Fate of Orally Administered Triethylenetetramine Dihydrochloride: A Therapeutic Drug for Wilson's Disease

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KODAMA, H., MEGURO, Y., TSUNAKAWA, A., NAKAZATO, Y., ABE, T. and MURAKITA, H. Fate of Orally Administered Triethylenetetramine Dihydrochloride: A Therapeutic Drug for Wilson’s Disease. Tohoku J. Exp. Med., 1993, 169 (1), 59-66 — Triethylenetetramine dihydrochloride (TETA) is a therapeutic drug for Wilson’s disease. We developed a simple fluorometric method for detection of TETA in biological fluids by using high-performance liquid chromatography (HPLC), and examined TETA concentrations in the serum and urine of two healthy adults who were given TETA orally. No TETA peak was detected in the serum. The amount of TETA in the urine of the two adults was only 1.6 and 1.7% of the dose administered. However, a large unidentified peak appeared in the urine after oral administration. This peak was not observed in a mixture of TETA and control urine or in urine before TETA administration. When the urine after TETA administration was analyzed after hydrolysis with HCl, the unidentified peak disappeared, while the TETA peak increased. These findings indicate that the substance which yielded the unidentified peak is a metabolite of TETA, suggesting that most of the TETA administered is metabolized and then excreted in the urine. —— triethylenetetramine dihydrochloride; Wilson's disease; analytical method; urine; TETA

Wilson’s disease is a genetic disorder characterized by the accumulation of excessive amounts of copper in the tissues, such as the liver, brain and kidney. The major clinical manifestations of this disease are liver cirrhosis and neurological disorders which subsequently lead to death. The standard treatment of Wilson’s disease involves the oral administration of D-penicillamine, a chelating agent. D-penicillamine is absorbed through the intestine, traps copper in the tissues by chelating, and then it is excreted in the urine as D-penicillamine-copper chelate (Wiesner et al. 1981; Klaassen 1990). Although the oral administration

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D-penicillamine is considered to be the most effective treatment for the disease, it has been reported to induce many adverse effects. Intolerance to D-penicillamine often results in discontinuation of this therapy (Danks 1989).

In 1985, the U.S. Food and Drug Administration approved triethylenetetramine dihydrochloride (TETA), another chelating agent, as an orphan drug for penicillamine-intolerant patients with Wilson's disease. Urinary copper excretion is increased by the oral administration of TETA (Dubois et al. 1970, 1990; Walshe 1973, 1982; Harders and Cohnen 1977). This suggests that orally administered TETA is absorbed through the intestine and then excreted in the urine as TETA-copper chelate. However, little is known about the in vivo metabolism of orally administered TETA. In addition, TETA administration was observed to cause mild anemia in a very small number of patients with Wilson's disease (Walshe 1982; Scheinberg et al. 1987). Therefore, it is important to the evaluation of the therapeutic and adverse effects of TETA to elucidate the in vivo metabolism of orally administered TETA.

In the present study, we developed a rapid and simple method for detection of TETA by reversed-phase ion-pair high-performance liquid chromatography (HPLC) coupled with fluorometric detection by post-column derivatization to analyze TETA concentrations in serum and urine. We report here that only a small part of the TETA administered is excreted as intact TETA in the urine after oral administration to human, and that most of the TETA administered is excreted as a metabolite which seems to have no ability to chelate copper.

**Materials and Methods**

TETA obtained from Kanto Chemical Co., Inc. was purified by the method described by Dixon et al. (1972). This was used as the standard in the analytical experiment and for oral administration.

*Effect of trichloroacetic acid (TCA) on TETA-copper complex*

Serum and urine samples were deproteinized with TCA before HPLC analysis. We then examined the effect of TCA on TETA-copper complex. TETA-copper complexes were prepared according to the method described by Hansen et al. (1985), i.e., each 3 ml of TETA standard solution (20-200 µg/ml) was mixed with 1 ml of cupric sulfate (5 mg/100 ml). One ml of 10% TCA was added to these mixtures. TETA-copper complex was assayed by measuring absorbance at 599 nm (Hansen et al. 1985).

