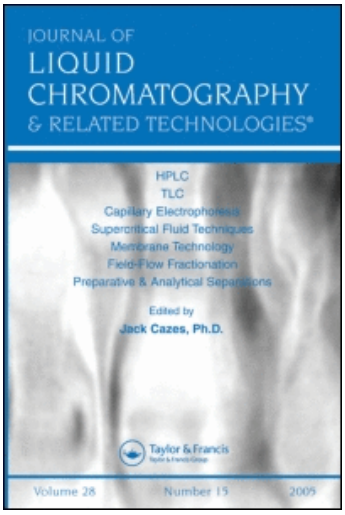

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DETERMINATION OF CHLORMETHIAZOLE IN BLOOD BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A simple and sensitive method is described for the determination of chlormethiazole in blood. Separation and quantitation were performed by reversed-phase high performance liquid chromatography utilizing ultraviolet detection at 254 nm. The internal standard, 2-amino-4-methylthiazole, was used as an aid to quantitation. The blood sample was deproteinized with phosphotungstic acid reagent and the clear supernatant obtained was directly analysed in the chromatographic system. Limit of detection was about 0.25 µg/ml blood. Recovery of chlormethiazole from blood was 73 ± 2%. The mean between-run coefficient of variation was 4.9% over the range of 0.25-32.0 µg/ml, whereas the within-run coefficient of variation at 0.25 µg/ml was 6.5%. The scale and sensitivity of detection were found to be suitable for use in pharmacokinetic studies in the rat.

INTRODUCTION

Chlormethiazole(CMZ) is a derivative of the thiazole part of thiamine, and has sedative hypnotic and anticonvulsant effects. CMZ

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is used mainly in the treatment of alcohol withdrawal symptoms, and as an anticonvulsant for treatment of conditions characterized by anxiety and agitation(1-5).

The pharmacokinetics of CMZ in healthy human volunteers following an intravenous infusion or oral administration have been reported. In these studies, CMZ in serum or plasma was quantitated by gas chromatography(6-9) and gas chromatography-mass spectrometry (3,10) which provided specificity and sufficient sensitivity. The main deficiency of these methods is the requirement for rather time-consuming extraction, purification and concentration procedures.

In this paper, we describe a simple and sensitive method for the determination of CMZ in blood using reversed-phase high performance liquid chromatography(HPLC). The applicability of the method is illustrated by a basic pharmacokinetic analysis after a single intraperitoneal administration of a typical experimental dose of CMZ to rats.

MATERIALS AND METHODS

Reagents and Materials

CMZ ethandisulphonate(Heminevrin®, Astra, Sodertalje, Sweden), 2-amino-4-methylthiazole(AMZ, A6-600-6, Aldrich, Milwaukee, WI, U.S.A.), HPLC grade acetonitrile(A-998, Fisher, Fair Lawn, NJ, U.S.A.), potassium phosphate monobasic(P-284, Fisher) and phosphotungstic acid(PTA) reagent(RO-1196, BDH, Toronto, ONT, Canada) were used. Water was deionized and then triple distilled.

Apparatus

HPLC determinations were performed with a Beckman Model 330 Isocratic Liquid Chromatograph, a Model 110A Pump, a Model 160 Ultraviolet Detector operating at 254 nm with the absorbance detector sensitivity set at 0.005 AU Full Scale, and a Hewlett Packard 3390A Recording Integrator. Separation of CMZ was performed on a 4.6 mm i.d. x 250 mm Altex Ultrasphere ODS column(C_{18} reversed-phase, particle size 5 μm) with isocratic elution. A 45:55 v/v mixture of acetonitrile and potassium phosphate monobasic 0.025 M in water(pH 4.6) was used as a mobile phase. The phosphate buffer was filtered through a 0.45 μm filter(Millipore, Bedford, MA, U.S.A.) prior to mixing. A flow rate of 2.0 ml/min(2500 p.s.i.) at ambient temperature was employed in the present study.

