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DETERMINATION IN PLASMA OF A NEW ANTIEPILEPTIC DRUG, *dl*-(5α,9α,11*S**)-5,6,9,10-TETRAHYDRO-N,N-DIMETHYL-5,9-METHANOBENZOCYCLOOCTEN-11-AMINE HYDROCHLORIDE, AND ITS N-DESMETHYL METABOLITE BY LIQUID–SOLID EXTRACTION AND CAPILLARY GAS CHROMATOGRAPHY

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SUMMARY

We have developed a sensitive and accurate method for the determination in plasma of the antiepileptic drug *dl*-(5α,9α,11*S**)-5,6,9,10-tetrahydro-N,N-dimethyl-5,9-methanobenzocycloocten-11-amine hydrochloride and its N-desmethyl metabolite. The extraction procedure utilizes base-treated disposable C_2 solid-phase columns, with the analyte eluted with organic solvent. Nitrogen-selective gas chromatography is used for detection. Linear regression analysis showed that the method is linear between 4 and 1500 ng/ml for the parent drug and between 8 and 3000 ng/ml for the N-desmethyl metabolite. Intra- and inter-day variability, as shown by the coefficient of variation, is less than 8% for both compounds. The method is applicable to routine plasma determination of both these compounds in clinical pharmacokinetic studies.

INTRODUCTION

The goal of the Antiepileptic Drug Development (ADD) Program of the National Institute of Neurological and Communicative Disorders and Stroke

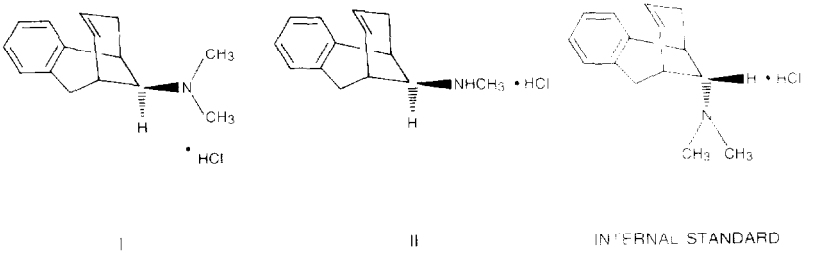

Fig. 1. Chemical structures of compound I, its N-desmethyl metabolite, compound II, and internal standard.

is to facilitate the development of more effective drugs for the treatment of epilepsy. One candidate compound now under investigation in this program is the tertiary amine *dl*-(5α,9α,11*S**)-5,6,9,10-tetrahydro-N,N-dimethyl-5,9-methanobenzocycloocten-11-amine hydrochloride (compound I, Org 6370) and its desmethyl metabolite (compound II, Org 6363), a secondary amine (Fig. 1). There is currently no published procedure for the extraction of these compounds. A preliminary report on the use of liquid–liquid extraction combined with gas chromatography (GC) indicates that compound I can be quantitated precisely and accurately by simple single-step partitioning [1], but this method presents problems. Namely, quantitation of the N-desmethyl metabolite is much less accurate and precise than with the parent compound, and the extracts tend to be quite dirty.

Our method uses a liquid–solid technique for extraction followed by GC analysis. It is convenient for extracting both tertiary and secondary amines and is less time-consuming and gives cleaner chromatograms than liquid–liquid extraction. The procedure may also find application in the extraction of other secondary and tertiary amine compounds from plasma matrices [2, 3]. Adsorptive losses of these amine compounds are a primary concern since phase 1 clinical pharmacological studies have shown that the expected plasma drug levels would be very low. In our previous experience with basic amines, they tended to disappear in the extraction at low concentrations (ng/ml). With the method described in this report, however, these amines can be quantitated routinely with good day-to-day reproducibility. This report also briefly describes the pharmacokinetic profile of compound I and its metabolite.

EXPERIMENTAL

Chemicals

The reference standards, compounds I and II, and the internal standard (Fig. 1) were supplied by Organon International (Oss, The Netherlands).

