CHROMBIO. 491

# SIMULTANEOUS QUANTITATION OF CHLORMETHIAZOLE AND TWO OF ITS METABOLITES IN BLOOD AND PLASMA BY GAS—LIQUID CHROMATOGRAPHY

S. EDMUND TSUEI and JACK THOMAS

*Department of Pharmacy, University of Sydney, Sydney, N.S.W. 2006 (Australia)*

and

ROGER L. NATION*

*Perinatal Pharmacology Laboratory, Foundation 41, The Women's Hospital, Crown Street, Sydney, N.S.W. 2010 (Australia)*

SUMMARY

A simple and rapid gas chromatographic method for the quantitation of chlormethiazole and two of its pharmacologically active metabolites, 5-acetyl-4-methylthiazole and 5-(1-hydroxyethyl)-4-methylthiazole, in plasma and blood is described. The total analysis time is less than 20 min for a single sample. The method requires 50—500 $\mu$l of plasma or 1 ml of blood. The compounds are detected with a nitrogen—phosphorus detector. An internal standard technique is used for the quantitation. Calibration data are linear over the range 32—2376 ng of chlormethiazole and a similar range of the metabolites in plasma. The method may be used for pharmacokinetic studies.

INTRODUCTION

Chlormethiazole [5-(2-chloroethyl)-4-methylthiazole, CTZ] is an anticonvulsant with sedative and hypnotic properties. It has been used extensively in the treatment of withdrawal symptoms of alcoholism. Recently, several authors have recommended its use in obstetrics as a sedative and hypnotic during labour [1, 2], and as a sedative—anticonvulsant in the treatment of eclampsia and severe pre-eclampsia [3—5]. The disposition kinetics of chlormethiazole in mother and infant in the perinatal period are under investigation in this laboratory.

Ethical considerations dictate that the minimum volume of blood be withdrawn from a patient for the purpose of drug analysis. This is especially true in the field of perinatal medicine since the mothers are subjected to additional blood loss at delivery and the total blood volume of infants is relatively small.

---

*To whom correspondence should be addressed.

Therefore, a sensitive method of drug analysis requiring a minimum volume of blood or plasma is of paramount importance.

Two metabolites of CTZ, 5-acetyl-4-methylthiazole (AMT) and 5-(1-hydroxy-ethyl)-4-methylthiazole (HEMT), have been reported to be pharmacologically active [6] and plasma levels of them in humans have been documented [7]. Therefore, it is desirable to follow the time course of production and elimination of these compounds. Additionally, in seeking a relationship between plasma concentration and pharmacological effect, it is necessary to quantitate these two metabolites as well as chlormethiazole.

Several methods [7—10] have been reported for the quantitation of chlormethiazole in biological fluids. These methods use gas—liquid chromatography [8—10] or mass fragmentography [7, 8]. Only the mass fragmentographic method reported by Nation et al. [7] allows simultaneous quantitation of chlormethiazole and its two metabolites, AMT and HEMT. All of these methods use relatively large volumes of plasma or blood and involve a time-consuming evaporation step prior to chromatographic analysis. This paper describes a simple and rapid gas chromatographic assay for the quantitation of chlormethiazole and two of its metabolites in plasma and blood.

EXPERIMENTAL

*Reagents and materials*

Chlormethiazole ethanedisulphonate and bromomethiazole [5-(2-bromo-ethyl)-4-methylthiazole, BTZ] ethanedisulphonate were gifts from Astra Chemicals (Sydney, Australia). The two metabolites of chlormethiazole, 5-acetyl-4-methylthiazole and 5-(1-hydroxyethyl)-4-methylthiazole, were synthesized according to the method described by Moore et al. [11]. The diethyl ether (anaesthetic grade, B.P.) used for extraction was freshly distilled in glass every morning. All other reagents were of analytical grade and were used without further purification. All the glassware was cleaned with a chromic acid mixture, rinsed with distilled water, and oven dried.

