Determination of Triethylenetetramine in Plasma of Patients by High-Performance Liquid Chromatography

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A sensitive and simple fluorometric method for the determination of *N,N'*-bis(2-aminoethyl)-1,2-ethanediamine dihydrochloride (triethylenetetramine) in human plasma by high-performance liquid chromatography is described. Free triethylenetetramine (TETA) obtained by passing the TETA-copper chelate compound through a solid-phase cation exchange resin was converted to its fluorescamine derivative in the presence of ethylenediaminetetraacetic acid to mask the interfering metal ions in the reaction solution, and the derivatives were separated on a nitrile high-performance liquid chromatograph column (Nucleosil 5-CN) using isocratic elution. The plasma levels of TETA were measured in eight patients receiving treatment for excess copper. Absorption rates of TETA were relatively slow and the peak levels were significantly different among patients. The bioavailability of TETA in the rat was also examined and the ratio of intestinal absorption was extremely low.

Keywords triethylenetetramine; Wilson's disease; plasma level; HPLC; fluorescamine

Introduction

Triethylenetetramine dihydrochloride (*N,N'*-bis(2-aminoethyl)-1,2-ethanediamine 2HCl, TETA), a selective chelating agent for copper, increases the urinary excretion of excess copper and is used for the treatment of Wilson's disease. It has been reported that TETA is a safe and highly effective drug for Wilson's disease.[1-4] Another study, however, indicated that TETA is teratogenic in rats at dosages similar to those used clinically.[5] Despite these implications, an estimation of the effective dose and pharmacokinetic behavior of TETA has been difficult to obtain since a sensitive determination method for plasma TETA has not been developed. Therefore, a direct analytical technique for the determination of TETA which forms a chelate compound with copper in plamsa is desirable in order to establish recommendations for the safe use of the drug.

In the present study, we developed a determination method of TETA associated with copper in plasma by high-performance liquid chromatography (HPLC) and measured the plasma levels of TETA in patients.

Materials and Methods

Reagents Triethylenetetramine dihydrochloride was obtained by partial neutralization of the polyamine base form (Wako Pure Chemical Ind., Osaka, Japan) with hydrochloric acid followed by crystallization from an ethanolic solution according to Dixon *et al.*[6] Electron impact mass spectra were obtained using a solid probe (Hitachi RMU-6MG). Molecular ion and fragmentation ions were consistent with the polyamine structure, and mass spectra of purified TETA agreed well with those of the reagent obtained from Aldrich Chemical Co., (Wisconsin, U.S.A.). Deionized and distilled water produced by an automatic distillation apparatus (Sakura Seiki Co., Tokyo, Japan) was used to prepare the HPLC mobile phase and in reagent preparations in the present study. Bond-Elut SCX (Analytichem International, Harborcity CA, U.S.A.) were washed with 5 ml of distilled water before use. Plasma samples were obtained from patients in Hokkaido University Hospital with permission and from healthy volunteers in our laboratory. Fluorescamine (FA) was obtained from Japan Roche (Tokyo, Japan). All chemicals were of reagent grade and used without further purification.

Apparatus A liquid chromatograph (Hitachi 655A-11) equipped with a multi-wavelength fluorometric detector (Jasco 820-FP) and sample injector (Rheodyne 7125) was used. For the stationary phase, a nitrile column (Nucleosil 5-CN, 25 cm × 4 mm i.d., particle size 5 μm, Macherey, Nagel & Co., Duren, G.F.R.) was used, and the column was maintained at 40 °C using a constant temperature water bath circulator. The mobile phase was 73% salt solution and 27% acetonitrile and pH of the mixture was adjusted to 6.0 with 2 M sodium hydroxide. The salt solution consisted of 0.14 M ammonium chloride, 48 mM sodium benzenesulphonate and 9.2 mM acetic acid. The flow rate was 0.5 ml/min and the pressure was approximately 70 kg/cm^2. Fluorescence measurement was made with the excitation set at 380 nm and emission at 485 nm.

