ited by such agents. In fact, the decreased emergence of resistants and lessened minimum concentrations of a drug to affect bactericidal action may indicate the advantages of such combinations.

The emergence of resistant organisms at earlier times with lower bactericidal concentrations of aminosidine (Fig. 1) implies that resistance is acquired in the presence of subbactericidal concentrations of the drug. It is most probably a property exhibited by generating organisms with aminosidine-lessened rates of generation and is consistent with the argument given (4) that altered ribosomes may be produced only by growth in the presence of drug. Aminosidine concentrations of 15 and 25 μg/ml, typical of the plasma levels achieved by the recommended therapeutic regimens (8), caused a rapid initiation of microbial death and did not permit that growth of resistants within 24 hr observed for concentrations below 7 μg/ml. This phenomenon was also observed (7) when penicillin or kanamycin was added to organisms previously treated with bacteriostatic agents, although the final killing rate was also not modified by such prior treatment.

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GLC Determination of Carbamazepine Suitable for Pharmacokinetic Studies

ANDRÉ GÉRARDIN [x], FRANÇOISE ABADIE, and JOËLLE LAFFONT

Abstract □ A GLC determination of carbamazepine in plasma and urine is described. It is performed by injecting the cyano derivative, which, in contrast to carbamazepine, has good GLC properties. The method is suitable for pharmacokinetic studies, since it is specific and accurate down to 0.27 μg/ml.

Keyphrases □ GLC—analysis, carbamazepine derivative, plasma and urine □ Anticonvulsants—carbamazepine derivative, GLC analysis, plasma and urine □ Carbamazepine—derivatization, GLC analysis, plasma and urine

Many GLC methods have been described for the determination of anticonvulsants in plasma or serum. Most of them are concerned with the monitoring of the effects of antiepileptic therapy. Some of these methods are suitable for the determination of carbamazepine[1] (I), which is injected either directly or after derivatization (1–12).

Reports on the GLC behavior of I indicate that its thermal stability is weak (6, 8, 9, 13, 14). As a result, some workers have used derivatives such as silylated I (6), dibenz[*b,f*]azepine, which is the thermal decomposition product of I (9), or its methylated derivative, obtained by flash reaction with a quaternary ammonium hydroxide (10). However, flash-reaction techniques sometimes require very precise conditions and may lead to poorly reproducible results. Moreover, these derivatives apparently cannot be obtained in a good state of purity (9). Recently, dimethylformamide dimethylacetal was reported to react with I, giving a derivative suitable for GLC (12).

These techniques generally meet clinical requirements, since the plasma concentrations in patients under continuous therapy (usually 2–10 μg/ml) do not require a high sensitivity. A method for the determination of I in plasma by mass fragmentography has been reported (14). The structure of the selected internal standard is closely related to I to compensate for its loss by thermal decomposition by an equivalent loss of the internal standard.

This paper describes a GLC method for the accurate and sensitive determination of I in plasma and urine, suitable for pharmacokinetic studies and bio-

[1] Tegretol, Geigy.

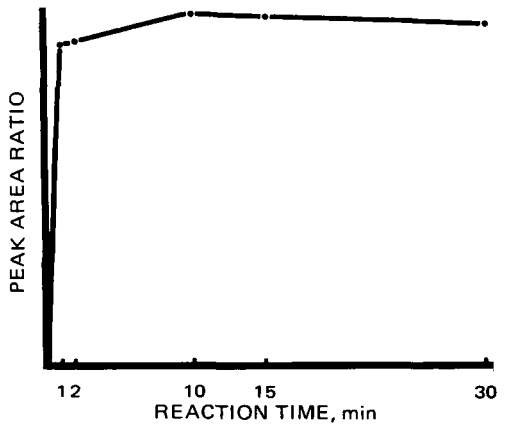


Figure 1—*Kinetics of the reaction according to Scheme I and the conditions given in* Experimental. *The internal standard used was a saturated hydrocarbon.*

availability assays. It involves the formation of 5-cyano-5*H*-dibenz[*b*,*f*]azepine, which is a stable derivative of low polarity, and does not require such expensive equipment as GLC–mass spectrometry.

EXPERIMENTAL

Reagents and Materials—The solvents[2] were analytical grade. Carbon disulfide and methylene chloride were used without further purification. All other solvents were distilled before use. Trifluoroacetic anhydride[3] and triethylamine[3] were used as 10% (v/v) solutions in methylene chloride. Fresh solutions were prepared daily. 10-Methoxycarbamazepine[4] was the internal standard. A 2.5-μg/ml stock solution in methylene chloride was prepared and stored at 4°.

Instrumentation—The GLC determinations were carried out with a gas chromatograph[5] equipped with a flame-ionization detector. The columns were 2-m long glass tubes packed with 5% cyanopropylphenylmethyl silicone[6] on hexamethyldisilazane-treated Chromosorb W (90–115 mesh). The column temperature was 270°. The retention time of the I derivative was about 5 min. Its relative retention volume by comparison with the internal standard was 0.62.