*Extraction from blood or plasma*

Aliquots of plasma (50—500 $\mu$l) or blood (1 ml) were pipetted into 15-ml glass centrifuge tubes. In the case of plasma volumes smaller than 500 $\mu$l, sufficient distilled water was added to bring the total volume up to 500 $\mu$l. Distilled water (700 $\mu$l) was added to the tubes containing aliquots of whole blood since this resulted in more uniform mixing. After the addition of an aqueous solution of the internal standard (2.5 $\mu$g of bromomethiazole ethanedisulphonate per 25 $\mu$l), 200 $\mu$l of water, 100 $\mu$l of phosphate buffer (pH 7.0, 0.1 $M$), and 5 ml of ether, each tube was closed with a PTFE-lined screw-cap. The tubes were shaken on a vortex mixer for 2 min. After centrifugation at 1500 $g$ for 5 min, the organic phase was transferred by disposable pipette to another centrifuge tube containing 500 $\mu$l of HCl (1 $M$). The mixture was shaken and centrifuged as described above. The organic phase was aspirated off and discarded. Following the addition of 100 $\mu$l of 10 $M$ NaOH, the mixture was extracted with 1 ml of ether as described previously. After centrifugation, 5—10 $\mu$l of the ethereal extract were injected into the gas chromatograph. Fig. 1 gives a schematic outline of the extraction procedure.

Plasma (+enough water to give 500 µl)
or blood (1 ml diluted with 700 µl of water)

+ internal standard
+ water (200 µl)
+ phosphate buffer (pH7.0) 0.1 M (100 µl)
+ ether (5 ml)

Vortex 2 min
Centrifuge

Discard aqueous layer          Ethereal extract

Vortex 2 min          + 1M HCl (500 µl)
Centrifuge

Discard ether layer

Aqueous extract

Vortex 2 min          + 10 M NaOH (100 µl)
Centrifuge          + ether (1 ml)

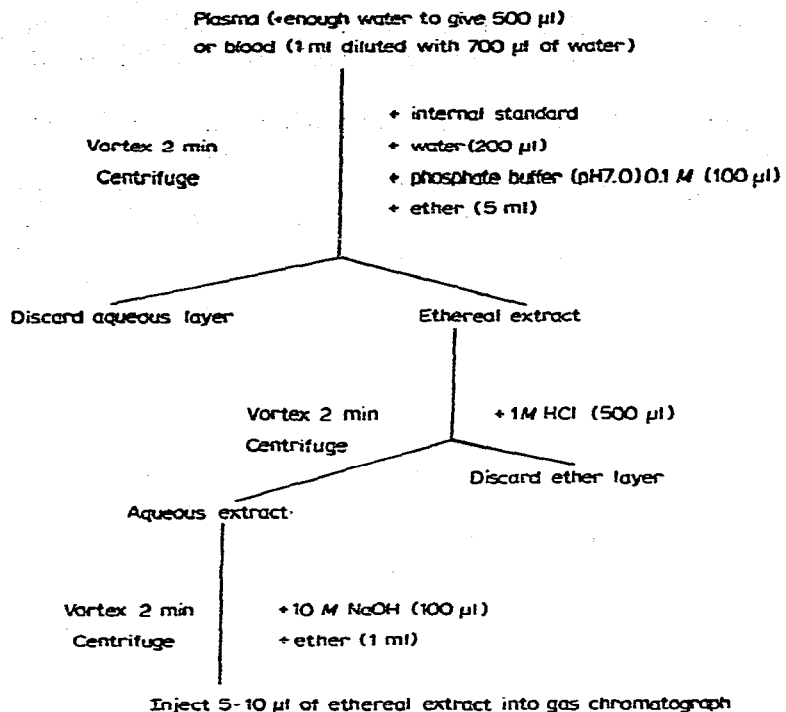Inject 5-10 µl of ethereal extract into gas chromatograph

Fig. 1. Schematic diagram for the extraction of CTZ, AMT and HEMT from blood and plasma.

## Chromatography

The chromatographic system consisted of a Hewlett-Packard Model 5730A gas chromatograph equipped with a Hewlett-Packard Model 18789A nitrogen—phosphorus detector. A glass column (1.5 m X 3 mm I.D.) silylated with 3% N,O-bis-(trimethylsilyl)trifluoroacetamide in toluene and packed with 5% OV-7 (Pierce Chemicals, Rockford, Ill., U.S.A.) on Gas-Chrom Q (100—120 mesh, Applied Science Labs., State College, Pa., U.S.A.) was used. The temperature of the injection port and detector was maintained at 200° and the oven at 145°. The nitrogen carrier gas flow-rate was 30 ml/min and the hydrogen and air flow-rates were 3 ml/min and 75 ml/min, respectively. The detector voltage (16—22 V) was adjusted to give a 10% recorder deflection at an attenuation setting of 32.

