Determination of Trichlormethiazide in Human Plasma and Urine by High-Performance Liquid Chromatography

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Key Words
Column liquid chromatography
Trichlormethiazide
Electrochemical detection
Diuretics in plasma and urine

Summary
This paper describes a high-performance liquid chromatographic (HPLC) assay method for the determination of trichlormethiazide (TCM) in human plasma and urine. After extraction and separation on an ODS column TCM in plasma was detected by oxidation in an electrochemical detector (ECD) by a porous graphite electrode. The sensitivity was better than HPLC with UV detection, enabling the determination of 2 ng ml⁻¹ TCM in human plasma. This method also allows determination of TCM at higher concentrations by exchanging the UV for the electrochemical detector. To study the pharmacokinetics, TCM in plasma and urine was assayed with coefficients of variation in the range 2–3 %. The method has the advantages of high sensitivity for plasma assay and high precision with a simple procedure for both plasma and urine samples. Small samples of 0.5 ml plasma per assay also reduced the total volume of plasma needed.

Introduction
Thiazides having 3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfonamide 1, 1-dioxide as a common ring structure with substituents at the 2-, 3- and 6-position, have been used as diuretic and antihypertension drugs. With increasing clinical application, many assay methods have been developed for them. HPLC has been used as the most reliable method for high specificity and sensitivity [1–6]. Of the thiazides, trichlormethiazide (TCM, Figure 1) is the most widely used. Recently, lowering the dosage of TCM to less than 4 mg has been examined in trying to reduce side effects without loss of drug efficacy. Meyer and Hwang [7] have established an HPLC method enabling detection of 10 ng ml⁻¹ TCM in plasma as the lower limit of assay and this has been applied to human samples obtained after administration of 8 mg doses. A more sensitive method has been reported by Orita et al. [8] to determine more than 5 ng ml⁻¹ TCM using another HPLC system. However, these methods are not suitable for the assay of plasma samples from humans given TCM doses of less than 4 mg.

We tried to develop a more sensitive assay and were able to establish an HPLC method to detect TCM with an electrochemical detector (ECD). Our method permits assay of 2 ng ml⁻¹ TCM in plasma, in addition to the advantages of employing a smaller sample, 0.5 ml, than other methods (2–3 ml) and a simpler procedure. We used the method to assay plasma samples collected after administration of 2 mg and 4 mg of drug. Urine samples were assayed using an HPLC system with a UV detector instead of the ECD because they had higher concentration of TCM.

Experimental
Materials and Reagents
Trichlormethiazide was supplied by our laboratories. Acetonitrile, methanol, tetrahydrofuran, distilled water and ethyl acetate (HPLC grade) were from Wako Pure Chem. Ind. (Osaka, Japan). All other reagents were of JIS (Japan Industrial Standard) special grade.

Apparatus
The chromatographic system consisted of a Shimadzu LC-5A or LC-6AD pump (Shimadzu, Japan) connected with a WISP 710B or 712A automatic injector (Waters Assoc., USA) with a cooling unit (15 °C). Detection of TCM in plasma was carried out at 0.8 V with a Model 5100A Coulochem electrochemical detector (Niko Bioscience, Japan) and a Shimadzu SPD-6AV variable wavelength UV detector was used at 268 nm for urine assay. Data-handling was by a Shimadzu Chromatopac C-R3A or C-R4A data processor.

In the pretreatment of samples, we used a refrigerated centrifuge Model 05PR-2 (Hitachi, Japan) at 15 °C. A KM shaker, Type V-S (Iwaki, Japan), an Eyela Metal Block Bath Model EMG-2 (Tokyo Rikakikai, Japan) at 35 °C and a Touch Mixer Model MT-51 (Yamato Scientific, Japan) were used.
All glass tubes employed in the extraction and evaporation procedures in the plasma assay were rinsed with methanol and dried at room temperature. The 0.25 ml tubes from Wakenyaku Co. (Japan) were used in the automatic injector.

For cyclic voltammetric measurements, a Model VMA-010 cyclic voltammetric analyzer (Yanagimoto, Japan) was used and to record the cyclic i vs. E curve a Model WX-1000 x-y recorder (Graphic, Japan) was used. A glassy carbon disk 3 mm diameter was used as a working electrode. The surface of the disk was polished to a mirror finish with alumina powder (0.05 μm) on an acrylic resin plate before use. An Ag/AgCl electrode and a platinum wire were used as reference and auxiliary electrode, respectively.

