Journal of Chromatography, 415 (1987) 357–364
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3512

IMPROVED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC PROCEDURE FOR THE DETERMINATION OF CHLORMETHIAZOLE LEVELS FOLLOWING SOLID-PHASE EXTRACTION FROM PLASMA

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(Received October 9th, 1986)

SUMMARY

An improved high-performance liquid chromatographic method for the determination of chlormethiazole levels in plasma is described. The drug is extracted from plasma using commercially available reversed-phase extraction columns; recovery values obtained using Sep-Pak C_{18} and Bond Elut C_1, C_2, C_4, C_6, C_8, C_{18} columns are compared. Separation was achieved by reversed-phase chromatography, using a mobile phase consisting of 0.025 *M* sodium acetate buffer, pH 4.5–acetonitrile (67:33) at a flow-rate of 1.6 ml/min in conjunction with a 15-cm Jones Chromatography Apex ODS column. The analytical column was protected by a Waters Assoc. Guard-Pak module containing a Guard-Pak CN insert. Using ultraviolet detection at 254 nm chlormethiazole levels in the region of 50 ng/ml can be measured with only 500 μl of plasma.

INTRODUCTION

Chlormethiazole, 5-(2-chloroethyl)-4-methylthiazole, is a drug possessing anticonvulsant, sedative and hypnotic properties [1,2]. In addition to extensive use in the management of alcohol withdrawal symptoms [3], chlormethiazole (CMZ) is increasingly used by obstetricians as a sedative–hypnotic during labour [4] and as a sedative–anticonvulsant in the treatment of pre-eclamptic toxaemia [5,6]. The disposition of CMZ in mother and infant subsequent to its use in high-risk pregnancies continues to be investigated in this laboratory.

In an earlier publication we described a simple high-performance liquid chromatographic (HPLC) method for the determination of CMZ in plasma [7]. Alternative methods for the quantitation of CMZ in biological samples, which were reviewed in the previously mentioned paper, are exclusively gas chromatographic (GC) [8–10] or gas chromatography–mass spectrometric (GC–MS) [11,12].

0378-4347/87/$03.50

Two metabolites of CMZ, 5-acetyl-4-methylthiazole (AMT) and 5-(1-hydroxyethyl)-4-methylthiazole (HEMT), together with the parent drug, were measured simultaneously in plasma by Nation et al. [12] and Tsuei et al. [10]. However, the importance of measuring these metabolites is doubtful since, on the basis of MS evidence following GC Tsuei [13] has more recently suggested that all or part of the HEMT measured might have been formed in vitro during the extraction procedure from other oxygenated metabolites of CMZ. Furthermore, AMT and HEMT possess less than 15% of the anticonvulsant effect of the parent drug and have no sedative or hypnotic properties [14].

The HPLC method described here, therefore, has concentrated on providing a more rapid and sensitive procedure for the determination of the parent drug in plasma. The first objective has been achieved by selecting a shorter, more efficient column (15 cm Apex ODS, 5 μm) to provide rapid separation and the second by adopting solid-phase extraction in preference to the protein precipitation procedure previously employed [7].

EXPERIMENTAL

Reagents

Glacial acetic acid (Aristar quality) and sodium acetate (Analar grade) were purchased from BDH (Poole, U.K.). Acetonitrile (HPLC S grade) and methanol (HPLC grade) were obtained from Rathburn (Walkerburn, U.K.). Chlormethiazole edisylate was a gift from Astra Pharmaceuticals (St. Albans, U.K.). Nitrazepam was donated by Roche (Welwyn Garden City, U.K.). Bovine serum albumin (fraction V) was supplied by Sigma London (Poole, U.K.).

Extraction columns

A variety of commercially available solid-phase extraction columns were evaluated. Sep-Pak C_{18} cartridges supplied by Waters Assoc. (Hartford, U.K.) were used in conjunction with a Sep-Pak cartridge rack to facilitate rapid sample handling. In addition, a number of 2.8-ml capacity Bond Elut extraction columns containing a range of reversed-phase packings, including C_{18}, C_8, C_6, C_4, C_2 and C_1, were tested. These extraction columns were used in combination with a Vac Elut vacuum apparatus to provide efficient sample processing. Bond Elut extraction columns and the Vac Elut vacuum apparatus are manufactured by Analytichem International (Harbor City, CA, U.S.A.) and supplied by Jones Chromatography (Llanbradach, U.K.).

