GLC Determination of 7-Chloro-5,11-dihydrodibenz[*b,e*][1,4]-oxazepine-5-carboxamide in Serum or Plasma

A. O. NIEDERMAYER ˣ **and R. G. KOSKI**

Abstract □ This report describes the isolation, derivative formation, GLC, and quantitation of unmetabolized 7-chloro-5,11-dihydrodibenz[*b,e*][1,4]oxazepine-5-carboxamide (I) in blood serum or plasma. Carbamazepine is used as the internal standard to compensate for losses of I during extraction and handling. Essentially complete recovery (100 ± 6%) was demonstrated over a concentration range of 1–30 μg of I/ml of serum.

Keyphrases □ 7-Chloro-5,11-dihydrodibenz[*b,e*][1,4]oxazepine-5-carboxamide—GLC, analysis in serum and plasma as 5-nitrile derivative □ GLC—analysis, 7-chloro-5,11-dihydrodibenz[*b,e*]-[1,4]oxazepine-5-carboxamide in serum and plasma as 5-nitrile derivative

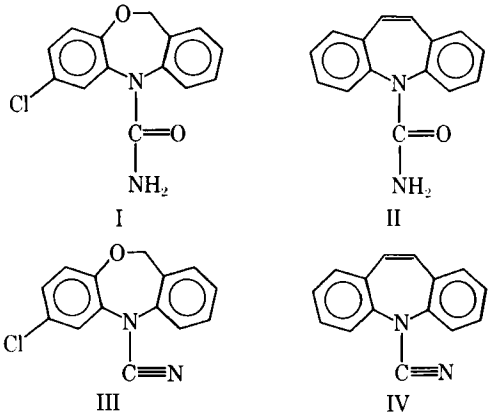
7-Chloro-5,11-dihydrodibenz[*b,e*][1,4]oxazepine-5-carboxamide (I) (1) exhibits antidepressant properties related to those of imipramine and anticonvulsant properties like those of carbamazepine (II). The pharmacological evaluation of I and a comparison of it with carbamazepine both require determination of the blood concentration of I.

The structural similarity of I and II suggested that a procedure for isolating carbamazepine from serum (2) might be useful in isolating I. It also appeared that carbamazepine might be used as an internal standard in any quantitative assay for I. Both premises proved to be correct.

The search for derivatives of I and II that are easier to chromatograph and assay than the parent compounds led to the synthesis of nitriles III and IV of I and II, respectively. These compounds were characterized by mass spectrometry, NMR spectroscopy, and IR spectrophotometry.

The flame-ionization response of the 5-nitrile derivative (III) was used successfully in the GLC determination of unmetabolized I in plasma and serum.

This methodology will prove useful in comparative bioavailability studies[1] in sheep, dogs, and humans, as well as for ascending-dose oral safety and short-term, multiple-dose tolerance studies[1] in humans.



EXPERIMENTAL

Reagents—Chloroform, methanol, *n*-hexane, and benzene, all chromatographic quality, were used without further purification. Trimethylamine[2] (0.2–0.5 *M*) in benzene was prepared by bubbling the amine through chilled benzene (dried for at least 24 hr over molecular sieve) at a slow rate until the desired concentration, as determined by perchloric acid titration, was achieved. The refrigerated reagent was found to deteriorate slowly, and its concentration was checked monthly by titration.

Ammonium hydroxide (5%) was prepared by diluting 17 ml of the concentrated reagent to 100 ml with water. Heptafluorobutyric anhydride[3], obtained in 1-ml ampuls, was stored refrigerated in a glass-stoppered tube after opening and was used for not more than 2 weeks. Compound I[4] and carbamazepine[5] were prepared as methanolic solutions with concentrations near 340 and 240 μg/ml, respectively. Phosphate buffer (pH 7.2) was prepared according to USP XVIII.

Hydrochloric acid (0.25 *N*) was prepared by diluting 21.4 ml of the concentrated reagent to 1 liter with distilled water. Tribasic potassium phosphate (0.2 *M*) was made by dissolving 4.2 g of K_3PO_4 in sufficient distilled water to yield 100 ml of solution. The 1% OV-17 on 80–100-mesh Chromosorb G (HP)[6] was used as purchased.