Chloroform and methanol were obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.) and used without further purification. Ethyl acetate, anhydrous, > 99% purity, Gold Label (packaged under nitrogen), was obtained from Aldrich (Milwaukee, WI, U.S.A.). Sodium hydroxide was analytical-reagent grade from J.T. Baker (Phillipsburg, NJ, U.S.A.). All water was pretreated with the Milli-Q water system (Millipore, Bedford, MA, U.S.A.).

The glassware silylating agents, Col-Treet and Glas-Treet, were obtained from Regis (Morton Grove, IL, U.S.A.).

Absolute ethanol was supplied by Aaper Alcohol and Chemical (Louisville, KY, U.S.A.).

Preparation of standards and solutions

Stock solutions of compounds I and II and the internal standard were individually prepared in ethanol (1 μg/μl). Two sets of working standards were prepared from the stock solutions. For quantitation of samples obtained after a single dose of compound I, working standards contained 0.5 ng/μl I and 0.25 ng/μl II or 5.0 ng/μl I and 2.5 ng/μl II. For the analysis of steady-state samples, in which plasma levels of the parent drug were high and levels of the metabolite were even higher, working standards contained 1.0 ng/μl I and 2.0 ng/μl II or 10.0 ng/μl I and 20.0 ng/μl II. The internal standard solutions were either 5.0 or 0.5 ng/μl depending on the standard curve generated. These solutions were stored at 4°C in PTFE-sealed amber glass vials.

The eluting solution for the solid-phase column was a mixture of methanol—chloroform (1:3, v/v). This solution must be prepared at least one day prior to use.

Sodium hydroxide solutions, 0.1 and 0.2 *M*, were prepared daily

Apparatus and instrumental parameters

A Hewlett-Packard Model 5880A gas chromatograph (Hewlett-Packard, Avondale, PA, U.S.A.) was equipped with a Model 7672A automatic sampler, splitless injector and nitrogen—phosphorus detector. High-performance, 25 m × 0.20 mm I.D., cross-linked 5% phenylmethylsilicone (0.33-μm film) fused-silica capillary columns (Hewlett-Packard) were used for the majority of the analyses. In those cases where high sensitivity and maximal resolution were required, such as with samples containing less than 5–10 ng/ml I, a Hewlett-Packard Ultra column of the same description as the high-performance column was used.

The carrier gas was helium adjusted to deliver a column flow rate of ca. 1 ml/min. Make-up gas flow-rate was set at 20 ml/min. Detector gases flow-rates were set at 4 ml/min for hydrogen and 75 ml/min for air. The detector temperature was 300°C and the injection port temperature 150°C.

For the analysis, the oven temperature was 70°C for 1 min, programmed at 20°C/min to 180°C with a 4-min final hold time. Analysis was followed by rapid temperature programming to 290°C with a 3-min posthold time for final burn-off. The splitless injector inlet purge delay time was set at 1 min

The liquid—solid extraction procedure was carried out on either 1-ml or 3-ml C_2 Bond-Elut disposable solid-phase columns (Analytichem International, Harbor City, CA, U.S.A.). Extractions from more than 0.5 ml of plasma required the larger-size 3-ml column because of column occlusion by plasma constituents.

Vacuum was applied to the C_2 columns by a Baker-10 SPE system (J.T. Baker).

Final evaporation of solvents was carried out on a Haake-Buchler (Saddle Brook, NJ, U.S.A.) evaporator under water aspiration.

Deactivation of glassware

Owing to the highly adsorptive nature of the secondary amine, some of the

glassware required pretreatment with silylating agent. The glass culture tubes used for the final evaporation were rinsed with methanol, oven-dried, treated with Glas-Treet for 10 min, rinsed again with methanol and oven-dried at 150°C overnight.

The quartz glass splitless injection liners were acid-washed, rinsed with water, then methanol and oven-dried. This was followed by Col-Treet treatment for 10 min, a hexane wash and a final drying at 150°C. After this initial silylation procedure, the liners were cleaned with a pipe cleaner and hexane after each series of analyses.