*HPLC analysis of TETA*

A series of standard solutions of TETA was prepared as described above. HPLC analyses of TETA were carried out by reversed-phase ion-pair HPLC coupled with fluorometric detection by post-column derivatization. Fig. 1 shows the system, which consists of two high-pressure micro-pumps (LC-6A; Shimadzu Co., Tokyo), one for propelling the mobile phase and the other for the reaction reagents; a stainless steel column (Capcellpak C18, 4.5×150 mm, Shiseido Co., Tokyo) in a column oven (45°C) equipped with an injector; 0.5×800 mm of coiled stainless steel in the column oven; and a fluoromonitor (RF-530; Shimadzu Co.) equipped with a 75 W xenon lamp and a data processing unit (C-R6A; Shimadzu Co.). A mobile phase consisting of 70 mM phosphate buffer containing 6 mM...
sodium pentanesulfonate, pH 2.1, was delivered at the rate of 1 ml/min. A solution of 0.2 M borate buffer, pH 10 containing 0.08% O-phthalaldehyde and 0.1% N-acetyl-L-cysteine was introduced into the eluate at a flow rate of 0.4 ml/min. The resulting mixture was heated while passing through stainless tubing and then monitored at a wavelength of 450 nm using 348 nm as the excitation wavelength. Twenty μl of sample were injected into the HPLC system.

Recovery of TETA in the serum and urine

Fifty μl of TETA standard solution (500 μg/ml) was mixed with each 200 μl of pooled serum or urine. The mixtures were added to 250 μl of 10% TCA solution, stirred, and then centrifuged. The supernatants were analyzed using the HPLC system.

Detection of orally administered TETA in the serum and urine

After an overnight fast, two healthy adults, who gave their informed consent, took a 30 mg/kg oral dose of TETA. These volunteers had lunch 4 hr after the dose. Serum samples were collected every 30 min for 4 hr, and urine samples were collected every 2 hr for 8 hr. The samples were kept frozen at −20°C until used. The serum and urine were added to an equal volume of 10% TCA solution and centrifuged. After centrifugation, the supernatants were analyzed using the HPLC system.

Acid hydrolysis of the urine

The urine after the oral administration of TETA was hydrolyzed overnight by heating at 110°C with 1 N HCl (final concentration) in a screw-capped test tube placed in a thermoblock. The hydrolysates were cooled and added to an equal volume of 10% TCA solution and centrifuged. The supernatants were analyzed using the HPLC system.
**RESULTS**

*Effect of TCA on TETA-copper complex*

The absorbance of the mixture of TETA and the copper solution at 599 nm increased in proportion to the TETA concentration, showing the formation of TETA-copper complex. The correlation coefficient between TETA concentration and absorbance was 0.999. On the other hand, no absorbance was detected when TCA was added to the mixture. This shows that TCA releases copper from TETA-copper complex.

*HPLC analysis of TETA*

Fig. 2a shows a chromatogram of a TETA standard solution. The peak area increased in proportion to the TETA concentration in the range of 10-250 µg/ml (correlation coefficient: 0.999). When TETA-copper complex was analyzed by the HPLC system after TCA treatment, a TETA peak was detected.

When serum and urine were injected into the HPLC after TCA treatment,
several peaks appeared. However, no substance which interfered the identification of TETA eluted around the retention time of TETA (Fig. 2b and c). When TETA mixed with the serum or urine was injected after TCA treatment, a peak corresponding to TETA appeared. Recovery values of TETA from the serum and urine calculated from the peak area compared with that of the standard were 97±5 and 97±4% (n = 6, mean±s.d.), respectively.