Equipment

The Waters Assoc. high-performance liquid chromatograph consisted of a Model 6000A constant-volume pump in combination with a U6K universal loop injector and a Model 440 UV detector operating at 254 nm with a sensitivity setting of 0.01 a.u.f.s. Chromatograms were obtained by connecting the detector output to a 10-mV Linseis Model LS24/80/80 two-pen recorder which was used with a chart speed of 200 mm/h.

Chromatography

A Jones Chromatography Apex ODS column (15 cm × 4.5 mm I.D., fully end-capped, 5 μm spherical octadecylsilane-bonded silica) protected by a Waters Assoc. Guard-Pak module containing a Guard-Pak CN insert, was used in combination with a mobile phase consisting of 0.025 *M* sodium acetate buffer, pH 4.5–acetonitrile (67:33). The mobile phase was prepared fresh daily and filtered through a 0.22-μm Millipore filter (Durapore Type GVWP) prior to use. Chromatography was performed at ambient temperature using a flow-rate of 1.6 ml/min which produced a back-pressure in the region of 6.80 MPa (1000 p.s.i.).

Preparation of internal standard solution

The internal standard, nitrazepam, was dissolved in ethanol to provide a stock solution containing 100 μg/ml. The working internal standard concentration of 4 μg/ml was achieved by taking a 4-ml aliquot of this stock solution and diluting with water to a final volume of 100 ml. This was incorporated into the sample as described in the extraction procedure.

Procedure

Rapid extraction of CMZ from plasma was achieved using octadecylsilane-bonded silica columns (Sep-Pak C_{18}). These were conditioned immediately prior to use by drawing 5 ml of methanol, followed by a similar volume of water, through the column under vacuum. On releasing the vacuum, 500 μl of plasma followed by 75 μl of the internal standard solution were loaded onto the Sep-Pak C_{18} column. After standing for 1 min the sample was drawn through the column by re-applying the vacuum. The vacuum was again released to allow a short equilibration period (2 min) before proceeding to the washing stage. Washing was accomplished by sucking 2.5 ml of water, followed by 2.5 ml of water–acetonitrile (75:25), through the column under vacuum. The solid-phase extraction column was then eluted with methanol. This was a two-stage process, column eluent following elution with the first 250 μl of methanol was discarded and the bulk of the drug was subsequently recovered in the column eluent obtained after eluting with a further 500 μl of methanol. This fraction was collected and, following mixing (vortex for 10 s), 50-μl aliquots were injected directly into the chromatograph.

Preparation of calibration standards

Calibration standards were prepared in plasma by making appropriate additions of aqueous solutions of chlormethiazole edisylate, containing 10 and 100 μg/ml, to provide samples with concentrations of 125, 250 and 500 ng/ml, 1, 10 and 20 μg/ml. These calibration standards were subjected to the previously described extraction procedure and injected into the chromatograph. Following analysis, graphs comparing peak-height ratio (CMZ/internal standard) with the actual concentration of chlormethiazole edisylate present were constructed.

Recovery experiment

Samples for evaluating extraction recovery were prepared in plasma and 3% bovine serum albumin (BSA) by adding aqueous CMZ to produce a final con-

