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Liquid chromatographic determination of triethylenetetramine in human and rabbit sera based on intramolecular excimer-forming fluorescence derivatization

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Abstract

A highly selective and simple fluorimetric liquid chromatographic method for the determination of triethylenetetramine (TETA), a therapeutic drug for Wilson's disease, in human and rabbit sera is described. This method is based on intramolecular excimer-forming fluorescence derivatization, which allows spectrofluorometric discrimination of polyamino compounds from monoamino species, followed by liquid chromatography. TETA and 1,6-hexanediamine (internal standard) were converted to the corresponding excimer-forming derivatives with a pyrene reagent, 4-(1-pyrene)butyric acid *N*-hydroxysuccinimide ester. The derivatives were separated within 20 min on a reversed-phase column using isocratic elution and detected spectofluorometrically at 480 nm with excitation at 345 nm. This method was successfully applied to the monitoring of TETA in human and rabbit sera with a simple pretreatment. The detection limit for TETA in serum was 18 ng/ml (0.13 nmol/ml) corresponding to 0.2 pmol on column at a signal-to-noise ratio of 3.

Keywords: Derivatization, LC; Triethylenetetramine

1. Introduction

Wilson's disease is a genetic disorder characterized by the accumulation of excessive amounts of copper in some organs, such as liver, brain, cornea and kidney, and its major clinical manifestations are liver cirrhosis, advanced extrapyramidal symptom and Kayser-Fleisher rings, which subsequently lead to death [1–3]. Wilson's disease has been treated with oral administration of triethylenetetramine dihydrochloride (*N*,*N*′-bis(2-aminoethyl)-1,2-ethanediamine 2HCl, TETA·2HCl) which chelates copper ions to increase their urinary excretion of the ion. Even though TETA is relatively safe and effective among the chelating agents for Wilson's disease [4,5], it still causes mild anemia in some patients [6], probably depending on individual differences in

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disposition of TETA. Therefore, drug monitoring in each patient is necessary for good therapy.

Several liquid chromatographic (LC) methods coupled with fluorescence [7–10] or conductometric [11] detection have been developed for the determination of TETA in human serum. The fluorometric method by precolumn derivatization using fluorescamine is highly sensitive and selective, but it requires a rather complicated clean-up procedure using solid-phase extraction on a cation-exchange cartridge [7]. On the other hand, the postcolumn fluorescence method using *o*-phthalaldehyde requires only simple deproteinization as a sample pretreatment, but the resulting chromatograms are rather complicated due to the presence of many endogenous amino compounds [8]. The conductometric method does not have enough sensitivity to be applied to drug monitoring in serum.

The aim of the present study was to develop a highly sensitive, selective and simple method for the determination of TETA in serum. Recently, we have developed a highly selective fluorescence derivatization technique for endogenous polyamines (putrescine, cadaverine, spermidine, and spermine) and basic amino acids (lysine and ornithine), in which the polyamino compounds were converted to the corresponding polypyrene-labeled derivatives by reaction with 4-(1-pyrene)butyric acid *N*-hydroxysuccinimide ester (PSE) [12,13]. The derivatives can emit intramolecular excimer fluorescence (450–550 nm), which can clearly be discriminated from the normal fluorescence of pyrene (370–420 nm). Since the chemical structures of TETA and the endogenous polyamines resemble each other closely, we applied the excimer-forming derivatization technique to the determination of TETA by optimizing derivatization and LC separation conditions. In the established method, TETA was converted to the polypyrene-labeled derivative by reaction (90 °C, 30 min) with PSE and the derivative afforded the intramolecular excimer-fluorescence in the LC mobile phase. The method was successfully applied to the determination of TETA in human and rabbit sera with a simple pretreatment, deproteinization with organic solvents (acetonitrile and tetrahydrofuran, (THF)). 1,6-Hexanediamine (HDA) was used as an internal standard (I.S.).