Apparatus—The gas chromatograph[7] was equipped with a 1.7-m (5.5-ft) × 0.3-cm (0.125-in.) o.d. glass column filled with OV-17 packing and had both flame-ionization and ^{63}Ni electron-capture detectors. A multistation evaporator[8] was used for solvent stripping during isolation and purification. The centrifuge was capable of handling 50-ml tubes and of attaining speeds of at least 1500 rpm. The typical laboratory glassware used included 2.5-, 13-, 15-, and 35-ml centrifuge tubes, Pasteur 14.6-cm (5.75-in.) disposable pipets, and 10-, 50-, and 250-μl syringes[9].

GLC Conditions—Operating temperatures for the inlet, column, and flame-ionization detector were 240, 210, and 270°, respectively. Helium at 30 psig was applied at the column head. The flow of air and hydrogen for the flame detector was optimized. The electrometer input sensitivity was adjusted to $\sim 2 \times 10^{-10}$ Å, and output attenuation was applied as necessary. The samples injected never exceeded 1 μl. Under the stated conditions, the retention times for the nitriles corresponding to carbamazepine and I were ~9 and 12 min, respectively (Fig. 1).

Newly prepared columns were conditioned for at least 16 hr under operating conditions. In this time, a stable baseline was achieved and the subsequent chromatograms appeared completely satisfactory.

The ^{63}Ni electron-capture detector was operated in dc and pulsed modes, using nitrogen and argon with 5% methane as the respective carrier gases.

Standard Preparation—Exactly 40 μl each of internal standard solution (carbamazepine) and standard solution (I) were transferred by syringe into 2.5-ml glass-stoppered centrifuge tubes. The solvent was removed by placing the tube in a 55° water bath[8] and flushing its head space with a gentle stream of nitrogen gas to remove the solvent vapor.

[1] Articles in preparation.
[2] Matheson Gas Products, East Rutherford, N.J.
[3] Pierce Chemical Co., Rockford, Ill.
[4] E. R. Squibb & Sons, Laurenceville, N.J.
[5] Geigy Pharmaceuticals, Ardsley, N.Y.
[6] Regis Chemical Co., Chicago, Ill.
[7] Model 900, Perkin-Elmer Corp., Norwalk, Conn.
[8] "N-EVAP," Organomation Associates Inc., Shrewsbury, Mass.
[9] Hamilton Co., Reno, Nev.

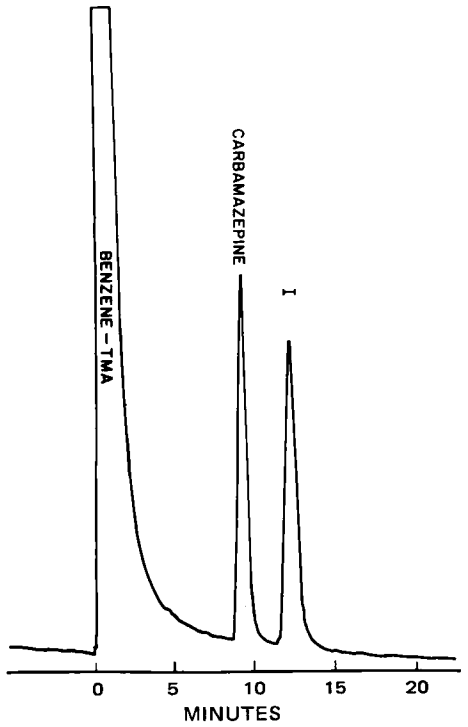


Figure 1—*Chromatogram of nitrile derivatives of I and carbamazepine.*

Sample Preparation (Extraction and Cleanup)—Exactly 5 ml of cell-free serum or plasma, obtained by standard techniques, or fraction thereof diluted to 5 ml with water was pipetted into a 35-ml centrifuge tube. Then 40 μl of internal standard solution was added by microsyringe, followed successively by 1 ml of pH 7.2 phosphate buffer and 13 ml of chloroform. The tube was shaken gently for 15 min but vigorously enough to ensure thorough mixing of the phases and was then centrifuged for 5 min at 1500 rpm. Occasionally, an emulsion formed but it was dispersed by stirring with a glass rod and recentrifugation.