The glassware was cleaned in the usual way and immersed in an ultrasonic bath of chloroform for 15 min. The columns used in the purification step were made up from Pasteur pipets (capillary tips cut off), packed (3 cm high) with silica gel for adsorption column chromatography[7] (activity grade 2). They were immersed in a mixture of ether–pentane (1:1), and the bubbles were drawn off by suction. The prepared columns were stored at a temperature lower than 22°.

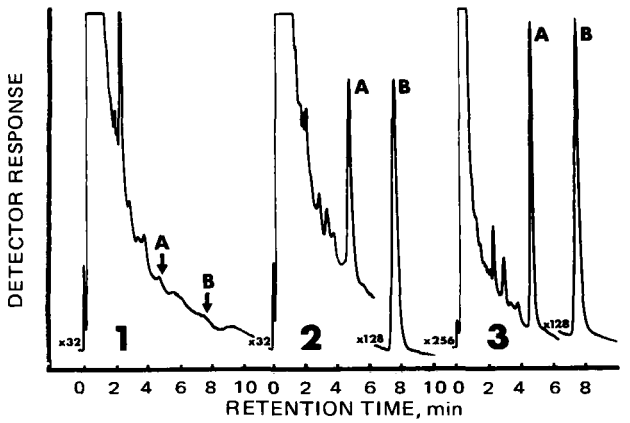


Figure 2—*Examples of chromatograms. Key: 1, plasma blank; 2, 0.2 μg of I/ml of plasma; 3, 2 μg of I/ml of plasma; A, I derivative; and B, internal standard derivative.*

[2] E. Merck AG, Darmstadt, Germany.
[3] Fluka AG, Buchs, Switzerland.
[4] Ciba-Geigy, Basle, Switzerland.
[5] Fractovap model 2400 T, Carlo Erba, Milan, Italy.
[6] Applied Science Laboratories, State College, Pa.

Table I—Recovery Experiments[a]

Added, μg/ml	*n*	Mean Recovered, μg/ml	Mean (±*SD*) Recovery, %
0.2	3	0.21	104 (±5.8)
0.5	2	0.48	96
1.2	8	1.22	102 (±4.0)
3.0	2	3.07	102
6.0	2	5.95	99
		Overall	101.3 (±4.7)

[a] The plasma samples were added to known amounts of I.

Procedure—One milliliter of plasma (or urine), 2 ml of ether, 1 ml of internal standard solution in methylene chloride, and 0.25 ml of 30% sodium hydroxide were introduced into a 10-ml ground-glass-stoppered centrifuge tube. The mixture was shaken for 5 min and centrifuged, and the organic phase was removed and evaporated under nitrogen.

The residue was dissolved in 200 μl of ether–pentane (1:1) and transferred to a small column packed with silica gel (described previously). Four milliliters of ether–pentane (1:1) was eluted and discarded, followed by 4 ml of acetone which was collected in a 10-ml glass centrifuge tube and evaporated to dryness under nitrogen.

The residue was dissolved in 100 μl of triethylamine, and 50 μl of trifluoroacetic anhydride was added. The stoppered tube was allowed to stand for 10 min and then 2 ml of hexane and 1 ml of 0.25 *M* sodium hydroxide were added. The mixture was shaken for 3 min and centrifuged. The organic phase was transferred to a conical glass tube and evaporated under nitrogen. The residue was dissolved in 100 μl of carbon disulfide, and 1–5 μl was injected into the gas chromatograph.

The concentrations were read from a calibration curve obtained from methylene chloride solutions containing known amounts of I and the same amount of internal standard as that used for the samples. These solutions were evaporated to dryness under nitrogen, and the residues were derivatized as described.

RESULTS AND DISCUSSION

Derivatization—The I derivative was synthesized (Scheme I) on a preparative scale and identified as Compound II by mass spectrometry.

Anal.—Calc. for $C_{15}H_{10}N_2$: C, 82.54; H, 4.62; N, 12.84. Found: C, 82.32; H, 4.66; N, 12.84.

The internal standard undergoes a similar reaction. A series of samples, containing known amounts of I, was prepared to determine the optimal reaction time. The results (Fig. 1) indicate that the formation of II was completed within a few minutes. The yield,

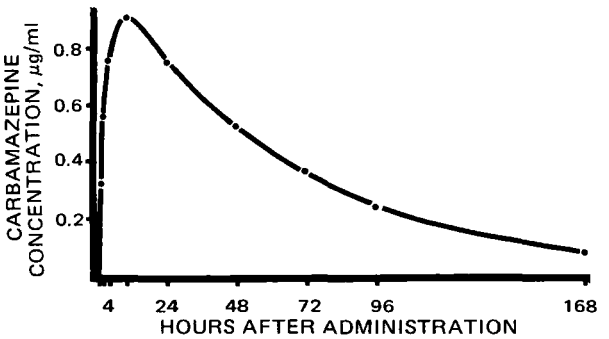


Figure 3—*Plasma concentrations of carbamazepine* versus *time in Subject 6 after the oral administration of a single 100-mg dose.*

[7] M. Woelm, Eschwega, Germany.

