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Tranexamic Acid Analysis as Ethoxycarbonyl-*tert*-butyldimethylsilyl Derivatives by Gas Chromatography–Tandem Mass Spectrometry

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Tranexamic acid (TA) as a synthetic derivative of lysine is a potent antifibrinolytic drug used to reduce bleeding during surgeries including dental extraction. The efficacy of TA administration in ovarian cancer and melasma has also been reported in a previous study. In this study, the mass spectral data set of TA as ethoxycarbonyl-tert-butyldimethylsilyl derivatives was newly constructed by gas chromatography-tandem mass spectrometry in multiple reaction-monitoring mode. This analytical method was validated with good linearity ($r > 0.9988$), limit of detection (LOD, 0.22 ng/mL), limit of quantification (LOQ, 0.72 ng/mL), relative standard deviation (1.3 to 4.8%), and relative error (−6.8 to 3.5%) under optimal conditions. The LOD and LOQ were assessed to be lower than other reported methods. Therefore, the present method will be useful for monitoring of TA in the pharmacological and cosmetic fields.

Keywords: Tranexamic acid, Ethoxycarbonyl-*tert*-butyldimethylsilyl derivatives, Gas chromatography-tandem mass spectrometry

Introduction

Tranexamic acid (TA, Figure 1) as a synthetic derivative of lysine is known as potent antifibrinolytic drug.[1–4] TA interacts at the lysine-binding site of plasminogen molecules during fibrinolysis, which inhibit plasminogen activators from converting plasminogen to plasmin.[5,6] It was reported that regulated TA administration reduces the bleeding during surgery and dental extraction.[7] Early administration of TA was reported to decrease morbidities in women with post-partum hemorrhage.[8] In ovarian cancer patients, treatment with TA showed inhibition of malignant cells and marked reduction in the ascites.[9] Furthermore, TA plays an important role in the treatment of melasma[10] where plasmin indirectly inhibits melanin synthesis.[11] Topical application of TA showed a dose-dependent preventative effect in UV-exposed skin and reduction of arachidonic acid-induced pigmentation.[12] In previous reports, quantitative analysis of TA has been performed using various UV-active derivatives by high-performance liquid chromatography (HPLC) for various biological matrices.[13–23] Since TA analysis without any derivatization is difficult for UV-detection, most methods have been performed by liquid chromatography equipped with mass spectrometry. Some derivatization methods using ethyl chloroformate (ECF) and heptafluorobutyric acid (HFBA) have also been reported for TA analysis by gas chromatography–mass spectrometry (GC–MS).[24,25] However, ethoxycarbonylation (EOC) combined with *tert*-butyldimethylsilylation (TBDMS) for quantitative analysis of TA has not been attempted by GC-tandem mass spectrometry (GC–MS/MS). The GC–MS/MS analysis requires pre-derivatization process of analytes to block all these active protons. In this study, EOC-TBDMS has a good advantage due to its enhanced MS property compared with that of single EOC derivative. Also, GC–MS/MS in multiple reaction monitoring (MRM) mode is selective, sensitive, and accurate. Previously, we reported amino acid (AA) profiling analysis as EOC-TBDMS derivatives, which was effective for biological fluids by GC and GC–MS.[26–28] Therefore, as a new approach in the current study, we developed a method for TA analysis as EOC-TBDMS derivatives in GC–MRM–MS/MS. A new mass spectral data of TA was established as EOC-TBDMS derivatives. Finally, we validated the

Figure 1 Chemical structure of TA.

current method under optimal conditions, which will be useful for TA monitoring and quality control in the pharmacological and cosmetic fields.

Experimental

Chemical and Reagents. The TA standard, 6-aminocaproic acid as an internal standard (IS) and ethyl chloroformate (ECF) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mouse balb/C serum was purchased from Innovative Research (Novi, MI, USA). *N*-Methyl-*N*-*tert*-butyldimethylsilyl trifluoroacetamide (MTBSTFA) was obtained from Pierce (Rockford, IL, USA). Toluene, diethyl ether, ethyl acetate, and dichloromethane were purchased as pesticide-grade from Kanto Chemical (Tokyo, Japan). Syringe-Driven Filter Unit (0.45 μm pore size) was purchased from Merck Millipore (Darmstadt, Germany). All other chemicals used were of analytical grade.