The entire lower solvent phase was transferred by pipet into a 15-ml glass-stoppered centrifuge tube to effect complete removal of solvent (55° water bath and stream of nitrogen). To the resulting residue was added 3 ml of methanol, 2 ml of 0.25 *N* HCl, and 5 ml of *n*-hexane. After 5-min applications of shaking and centrifugation of the tube, the upper solvent phase was discarded. The wash cycle was repeated once with fresh hexane.

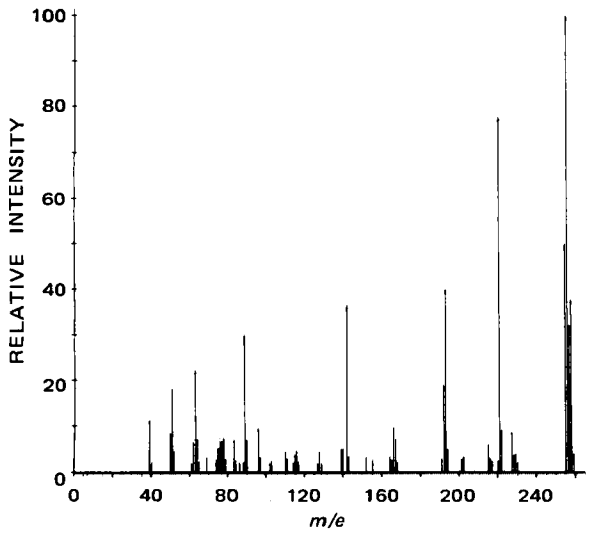


Figure 2—*Mass spectra of III.*

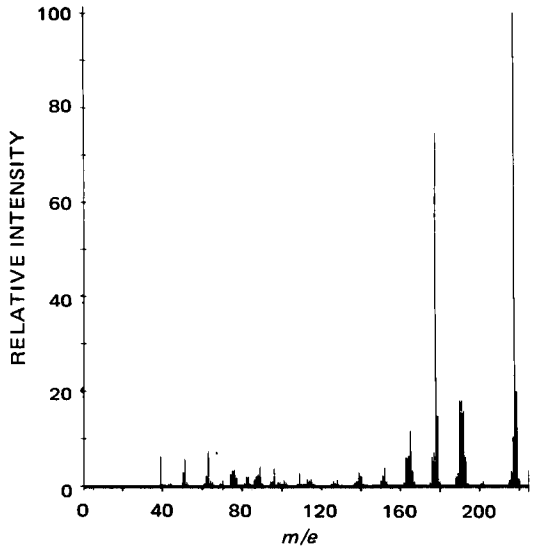


Figure 3—*Mass spectra of carbamazepine reaction product.*

The lower aqueous methanolic hydrochloric acid solution was transferred with a disposable pipet into a clean 13-ml centrifuge tube and extracted with 5 ml of chloroform for 5 min. After 3 min of centrifugation, the upper aqueous phase was discarded. The remaining chloroform solution was washed for 3 min with 5 ml of 0.2 *M* tribasic potassium phosphate and was then centrifuged. After centrifugation, the aqueous phase was discarded.

The washed chloroform extract was transferred in three aliquots into a 2.5-ml glass-stoppered centrifuge tube with intermittent solvent stripping (55° water bath and stream of nitrogen). Finally, the inside of the tube was twice alternately rinsed with a 1-ml aliquot of chloroform and restripped to concentrate the residue in the tube bottom.

Derivative Formation (Standards and Samples)—The residue of standard or sample contained in the 2.5-ml glass-stoppered centrifuge tube was dissolved in 100 μl of trimethylamine–benzene reagent. Five microliters of heptafluorobutyric anhydride was added, and the solutions were thoroughly mixed. The tube was tightly stoppered, and the solutions were allowed to react at 55° for 10 min.