Detection of orally administered TETA in the serum and urine

No TETA peak was observed in the serum samples before or after TETA administration. On the other hand, a clear TETA peak appeared in the urine samples after administration. Moreover, a large peak following the TETA peak was also observed in all 4 urine samples after oral administration of TETA (Fig. 3b). The peak was not found in the urine before TETA administration and the peak area was largest 2-4 hr after administration. When the urine was injected into the HPLC after hydrolysis with HCl, the unidentified peak disappeared (Fig. 3c). The peak corresponding to TETA, on the other hand, increased.

Fig. 3. HPLC chromatograms of standard TETA solution (a) and urine from 2 to 4 hr after administration (b). A large peak appeared in the urine (b, *). The urine was hydrolyzed with HCl before HPLC analysis (c).
Cumulative amount of urinary TETA

Fig. 4 shows the results for cumulative excretion of TETA 8 hr after the administration. The cumulative amount of TETA in the two healthy adults was 1.6 and 1.7%, respectively, of the dose administered, and the most amount was excreted in the first 4 hr.

Discussion

In the HPLC system developed in the present study, the increase of peak area corresponding to TETA was proportional to the concentration of TETA. When the TETA standard mixed with pooled serum or urine was analyzed by the HPLC after TCA treatment, it was recovered with a high recovery rate. No overlapping between the TETA peak and peaks from the serum and urine was observed. Miyazaki et al. (1990) reported an HPLC method for detection of TETA in the serum. Their method is more sensitive than the present method, because they used the pre-column derivatization of TETA. However, pre-column derivatization requires complex treatments of the sample before analysis. In the present study, we applied the HPLC system coupled with on-line post column derivatization of TETA and fluorometric detection. In this method, samples can be analyzed just after removal of protein by simple TCA treatment. Thus it seems to be more applicable to routine clinical use.

In the HPLC analyses of TETA in the serum and urine of adults who orally ingested TETA, while no peak corresponding to TETA was observed in the serum, small amounts of TETA were detected in the urine. The 8 hr TETA excretion by the 2 subjects was only 1.6 and 1.7%, respectively, of the administered dose. This finding apparently suggested that TETA was hardly absorbed through the digestive tract. However, a large unidentified peak, which was observed in the
chromatograms of the urine analysis, does not support the low absorption. The peak was not observed in the mixture of TETA and normal urine or the urine sampled before TETA administration. Moreover, when the urine exhibiting this peak was analyzed after hydrolysis with HCl, the large unidentified peak disappeared. In contrast, the peak corresponding to TETA increased. These facts show that the substance in the urine which yielded the large unidentified peak is a metabolite of TETA. In other words, orally administered TETA is absorbed through the digestive tract, metabolized to the substance which produces the large unidentified peak in the HPLC, and is then excreted in the urine.

When the TETA standard was treated with HCl under the same conditions, the TETA peak decreased considerably in the HPLC analysis, showing that TETA is decomposed by HCl hydrolysis. Therefore, the amount of TETA converted to the unidentified metabolite could not be quantified. However, the large peak area compared with that of TETA in the urine suggests that a considerable amount of the orally administered TETA is absorbed through the digestive tract, but that most of the absorbed TETA is converted into the unidentified metabolite, and then excreted in the urine. Kobayashi et al. (1990) examined the absorption and excretion of orally administered TETA in rats and reported a similar phenomenon.

TETA is known to chelate copper. Thus the TETA absorbed from the digestive tract is expected to eliminate excessive copper in the tissues of patients with Wilson’s disease. However, the copper level of urine excreted after oral administration of TETA in the patients is unexpectedly low (Dubois et al. 1970; Walshe 1973; Harders and Cohnen 1977; Haslam et al. 1980; Saito et al. 1991). Siegemund et al. (1991) reported that the increase of copper level in the urine was 6.6±4.1 μmol even when 1.2 g of TETA was orally administered. These findings along with the results of the present study and that of Kobayashi et al. (1990) suggest that orally administered TETA in human is absorbed through the digestive tract, but that most of it is readily converted into a metabolite which loses its ability to chelate copper, and is then excreted into the urine. Determination of the chemical structure of the unidentified metabolite of TETA is under way.

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References