Table II—Cross-Check of Methods[a]

Plasma			Urine		
Hours	Isotope Dilution, μg/ml	GLC, μg/ml	Hours	Isotope Dilution, μg/ml	GLC, μg/ml
24	4.28	4.01	0–24	1.28	1.28
48	2.85	3.05	24–48	1.00	1.02
72	2.21	2.25	48–76	0.71	0.72

[a] A volunteer was given a single 400-mg dose of ^{14}C-carbamazepine orally. Samples of blood and urine were collected at the indicated times.

as measured with ^{14}C-carbamazepine, was 85% and did not depend on the amount of carbamazepine. Compound II is stable and can stand as a dry residue for hours before being injected.

GLC—The thermal decomposition of I was thoroughly checked before its derivative was investigated. Various conditions recommended in the literature were carefully reproduced. In addition to the presence of a small peak with the same retention time as dibenzazepine, it was observed that the tailing of the solvent peak was smaller when the pure solvent was injected. This result might imply a partial decomposition of I during GLC. It was confirmed by injecting a larger amount of I (1 μg), which permitted the tailing of the solvent peak to be reduced by increasing the attenuation of the signal. Under these conditions, the chromatogram showed a different baseline level before and after the peak, which is characteristic of this kind of decomposition.

When II was injected, there was no difference in the baseline levels. The peak corresponding to 5 ng was nearly symmetrical, and the response of the flame-ionization detector proved to be linear between 5 ng and 0.5 μg.

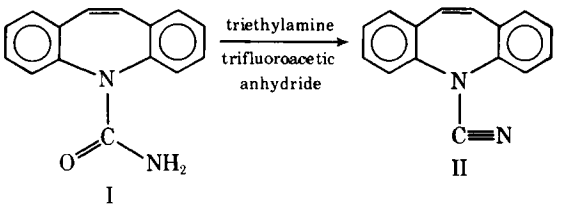
Extraction and Purification—Upon extraction of I, the yield was measured at a plasma concentration of 1 μg/ml and found to be 95%. The crude extracts must be purified to remove cholesterol and other plasma components of low polarity. The absolute yield of the purification step was 87% as measured by adding the internal standard after column chromatography and making the comparison with a pure solution of the two compounds in equal amounts. Experiments showed that the loss was exactly compensated for because the internal standard was lost to the same extent. A series of plasma samples, containing known amounts of I, was prepared to determine a calibration curve; this curve proved to be linear. Therefore, the overall yield of the procedure is not dependent on the concentration of I in the plasma.

Calculations—The calculations were made on the basis of the areas of the peaks. Their ratio remained constant whether I and the internal standard were added to plasma, urine, water, or an organic solution. Furthermore, the ratio remained the same when the internal standard was introduced into the solvent instead of the plasma. Therefore, for convenience, it was decided to use a solution of the internal standard as the extractive solvent and to make the calculations by comparison to a calibration curve obtained from organic solutions containing the same amount of internal standard and known amounts of I.

Recovery Experiments—Plasma samples containing known amounts of I were analyzed according to the described method. Examples of chromatograms are given in Fig. 2. In the blank, the small peak with nearly the same retention time as II corresponds to less than 20 ng/ml of I. The results (Table I) indicated that recovery of I from plasma is quantitative down to 0.2 μg/ml.

Specificity Experiments—A volunteer was given 400 mg of ^{14}C-carbamazepine orally. Samples of blood and urine were collected and analyzed by both inverse isotope dilution and GLC. The results (Table II) indicated a good agreement between the two methods.

The fate of carbamazepine-10,11-epoxide (the main metabolite of I in plasma) during the analytical procedure was investigated. It was shown by elemental analysis and mass spectrometry to undergo a reaction similar to I.



Scheme I—Derivatization of carbamazepine for GLC determination

Anal.—Calc. for $C_{15}H_{10}N_2O$: C, 76.91; H, 4.30; N, 11.96. Found: C, 76.75; H, 4.41; N, 11.87.

However, no peak was obtained with these GLC conditions.

Suitability for Pharmacokinetic Studies—Six healthy volunteers were given I orally in single doses. Blood was collected at different times in heparinized tubes, and plasma samples obtained by centrifugation were kept frozen until analysis. An example of the plasma concentration curves calculated from the results by a digital computer is shown in Fig. 3. The deviation of the experimental points from the calculated curve remains, in all cases, small. Each determination was made in duplicate. The results corresponding to the lowest plasma concentration were 0.084 and 0.078 μg/ml. An extensive study of the pharmacokinetic behavior of I in humans is underway and will be the subject of a future paper.

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