After the reaction mixture had been cooled to room temperature, 0.5 ml of water was added and the two-phase system was thoroughly shaken. Following the addition of 0.5 ml of 5% NH_4OH, the tube was shaken again. After centrifugation, the lower aqueous phase was withdrawn almost completely by means of a capillary pipet; the upper solvent phase was lowered into the constricted part of the tube to facilitate sampling for chromatography.

Quantitation—After the peaks attributed to carbamazepine and I were identified by their retention times, baselines were interpolated and either peak height or area under the peak was measured. Then:

$$R_s = \frac{H_s}{H_{is}} \text{ and } R_u = \frac{H_u}{H_{is}} \qquad \text{(Eq. 1)}$$

where R_s is the ratio of standard peak height (H_s) to internal standard peak height (H_{is}), and R_u is the ratio of unknown peak height (H_u) to internal standard peak height (H_{is}). Finally:

$$\begin{matrix}\text{micrograms of I}\\ \text{per milliliter of}\\ \text{serum}\end{matrix} = \frac{R_u}{R_s} \times \frac{\text{micrograms of I in standard}}{\text{milliliters of serum taken}} \qquad \text{(Eq. 2)}$$

RESULTS AND DISCUSSION

Selection of Extraction and Cleanup Procedure—The structural similarity of carbamazepine and I made Kupferberg's extraction methodology (2) appear to be applicable to the extraction of I from serum. When ^{14}C-labeled I was added to human serum and its recovery from various stages of Kupferberg's extraction pro-

Table I—Recovery of I from Spiked Serum

I Added, μg	I Recovered, μg	Recovery, %
5	4.5	90
13.9	13.9	100
20.1	19.1	95
26.0	25.4	98
39.0	37.1	95
40.2	39.3	98
52.0	51.3	99
93.8	82.2	98
157.5	159.4	101

cedure was monitored by scintillation counting, the recovery of I was essentially quantitative.

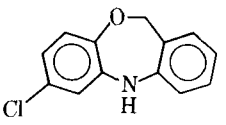
Selection of Internal Standard—The structural similarity of carbamazepine and I is the prime reason for the choice of carbamazepine as the internal standard. The comparable chemical and physical properties of the two compounds ensured similar behavior during isolation. When added to the serum early in the assay, the internal standard compensates for I losses due to partitioning and handling. As a result, the need for accuracy in measuring aliquots for chromatographic injection is reduced. The chromatographic system chosen produced an adequate separation of the pertinent components. The cleanup procedure removed all interferences.

Derivative Formation—Several chromatographic procedures were studied and rejected. Chromatography of underivatized I suffered from problems observed (3) with carbamazepine—*viz.*, absorption, decomposition, and chromatographic peaks unsuitable for quantitation. Formation of the trimethylsilyl derivative by standard techniques was rapid, but the derivatives exhibited poor stability. Furthermore, conflicting peaks were evident in serum extracts, precluding quantitation of I.

The anticipated need for high sensitivity led to consideration of electron-capture detection and the feasibility of preparing an electron-capturing derivative of I. Heptafluorobutyramides exhibit great electron affinity and have been used as amine derivatives (4). Reaction of heptafluorobutyric anhydride with amides was unreported.

The unanticipated reaction products produced by the attempted heptafluorobutyrylation of I and carbamazepine chromatographed well (Fig. 1). However, it was quickly established that they had little electron-capturing capability. Neither dc nor pulsed-mode operation of the detector yielded responses comparable in sensitivity to those of flame ionization. Mass spectrometry of the I derivative (Fig. 2) failed to reveal the expected molecular ion at *m/e* 470. This failure is consistent with the lack of electron-capturing capability of the compound. The molecular ion at *m/e* 256 is 18 mass units less than the molecular weight of I, possibly the result of dehydration. As expected for a chlorine-containing molecule, a second ion was evident at *m/e* 258 with one-third the intensity. The analogous derivative of carbamazepine revealed a molecular ion at *m/e* 218, also indicative of the corresponding 5-nitrile (Fig. 3).

