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Electron-Capture Detector GLC Technique for Estimating Tocainide in Biological Fluids

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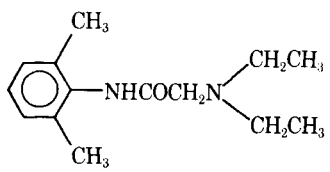
Received September 20, 1976, from the *Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, British Columbia, Canada.* Accepted for publication May 2, 1977.

Abstract □ A sensitive and specific electron-capture detector GLC method capable of detecting picogram quantities of tocainide, a lidocaine analog, in biological fluids was developed. This method consists of extracting the compound into methylene chloride and derivatizing with heptafluorobutyric anhydride to form the monoheptafluorobutyryl derivative. The derivative formation was confirmed by GLC–mass spectrometry. Quantitative estimation was performed using 1-bromonaphthalene as an internal standard. The minimum detectable level by the electron-capture method was approximately 30 pg/injection as opposed to approximately 3 ng/injection with a flame-ionization detector. Linear response was observed in the range from 50 pg to 3 ng using an electron-capture detector. No interference from endogenous substances was observed.

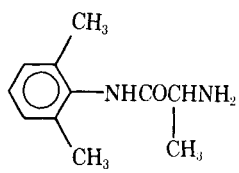
Keyphrases □ Tocainide—electron-capture GLC analysis in biological fluids □ GLC, electron capture—analysis, tocainide in biological fluids □ Cardiac depressants—tocainide, electron-capture GLC analysis in biological fluids

Lidocaine (I), a commonly used drug in acute coronary care for the treatment of acute myocardial infarction (1), has a prompt onset of action and elicits minimal hemodynamic disturbances (2). In spite of these advantages over other commonly used antiarrhythmic agents such as propranolol, procainamide, and quinidine (3), lidocaine is not an ideal antiarrhythmic agent. Its undesirable features include a short biological half-life ($t_{1/2}$ = 90 min) (4), inactivation by first-pass metabolism (5), and formation of toxic metabolites (6).

Tocainide[1,2], 2-amino-*N*-(2,6-dimethylphenyl)propanamide (II) hydrochloride, a primary amine analog of lidocaine, has antiarrhythmic activity without the disadvantages of lidocaine therapy. Tocainide exhibits a biological half-life of approximately 11 hr in normal healthy volunteers (7), oral effectiveness (8), and total availability after oral administration (9).



I



II

A flame-ionization detector GLC assay method was developed for the separation and quantitation of this compound in biological fluids[3]. Flame-ionization detectors are inherently less sensitive than electron-capture or nitrogen-specific detectors and, as a consequence, relatively large volumes of biological samples (1–5 ml of blood) are required for quantitation of compounds. Unfortunately, the sample volume required for the flame-ionization detector method is greater than what can be routinely obtained from small animals when serial samples are taken.

The limitations of the flame-ionization detector assay technique necessitated the development of a more sensitive assay method for the quantitative analysis of II. It was desirable to convert II to a suitable derivative that is highly electronegative and amenable to electron-capture detection. Several derivatizing agents have been used in the quantitative analysis of primary amines (10). In the present study, heptafluorobutyric anhydride satisfied the requirements.

EXPERIMENTAL

Materials—The hydrochloride salt of II was used. The solvents, methylene chloride[4], hexane[4], and benzene[4], were either pesticide grade[5] or distilled in glass.

[1] Astra Pharmaceutical Products, Framingham, Mass.

[2] Previous publications referred to this compound as W36095-HCL.

[3] Dr. David Lalka, Astra Pharmaceutical Products, Framingham, Mass., personal communication.

[4] Caledon, Georgetown, Ontario, Canada.

[5] Nanograde, Mallinckrodt Chemical Works, St. Louis, Mo.