2. Experimental

2.1. Chemicals and solutions

All chemicals and solvents were of the highest purity available and were used as received. Deionized and distilled water was used for all aqueous solutions. TETA·2HCl was purchased from Sigma (St. Louis, MO, USA), and a therapeutic capsule containing 250 mg TETA·2HCl (metalite capsule, Tsumura, Tokyo, Japan) was used for the drug monitoring studies. HDA was obtained from Kanto Chemical (Tokyo, Japan). PSE was purchased from Molecular Probes (Eugene, OR, USA) and used without further purification. TETA stock solution (1.0 mg/ml, 6.84 m*M*) was prepared by dissolving TETA·2HCl (15 mg) in water (10 ml) and stored at −20 °C until use. HDA stock solution (116 μg/ml, 1 m*M*) was prepared by dissolving HDA·2HCl (1.89 mg) in water (10 ml) and stored at −20 °C until use. PSE solution (5 m*M*) prepared in acetonitrile was usable for at least a week when stored at −20 °C.

2.2. Apparatus

The LC system consisted of a Shimadzu (Kyoto, Japan) LC-6A pump controlled by a SCL-6A system controller, a Rheodyne (Cotati, CA, USA) 7125 manual sample injector equipped with a 20-μl sample loop, a Radial Pak C_{18} reversed-phase column (100 mm×8 mm I.D., particle size 5 μm; Waters, Milford, MA, USA), a Shimadzu RF-535 fluorescence detector and a Shimadzu C-R3A integrator. The LC mobile phase was a mixture of acetonitrile and 10 m*M* ammonium acetate (3:1, v/v), and was delivered at a flow rate of 2.0 ml/min. The column temperature was ambient (25 °C) and the pressure was ~60 kg/cm^2. The mobile phase was filtered through a 0.22-μm membrane filter (polyvinylidene fluoride, Millipore, Bedford, MA, USA). The fluorescence detector was operated at the excitation and emission wavelengths of 345 and 480 nm, respectively, and the slitwidths of both the monochromators were set at 18 nm. For comparative studies, monomer fluorescence was monitored at the excitation and emission wavelengths of 345 and 375 nm, respectively.

2.3. Standard derivatization procedure

To 25 μl of TETA solution placed in a 1.5-ml screw-capped tube, were added 25 μl of 10 μ*M* HDA, 50 μl of acetonitrile–THF (1:1, v/v), 10 μl of 25 m*M* disodium hydrogen phosphate and 100 μl of 5 m*M* PSE. The tube was tightly sealed and then heated at 90 °C for 30 min in a block heater (Eyela, MG-2100, Tokyo, Japan). After cooling in ice-water, a 20-μl portion of the final reaction mixture was injected into LC. To prepare the reagent blank, 25 μl of water in place of the TETA solution was subjected to the same procedure. The net peak height ratio of TETA to HDA was used for the quantitation.

2.4. Sample preparation in drug monitoring study

2.4.1. Human

A healthy male volunteer (41 years old, 60 kg) was orally administered TETA·2HCl as six 250 mg capsules with 250 ml of water at 8 a.m. after a light breakfast at 7 a.m. Serial sampling of venous blood (3–5 ml) was done 10 min before and at 0.5, 1, 2, 3, 4, 6, 8 and 24 h after the administration. Each sample was allowed to stand for ~2 h at room temperature (25 °C) and then centrifuged at 1000 *g* for 10 min. Aliquots of the serum samples were transferred to respective screw-capped tubes and kept frozen at −20 °C until use. After an interval of 6 days, the same dose was given to the same volunteer at 8 a.m. after an overnight fast for 10 h, and the same sampling program and serum preparation procedures were carried out.

2.4.2. Rabbit

Three white rabbits (3.4–3.6 kg) were orally given TETA·2HCl (150 mg/kg weight) as a 150 mg/ml solution using a sonde after an overnight fast, and they were given feed at 6 h after the dosing. The blood (1–3 ml) was withdrawn from the marginal ear vein. The serial sampling program and serum preparation method were the same as in human.

