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DETERMINATION OF THIAMAZOLE IN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A simple and sensitive method for the determination of thiamazole in serum by high-performance liquid chromatography with electrochemical detection is described. Thiamazole in serum was quantified without an extraction procedure at concentrations down to 10 ng/ml. This method was applied to determine the serum concentration of the drug in two healthy volunteers given a single oral dose of 10 mg of thiamazole. The concentration of the drug reached a maximum at 3—4 h after the oral dose and two elimination phases were observed.

INTRODUCTION

Thiamazole (1-methyl-2-mercaptoimidazole), an inhibitor of thyroid hormone synthesis, is widely used for the treatment of hyperthyroidism. Its chemical structure is shown in Fig. 1.

Many methods for the determination of thiamazole in plasma and other biological fluids have been reported. Alexander et al. [1] and Lazarus et al. [2] measured the radioactivity of ^{35}S-labelled thiamazole and carbimazole in man. McAllister [3] and Pittman et al. [4] determined the plasma concentration of

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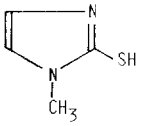

Fig. 1. Chemical structure of thiamazole.

thiamazole colorimetrically. However, in these methods the concentration of the drug was likely to be overestimated due to the presence of its sulphur-containing metabolites. On the other hand, such chromatographic methods as thin-layer chromatography (TLC) [5, 6], gas—liquid chromatography (GLC) [6, 7], high-performance liquid chromatography (HPLC) [8—12] and gas chromatography—mass spectrometry (GC—MS) [13] have been applied in determining thiamazole extracted from biological fluids and tissues. The drug concentrations reported seem to be variable when employing the above-mentioned chromatographic extraction procedures. An assay method using nuclear magnetic resonance (NMR) was applied for the drug only in pure and tablet formulation [14].

In any case, the above conventional methods may not be appropriate to monitor the therapeutic plasma levels after administration of the usual maintenance dose of thiamazole.

This paper reports an HPLC assay employing electrochemical detection for quantification of thiamazole in human serum without an extraction procedure.

EXPERIMENTAL

Materials

Thiamazole and its metabolite, 3-methyl-2-thiohydantoin, were kindly provided by Chugai Pharmaceutical (Tokyo, Japan). The tablets of thiamazole (Mercazole®) were obtained commercially from Chugai Pharmaceutical. All the other chemicals used were of analytical-reagent grade.

Preparation of serum samples

To 0.1 ml of serum was added 0.3 ml of methanol containing 48 ng of *p*-hydroxyanisole, as an internal standard, and mixed on a vortex mixer for 1 min. After standing for 30 min at room temperature the mixture was centrifuged at 10 000 *g* for 10 min. Aliquots (10—20 μl) of the deproteinized supernates were injected onto the HPLC column.

Chromatographic system

The HPLC system was as follows: a solvent delivery pump (Waters Assoc., Model M-6000), a septumless injector (Waters Assoc. Model U6K), a prepacked 10 cm × 8 mm I.D. Radial Pak cartridge C_{18} (10 μm particle size) HPLC column operated in a radial compression module RCM-100 (Waters Assoc.) and an electrochemical detector (Bioanalytical Systems) using a glassy carbon working electrode and an Ag/AgCl reference electrode. The electrochemical potential of the working electrode was set at +0.70 V versus Ag/AgCl reference electrode. To decrease the variance of the electrochemical response the

detector was maintained at 20°C. The mobile phase consisted of 0.01 *M* ammonium phosphate containing EDTA · 2Na (1 m*M*) and methanol (8%), and the pH was adjusted to 4.00 with phosphoric acid. The mobile phase, prepared daily, was filtered through a membrane filter (Sartorius, 0.2 μm) and degassed by sonication before use. The flow-rate was 3.0 ml/min. Injections were made with a 10—20 μl Hamilton syringe.