Table I—Recovery of II in Rat Plasma as Determined by the Electron-Capture Detector GLC Analysis

Amount of II Added to 0.1 ml of Plasma, ng	*n*	Amount of II Recovered after Extraction, ng	Mean Recovery, %	*SD* of Percent Recovery
0.1751	1	0.1262	72.1	—
0.3501	6	0.2237	63.90	10.596
0.7002	5	0.4607	65.79	4.984
1.0503	6	0.7333	69.82	7.794
1.4004	6	0.9118	65.11	2.400
1.7505	3	1.0181	58.16	0.675
2.1006	2	1.3299	63.31	2.200
2.4507	6	1.8767	76.58	1.855
2.8008	4	1.9303	68.92	0.728
Mean recovery			67.08	5.43

Heptafluorobutyric anhydride[6] was stored in tightly stoppered bottles at 5° in a refrigerator. Once an ampul of heptafluorobutyric anhydride was opened, the contents were used within 3 days or discarded.

The internal standard solution consisted of 1-bromonaphthalene[7] in benzene when the electron-capture detector was used and an aqueous solution of glycinexylidide[1] when the flame-ionization detector was used.

Sodium hydroxide[8] (1 *N*) was used to alkalinize the solution prior to extraction.

GLC—All routine analyses were conducted using a reporting gas chromatograph equipped with a ^{63}Ni-electron-capture detector[9] or dual flame-ionization detectors[10]. The glass column, 1.8 m × 2 mm i.d., contained 3% OV-17 coated on 80–100-mesh Chromosorb W (HP).

All columns were conditioned for 24 hr at 100°, and then the oven temperature was programmed to increase by 0.1°/min up to 280°. The oven temperature was allowed to remain at 280° for approximately 24 hr to complete the conditioning process. The flow of carrier gas during the conditioning period was 7 ml/min.

The electron-capture detector method was developed using a 95% argon–5% methane carrier gas mixture; helium was used as the carrier gas for the flame-ionization detectors. The gas flow rates for the flame-ionization detector were: carrier gas, 40 ml/min; hydrogen, 40 ml/min; and air, ~300 ml/min. For the electron-capture detector, the carrier gas flow rate was 40 ml/min.

The operating conditions with both procedures were: injection port temperature, 200°; oven, 180°; and detector, 300°.

Mass Spectrometry—A GLC–mass spectrometer[11] (electron impact) was used under the following conditions: energy of the ionization beam, 70 ev; electron multiplier voltage, 2 kv; analyzer temperature, 75°; and separator oven temperature, 180°. Sample introduction was carried out by a GLC procedure on a glass column, 1.8 m × 2 mm i.d., packed with 3% OV-17 on 80–100-mesh Chromosorb W (HP) under the following conditions: injection temperature, 180°; oven temperature, 160°; and helium (carrier gas) flow rate, 25 ml/min.

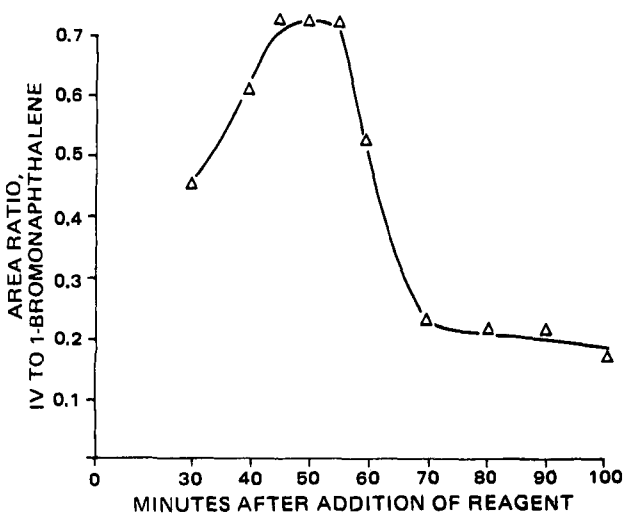


Figure 1—*Reaction kinetics of II at 55°. Plot of area ratio of the derivative formed (IV) to 1-bromonaphthalene as a function of time using 2.53 ng of II and 1.83 ng of 1-bromonaphthalene.*

[6] Pierce Chemical Co., Rockford, Ill.
[7] ICN Pharmaceuticals Inc., Plainview, N.Y.
[8] Mallinckrodt Chemical Works, St. Louis, Mo.
[9] Hewlett-Packard model 5833A reporting gas chromatograph.
[10] Hewlett-Packard model 5830A reporting gas chromatograph.
[11] Finnigan 9500 automated GC/MS, Finnigan, Palo Alto, Calif.