2.5. Determination of TETA in serum sample

To 25-μl of serum placed in a 1.5-ml test tube, were added 10 μl of 7.5 μ*M* HDA, 15 μl of 1 m*M* EDTA (pH 7.5), 25 μl of water and 75 μl of acetonitrile–THF (1:1, v/v). After vortex mixing (~10 s), the mixture was allowed to stand for 10 min to complete the deproteinization. The mixture was centrifuged at 1000 *g* for 1 min, and the supernatant (100 μl) was transferred to a 1.5-ml screw-capped tube. To the solution were added 10 μl of 25 m*M* disodium hydrogen phosphate and 100 μl of 5 m*M* PSE. The mixture was subjected to the derivatization reaction (90 °C, 30 min) and LC according to the standard procedure (Section 2.3).

To establish the calibration graph, 25 μl of serum was replaced with 25 μl of the pooled drug-free serum fortified with TETA (1, 2.5, 5, 10, 25, 50 and 100 μg/ml serum). The net peak height ratios of TETA to I.S. were plotted against the concentrations of TETA added.

2.6. Validation of the methods

The validation parameters (precision (intra-day and inter-day), accuracy, linearity, limit of detection and recovery) of the present method were determined throughout the full analytical procedures (sample pretreatment, derivatization and HPLC) using the validation sample (blank serum fortified with TETA). The intra-day precision at concentration levels of 2.0 and 0.8 μg/ml for TETA in serum was assessed by analysis in five replicates on the same day for each level. The inter-day precision at concentration level 2.0 μg/ml was assessed by analysis in triplicate on 5 different days within 1 month. The accuracy was expressed as a percent deviation of the determined concentration from theoretical concentration. The linearity of the method for TETA was checked by preparing calibration graph in triplicate at seven different concentrations, 1, 2.5, 5, 10, 25, 50 and 100 μg/ml, and by determining the response factor (the ratio of peak height to concentration) at each point. The equation of the calibration line was calculated by least-squares linear regression. The limit of detection (LOD) was determined as the lowest concentration yielding a signal-to-noise ratio of 3. Recovery was determined in triplicates at a concentration of 10 μg/ml, by comparing the peak heights of TETA added to the validation sample before and after deproteinization.

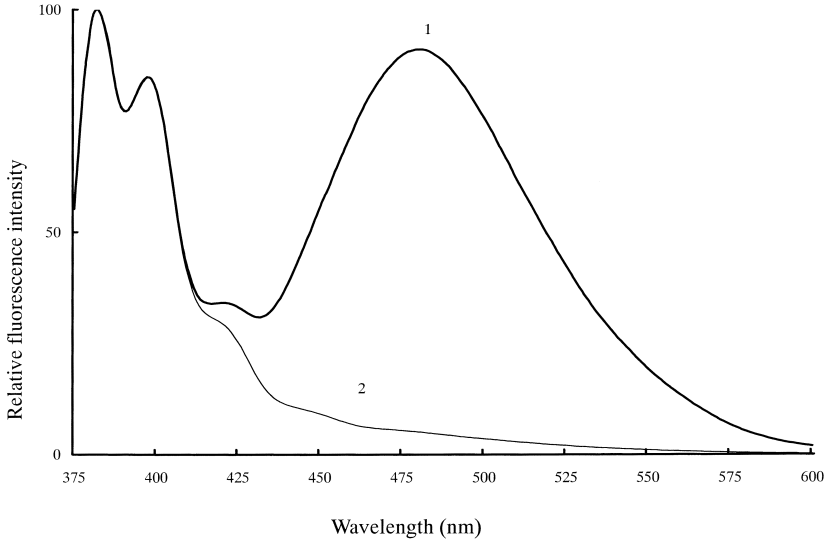

Fig. 1. Fluorescence emission spectra of the column eluate for TETA and PSE. Curve 1: 46 nmol/ml TETA were treated according to the derivatization procedure, and the eluate from the HPLC column was subjected to spectrofluorometry; curve 2: 11 nmol/ml PSE dissolved in the LC mobile phase. Each spectrum was normalized to the first peak at 382 nm.

