indicates that the method has satisfactory sensitivity for a plasma 25(OH)D_3 assay (normal range: 10 – 40 ng mL^{-1}) and exceeds the commercially available ^{125}I-radioimmunoassay in sensitivity.[9] An additional eight plasma samples were analyzed, and the resulting 25(OH)D_3 levels were compared with those obtained by the LC/positive APCI-MS/MS method after derivatization with DMEQTAD (DMEQTAD-LC/positive APCI-MS/MS method), which had already been well validated.[2] The 25(OH)D_3 levels measured by the present method (x) correlated well with the assay values obtained with the DMEQTAD-LC/positive APCI-MS/MS method (y); y = 0.9984x + 0.0267 (correlation coefficient, 0.985). These data indicate that the present method is reliable for measuring 25(OH)D_3 in plasma. Furthermore, the method has satisfactory selectivity and throughput of analysis; no chromatographic purifications are necessary for sample pretreatment, and the analysis time from one injection to the next is also short (within 7 min).

Conclusions

A highly sensitive method based on the deivatization of D compounds with NPTAD coupled with LC/MS was developed. The derivatization introduced a nitrophenyl group, a highly electron-affinitive moiety, into the molecule that significantly enhanced the sensitivity in ECAPCI-MS. The LOD of the newly developed method is almost the same as that of the previous method using DMEQTAD,[2] but the new method is expected to be effective in analyses of D compounds in complex biological matrices, because it is usable in the negative-ion mode, which gives low background noise.

Acknowledgements

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