Assay Procedure A hundred microliters of distilled water and 1 ml of acetonitrile were added to 0.5 ml of plasma in a glass centrifuge tube. The mixture was shaken briefly on a Vortex mixer and then centrifuged at 1000 × *g* for 5 min. The supernatant (1.2 ml) was loaded onto the SCX column and the column was washed by passing through it 3 ml of distilled water, 2 ml of 1 M potassium chloride and 3 ml of 2 M potassium chloride, and then TETA was eluted with 1 ml of 4 M potassium chloride. To 0.2 ml of eluate, 0.6 ml of 0.1 M phosphate buffer (Na_3PO_4–Na_2HPO_4, pH 9.5) and 0.1 ml of 0.15 M ethylenediaminetetraacetic acid (EDTA 3Na) in the same buffer were added. To the mixture, 0.1 ml of 10 mM FA in acetonitrile was added and mixed vigorously for 1 min on a Vortex mixer. After the reaction ceased and excessive FA decomposed completely (20 min), 50 μl of 0.25 mM α-naphthylamine in methanol was added to the mixture as an internal standard. Usually, 20—50 μl of the sample solution was injected into the HPLC system.

Free TETA, TETA which does not take part in the chelation, could be detected by the addition of 0.1 M phosphate buffer (pH 9.5), EDTA and FA to 0.2 ml of deproteinized supernatant according to the procedure mentioned above.

Standard Curve Standard solutions containing 1.5, 2.5, 5, 10 and 15 μg/ml TETA in distilled water were prepared and 0.1 ml increments of these samples were pipetted to centrifuge tubes containing 0.5 ml of drug-free plasma. One ml of acetonitrile was added and samples were processed as described above. The ratio of the peak height of FA derivatives to that of α-naphthylamine (internal standard) was used to construct a calibration graph.

Bioavailability in Rats Male Wistar rats (300—320 g) were fasted for 18—21 h before the experiments. Water was allowed *ad libitum*. TETA was administered orally (25 mg/kg) or intrajugularly (100 μg/body) as a solution in physiological saline. Rats were anesthetized with diethyl ether or sodium pentobarbital (i.p., 3 mg/100 g body weight) before the oral or intravenous administration, respectively. In the case of oral administration, rats were anesthetized with diethyl ether at blood-collecting time. About 0.5 ml of blood was collected by cardiac puncture at 0.5, 1, 2, 3, 4 and 6 h post-dosing. Bioavailability was measured by comparing the respective area under the plasma levels with the time curve from 0 to 6 h (AUC_{0-6h}) after oral and intravenous administration.

$$\text{bioavailability} = \frac{\text{each of } (AUC_{0-6h})_{p.o.}/\text{dose}_{p.o.}}{\text{mean of } (AUC_{0-6h})_{i.v.}/\text{dose}_{i.v.}}$$

AUC_{0-6h} was calculated by the trapezoidal rule.

Results

Effect of Iron and Zinc Ion on the Formation of TETA-Copper Chelate It is well known that iron (Fe^{3+}) and zinc (Zn^{2+}) ion also form chelates with TETA and that there is no remarkable difference in the plasma concentration between copper, iron and zinc ions. Therefore, the interference of iron and zinc ions on the formation of TETA-copper chelate was examined. Figure 1 shows the effect of the concentration of ferric chloride or zinc sulphate on the ultraviolet (UV) absorption of TETA-copper chelate compound at 254 nm. TETA-copper chelates have UV λ_{max} at 254 nm while neither TETA-iron nor TETA-zinc chelate compound has any UV absorption at 254 nm. It was found that 90% and 70% of the TETA-copper chelates remained in the presence of 6 times the amount of zinc and 6 times the amount of iron ions. These results are consistent with those of May and Williams[7] in that TETA is a strong and selective chelating agent for copper ion. These results suggest that the TETA-copper chelate compound would be formed preferentially in plasma.