Table II—Plasma Levels of II Using 0.05 ml of Plasma in a Rat that Received 20 mg/kg iv

Minutes	Plasma Level, μg/ml
30	2.697
60	1.831
90	1.664
150	1.528
180	1.097

Extraction Procedure—To 0.1 ml of plasma or aqueous solution containing the drug, 1 ml of 1 *N* sodium hydroxide was added. The total volume was then made up to 2 ml with distilled water. A 10-ml volume of methylene chloride was added, and the tubes were shaken in a wrist-action shaker[12] for 15 min. After centrifugation to separate the layers, 7 ml of organic phase was transferred to a 15-ml centrifuge tube. The organic layer was evaporated to dryness under a low stream of nitrogen.

The residue was dissolved in 1 ml of hexane, and 10 μl of the derivatizing agent, heptafluorobutyric anhydride, was added. Then the tube was shaken thoroughly in a vortex mixer. The mixture was then incubated at 55° for 50 min in a constant-temperature oven. After completion of the reaction, the tube was cooled to room temperature and the solution was evaporated to dryness under a low stream of nitrogen.

The residue was dissolved in 100–200 μl of benzene containing the internal standard (0.63 ng/μl). A 3-μl aliquot of the sample was then injected into the gas–liquid chromatograph, and the response was noted.

Recovery Studies—A known amount of II was added to 0.05 or 0.1 ml of rat plasma and extracted by the described procedure. The base was obtained from the hydrochloride salt by extraction with benzene after the addition of sodium hydroxide. The solvent was then carefully evaporated, and the base remained as a solidifying oil. The base was dried in a vacuum oven at 30° for a minimum of 24 hr before use.

The purity of the base so obtained was determined by differential scanning calorimetry[13] and by a melting-point determination using a hot-stage microscope. Percentage recoveries were calculated from the standard curve of the base, which was derivatized without any extraction. The recovery values are given in Table I.

Biological Measurements of II in Plasma—The application of the technique developed for the determination of *in vivo* levels of II was demonstrated by studying the elimination of the compound in the rat. A freshly prepared solution of II was injected into a male Wistar rat (300 g) through the tail artery as a single bolus of 20 mg/kg.

Blood samples were taken at regular intervals of time through a jugular vein cannula, and the plasma obtained was used for the analysis of II. Representative plasma level data obtained are listed in Table II.

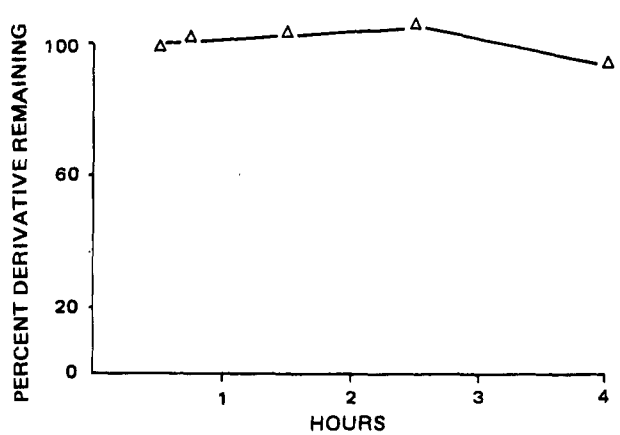


Figure 2—*Stability of IV at room temperature.*

[12] Burrell Corp., Pittsburgh, Pa.
[13] Model 1B differential scanning calorimeter, Perkin-Elmer, Norwalk, Conn.