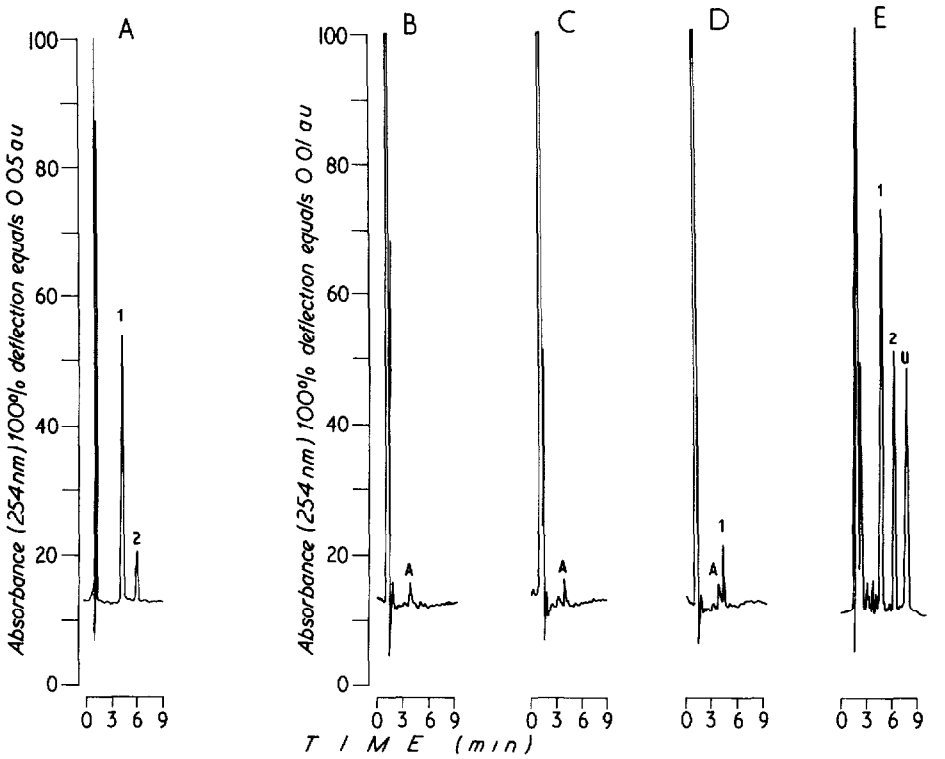

Fig. 1. (A) Chromatogram of extracted plasma supplemented with authentic chlormethiazole edisylate (10 μg/ml). The retention times for the drug and the internal standard (nitrazepam) are 4.13 and 5.93 min, respectively. (B) Chromatogram of extracted blank plasma. The small peak A with a retention time of 3.90 min appears to be an artefact. (C) Chromatogram of extracted water showing presence of artefact (peak A). (D) Chromatogram of extracted plasma which was subsequently supplemented with CMZ to illustrate baseline separation of drug and artefact (peak A). (E) Chromatogram of extracted plasma from a patient following intravenous CMZ. Peak U represents an unknown component found in some patient plasma samples. Peaks: 1 = chlormethiazole edisylate; 2 = nitrazepam, internal standard.

centration of 10 μg/ml in both cases. In addition to a comparison of Sep-Pak C_{18} cartridges with Bond Elut C_{18} columns, a variety of bonded phases (Bond Elut C_8, C_6, C_4, C_2 and C_1) were also tested. Samples extracted using Sep-Pak C_{18} cartridges were processed by the previously described method. Bond Elut extraction columns were conditioned in accordance with the manufacturer's instructions, otherwise they were treated in a similar manner to the Sep-Pak C_{18} cartridges. Following extraction, samples were injected into the chromatograph and the peak heights obtained compared with those from injections of standard solutions.

RESULTS

Fig. 1A illustrates the chromatogram obtained from extracted plasma supplemented with authentic chlormethiazole edisylate at a concentration of 10 μg/ml.

TABLE I

RETENTION TIMES (t_R) OF SOME WIDELY USED DRUGS AND MEDICATIONS

Compound	t_R (min)	Compound	t_R (min)
Hydrochlorothiazide	<2	Cortisol	2.48
Methazolamide	<2	Prednisolone	2.48
Theophylline	<2	Vitamin K_1 (Konakion)	2.63
Caffeine	<2	Prednisone	2.63
Acetylsalicylic acid	<2	Trimethoprim	2.93
Acetazolamide	<2	Carbamazepine	4.05
Benzylpenicillin	<2	Chlormethiazole	4.13
Chlorothiazide	<2	Phenytoin	4.58
Propazolamide	<2	Ethoxyzolamide	5.25
Ampicillin	<2	Nitrazepam	5.93
Cefuroxime	<2	Clonazepam	6.15
Acetaminophen	<2	Diazepam	14.4
Salicylic acid	<2	Vitamin E	N.D.*
Vitamin A	2.25	Netilmicin	N.D.*
Phenobarbitone	2.48	Valproic acid	N.D.*

*No peak detected up to 20 min.