Preparation of Standard Solutions. Stock solutions of TA and IS standards were prepared at 10 μg/μL in 0.1 M HCl. Then, they used to prepare TA and IS working solution (0.1 μg/μL) with 0.1 M HCl. All standard solutions prepared were stored at 4°C.

Gas Chromatography-Tandem Mass Spectrometry. The GC-MRM-MS/MS analysis was performed with a GC–MS-TQ8040 interfaced to triple quadrupoles mass spectrometer with electron impact mode in 70 eV, equipped with Ultra-2 (5% phenyl–95% methylpolysiloxane bonded phase; 25 m × 0.20 mm i.d., 0.11 μm film thickness) cross-linked capillary column (Agilent Technologies, Palo Alto, CA, USA). Injector, interface, and ion source temperatures were maintained at 260, 300, and 230 °C, respectively. Helium was used as the carrier gas at a flow rate of 0.5 mL/min in a constant flow mode. Samples (ca 1.0 μL) were introduced by AOC-20i auto-injector and AOC-20s auto-sampler in the split-injection mode (10:1). Oven temperature was initially set at 180°C for 1 min, and programmed to 300°C, at a rate of 30°C/min with holding time for 5 min. The mass range scanned was 45–600 u at a rate of 3333 u/s. In the MRM mode, one precursor ion of TA and IS, and three characteristic ions of each TA and IS, were used for peak identification and quantification. All GC-MRM-MS/MS runs were performed in triplicate.

Sequential EOC-TBDMS Derivatives. TA and IS working standard solutions were used for optimization in the reaction. A two-phase extractive EOC reaction was immediately performed by mixing for 10 min with ECF (20 μL) in the dichloromethane phase (1 mL) after adjusting pH ≥ 12 with 5.0 M sodium hydroxide in aqueous phase. The aqueous phase solution was then acidified with 10% sulfuric acid, followed by saturation with sodium chloride. The EOC derivatives were sequentially extracted with diethyl ether (3.0 mL) and ethyl acetate (2.0 mL); the combined extracts were evaporated under a gentle nitrogen stream at 40°C. For

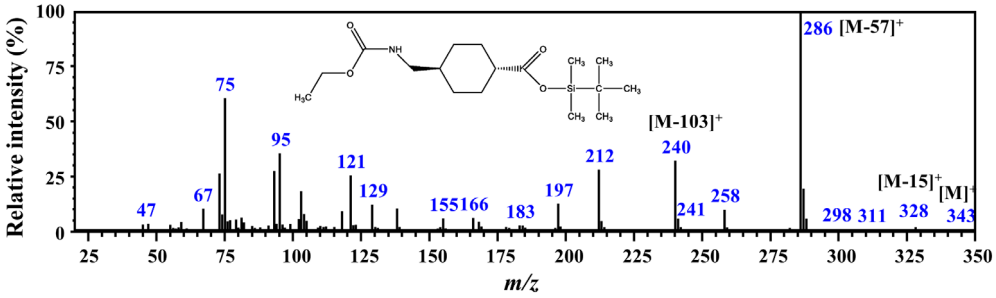

Figure 2 Electron-impact mass spectrum of TA as EOC-TBDMS derivatives.

Table 1. Mass spectral data of TA as EOC/TBDMS derivatives.

	Mass spectral data set[a]				
Analyte	Molecular ion $[M]^+$	Base ion $[M-57]^+$	$[M-15]^+$	$[M-103]^+$	Other characteristic ions
Tranexamic acid	343 (1)	286 (100)	328 (2)	240 (32)	75 (59), 95 (35), 212 (27), 93 (27), 73 (25), 121 (25), 287 (19), 103 (18), 197 (12), 129 (12), 67 (10), 138 (10), 258 (10), 118(9), 104 (8), 74 (7), 81 (6), 241 (6) 166 (6), 155 (6), 288 (6), 79 (5), 102 (5), 77 (5), 105 (5), 76 (5), 213 (5), 59 (4), 168 (4), 82 (4)

[a] *m/z* values with relative abundances of ions (%) in parentheses.

TBDMS derivatization, the prepared EOC derivatives were reacted with MTBSTFA (20 μL) in toluene (20 μL) at 60 °C for 30 min for the GC–MRM–MS/MS analysis.