Calibration curve

Standard solution was prepared by dissolving 5 mg of thiamazole in 25 ml of methanol. The stock solution of the internal standard was prepared by dissolving 3 mg of *p*-hydroxyanisole in 25 ml of methanol; this stock solution was diluted before use. To provide the calibration standards used in this study, 0.1 ml of methanol containing 2—80 ng of thiamazole and 0.2 ml (48 ng) of *p*-hydroxyanisole were added to blank serum (0.1 ml). The assay of samples was performed as described above, and the calibration curve was obtained by plotting the peak height ratios of thiamazole to *p*-hydroxyanisole against the concentration of thiamazole over the range 5—200 ng/ml.

Recovery and reproducibility

For the determination of recovery six replicate samples at levels of 50 ng/ml and 200 ng/ml for thiamazole were spiked in fresh blank serum, and assayed with the same procedure as described for preparation of samples. The peak height ratios of thiamazole to the internal standard obtained from these samples were compared with those of standards in methanol.

Reproducibility was examined by assaying six preparations of the serum sample spiked with 53.10 ng/ml thiamazole.

Analysis of serum samples

Two healthy male volunteers, weighing 56 and 58 kg, participated in this study performed under medical supervision. They were orally given two 5-mg tablets of a commercial formulation of thiamazole with 400 ml of water. A control blood sample was taken just before the administration of the drug, and then at various time intervals between 30 min and 48 h. All the samples were immediately centrifuged and the serum samples were stored at −70°C until analysis.

RESULTS AND DISCUSSION

Many studies using TLC, GLC and HPLC to determine thiamazole concentrations in biological fluids and tissues have been reported. Stenlake et al. [6] found that the densitometric method was capable of detecting 0.1 μg of thiamazole on a thin-layer plate. They also reported that the densitometric method was better suited to routine use although the GLC method was more sensitive. Bending and Stevenson [7] measured thiamazole using GLC with thermionic nitrogen—phosphorus detection and showed that the limit of detection was 30 ng/ml of plasma. HPLC with ultraviolet detection was employed by Skellern and co-workers [8—12] to detect thiamazole and its metabolite; thiamazole in plasma was detectable above a concentration of 100

ng/ml. When the usual therapeutic dose of thiamazole is administered the concentration of the drug in plasma is often less than 100 ng/ml. Therefore it is necessary to develop a method that is specific and reproducible enough to determine concentrations lower than 100 ng of thiamazole per ml.

Electrochemical characteristics

The oxidative characteristics of thiamazole were estimated at various working electrode potentials in the range +0.30 to +0.90 V. Fig. 2 illustrates the electrochemical response versus applied potential for thiamazole and two compounds. For thiamazole, an increase in applied potential in the range +0.4 to +0.70 V resulted in an increase in current, and it showed a plateau level above +0.70 V. In order to minimize the variance of response and to increase the electrochemical specificity a potential was set at the lower end of the plateau range, +0.7 V. As an internal standard *p*-hydroxyanisole was chosen in preference to 3-hydroxypyridine because of its constant response above +0.60 V.

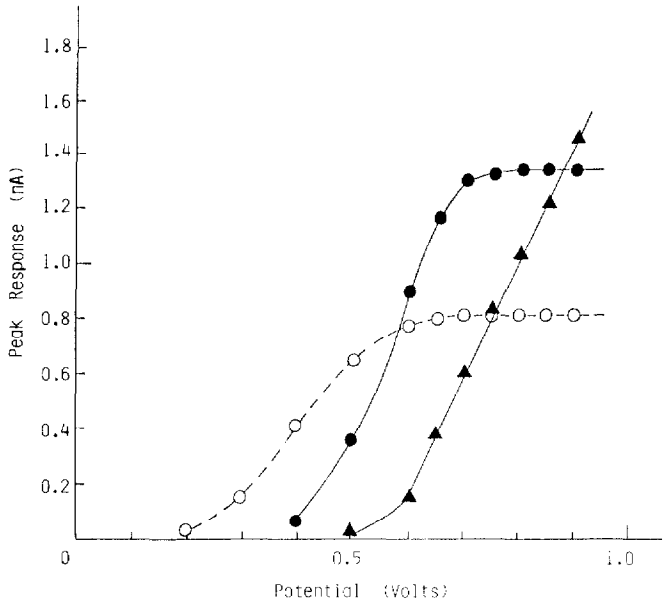

Fig. 2. Electrochemical responses (peak height) of thiamazole (●), *p*-hydroxyanisole (○) and 3-hydroxypyridine (▲) at various potential settings. Chromatographic conditions: column, Radial Pak cartridge C_{18} (10 cm × 8 mm I.D., particle size 10 μm); mobile phase, ammonium dihydrogen phosphate (0.01 *M*) with EDTA • 2Na (1 m*M*)—methanol (92:8); pH 7.5; flow-rate, 3.0 ml/min. Detector was maintained at 20°C.