Preparation of Standards

Standard solutions of CMZ base(0.16 mg/ml) and AMZ(0.1 mg/ml) were prepared in water and stored subsequently at +4°C. The appropriate concentrations of standard solutions were prepared by diluting the stock solutions in water.

Extraction Procedure

A series of spiked rat blood samples(0.4 ml) in polypropylene tubes was prepared by mixing 0.1 ml blood with 0.3 ml water containing varied amounts of CMZ, from 0.025 to 3.2 μg(corresponding to 0.25-32.0 μg/ml blood) and 1.0 μg AMZ as an internal standard. A spiked human blood sample containing 0.05 μg CMZ(corresponding to 0.5 μg/ml blood) and 1.0 μg AMZ was prepared as above. The samples

were deproteinized with 25 µl PTA reagent for 90 min at room temperature and then centrifuged for 15 min at 31,550 x g. Twenty µl of the clear supernatant obtained(pH 4.0-4.5) was injected into the HPLC system with a 50 µl Hamilton syringe.

Standard Curve

Known amounts of CMZ(0.025-3.2 µg) in 0.4 ml aliquots of the spiked blood samples were taken through the entire procedure, AMZ (1.0 µg) being added to each sample as an internal standard. An identical set of CMZ samples was made up in 0.425 ml water with no blood or PTA reagent. To construct the standard curve, the CMZ/AMZ response ratios were plotted against the concentrations of CMZ, in µg/ml. Actual amounts of CMZ injected into the HPLC system were 1.2-150.6 ng, while the amount of AMZ was 47.1 ng.

Animal Experiments

To study the suitability and applicability of the method to animal studies, male rats weighing 320-370 g(CBL, Montreal, QUE, Canada) were injected intraperitoneally(i.p.) with 43 mg/kg CMZ base (in 1.0 ml saline/100 g body weight, pH 7.0). Rats were housed under constant environmental conditions and food was removed 24 hrs prior to the administration of CMZ. Blood samples(0.05 ml) from the tail vein were collected into polypropylene tubes containing 0.35 ml water at various time intervals after administration. The samples were analysed as described above for the spiked samples.

RESULTS AND DISCUSSION

CMZ and its internal standard AMZ were clearly separated in the chromatograms obtained with standard solutions as well as with blood

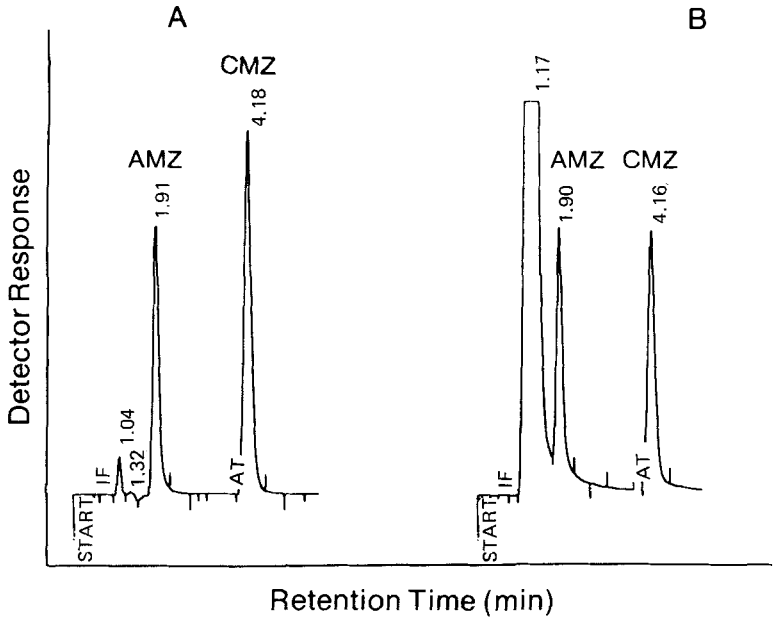

Fig. 1. Isocratic separation of CMZ and its internal standard AMZ. A, chromatogram of CMZ and AMZ standards in water. B, chromatogram from rat blood sample showing peaks of CMZ(9.6 ng) and of internal standard, AMZ(47.1 ng).