Method Validation and Application for TA Analysis in Serum Sample as EOC-TBDMS Derivatives. Mouse balb/C serum was used for matrix validation and application in this study. A serum sample (20 μL) containing 0.5 μg of IS was vortex-mixed with acetonitrile (0.1 mL) for 3 min. Then, mixture was diluted with 0.88 mL of distilled water and centrifuged (15 000 rpm, 15 min) to precipitate proteins. The supernatant phase was subjected to the aforementioned EOC-TBDMS reactions prior to GC–MRM–MS/MS analysis. Linearity, repeatability (%RSD, relative standard deviation), accuracy (%RE, relative error), limit of detection (LOD), and limit of quantification (LOQ) were evaluated for method validation in serum sample with triplicate runs. Seven calibration points were performed in the range of 0.1250 μg to 1.50 μg/mL, and each calibration sample was combined with IS of 0.5 μg/mL in serum of 20 μL. The values of slope, intercept, and correlation coefficient (r) were determined to linearity evaluation using least-squares regression analysis for the calibration curve constructed, which based on peak area ratios relative to IS. The LOD and LOQ for TA were calculated at three- and 10-times value of the standard deviation of blank divided by slope of calibration curve, respectively. The repeatability of intra- and inter-day were obtained in triplicates under optimal conditions. Accuracy was evaluated by comparing the spiked amount value and calculated amount value. Repeatability and accuracy were performed in triplicate under optimal conditions at three different concentrations of 0.25, 0.75, and 1.25 μg/mL.

Table 2. Ion transitions of TA and IS as EOC-TBDMS derivatives.

	Ion transitions		
Analyte	Precursor ion (m/z)	Product ions (m/z)	CE (V)
Tranexamic acid	286	75[a], 121[b], 93[b]	24, 9, 15
6-Aminocaproic acid (IS)	260	214[a], 171[b], 75[b]	3, 9, 24

[a] Quantification ion (Underline).
[b] Identification ion.

Results and Discussion

Optimal Conditions for TA Analysis in Serum by GC–MRM–MS/MS. The TA analysis as EOC-TBDMS derivatives was performed under optimal conditions. The EI mass spectral data of TA as EOC-TBDMS derivatives was presented in Figure 2 and Table 1. We identified the molecular ion as m/z 343. By applying scan mode, the characteristic fragment ions of EOC-TBDMS were identified as m/z 328 $[M-15]^+$, m/z 286 $[M-57]^+$, and m/z 240 $[M-103]^+$ (Table 1 and Figure 2).

The ion m/z 286 $[M-57]^+$ was selected as the precursor ion of TA due to characteristic ion with major intensity as

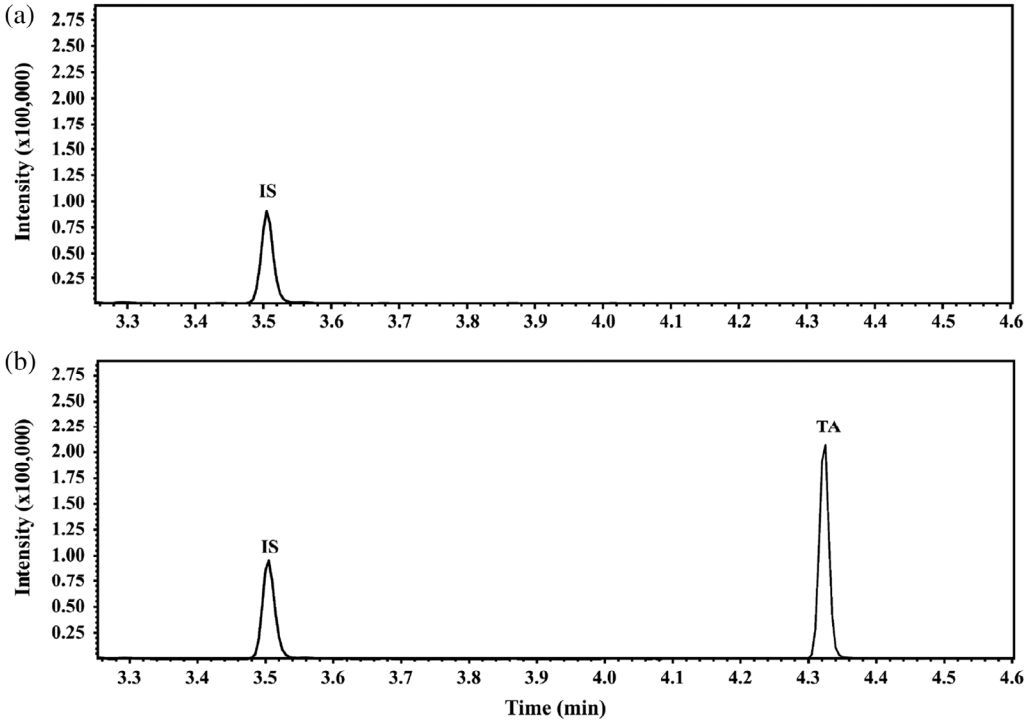

Figure 3 MRM chromatograms of TA and IS in blank serum (a), and serum spiked with standards (b) as EOC-TBDMS derivatives by GC–MS/MS.