Electrochemical responses were affected by temperature fluctuations. The observed peak height of thiamazole changed by about 20% when the ambient temperature varied by 5°C. When the room temperature was held constant at 25°C, the peak heights were reproducible. The metabolite of thiamazole, 3-methyl-2-thiohydantoin, was detected in the plasma of patients receiving thiamazole using the method of HPLC with ultraviolet detection at 254 nm [11]. But this metabolite was electrochemically inactive.

Preparation of samples

Deproteinization was carried out with methanol, N,N-dimethylformamide and 30% perchloric acid. The method using methanol was simple and preferable, while the supernates containing N,N-dimethylformamide or perchloric acid caused electrochemical noise.

Many reports on the analysis of thiamazole described that pretreatment of serum samples was performed by extraction with organic solvents before injection onto the HPLC column. We tried to extract thiamazole with chloroform and ethyl acetate. The recoveries from serum using chloroform and ethyl acetate were 64.4% and 56.0%, respectively. Thus, neither of these extracting solvents was suitable because of poor and variable yields. Considering these observations, we elected to prepare our samples by deproteinization instead of employing an extraction procedure. If the concentration of thiamazole in the deproteinized supernate was lower than 5 ng/ml, 0.2 ml of the serum and 0.2 ml of methanol containing the internal standard were mixed and centrifuged 1 h later.

High-performance liquid chromatography

Various methanol—0.01 *M* ammonium dihydrogen phosphate systems were used as the mobile phase for HPLC. The methanol concentration and pH of the mobile phase were varied to separate thiamazole from serum constituents (Fig. 3). The electrochemical sensitivity of thiamazole was higher in a neutral mobile phase (pH 7.5) than in an acidic mobile phase (pH 4.0), but in neutral mobile phase thiamazole could not be completely separated from the serum constituents. The acidic mobile phase (pH 4.0) resulted in a good resolution of