3. Results and discussion

3.1. Excimer fluorescence from TETA derivative

Fig. 1 shows normalized fluorescence emission spectra of the column eluate for TETA peak and PSE dissolved in the LC mobile phase. PSE gave only normal (monomer) fluorescence (370–420 nm), whereas the pyrene derivative of TETA in the peak eluate emitted excimer fluorescence (450–550 nm). The shape of the spectrum for TETA did not changed when the solution was diluted 10 times with the mobile phase. These observations imply that the produced TETA derivative under the present conditions is a polypyrene labeled compound and affords intramolecular excimer fluorescence as is the case with the polypyrene derivatives of polyamines [12,13] and dipyrenylalkanes [14,15]. Thus, the present derivatization allows highly selective determination of TETA by measuring the intramolecular excimer fluorescence of the TETA derivative, which was clearly discriminated from normal pyrene fluorescence of PSE and PSE-labeled monoamino concomitants. The structure of the TETA derivative remained unknown.

3.2. LC separation

Since monopyrene compounds also afford intermolecular excimer fluorescence in their highly concentrated solutions (greater than 1 m*M*) [15], PSE and its hydrolysate (4-(1-pyrene)butyric acid) should be separated from the TETA derivatives by LC. The separation conditions were studied by the combinatorial optimization of stationary phase (C_{18}, C_8 and C_4) and mobile phase (organic modifier and buffer). The best separation was achieved on a Radial Pak C_{18} reversed-phase column by isocratic elution using acetonitrile–10 m*M* ammonium acetate (3:1, v/v) as a mobile phase. A typical chromatogram obtained with a standard mixture of TETA and HDA (I.S.) is shown in Fig. 2A. They gave the respective single peaks and had retention times of 12.3 and 17.6 min, respectively. In contrast, when monomer fluorescence was monitored at emission wavelength of 375 nm, large peaks of PSE, 4-(1-pyrene)butyric acid and PSE-related impurities interfered with observation of the peaks for TETA and HDA (Fig. 2B). Thus, this method is highly selective and applicable to biological samples, which should contain miscellaneous monoamino compounds.

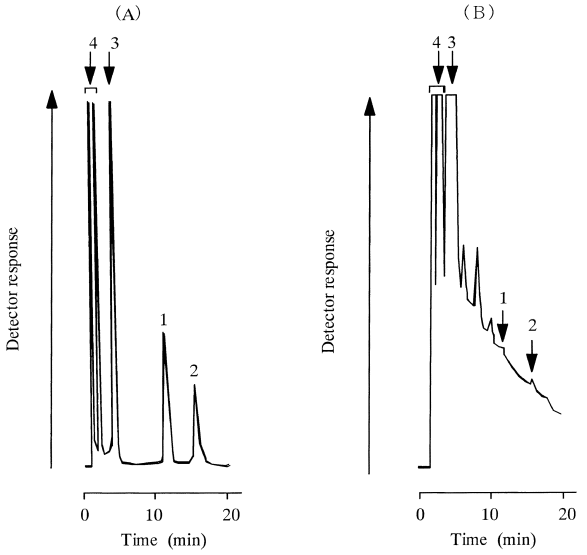

Fig. 2. Chromatograms obtained with a standard mixture of TETA and HDA. Concentrations (pmol/injection): TETA, 163; HDA, 23.8. Fluorescence detection: (A) ex 345 nm, em 480 nm; (B) ex 345 nm, em 375 nm. Peaks: 1=TETA; 2=HDA; 3=PSE; 4=4-(1-pyrene)butyric acid and impurities in PSE.