samples(Fig. 1). The chromatogram obtained with a human blood sample showed the same result. No interfering endogenous compounds were found on the chromatograms obtained with the blank rat or human blood. Under the present conditions, the elution volume for CMZ(retention time, 4.2 min) and the internal standard AMZ(retention time, 1.9 min) were 8.4 and 3.8 ml, respectively. Maximum absorbance of CMZ and AMZ were found to occur at 252 and 257 nm, respectively, and in all subsequent studies these were analysed at 254 nm.

Fig. 2 shows the linearity of the plot of peak area response ratios(CMZ/AMZ) with increasing concentrations of CMZ. A linear

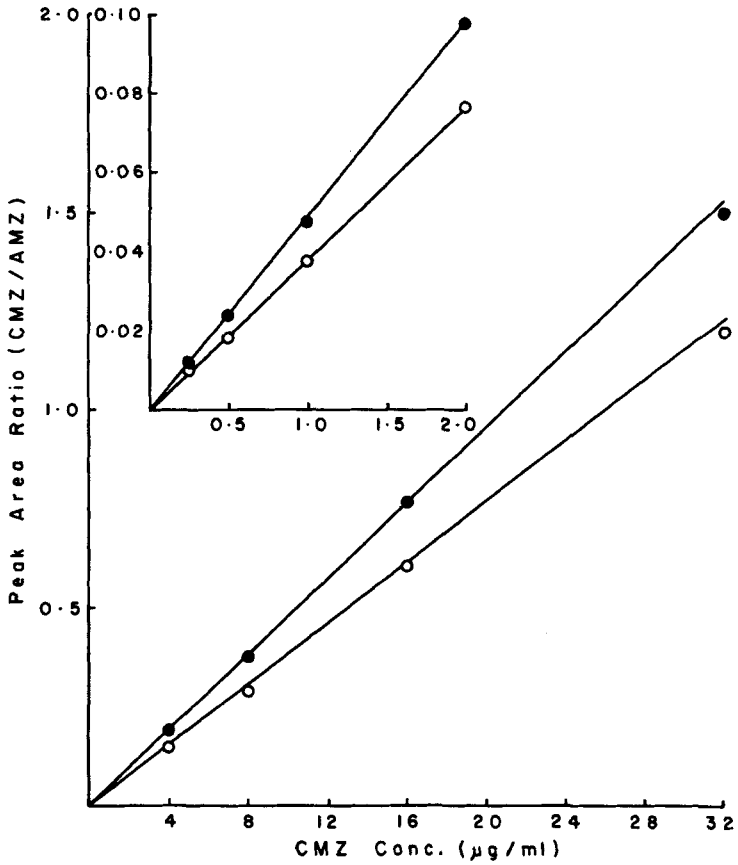

Fig. 2. Calibration graph of peak area ratios of CMZ vs. its internal standard AMZ plotted against the amounts of CMZ, μg/ml. Closed circles indicate values obtained with standard solutions in water; open circles, in rat blood.

regression analysis from blood indicated the correlation coefficient, 0.9999; slope, 0.0381; y-intercept, -0.0005. The practical detection limit was set at 0.25 μg/ml (i.e., to give an integrator peak height of at least 1/10 of full scale deflection). The mean between-run coefficient of variation recovered from blood was 4.9% over the

range of 0.25-32.0 µg/ml (in all concentrations, n = 7), whereas the within-run coefficient of variation at 0.25 µg/ml (n = 10) was 6.5%. Residue analysis indicated that recovery of CMZ calculated for peak areas by comparison with results obtained with water samples over the range of 0.25-32.0 µg/ml was 73 ± 2% ($\bar{x}$ ± SD).