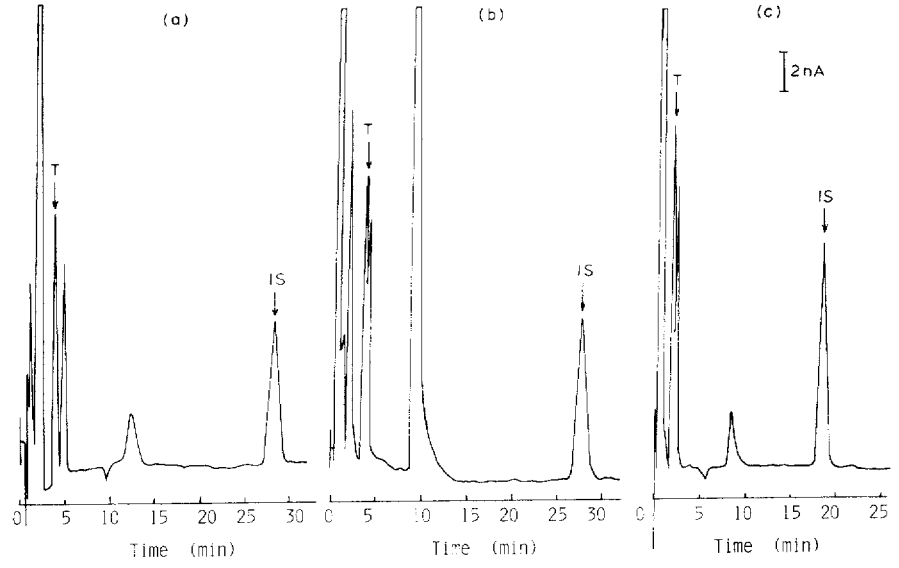

Fig. 3. Chromatograms of deproteinized serum (0.4 ml) spiked with thiamazole (T, 10 ng) and *p*-hydroxyanisole (IS, 40 ng) in three mobile phases: the volume ratios of ammonium dihydrogen phosphate (0.01 *M*) with EDTA · 2Na (1 m*M*) to methanol and pH are: (a) 92:8, pH 4.00; (b) 92:8, pH 7.46; (c) 88:12, pH 4.00. Electrode potential was +0.70 V versus Ag/AgCl reference electrode. Other chromatographic conditions as in Fig. 2.

thiamazole and the eluting components of serum. The retention time of thiamazole was influenced by the concentration of methanol in the mobile phase, ranging from 3% to 12%. An increase in the methanol concentration caused a decrease in the retention time of thiamazole. To separate the thiamazole peak completely from neighbouring peaks of serum constituents, methanol—0.01 *M* ammonium dihydrogen phosphate (8:92, pH 4.00) was employed as the mobile phase. EDTA · 2Na (1 m*M*) was added to decrease background electrical noise. The retention times of thiamazole and *p*-hydroxyanisole under these condition were 4 and 28 min, respectively.

Fig. 4 shows chromatograms of serum of a volunteer before and after the administration of thiamazole. It can be seen that the components of serum did not interfere in the analysis of thiamazole.

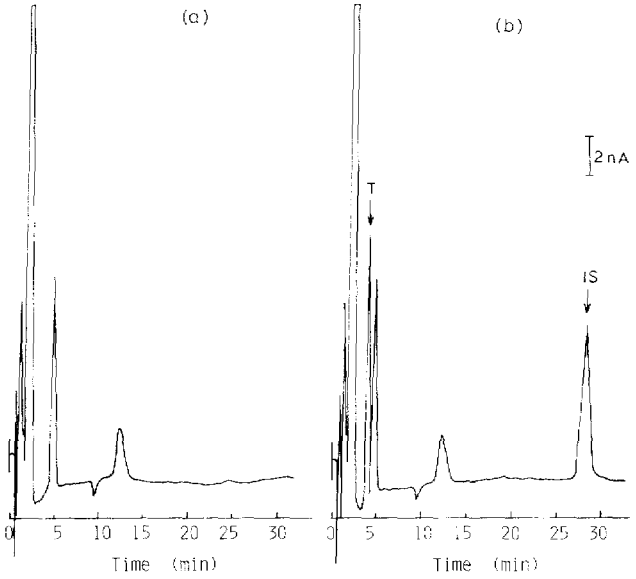

Fig. 4. Chromatograms of deproteinized serum samples from a volunteer receiving 10 mg of thiamazole orally. (a) Control serum; (b) test serum. Peaks: T = thiamazole; IS = *p*-hydroxyanisole. Mobile phase, ammonium dihydrogen phosphate (0.01 *M*) with EDTA · 2Na (1 m*M*)—methanol (92:8); pH 4.00. Other chromatographic conditions as in Fig. 2.

Calibration curve

The calibration standards were prepared with blank serum by adding thiamazole in concentrations ranging from 5 to 200 ng/ml and a fixed amount of *p*-hydroxyanisole as internal standard. The linear regression equation for calibration (peak height ratio versus concentration, y) was $y = 0.032x - 0.026$ (correlation coefficient of 0.9999) for thiamazole. Good linearity was obtained for thiamazole over the range 5 ng/ml to 2 μg/ml. Under these conditions, thiamazole in serum was detectable at a concentration as low as 10 ng/ml. The calibration curve was prepared daily.

Recovery and reproducibility

Known amounts of thiamazole were added to blank serum samples and the whole analytical procedure was performed to determine the recovery rates.

TABLE I

RECOVERY OF THE METHOD FOR THE DETERMINATION OF THIAMAZOLE IN SERUM

The concentrations were obtained by comparing the peak height ratios of the samples to those of the standard solutions.