Peaks corresponding to CMZ and the internal standard (nitrazepam) are present with retention times of 4.13 and 5.93 min, respectively. Comparison of these values with the retention times of a number of commonly prescribed drugs and preparations used in paediatric medicine listed in Table I, indicates that, with the exception of carbamazepine, these medications should not interfere with the quantitation of CMZ using this method when administered concomitantly. The chromatogram represented in Fig. 1B is that of extracted blank plasma; apart from the small peak with a retention time of 3.90 min, the blank is free from interfering components. This small peak appears to be an artefact since it is also present in chromatograms obtained when water is substituted for plasma (see Fig. 1C). However, this peak does not interfere with the determination of CMZ in plasma because baseline separation of the two peaks is achieved (see Fig. 1D). The chromatogram shown in Fig. 1E is that of an extracted plasma sample from a patient following intravenous administration of CMZ. In addition to the expected CMZ and internal standard peaks, a large number of plasma samples produced a peak with a retention time of 7.50 min. The identify of this peak is not known, but it may not be related to CMZ since it is not present in all patient samples.

The recovery of CMZ from plasma and 3% BSA using solid-phase extraction columns varied considerably with the type of cartridge and the choice of bonded phase. The results of the extraction recovery experiment, outlined in Table II, show that recovery values ranging from approximately 56 to 88% were obtained. Although Sep-Pak C_{18} cartridges provided higher recovery values than the corresponding Bond Elut C_{18} extraction columns, maximum efficiency was achieved using Bond Elut C_6 columns.

Calibration curves were obtained by comparing the peak-height ratio (CMZ/internal standard) with the actual concentration of chlormethiazole edi-

TABLE II

RECOVERIES OF CHLORMETHIAZOLE EDISYLATE AND NITRAZEPAM FROM 3% BSA AND PLASMA USING SOLID-PHASE EXTRACTION COLUMNS

C V. = coefficient of variation.

Extraction column	Carbon loading (%)	Carbon number	3% BSA				Plasma			
			CMZ		Nitrazepam		CMZ		Nitrazepam	
			Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)	Recovery (%)	C.V (%)	Recovery (%)	C.V. (%)
Sep-Pak C_{18}	10-11	18	77.46	2.66	83.48	1.93	77 38	4.70	98.04	6.18
Bond Elut C_{18}	18.0	18	56.86	2.53	82.50	7.80	60.68	2.11	81.38	2.04
Bond Elut C_8	12.5	8	66.74	5.44	85 50	6.02	64.98	5.50	89.28	5.99
Bond Elut C_6	10.0	6	85.22	4.29	96.80	3.97	87.57	8.52	102.50	6.13
Bond Elut C_4	8.5	4	77.70	3.43	90.72	3.27	79.45	4.37	94.40	6.44
Bond Elut C_2	6 0	2	74.40	4.53	89.43	4.57	71.44	7.50	94.18	3.51
Bond Elut C_1	4.5	1	71.82	6.70	92.44	1.84	74.70	5 34	88.90	5.57

sylate in supplemented plasma following extraction using Sep-Pak C_{18} cartridges. The relationship was linear over the concentration range 0–20 μg/ml with a correlation coefficient (r) of 1.000 and slope value of 0.55.

The precision of the method was established by simultaneously measuring CMZ levels in five replicate samples following extraction with Sep-Pak C_{18} cartridges. Further replicate samples were assayed at intervals for seven days. The concentration of CMZ added to these replicate samples was 10 μg/ml, and the concentration determined and coefficients of variation (C.V.) were 10.01 μg/ml and 4.70% and 10.15 μg/ml and 1.64% for intra-batch ($n=5$) and inter-batch ($n=7$) analyses, respectively.

DISCUSSION

The technique described for the determination of CMZ levels in maternal, cord and neonatal plasma is superior to our earlier method [7]. Significant improvements, both in speed of separation and sensitivity, were achieved by altering chromatographic parameters and adopting a solid-phase extraction procedure.

Selection of a shorter, more efficient column (15 cm, Jones Chromatography Apex ODS, 5-μm spherical particles) and modification of the mobile phase to 0.025 M sodium acetate, pH 4.5–acetonitrile (67:33) provided a retention time of 5.93 min for the longest retained component (internal standard) compared with 11.5 min using our earlier approach [7].