The in vivo study illustrated that the method was suitable for the pharmacokinetic study of CMZ following administration of a single dose (Fig. 3). The blood level of CMZ reached 0.7 ± 0.2 µg/ml ($\bar{x}$ ± SEM, n = 4-8) within 1 min after i.p. administration, and the

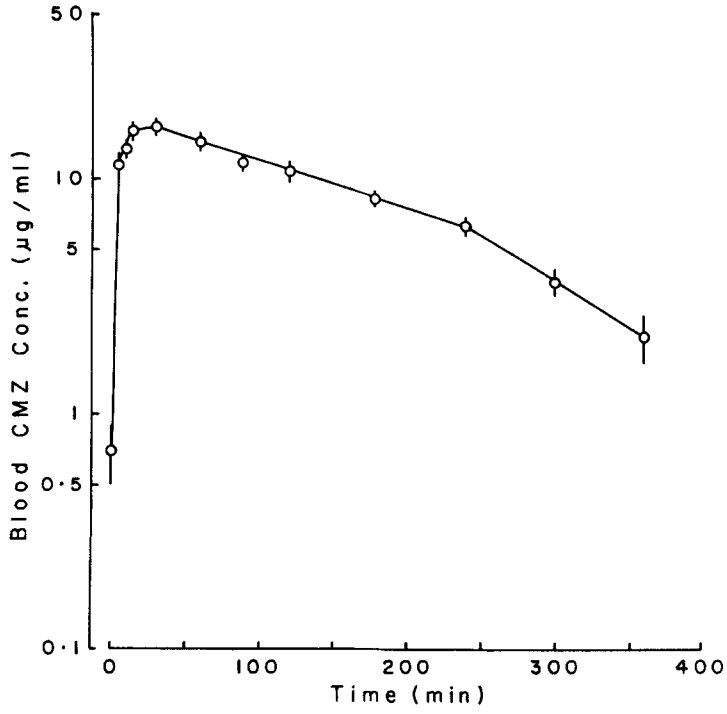

Fig. 3. The concentration of CMZ in blood of rats, after i.p. administration in a dose of 43 mg/kg. Results are the means ± SEM for 4-8 rats at each time interval.

peak level of 17.0 ± 1.2 μg/ml occurred at 30 min. The blood CMZ appeared to have a $t_{1/2}$ of about 3 hrs.

The presence of PTA reagent in samples of blood and water was found to interfere with the maximum absorption of CMZ and AMZ at 254 nm. Under the present conditions, the values obtained for peak area of CMZ and AMZ from blood samples with PTA reagent were 27 and 9% lower than those obtained with water samples with no PTA reagent. However, the ratios of peak area (CMZ/AMZ) remained constant, although 21% lower than the absolute values. Concentrated PTA reagent (25 μl) added to water samples containing CMZ and AMZ obscured the CMZ and AMZ peaks, and addition of PTA reagent diluted 1:10 to water samples resulted in a significantly decreased, though still detectable, response for CMZ (60% or higher). The amount of PTA reagent used in the present study was sufficient to deproteinize blood samples, but less than 25 μl (10-20 μl) did not produce complete deproteinization. Addition of CMZ before or after the deproteinization of blood samples with PTA reagent yielded the same quantitative results which indicated that there appeared to be no protein-binding interactions. The retention times and resolution of CMZ and AMZ were constant over 2000 sample runs on the same column (including the intermittent direct analyses of biogenic amines in brain tissue). Column rejuvenation to the same efficiency was accomplished by washing the column with 500-600 ml of methanol-water (40:60%, v/v) every 100 analyses. Storage of blood samples at +4°C for up to 2 months did not alter the quantitation of CMZ.

A simple and sensitive reversed-phase HPLC is an effective method to quantitate CMZ in blood and is practical for utilization in

the clinical laboratory. In the present study, no extraction, purification and concentration procedures of samples before the HPLC system were found to be necessary.

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