Selectivity Figure 2 shows the effect of the concentration of EDTA on the formation of the FA derivative of TETA (13.7 μM plasma). The formation of the FA derivative increased with increasing amount of EDTA and reached a plateau when the concentration of EDTA had increased to 5 mM in reaction solution. This increase in FA derivative formation might be due to the masking of interfering metal ions in the reaction solutions by EDTA.

Calibration curves obtained in the presence (0.1 ml of 0.15 M EDTA in the reaction solution) and absence of EDTA are shown in Fig. 3. In the presence of EDTA a straight line that coincides with the origin was obtained and linearity was demonstrated up to at least 0.183 mM (40.1 μg/ml plasma). A coefficient of variation at 0.5 μg/ml plasma was 3.3% ($n=5$). The limit of detection was 0.456 μM (100 ng/ml plasma) at a signal-to-noise ratio of 3.0.

In the absence of EDTA, on the contrary, the standard curve does not intersect the origin as shown in Fig. 3. These results (Figs. 2 and 3) indicate that the formation of FA derivative of TETA does not proceed completely unless interfering metal ions are removed from the reaction system.

Figure 4 shows chromatograms of TETA-free plasma, plasma sample spiked with 4.56 μM TETA and the plasma sample of a patient to whom TETA had been administered. FA derivative and internal standard were well separated from endogenous substances. The formation of FA derivative of TETA was almost the same within the pH range of 8.5—9.5 (8.5, 8.75, 9.0, 9.25, 9.5). The small unknown peak observed in the chromatogram is possibly due to another FA derivative of TETA as shown in the case of FA derivatives of biological polyamines.[8] The ratio of the large

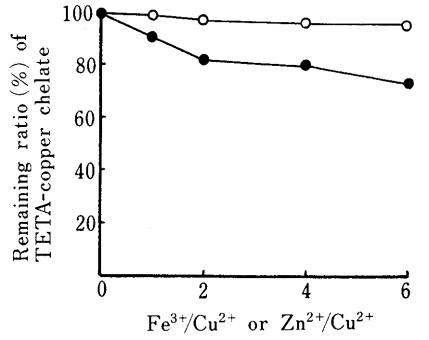

Fig. 1. Effect of Iron (●) and Zinc (○) Ions on the Formation of TETA-Copper Chelate

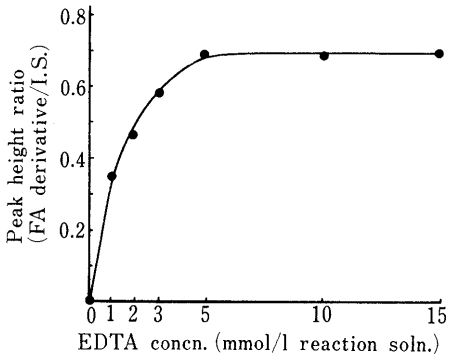

Fig. 2. Effect of the Concentration of EDTA on the Formation of the Fluorescamine Derivative of TETA

TETA sample was prepared according to the elution system for the plasma sample in the text.

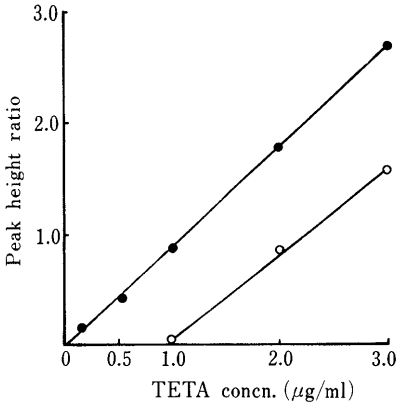

Fig. 3. Calibration Curves of TETA Obtained in the Presence (●) and Absence (○) of EDTA