Table 3. Linearity, limit of detection (LOD), limit of quantification (LOQ), repeatability, and accuracy of tranexamic acid as EOC-TBDMS derivatives by GC–MS/MS.

Analyte	Calibration range (μg/mL)	Linearity (r)	LOD[a] (ng/mL)	LOQ[b] (ng/mL)	Repeatability (%)[a]				Accuracy (%)[b]			
					Added (μg/mL)				Added (μg/mL)			
					Intra-day		Inter-day		Intra-day		Inter-day	
					0.25	0.75	1.25	0.25	0.75	1.25	0.25	0.75	1.25
Tranexamic acid	0.125–1.50	0.9988	0.22	0.72	1.8	1.4	1.3	2.3	4.8	4.5	2.2	−4.0	3.5	−6.7	−6.8	3.5

[a] Relative standard deviation (%RSD). [b] Relative error (%RE).

base peak, which was found to be very selective and stable in the detection of TA analysis. In GC–MS/MS, the precursor ion *m/z* 286 was fragmented to produce product ions in ranging from 3 to 45 V of collision energy (CE). Three product ions with the maximum intensity were selected for identification and quantification: *m/z* 75, 121, and 93, with a CE range of 9 to 24 V. The ion *m/z* 260 $[M-57]^+$ as precursor ion, *m/z* 214, 171 and 75 as product ions were selected for identification and quantification of IS. The quantification product ion was selected based on the criteria of ion intensity and potential interferences from other compounds; the selected product ion was denoted underlined in Table 2. MRM chromatograms of TA and IS in blank serum (3A) and serum spiked with standards (3B) showed as EOC-TBDMS derivatives by GC–MS/MS in Figure 3.

In the general EOC derivatization, excess ECF is required due to its instability in basic aqueous solution. However, this two-phase EOC derivatization in the dichloromethane phase is not needed excess ECF. Especially, EOC-TBDMS derivatives have a good advantage than EOC derivatives due to their enhanced MS property. In addition, this method by using tandem MS is much more accurate and selective in the presence of interference than previously reported method.[24]

Method Validation for TA Analysis by GC–MS/MS. The linearity, repeatability, accuracy, LOD, and LOQ were measured for method validation of TA analysis by GC–MRM–MS/MS. The correlation coefficient (r) is better than 0.9988 in the calibration curve ranging from 0.125 to 1.5 μg/mL. The repeatability and accuracy were measured with three different concentrations (0.25, 0.75, and 1.25 μg/mL), and was performed in triplicate on different three days. Intra-day repeatability (%RSD) and accuracy (%RE) varied from 1.3 to 1.8% and 2.3 to 4.8%, respectively, whereas the inter-day repeatability (%RSD) and accuracy (%RE) varied from 2.3 to 4.8% and −6.8 to 3.5%, respectively (Table 3). The LOD and LOQ were assessed as 0.22 and 0.72 ng/mL, respectively. The LOD and LOQ were assessed to be lower than other reported method.[24] When applied to serum samples with spiked of TA, TA was identified and quantified without interferences. Therefore, the present method will be reliable for the quantification of TA in serum samples.

Conclusion

We developed a method for TA analysis in serum as EOC-TBDMS derivatives by GC–MS/MS with MRM mode. In this study, the mass spectral data set of TA was newly constructed as EOC-TBDMS derivatives. This method was validated with good linearity ($r \geq 0.9988$), LOD of 0.22 ng/mL, LOQ of 0.72 ng/mL, intra-day repeatability (% RSD = 1.3 to 1.8), and accuracy (%RE = −4.0 to 3.5), as well as inter-day repeatability (%RSD = 2.3 to 4.8) and accuracy (%RE = −6.8 to 3.5) under optimal conditions. Thus, the present method will be useful for the

measurement and quality control of TA in the pharmacological and cosmetic fields.

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