Extraction

For the general extraction procedure, 1-ml C_2 columns were prewashed with two column volumes of methanol followed by two column volumes of water. Meanwhile, 0.5 ml each of plasma, 0.2 *M* sodium hydroxide and 150 ng of internal standard were combined and the mixture was briefly vortexed. One column volume of 0.1 *M* sodium hydroxide was then passed through the column. This was followed by the plasma mixture and a final rinse of one column volume of 0.1 *M* sodium hydroxide. The alkaline washes and the plasma application were carried out by applying a slow uniform pressure to the top of the column with a disposable syringe. This was to ensure that the flows through the columns at the critical analyte adsorption step were uniform. Calibration samples consisting of blank plasma spiked with standards were also run concurrently with the unknowns. Standards were 2, 10, 25, 50, 100, 250, 500 and 750 ng per 0.5 ml plasma for compound I and 4, 20, 50, 100, 200, 500, 1000 and 1500 ng per 0.5 ml plasma for compound II, with the addition of 150 ng internal standard. The columns were then centrifuged for 10 min at 5000 *g* followed by vacuum aspiration for 40 min. The compounds were then eluted from the column with 4 × 250 µl of methanol–chloroform (1 3, v v). The 1 ml of eluent was then evaporated to dryness at 40°C. Finally, the dried residue was reconstituted in 300 µl of ethyl acetate and 1 µl was used for analysis.

RESULTS AND DISCUSSION

The extraction of both amines using liquid–solid techniques indicates that these compounds interacted with Bond-Elut C_2 columns in unexpected ways. For example, with the application of these compounds under aqueous acidic conditions, no organic solvent could elute them off the column. The secondary amine also appeared to adsorb more readily to glassware. Alkaline conditions, however, allowed column application, washing and elution.

Various alkaline buffer solutions were tried for solid-phase column pretreatment and analyte application, but were found to leave a final residue. Therefore, sodium hydroxide solution was used even though there was the possibility that it would degrade the silica-based column sorbent. In fact, however, sodium hydroxide had minimal effects on the sorbent owing perhaps to the relatively brief period that the solid phase was exposed to sodium hydroxide. It was necessary to remove any remaining aqueous solvent from the solid-phase columns because of its deleterious effect on the GC capillary columns. While

small amounts (< 50 μl) of organic solvent could be used to flush any remaining aqueous solvent from the columns [2, 3], we found that some analyte was being co-eluted. Centrifugation followed by vacuum aspiration was sufficient to remove residual aqueous solvent.

Methanol was found to elute all compounds from the C_2 column, including interfering contaminants. Chloroform gave cleaner results but would not totally elute the secondary amine. Therefore, a combination of the two solvents was used for elution. Final evaporation of the eluent was also investigated and it was determined that evaporation with a stream of nitrogen led to a large loss of both compounds. Even with vacuum aspiration in silylated tubes, the compounds tended to diffuse up the walls of the tubes.

Methanol as a reconstituting solvent solubilized all the extracted compounds, but as an injection solvent it led to inconsistent chromatography, perhaps because of methanolic hydrogen bonding to the capillary column [4]. Ethyl acetate was finally chosen as the injection solvent, since it solubilized the amines and gave consistent chromatography. It was essential to use only the highest purity grade ethyl acetate, since these amines were easily degraded by impurities in the supposedly pure grades of ethyl acetate tested. Various ethyl acetate clean-up procedures [5] failed to eliminate this problem, which was solved only after using pure ethyl acetate packaged under nitrogen.

Representative chromatograms of plasma extracts are shown in Fig. 2. The plasma samples were obtained from healthy volunteers. Standard curves used spiked plasma from volunteers given placebo; blank blood bank plasma stored in plastic packets often generated interfering peaks.