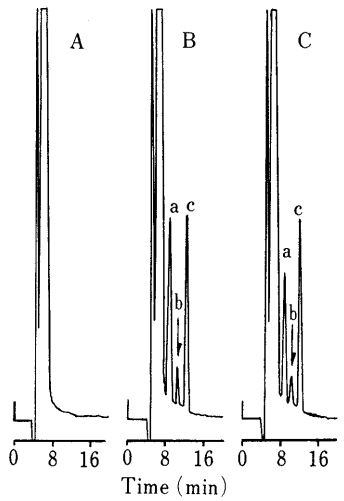

Fig. 4. Chromatograms of TETA-Free Plasma (A), Plasma Sample Spiked with 1.0 μg/ml (4.56 μM) TETA (B) and Plasma Sample after the Administration of TETA to a Patient (C)

Peaks: a = fluorescamine derivative of TETA, b = unknown peak, c = α-naphthylamine (internal standard). Detector sensitivity: attenuation 2, gain 10.

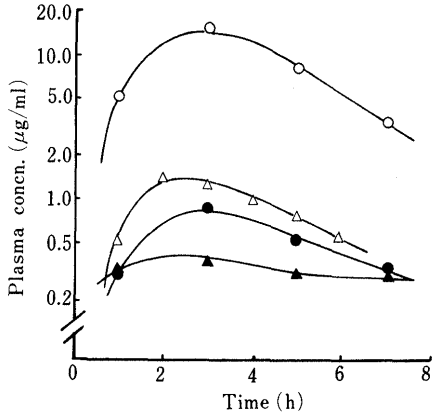

Fig. 5. Plasma Concentration–Time Profiles of TETA after Oral Administration of TETA to Four Male Patients

Dose: 8.3 mg per kg body weight.

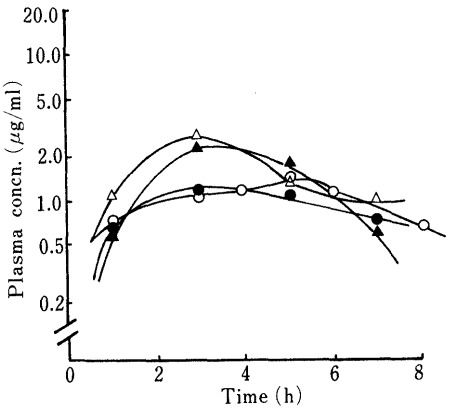

Fig. 6. Plasma Concentration–Time Profiles of TETA after Oral Administration of TETA to Four Female Patients

Dose: 8.3 mg per kg body weight.

peak based on the TETA derivative to the small unknown peak was almost the same in each chromatographic run.

Plasma Concentration Profile in Patients and Bioavailability in Rats The time course of plasma concentrations of TETA after oral administration to eight patients (approximately 8.3 mg per kg body weight) was monitored using the newly developed assay method. Plasma concentration–time profiles of four male and female patients are shown in Figs. 5 and 6, respectively. Absorption rates were relatively slow and plasma levels were significantly different among patients, especially among males.

Free TETA, TETA which does not take part in the chelation with metal ions, was not detected at any peak time in the eight patients. The greatest part of TETA in plasma is thus probably chelated with metal ion, especially copper.

Further, the bioavailability of TETA (0—6 h) was examined in the rat. The plasma levels *versus* time curves of three animals after intravenous administration were similar, but those of three animals after oral administration were different (data not shown). Therefore, bioavailability was estimated by comparing each *AUC* after oral administration and mean *AUC* after intravenous administration. Absorption rates were relatively slow and the bioavailability was extremely low (5.6%, 5.7% and 16.4%).