In our previous study, extraction of CMZ from plasma by conventional liquid–liquid extraction presented problems due to the volatility of the drug. Although CMZ was extracted quantitatively into chloroform, a considerable proportion was lost upon evaporation of the solvent during sample concentration. As a consequence, we adopted a protein precipitation procedure which, although quite adequate, resulted in dilution of the sample for analysis. However, solid-phase

TABLE III

LEVELS OF CHLORMETHIAZOLE EDISYLATE DETERMINED IN MATERNAL, CORD AND NEONATAL PLASMA USING HPLC

Patient	Plasma concentration chlormethiazole edisylate* (μg/ml)							$t_{1/2}$ (h)
	Maternal	Cord	Neonate					
			0.17 h	1 h	6 h	8 h	12 h	
1	4.85	3.80	—	3.70	2.85	—	1.95	11.95
2	3.35	3.95	—	2.35	1.50	—	0.90	7.97
3	7.40	9.85	—	—	3.45	—	2.25	9.75
4	4.65	5.10	—	5.05	4.35	—	2.25	9.24
5	3.60	3.10	1.80	1.70	—	0.85	—	7.14
6	2.95	1.95	—	0.85	0.35	—	—	4.00
Mean	4.47	4.63	—	2.73	2.50	—	1.84	8.34
S.D.	1.62	2.76	—	1.66	1.59	—	0.64	2.69
n	6	6	—	5	5	—	4	6

*1 g of chlormethiazole edisylate = 0.63 g chlormethiazole base.

extraction has provided a means of extracting CMZ from plasma without diluting the sample and with no requirement for solvent evaporation since the eluent from the extraction columns is suitable for direct injection into the chromatograph. A variety of extraction columns were evaluated using both plasma and 3% BSA supplemented with CMZ; the recovery values obtained from plasma and 3% BSA were comparable, ranging from 56 to 88% depending on the type of column and choice of bonded-phase. Bond Elut columns (2.8 ml capacity) containing 500 mg of sorbent were chosen in preference to the smaller capacity columns because they were considered to be more comparable with the Sep-Pak C_{18} cartridges which contain 350 mg of bonded phase. Highest recoveries were obtained with Bond Elut C_6, C_4 and Sep-Pak C_{18} cartridges which have carbon loading values in the region of 8.5–11% (see Table II). On the basis of these results, it appears that the major factor affecting recovery of both CMZ and the internal standard using solid-phase extraction columns is the percentage carbon loading of the bonded phase and that carbon number plays a secondary role. This would seem to suggest that non-hydrophobic interactions contribute significantly to the retention mechanism.

The changes in chromatographic conditions and extraction technique described have succeeded in improving sensitivity approximately four-fold, thus enabling drug levels in the region of 50 ng/ml to be measured using only 500 μl of plasma. In this study, routine analysis of patient samples was carried out exclusively using Sep-Pak C_{18} extraction columns. However, overall sensitivity of the method should be improved by using Bond Elut C_6 columns since the highest extraction efficiencies were obtained with these.

Clinical results obtained using this HPLC method are summarised in Table III. The mean maternal, cord and neonatal plasma levels are comparable with those

published earlier [7] and within the range of values obtained by others using alternative methods [5,6,9,11,13]. In all cases neonatal plasma CMZ levels followed a monoexponential decline with respect to time. The half-life ($t_{1/2}$) of the drug in neonates (mean: 8.34 h, range: 4.00–11.95 h, $n=6$) was in good agreement with $t_{1/2}$ values obtained by Moore [15] (mean: 9.19 h, range: 3.35–27.72 h, $n=8$) and by Tsuei [13] (mean: 7.88 h, range: 4.08–11.68 h, $n=8$) in similar patients.

Despite the absence of standardised doses, CMZ being administered according to individual requirements, there is good, general agreement between the clinical results obtained using the method described and those reported earlier [5,6,9,11,13,15]. Clearly, the present method provides a valid alternative to the earlier GC, GC–MS and HPLC procedures [7–12] for routine drug monitoring applications and for pharmacokinetic studies of CMZ.

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