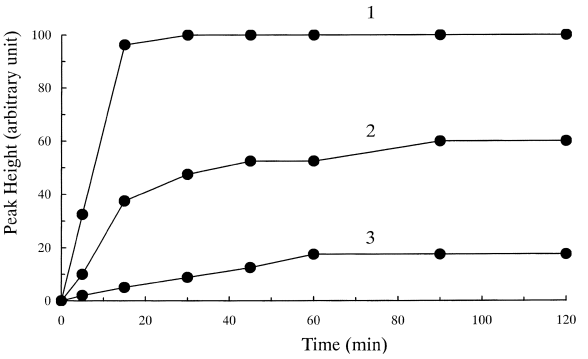

Fig. 3. Effect of the derivatization reaction time and temperature on the fluorescence derivatization. Curves: 1=90 °C; 2=70 °C; 3=50 °C.

3.3. Optimum derivatization conditions

Optimization studies were carried out to maximize the excimer fluorescence peak height for TETA and HDA. The derivatization reaction proceeded effectively in the presence of organic solvents, such as methanol, ethanol, acetonitrile, dimethylsulfoxide (DMSO), THF and their mixtures. The best results were obtained when a mixture of THF, acetonitrile and water (5:25:12, v/v) was used as a reaction solvent. PSE concentration of 2–10 m*M* in the reagent solution provided almost maximum peak heights for TETA and HDA; 5 m*M* PSE solution was used as optimum. The derivatization reaction was facilitated by the addition of organic or inorganic base. Among the examined bases or buffers (sodium acetate, disodium hydrogen phosphate, sodium sulfite, sodium carbonate, potassium carbonate and phosphate buffers (pH 7–12)), disodium hydrogen phosphate gave the most intense peaks for both the amines in the concentration range of 5–100 m*M*. With increasing reaction temperature in the range 0–90 °C the derivatization proceeded more rapidly (Fig. 3), but higher temperature (100–120 °C) caused degradation of the TETA derivative and the formation of artifact peaks in the chromatogram; heat at 90 °C for 30 min was selected for reproducible results. The polypyrene derivatives in the final mixture were stable for at least 72 h in daylight at room temperature.

The reproducibility of the derivatization was tested with respect to repeatability ($n=5$) using standard TETA solutions (1, 10 and 100 μg/ml). The relative standard deviations (RSDs) of the peak height were 7.3, 5.2 and 2.6%, respectively, and RSDs of the peak height ratio of TETA to HDA were less than 3.0%. The reproducibility is acceptable for the precise measurement of TETA.

3.4. Determination of TETA in human and rabbit sera

In order to evaluate the proposed method, it was applied to drug monitoring in serum samples. For deproteinization of serum samples, the addition of an acid (perchloric acid or trichloroacetic acid), an organic solvent (methanol, acetonitrile, THF or their mixture), and ultracentrifugation with Ultrafree-CL filter unit (30 000 NMWL, Millipore) were investigated in a preliminary recovery test. Of the methods, deproteinization by the addition of acetonitrile–THF (1:1, v/v) was the most suitable because the resulting deproteinizate itself consists of the above-mentioned optimized solvents for the derivatization

reaction. The recovery and reproducibility of the deproteinization procedure were satisfactory as described later (Section 3.5).

Since some metal ions other than copper ion also form fairly stable chelate compounds with TETA, the ions in the sample are expected to affect the derivatization of TETA. It has been reported that the ions interfered with the derivatization of TETA using fluorescamine as a label reagent, and the interference could be eliminated by the addition of EDTA [7]. In the present method, the addition of EDTA in the range 0.5–10 m*M* eliminated the interference from metal ions; 1 m*M* EDTA was added in the procedure. In the absence of EDTA, metal ions in a human serum sample and in the same sample fortified with metal ions (Fe^{2+}, Mn^{2+}, Zn^{2+} and Cu^{2+}(nmol/ml serum): 35, 20, 60 and 5, respectively) caused a decrease in the peak height of TETA (8 μg/ml serum) by 10.2 and 28.2%, respectively, in comparison with that its presence.