Discussion

A determination method for plasma TETA has been needed for pharmacokinetic study and the safe use of TETA. Several methods for the assay of TETA, a kind of polyamine, and biological polyamines have been described. Hansen *et al.*[9] reported a simple HPLC procedure for TETA and biological polyamines (spermidine and spermine) without formation of derivatives using conductivity detection. Although this method would be applicable for estimation of the drug's purity and stability in aqueous solution, the sensitivity is too low for clinical use. Several analytical methods have also been developed for biological polyamines in food and urine samples using gas chromatography (GC)[10] and HPLC[8,11–14] procedures; however, these procedures are very complex and time consuming. Kai *et al.*[15] reported an HPLC procedure for biological polyamines in human serum as their FA derivatives, using a reversed-phase column (Lichrosorb RP18). In preliminary experiments, however, it was observed that the peak of the FA derivative of TETA was extremely broad and tailed in comparison with those of polyamine derivatives. Moreover, TETA chelate compounds rendered it difficult to secure a fluorescent product.

In the present study, we were able to solve these problems and develop a determination method of TETA which chelates with copper in plasma as follows. First, TETA was separated from the TETA-copper chelate by passing deproteinized plasma through a cation exchange resin. Second, the metal ions which interfere with the formation of FA derivative of TETA were eliminated by adding the chelating agent, EDTA, to the reaction system. Third, fluorescent derivative of TETA was analyzed sensitively by HPLC with the use of a nitrile reversed-phase column eluted with an isocratic elution system.

In the above procedures, it was especially important to eliminate the inhibitory effect of the metal ions in the reaction system on the formation of FA derivative. It was found that TETA was separated from the TETA-copper chelate by passing it through the SCX column and that TETA could be recovered completely with 1 ml of 4 M potassium chloride. We ascertained that the copper ion was removed by washing the column with 3 ml of 2 M potassium chloride. However, we were unable to obtain the TETA standard curve that coincided with the origin, as shown in Fig. 3, in both plasma and plasma-free samples. In contrast, the standard curve of the potassium chloride-free TETA, TETA samples prepared in distilled water, did intersect with the origin. It was also observed that the degree of the FA derivative formation decreased with the increasing concentration of potassium chloride in the reaction system which had the same pH value, 9.5. In preliminary experiments, furthermore, it was found that Ni^{2+} and Zn^{2+} interfere with the reaction between FA and TETA. It is, therefore, reasonable to consider that several metal ions existing in the reagents, especially in 4 M potassium chloride, passed through the column and interfered with the FA derivative formation. We could eliminate the interfering effect of the metal ions by adding the chelating agent, EDTA, to the final reaction mixture (Figs. 2 and 3).

In the present study we used α-naphthylamine as an internal standard. We observed that 1,6-hexanediamine, a compound often used as an internal standard for the

determination of biological polyamines,[8,11,13,15] was not recovered completely and quantitatively from the SCX column with the elution medium for TETA, 1 ml of 4 M potassium chloride. Although the reproducibility of the FA derivative formation of 1,6-hexanediamine was good when this compound was added to the final reaction mixture, a greater amount of FA, a high-priced reagent, was required to get both the fluorescent derivatives of TETA and 1,6-hexanediamine. We therefore added α-naphthylamine, a fluorescent compound, to the final medium before each chromatographic run.

For the first time, we were able to measure the plasma concentration of TETA in patients using this newly developed assay method. The plasma levels of TETA were significantly different among patients and absorption rates of TETA relatively slow (Figs. 5 and 6). It was further found that the bioavailability of TETA in the rat was extremely low. Chung *et al.*[16] have proposed that polycation like spermine bind and lie flat on the surface of the vesicles of acidic phospholipids. We have ascertained that TETA, a polycation like spermine, binds to rat small intestinal brush border membrane vesicles (unpublished data). One reason for the variation of plasma levels and low bioavailability of TETA may be the binding property of TETA to the brush border membrane.

It has been difficult to estimate the relationship between the effective dose and the pharmacokinetic behavior of TETA, since no analytical method of the biological fluids of TETA has been developed. Our results suggest that the present method is useful for estimating an effective dose and for detailed pharmacokinetic studies of TETA.

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