Concentration spiked into serum (ng/ml)	Found (ng/ml)	Recovery (%)	Mean recovery ± S.D. (%)
53.1	52.0	97.8	
	60.7	114.3	
	57.6	108.5	
	59.1	111.2	
	52.2	98.4	
	56.2	105.8	106.0 ± 6.75
212.4	212.7	100.1	
	205.0	96.5	
	208.1	98.0	
	229.7	108.2	
	224.9	105.9	
	227.7	107.2	102.6 ± 5.05

The data are given in Table I. Reproducibility was calculated in six preparations of the serum sample spiked with 53.1 ng/ml thiamazole. The value obtained was 54.3 ± 1.56 ng/ml (mean ± S.D.) and the coefficient of variance was 2.9%.

Determination of thiamazole in serum of volunteers

Pharmacokinetic parameters of thiamazole have been described in several papers. Pittman et al. [4] and Vesell et al. [15] determined the concentration of thiamazole in plasma colorimetrically and the half-lives were 6.4—9.3 h in euthyroid volunteers, 6.9 ± 0.6 h in hyperthyroid patients and 13.6 ± 4.8 h in hypothyroid patients. Using the HPLC method, the half-lives of thiamazole in plasma were found to be 3.5—4.0 h in euthyroid volunteers [8, 16], 3.1 ± 0.8 h in hyperthyroid patients [11] and 2.0 ± 0.2 h in hyperthyroid pregnant patients [12].

We applied the present method to determine the serum concentration of thiamazole after a single oral dose to healthy volunteers. Fig. 5 shows typical thiamazole concentration—time profiles of the volunteers after a single oral administration of two 5-mg tablets. The serum concentration reached maximum values of 192 and 214 ng/ml at 3—4 h after administration. It declined slowly with time and was less than 100 ng/ml about 10 h later. Nevertheless we could detect the drug for about two days after the oral dose.

As shown in Fig. 5 the serum concentration—time profiles of thiamazole could be described by a two-compartment model, which includes an absorption phase and two elimination phases. The half-lives of the initial elimination phase were 2.9 and 3.2 h, and are similar to the parameters of previous reports using HPLC methods. In addition, a terminal elimination phase with half-lives of 17.0 and 30.2 h was observed. Our method has now been in continuous operation without endogenous interferences for over a year. A detailed pharmacokinetic study is in progress.

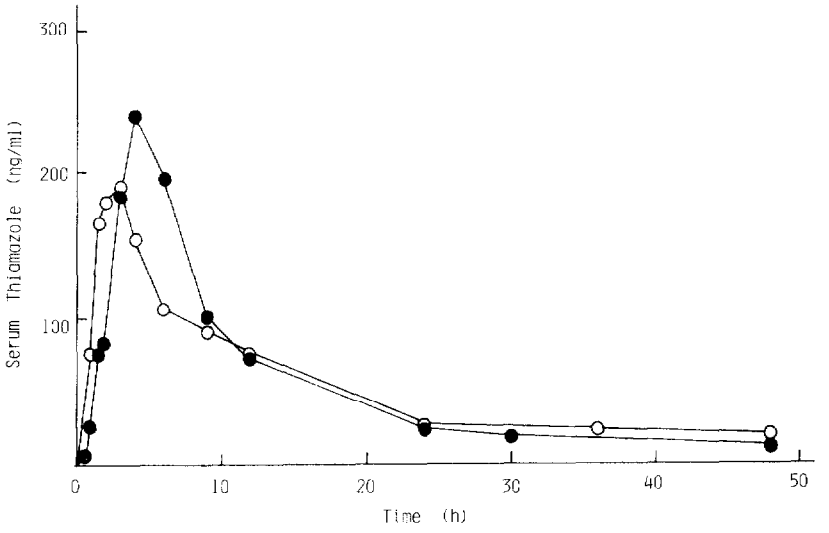

Fig. 5. Serum concentration—time profiles of thiamazole in two healthy volunteers following a single oral administration of thiamazole (10 mg).

This method will be sufficiently sensitive for monitoring the given maintenance dose of thiamazole. The role of the therapeutic drug monitoring laboratory in the appropriate adjustment of drug required subsequent to drug analysis needs to be investigated.

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