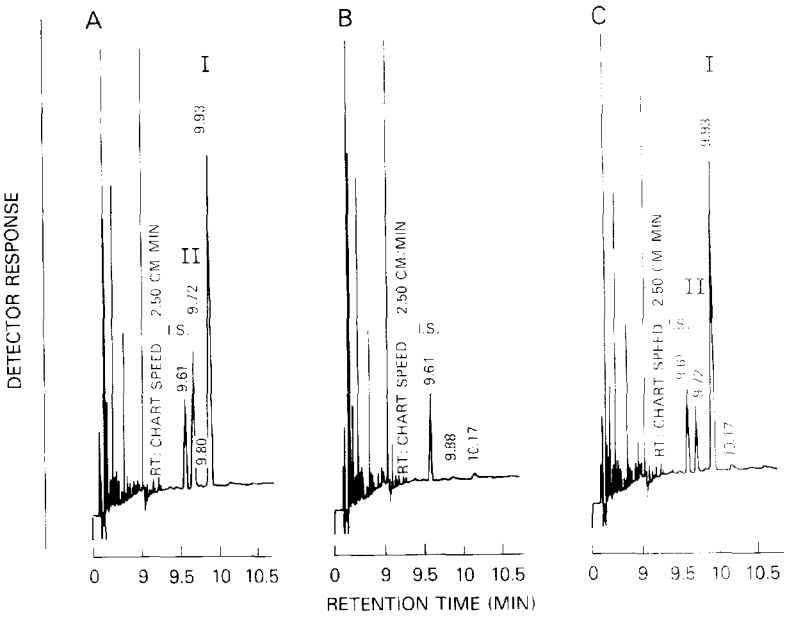

Fig. 2. Typical chromatograms of extracts from (A) plasma of placebo-treated volunteers spiked with 200 ng/ml I and 100 ng/ml II; (B) blank plasma of a healthy volunteer, and (C) plasma of the same volunteer obtained 6 h after a single 100-mg oral dose of compound I. The concentrations were 189.2 ng/ml for I and 51.8 ng/ml for II.

The coefficients of variation (%) for repeated injections of extracted samples are shown in Table I. Since GC column adsorption was a concern, all standards were injected before and after the run to ensure that the chromatographic conditions remained constant. If there was a change in peak-height ratios or in absolute height of the internal standard, it was symptomatic of a contaminated splitless liner and/or capillary front-end contamination. On rare occasions, the nitrogen–phosphorus collector also became contaminated and was washed in water, methanol and hexane, or replaced.

Results of multiple extractions of spiked blank human plasma on three separate days to check intra-day variability are shown in Table II. The values listed are twice the amount actually extracted, since 0.5 ml rather than 1 ml of plasma was used for analysis. The inter-day variability of these results is shown in Table III. The concentrations of I and II in these validation samples

TABLE I

COEFFICIENTS OF VARIATION OBTAINED FROM MULTIPLE GC INJECTIONS

Calculations are based on comparison of peak-height ratios (compound/internal standard)

Drug extracted (ng)	Drug injected (ng)	Number of injections	Coefficient of variation* (%)
Compound I			
10	0.033	12	0.00
100	0.333	13	1.85
250	0.833	10	0.89
Compound II			
20	0.067	12	3.69
200	0.667	13	2.66
500	1.667	10	1.33

*Coefficient of variation = S.D./mean × 100%.

TABLE II

INTRA-DAY ASSAY VARIABILITY

$\overline{X}$ = Mean of drug level values observed, obtained using multilevel standard calibration curves for quantitation. C.V. = Coefficient of variation.

Drug added (ng/ml)	Day 1			Day 2			Day 3		
	$\overline{X}$ ± S.D. (ng/ml)	*n*	C.V. (%)	$\overline{X}$ ± S.D. (ng/ml)	*n*	C.V. (%)	$\overline{X}$ ± S.D. (ng/ml)	*n*	C.V. (%)
Compound I									
30	30.5 ± 2.0	4	6.5	27.3 ± 1.2	4	4.5	30.7 ± 1.1	4	3.6
500	491.2 ± 13.6	4	2.8	504.2 ± 10.7	4	2.1	494.4 ± 17.2	4	3.5
1200	1265.3 ± 55.5	4	4.4	1240.7 ± 28.0	4	2.3	1169.6 ± 32.5	4	2.8
Mean C.V. (%)			4.6			3.0			3.3
Compound II									
60	67.0 ± 3.0	3	4.5	57.1 ± 1.8	4	3.2	61.5 ± 2.7	4	4.3
1000	1044.6 ± 19.0	4	1.8	974.7 ± 14.2	4	1.5	1056.2 ± 35.9	4	3.4
2400	2464.6 ± 165.7	4	6.7	2401.1 ± 96.5	4	4.0	2450.4 ± 64.3	4	2.6
Mean C.V. (%)			4.3			2.9			3.4