## Calibration and reproducibility

Aqueous solutions totalling 200 µl and containing known quantities of chlormethiazole ethanedisulphonate (equivalent to 32—2376 ng of the chlormethiazole base), AMT (37—2760 ng) and HEMT (29—2160 ng) were added to blank plasma samples (50—500 µl) in such a way that samples containing low levels of CTZ contained high levels of the metabolites, and vice versa. This was to mimic the clinical situation, in which, soon after starting infusion, CTZ concentrations are high while concentrations of the metabolites are low, and after cessation of infusion, the converse may be true. The samples were assayed for CTZ, AMT and HEMT as described earlier. Calibration curves were constructed by plotting

the ratios of the peak heights of CTZ, AMT and HEMT to that of BTZ (the internal standard) versus the amounts of added CTZ, AMT and HEMT, respectively.

To check the reproducibility of the analytical procedure, three different plasma calibration curves (each composed of 5 or 6 data points) were constructed on three different days using human plasma samples from five different sources: a healthy non-pregnant female and four healthy pregnant women near term. Calibration curves (each composed of three data points) using blood (1 ml) from two healthy pregnant women near term were similarly prepared on two different days over the range 316.8—3960 ng of CTZ, 368—4600 ng of AMT and 288—3600 ng of HEMT.

## Drug disposition study

Chlormethiazole ethanedisulphonate (Hemineurin®, Astra Chemicals) was administered by intravenous infusion to a pregnant patient near term (age 21 years, weight 70 kg) at a rate of 800 mg/h for the first 1.42 h and then at a rate of 120 mg/h for the next 6.28 h. Informed consent was previously obtained. Blood was withdrawn at intervals into disposable syringes (Monoject, Sherwood Medical Industries, Deland, Fla., U.S.A.) via a cannula inserted in an antecubital vein of the arm not receiving the infusion, and placed in plastic tubes containing 100 units of ammonium heparin and separation granules (Disposable Products, Sydney, Australia). The plasma was separated and stored frozen until time of analysis.

## RESULTS AND DISCUSSION

Under the chromatographic conditions described above, the retention times of AMT, HEMT, CTZ and BTZ were 4, 4.6, 6 and 9 min, respectively. Fig. 2 shows chromatograms of the extracts of plasma samples (500 $\mu$l) from a pregnant patient drawn immediately prior to commencement of chlormethiazole therapy, and at 0.38 and 4.92 h after the start of the infusion. Chromatograms obtained from extracts of blood were similar. The CTZ, AMT, HEMT and BTZ peaks were satisfactorily resolved from each other and were not interfered with by peaks of endogenous compounds in plasma or blood. In extracts of samples collected 1 h or more after commencement of therapy, there was a relatively small peak which chromatographed just before the AMT. This peak may represent another metabolite of CTZ as the patient was not taking any other medication. The identity of this compound is not known.