Fig. 4 illustrates chromatograms obtained with human sera collected before and after administration of TETA. The drug-free serum did not show any interfering peak, and the chromatograms are fairly simple in spite of simple pretreatment of the sample. Serum components in the derivatization mixture did not affect the retention times or fluorescence properties (peak heights, peak areas and fluorescence spectra) of the peaks of TETA and HDA.

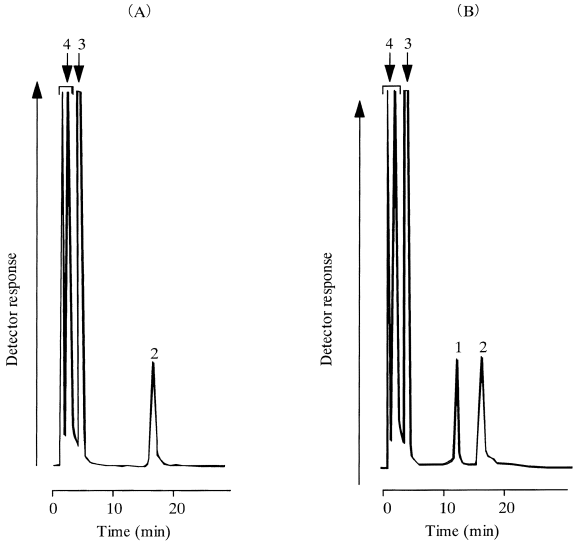

Fig. 4. Chromatograms obtained with human sera collected (A) before and (B) at 1 h after administration of TETA·2HCl (1500 mg). Peaks: 1=TETA; 2=HDA; 3=PSE and serum component(s); 4=4-(1-pyrene)butyric acid, impurities in PSE and serum component(s).

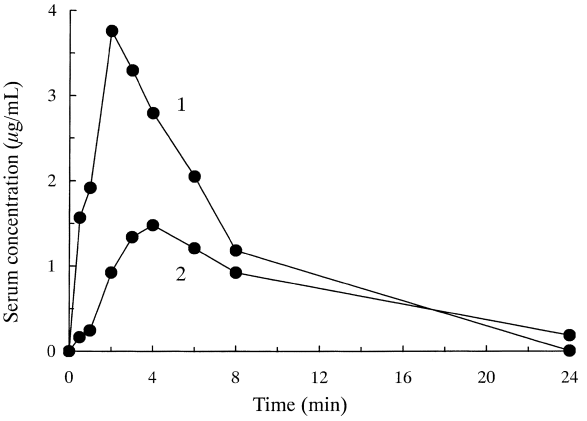

Fig. 5. Serum concentration–time profiles of TETA after oral administration of TETA·2HCl to a healthy volunteer (41 years old, 60 kg). Dose: 25 mg per kg body weight. Curves: 1=fasted; 2=non-fasted.

Figs. 5 and 6 show the serum concentration–time profiles of TETA after oral administration of the drug to a healthy man (TETA·2HCl, 1500 mg) and to three rabbits (150 mg per kg body weight). The concentrations of TETA reached a maximum at 2 h after oral administration in fasted state and then decreased. The maximum serum concentrations of TETA in rabbit serum and in human serum was 16.3

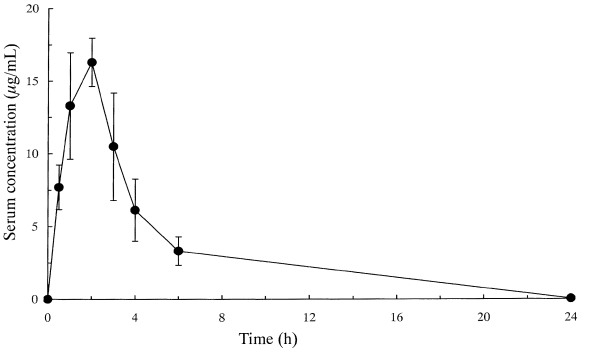

Fig. 6. Serum concentration–time profiles of TETA after oral administration of TETA·2HCl to three rabbits. Dose: 150 mg per kg body weight. Each value is mean±SD of three experiments.