TABLE III

INTER-DAY ASSAY VARIABILITY

Drug added (ng/ml)	Drug level observed* (mean ± S.D.) (ng/ml)	*n*	Coefficient of variation (%)
Compound I			
30	29.4 ± 2.1	12	7.1
500	496.6 ± 14.0	12	2.8
1200	1225.2 ± 56.0	12	4.6
Compound II			
60	61.4 ± 4.7	11	7.6
1000	1025.2 ± 43.8	12	4.3
2400	2438.8 ± 109.3	12	4.5

*The mean of all individual values from three days of determinations (from Table II). Values obtained using multilevel calibration curves for quantitation.

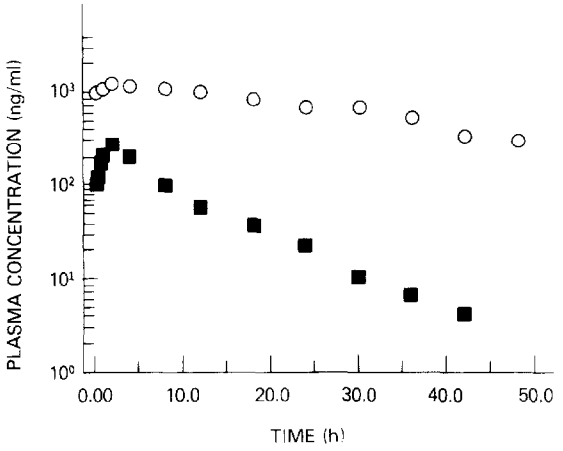

Fig. 3. Typical plasma concentration versus time curves for compounds I (■) and II (○) from a healthy volunteer showing withdrawal after receiving 100 mg of compound I four times a day for three weeks.

were chosen to fall in the low, medium and high range of the standard curves and to be representative of those expected in the volunteers taking the drug.

The following representative linear regression equations were obtained using peak-height ratios versus amount of drug (ng) added to standard-spiked blank plasma. Compound I: $y = 0.0064793x + (-0.01045)$, $r^2 = 0.9999$; compound II: $y = 0.0061931x + (-0.11571)$, $r^2 = 0.9988$. Although the correlations were generally good, multilevel point-to-point calibration was found to give better results at the lowest end of the standard curve simply because of the weighting of the high standards. Although not presented here, in cases where blood drug levels fall outside the range of standards, adjustments to the amounts of plasma used for extraction may be necessary. As long as the volume of plasma used for analysis is the same as the amount of 0.2 *M* sodium hydroxide (v/v) the standard curves will be linear. The procedure has been run

on various amounts of plasma ranging from 0.25 to 1.0 ml with comparable results.

Typical plasma decay curves from a healthy volunteer given 100 mg of compound I orally four times a day for three weeks is shown in Fig. 3. The levels of compounds I and II reached a peak of 279 and 1251 ng/ml, respectively. Apparent elimination half-lives were 7.4 and 21.5 h for each respective compound. Other work [6] indicates that plasma level determinations are possible after even a single 50-mg oral dose of compound I.

CONCLUSIONS

Disposable solid-phase columns are practical for routine analysis of nanogram quantities of both these anticonvulsant agents. The method is linear for both parent drug and metabolite throughout the dosage range evaluated and can be utilized in clinical pharmacokinetic studies of these compounds. The procedure is limited more by GC signal-to-noise than by compound loss during extraction, a primary concern. The nitrogen- phosphorus detector was highly sensitive to these amines, even in picogram quantities. Therefore, use of pure solvents and reagents and careful maintenance of the GC splitless insert, capillary column and nitrogen- phosphorus detector will allow accurate quantitation of these compounds throughout the expected range.

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