Both derivatives exhibited IR absorption at 2220 cm^{-1}, indicative of a nitrile, probably the result of dehydration. NMR indicated the absence of exchangeable NH protons, along with a methylene singlet at δ 5.25 and an aromatic proton multiplet between δ 6.80 and 7.60 to support the formation of the 5-nitrile. The use of acetic anhydride as a substitute for heptafluorobutyric anhydride in the dehydration reaction failed to produce the expected products. Trifluoroacetic anhydride, however, produced the nitriles in yields comparable to those obtained with heptafluorobutyric anhydride, but the use of heptafluorobutyric anhydride was continued for consistency.



V

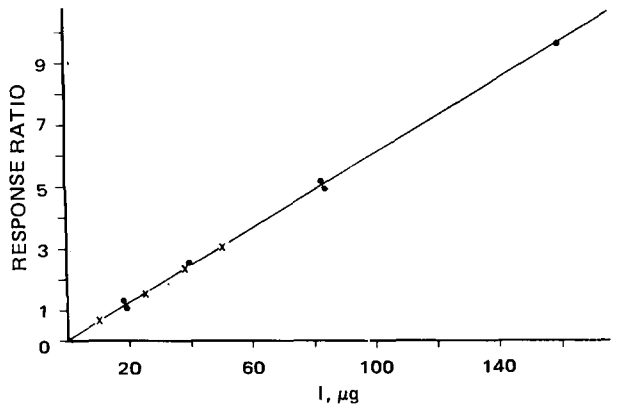


Figure 4—*Calibration curve of I.*

Linearity and Sensitivity—The linearity of the assay was first demonstrated for concentrations of I from 0 to 60 μg and then extended through 160 μg. The composite calibration curve is shown in Fig. 4. The extended linear range permits the assay of higher concentrations of I through signal attenuation rather than by rerunning a reduced size sample.

The response ratio (Fig. 4) represents the corrected I peak height response, for attenuation through ×8, related to the peak height of the internal standard. The concentrations are expressed as micrograms of I per sample (1–5 ml) of serum. The curve slope is ~16.5 (response per microgram of I), and deviation from the origin is negligible. A change in the concentration of internal standard obviously yields a different slope. In practice, the same internal standard solution is used for both standard and sample, and rigid control of its concentration is not necessary.

The limit of detection can be visualized on the calibration curve as 1–2 μg of I. When using 5 ml of serum, 0.2–0.4 μg/ml should be quantifiable. In practice, 0.2 μg/ml was readily achieved, since the sample background was negligible.

Specificity and Interference—Serum and plasma, known to be free of I or carbamazepine, were carried through the entire analytical procedure to demonstrate the absence of any normally occurring interference.

The precursor for I, 2-chloro-6,11-dihydrobenz[*b,e*][1,4]oxazepine (V), was tested for possible chromatographic interference during the quantitation of I. The nitrile derivative of I was unaffected by V. There was, however, chromatographic interference with the derivative of the internal standard, carbamazepine, since V is situated on its tail. The degree of interference was related to the condition and efficiency of the chromatographic column. The presence of V inflates the response of the carbamazepine, thus depressing the response ratio for I and leading to low biased assays. It is unlikely that significant quantities of the precursor V will ever be present during therapy, since it apparently is not a degradation product of I.

The anticoagulants potassium oxalate and edetic acid were examined for their effects on the assay of I. Both substances are commonly used to prevent coagulation of blood samples drawn by vein. At anticoagulant concentrations up to 2 mg/ml of whole blood (the concentration of anticoagulant usually employed), the recovery of I was essentially quantitative. There was no chromatographic evidence of either anticoagulant.

Recovery from Serum—The validity of any assay method for the blood level of a substance is the recovery of such material from fortified serum. This validation procedure was applied to the entire analytical range (Table I).

Stability of I in Frozen Serum—When 5-ml serum samples, containing 12.7 μg of I each, were stored frozen for 1, 3, 8, and 102 weeks, recoveries were 102, 106, 104, and 97%, respectively. Storage of serum for more than 8 weeks is not anticipated. The data for the 102-week period demonstrate that I can be recovered from serum stored that long.