and 3.76 μg/ml, respectively. The profiles are almost similar to those reported by other workers [7]. In a non-fasted human, the maximum concentration reached 1.45 μg/ml at 4 h after oral administration. These results are in good agreement with the previous report [16].

3.5. Method validation

The intra-day precision was established by repeated determinations ($n=5$) using the validation samples fortified with TETA at concentrations 2.0 and 0.8 μg/ml; the RSDs were 1.5 and 2.6%, respectively. The inter-day precision using the validation sample (2.0 μg/ml TETA) was 4.7%. The RSDs of the retention times for TETA in the intra-day and inter-day tests were 0.12 and 2.8%, respectively. Accuracy was determined by the above intra-day precision study; the average deviations from the theoretical values were 0.1 and −0.4% at TETA concentrations of 2.0 and 0.8 μg/ml, respectively. The relationship between the amounts of TETA and the peak height was linear over concentration range 0.15–15 μg/ml serum, which corresponded to 0.238–23.8 ng per 20-μl injection volume in LC. The linear correlation coefficient was 0.999 ($n=3$) and RSD of the mean response factors in the seven points (Section 2.6) was 4.4%. The detection limit (fmol per 20-μl injection volume, signal-to-noise of 3) for TETA was 200. The detection limit for TETA in serum was 18 ng/ml (0.13 nmol/ml) corresponding to 0.2 pmol on column at a signal-to-noise ratio of 3. This sensitivity is comparable to the most sensitive method reported so far [7]. The recoveries of TETA and HDA added to the validation sample (10 μg/ml each) were 94.4±2.7% and 89.3±1.3% (mean±SD; $n=5$), respectively.

Specificity of the present method was evaluated by applying many biologically important substances to this LC method. In the preliminary study by thin layer chromatography on a silica gel plate (5553 aluminum sheets, Merck, Darmstadt, Germany), other polyamino compounds tested (arginine, histamine, putrescine, cadaverine, spermidine and spermin) also reacted with PSE to afford the corresponding polypyrene-labeled products and generated the intramolecular excimer fluorescence. Putrescine and cadaverine afforded 33- and 36-times higher peaks than TETA at retention times of 14.1 and 16.5 min, respectively. The other amines gave no peak under the present LC conditions because they were not separated from the reagent blank or not eluted from the column. The following biological compounds having only one amino moiety or none in the molecule, at a concentration of 100 nmol/ml, did not afford any peak under the present conditions: the compounds tested were neutral and acidic amino acids, ammonia, acetylcholine, serotonin, catecholamines (epinephrine, norepinephrine, and dopamine), acids (α-ketoglutaric acid, phenylpyruvic acid, homovanilic acid, 5-hydroxyindole-3-acetic acid, and ascorbic acid), sugars (D-glucose, D-fructose, D-galactose, D-ribose, *N*-acetyl-D-glucosamine, maltose, and sucrose), nucleic acid bases (adenine, guanine, thymine, cytocine, and uracil), and other compounds (methanol, acetone, phenol, cholesterol, creatine, creatinine, and urea). These observation suggest that the present derivatization method is usefully selective for polyamino compounds.

4. Conclusions

The present excimer fluorometric LC method utilizing PSE only needs a one-step deproteination procedure for pretreatment of the serum sample. Moreover, it offers higher sensitivity to permit the quantification of TETA in 25 μl of human serum after oral administration of the drug. The method can thus be useful in the therapeutic and pharmaceutical investigation of TETA.

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