A summary of the composite calibration data in plasma is presented in Fig. 3. The calibration curves are linear ($r$ = 0.9997, 0.9996 and 0.9993 for CTZ, AMT and HEMT, respectively) and pass through the origin. Similar results were obtained from blood analyses ($r$ = 0.9985, 0.9974 and 0.9993 for CTZ, AMT and HEMT, respectively). The linearity of the calibration curves demonstrates also the reproducibility of the analysis as the calibration data were obtained on different days using samples from different sources. Slopes of the linear regression lines of the plasma calibration data from three different days are presented in Table I. The method allows quantitation of 32—2376 ng of CTZ, 37—2760 ng of AMT and 29—2160 ng of HEMT in plasma, and of 316.8—3960 ng of CTZ,
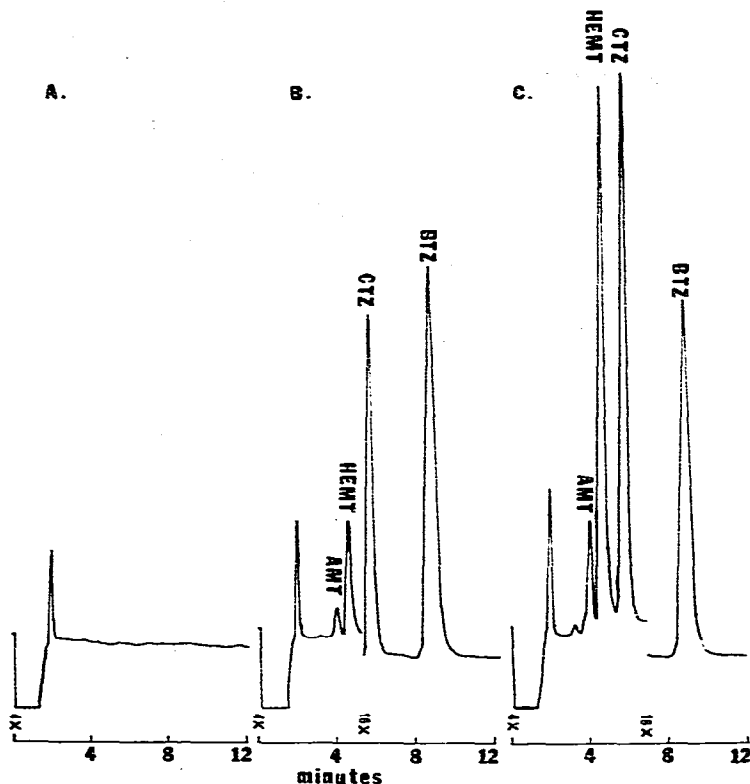
Fig. 2. Chromatograms of extracts of plasma samples (500 μl) from a pregnant patient near term. (A) Immediately prior to commencement of CTZ therapy. (B) 0.38 h after commencement of therapy (AMT, 61.4 ng/ml; HEMT, 191 ng/ml; and CTZ, 1759 ng/ml). (C) 4.92 h after commencement of therapy (AMT, 252 ng/ml; HEMT, 982 ng/ml; and CTZ, 753 ng/ml). Chromatographic conditions are given in text.

368—4600 ng of AMT and 288—3600 ng of HEMT in blood. The signal-to-noise ratio for 32 ng of CTZ was 25:1, which suggests that the method may be applicable to the analysis of considerably smaller amounts.

The major problem encountered in the development of this method was that of combining a chromatographic and detection system that provides high sensitivity and adequate separation between AMT, HEMT, CTZ and the solvent front. With conventional flame ionization detection, the solvent front corresponding to 10 μl of ether will override both the AMT and HEMT peaks and also interfere with the CTZ peak when relatively low levels of the compounds are involved. Sensitivity to low-molecular-weight substances such as CTZ, containing only six carbon atoms, is also low. The use of selective detectors can significantly reduce the interference by the solvent front. Electron-capture detection should give adequate sensitivity for CTZ and BTZ, but the metabolites, AMT and HEMT, which do not contain halogens, will not give adequate response. These problems were overcome by the use of a nitrogen—phosphorus detector (NPD). With the use of an NPD, ether only appeared as a short negative signal which did not interfere with the analysis, and high sensitivity was also achieved with the nitrogen-containing compounds involved.
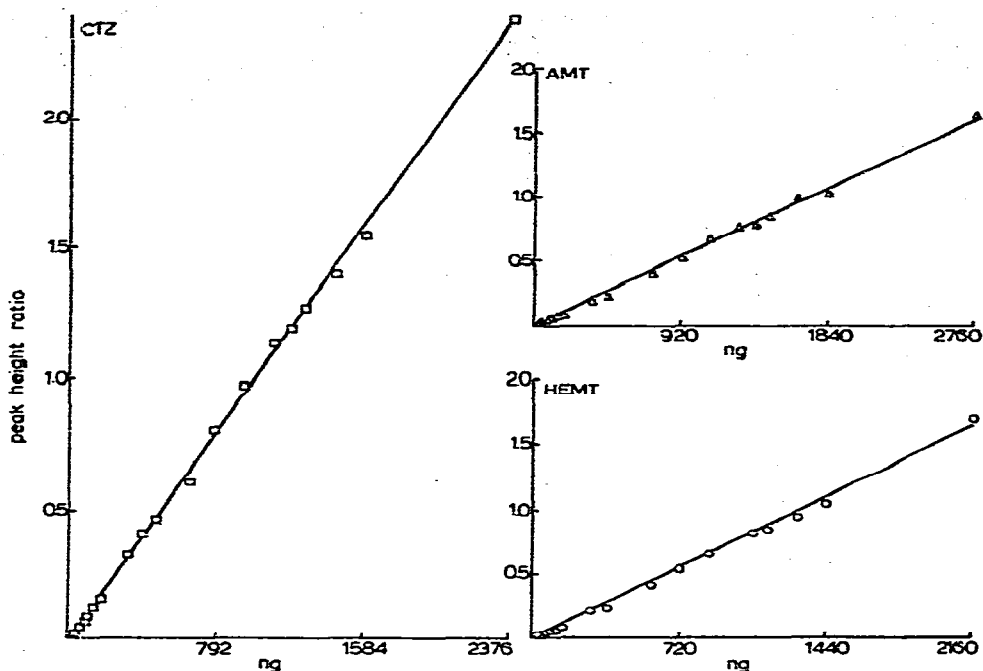
Fig. 3. Calibration data in plasma.