Recoverability of I from Urine—The described methodology was also applied to urine spiked with I. The recovery of I from frozen urine stored for 8 weeks was 97%. No drug could be found in the urine of several subjects given I orally, suggesting that I had been excreted as a metabolite or conjugate.

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Molecular Arrangement in Monolayers Containing Cholesterol and Dipalmitoyl Lecithin

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Abstract □ The molecular arrangement of dipalmitoyl lecithin and cholesterol in mixed monolayers was investigated with the aid of a physical model. The two lipids are miscible at the surface, but there is no indication of a specific interaction. In equimolar mixed monolayers at 25 and 37°, the lipids are in tail contact. Lecithin molecules are able to remain hydrated in the mixed monolayers at high values of surface pressure.

Keyphrases □ Monolayers—cholesterol and dipalmitoyl lecithin, molecular arrangement, force–area isotherms, model □ Cholesterol–dipalmitoyl lecithin monolayers—molecular arrangement, force–area isotherms, model □ Dipalmitoyl lecithin–cholesterol monolayers—molecular arrangement, force–area isotherms, model □ Films—molecular arrangement in cholesterol–dipalmitoyl lecithin mixed monolayers, model

The notion that monomolecular films might serve as a physical model for cellular membranes was first suggested by Langmuir (1) in 1917. Like a natural membrane, the monolayer is a coherent, organized, interfacial structure. Consequently, it is a particularly useful model system for obtaining information on the orientation and arrangement of membrane components.

Special significance has been ascribed to the behavior of monolayers containing both cholesterol and a phospholipid, such as lecithin, since these materials are known to be important constituents of biological membranes. Usually, investigators have reported the mean area per molecule (at constant surface pressure) for the mixture as a function of monolayer composition (2–7). In some cases, the mean molecular area has been a linear function of composition, indicating that each component occupied essentially the same molecular area in the mixed system as in a monolayer of the pure material.

However, deviation from linearity has been observed. The mean molecular area in such systems is less than that expected on the basis of the monolayer properties of the pure materials (2–7). These deviations have been interpreted in various ways. One view is that they are evidence of a significant interaction between the two lipids as a result of van der Waals' forces, configurational entropy effects, and alteration in water structure (3), leading to the conclusion that cholesterol must strengthen or stabilize the monolayer. The implication is that cholesterol also stabilizes biological membranes.

Another explanation of the reduction in mean area per molecule was put forth by Shah and Schulman (4), who attributed the effect to the formation of cavities between phospholipid molecules as a result of thermal motion of the fatty acyl chains. Cholesterol molecules were believed to be accommodated in the cavities. These authors noted that monolayers of pure dipalmitoyl lecithin at a surface pressure of 35 dynes/cm underwent a transition to the gel state and that compression to 40 dynes/cm resulted in the formation of a two-dimensional solid. The transitions were not observed in mixed monolayers containing cholesterol, which remained fluid at all values of surface pressure. It was concluded that cholesterol decreases monolayer cohesion and, by inference, that it acts as a "biological plasticizer" in natural membranes (4).

Gershfeld and Pagano (8) reviewed these theories and, based on the results of adsorption experiments, concluded that dipalmitoyl lecithin is immiscible with cholesterol at the surface and that a rational interpretation of the mixed monolayer experiments is not possible.

A physical model (the "heads-or-tails" model), which should help clarify the role of cholesterol, was recently suggested (9). It is based on the assumptions that lipids with long hydrocarbon chains are essentially vertically oriented and that the surface area of each molecule in a coherent monolayer conforms either to the area of the hydrocarbon tails (tail contact) or the hydrated polar head (head contact). According to this model, phospholipids in tail contact (*i.e.*, in a condensed surface state) do not exhibit a reduction in the mean molecular area in the presence of cholesterol, a result in accord with published findings (4, 5). But if the phospholipid molecules are extensively hydrated, cholesterol molecules may find free space between the hydrocarbon tails and thus require little