Columns and Mobile Phases

<table>
<thead>
<tr>
<th>Type</th>
<th>Diameter</th>
<th>Length</th>
<th>I.D.</th>
<th>Mobile Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>150 x 4.6 mm I.D. 5 μm</td>
<td>Capcell Pak SC-18 SG-type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine</td>
<td>250 x 4.6 mm I.D. 5 μm</td>
<td>Capcell Pak SC-18 SG-type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>0.05 M KH₂PO₄ (pH 4.5) / methanol (78/22, v/v)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine</td>
<td>0.01 M ammonium acetate / tetrahydrofuran / acetonitrile (71/16/13, v/v)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The column temperature was 35 °C for plasma assay and room temperature for urine assay. The flow rate of the mobile phase was 0.9 ml rain⁻¹ in both assays.

Assay Procedure

To 0.5 ml plasma sample (1.0 ml urine sample) in a 12 ml centrifuge tube were added 0.5 ml phosphate buffer (0.2 M) pH 7.5 and 6 ml ethyl acetate/1-chlorobutane [4/1, v/v]. The tubes were shaken for 10 min and centrifuged at 2000 g for 5 min. In the plasma assay, the organic layer (5 ml) was transferred to a 10 ml centrifuge tube and then 0.5 ml 0.5 M HCl added. The mixture was shaken for 5 min. After centrifugation at 2000 g for 5 min, 4 ml of the organic layer was transferred to a 12 ml centrifuge tube and evaporated to dryness under N₂ at 35 °C. The residue was dissolved in 200 μl 70 % methanol-H₂O with mixing for 15 sec, diluted with 200 μl H₂O, and mixed for 15 sec. A 100 μl portion of the mixture was injected into the column from an automatic injector. In the urine assay, the organic layer (4 ml) was transferred to a 12 ml centrifuge tube and evaporated to dryness under N₂ at 35 °C. The residue was dissolved in 250 μl of mobile phase with mixing for 15 sec, then a 25 μl portion was automatically injected into the column. The TCM peak areas were measured.

Concentration of TCM in plasma (ng ml⁻¹) =

\[ \text{Concentration found} \times \frac{6}{4} \times \frac{100}{95.6} \]

(Recovery ratio 95.6 %)

Concentration of TCM in urine (μg ml⁻¹) =

\[ \text{Concentration found} \times \frac{6}{4} \]

(Recovery ratio 100.0 %)

Calibration Curves

A 50 μl portion of each standard solution for plasma and urine assays was evaporated to dryness under N₂ at room temperature. The residue was dissolved in each mobile phase for two assays (200 μl and 250 μl). Each solution was injected in the same manner as described in the assay procedure. Standard solutions were prepared using acetonitrile to give concentrations of 10, 20, 40, 80, 120, 160 and 200 ng ml⁻¹ for plasma assay and 0.25, 0.5, 1, 1.5, 2 and 2.5 μg ml⁻¹ for urine assay.

Cyclic Voltammetry

Cyclic voltammetry was performed at a concentration of 5 mM TCM in 70 % methanol containing 0.05 M sodium perchlorate. The solution was stored in a 15 ml tube covered with a polytetrafluoroethylene cap through which the three electrodes were inserted. The voltammogram of the stationary solution was obtained at room temperature.

Hydrodynamic Voltammetry

A hydrodynamic voltammogram of TCM, using the HPLC system described in the assay procedure, was performed by varying the applied potential on the electrochemical cell. The current response was measured by replicate injections of a 10 ng TCM standard at various potentials from +0.5 to +0.85 V. The resulting peak areas were calculated.

Stability Measurement of TCM in Plasma and Urine

Two spiked solutions, 85.2 ng ml⁻¹ in plasma and 1.5 μg ml⁻¹ in urine, were prepared by mixing 0.1 volume of TCM standard solution with 9.9 volumes of human plasma and urine. The spiked plasma and urine were preserved in centrifuge tubes after separation into 0.5 ml and 1 ml portions, respectively. The samples were assayed after the specified times. The residual amounts of TCM were measured.

Results and Discussion

ECD Conditions for Plasma Assay

Amino groups are electrochemically active. Furosemide (Figure 1) was assayed recently by HPLC using an electrochemical detector (LC-EC) [9]; oxidation appears

![Figure 1](image)

Structures of trichlormethiazide and furosemide.