TABLE I

SLOPES OF THE LINEAR REGRESSION LINES OF THE PLASMA CALIBRATION
DATA FROM THREE DIFFERENT DAYS

| | Slope × 10⁴ | | |
| | CTZ | AMT | HEMT |
|---|---|---|---|
| Day 1 | 9.92 | 5.68 | 7.43 |
| Day 2 | 10.01 | 5.70 | 7.44 |
| Day 3 | 10.05 | 5.95 | 7.73 |

One disadvantage of an NPD is the relatively narrow range of linear detector response. If the final ethereal extract was concentrated to about 10 $\mu$l and the entire volume chromatographed, then the calibration data showed progressive negative deviation from linearity as the amount of the compounds was increased. Obviously, higher sensitivity can be achieved by either injecting a larger volume or concentrating the ethereal extract. For example, when the entire extract was concentrated down to 10 $\mu$l and injected, the signal-to-noise ratio was approximately 40:1 for 3.2 ng of CTZ. Conversely, quantities of the compounds beyond the upper limits of the calibration curves can be analyzed by either injecting smaller volumes or diluting the final ethereal extract. When injecting only 1% (10 $\mu$l) of the final 1-ml ethereal extract, the amounts of the compounds on column were in the linear range of detector response.

The use of various silicone phase column packings (OV-1, OV-7, OV-17, OV-25 and OV-225) was investigated. All these stationary phases gave adequate

separation of AMT, HEMT, CTZ and BTZ, with OV-7 and OV-225 giving the best peak shapes. A column packed with OV-7 was chosen since cyano-substituted silicone packings such as OV-225 are incompatible with the NPD.

The extraction of CTZ and its metabolites from plasma and blood using either hexane, ether or dichloromethane was investigated. Ether was found to be most suitable as it gave adequate and consistent extraction of all the compounds. Hexane and dichloromethane also gave adequate extraction of CTZ and BTZ. But the less polar hexane resulted in poor and non-reproducible extraction of HEMT and the same applied to extraction of AMT with the more polar dichloromethane. Also, use of dichloromethane may cause a reversible loss of detector sensitivity. The acid wash of the first ethereal extract is necessary to remove neutral compounds which interfere with the analysis. The selectivity of the extraction procedure and chromatographic system is demonstrated by the chromatograms in Fig. 2.

Reproducible quantitation of CTZ, AMT and HEMT was achieved by using BTZ, the bromo analog of CTZ, as the internal standard over the range of plasma volumes 50—500 $\mu$l and for 1 ml of blood. The ability to analyze very small volumes of plasma enables clinical monitoring and pharmacokinetic studies to be carried out in newborns as only micro volumes of blood can be sampled.

By using this method, the plasma levels of CTZ, AMT and HEMT were followed for 30 h in the perinatal period in a woman who required CTZ for sedation. The plasma concentration—time profile is presented in Fig. 4. These data
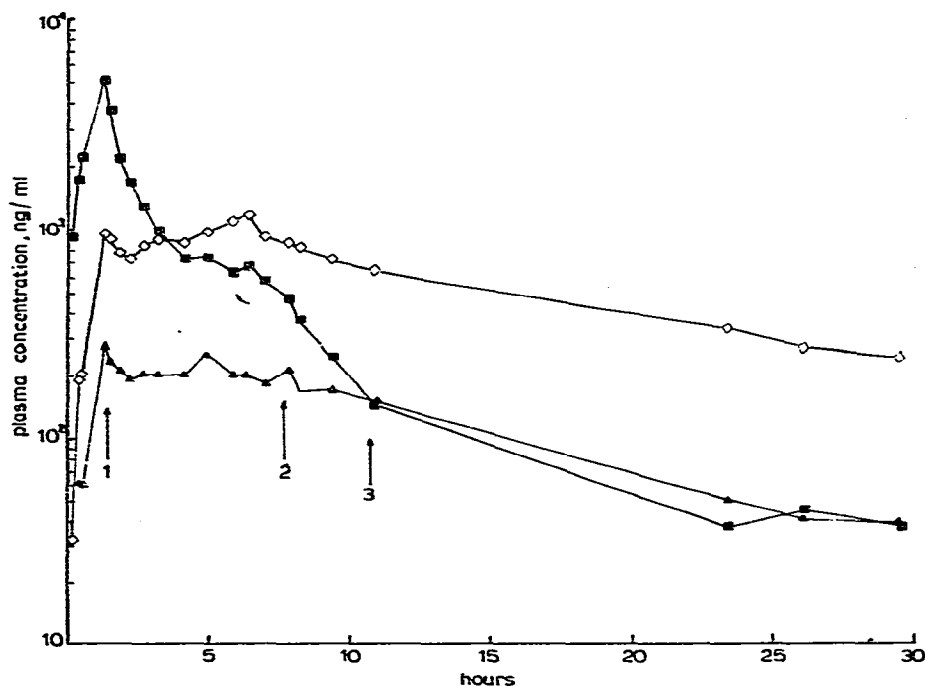


Fig. 4. Plasma concentration—time profiles of CTZ (■), AMT (▲) and HEMT (◊) in a woman in the perinatal period. CTZ was administered by intravenous infusion. Infusion commenced at a rate of 800 mg of chlormethiazole ethanedisulphonate per hour, changed to 120 mg per hour at (1) and stopped at (2). Childbirth occurred at (3).

were amenable to pharmacokinetic analysis and this will be reported elsewhere.

This chromatographic method is rapid and precise for the quantitation of CTZ, AMT and HEMT in plasma and blood. Analysis can be performed with 50—500 μl of plasma or 1 ml of blood. The sample preparation procedure is relatively simple requiring no evaporation step. The total analysis time for a single sample is less than 20 min.

REFERENCES

1  R.G. Condie and M.E. Tunstall, Acta Anaesthesiol. Scand., Suppl., 37 (1970) 192.
2  G.O. Hamann and F. Ebbesen, Ugeskr. Laeg., 135 (1973) 241.
3  G.M. Duffus, M.E. Tunstall and J. MacGillivray, J. Obstet. Gynaecol. Brit. Commonw., 76 (1969) 645.
4  T.J. Varma, J. Obstet. Gynaecol. Brit. Commonw., 79 (1972) 513.
5  E. Tischler, Aust. N.Z. J. Obstet. Gynaecol., 13 (1973) 137.
6  U.H. Lindberg, Acta Pharm. Suecica, 8 (1971) 39.
7  R.L. Nation, J. Vine, E.J. Triggs and B. Learoyd, Eur. J. Clin. Pharmacol., 12 (1977) 137.
8  K.G. Jostell, S. Agurell, L.G. Allgen, B. Kuylenstierna, J.E. Lindgren, G. Aberg and G. Osterlof, Acta Pharmacol. Toxicol., 43 (1978) 180.
9  E.P. Frisch and B. Ortengren, Acta Psychiat. Scand., Suppl., 42 (1966) 192.
10  R.G. Moore, E.J. Triggs, C.A. Shanks and J. Thomas, Eur. J. Clin. Pharmacol., 8 (1975) 353.
11  R.G. Moore, A.V. Robertson, M.P. Smyth, J. Thomas and J. Vine, Xenobiotica, 5 (1975) 687.