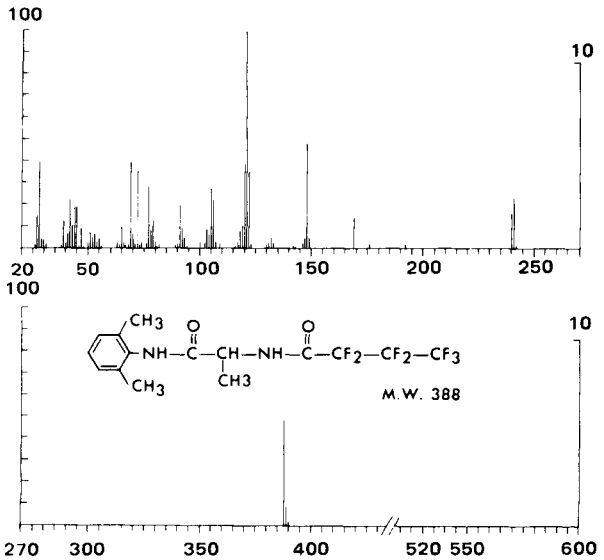


Figure 3—*Mass spectra of IV obtained by GLC–mass spectrometry after extraction from plasma of rat dosed with 20 mg of II/kg.*

RESULTS AND DISCUSSION

Selection of Derivatizing Agent and Solvent—Heptafluorobutyric imidazole[6], heptafluorobutyric anhydride, and pentafluorobenzoyl chloride[6] were examined as potential derivatizing agents. With pentafluorobenzoyl chloride, a low area ratio was obtained in the absence of a catalyst. Even though the addition of a catalyst (pyridine) did improve the yield of the derivative, it led to a number of background peaks in the chromatogram.

With heptafluorobutyric imidazole, precipitation of the reaction product occurred when hexane was used as the solvent; when methylene chloride was used, interfering peaks appeared preventing quantitation. Heptafluorobutyric anhydride was the most suitable and was used in later studies.

Careful selection of the solvent for the reaction is important for obtaining the optimum yield of the derivative. The choice, however, is limited to solvents that are inert to derivatization and that elute rapidly from the column. With a fixed concentration of II, various solvents were investigated for their suitability. The use of hexane resulted in a quantitatively higher area ratio of the derivative to the internal standard than did benzene or methylene chloride. Therefore, hexane was used as the solvent for the derivatization reaction.

Evaluation of Optimum Conditions—Both the temperature and time of the reaction are important in derivative formation. While the temperature was kept constant at 55°, the time for the reaction was varied from 0 to 100 min and the yield of the derivative, as observed by the area ratio of the derivative to the internal standard, was monitored. From Fig. 1, it is clear that the optimum yield was obtained around 45–55 min; 50 min was taken to be the optimum time in later studies.

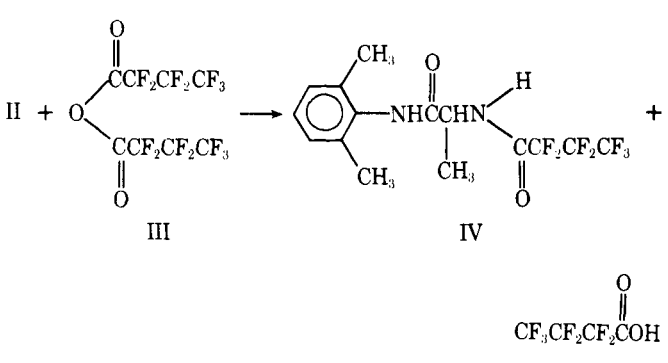
Figure 2 shows that the derivative formed was stable over 4 hr at 21.1°. In subsequent studies, the sample was injected into the gas chromatograph 30 min after the completion of the reaction time.

Under suboptimal conditions, the yield of the derivative was low and the peak obtained was not sharp and symmetrical, indicating incomplete derivative formation.

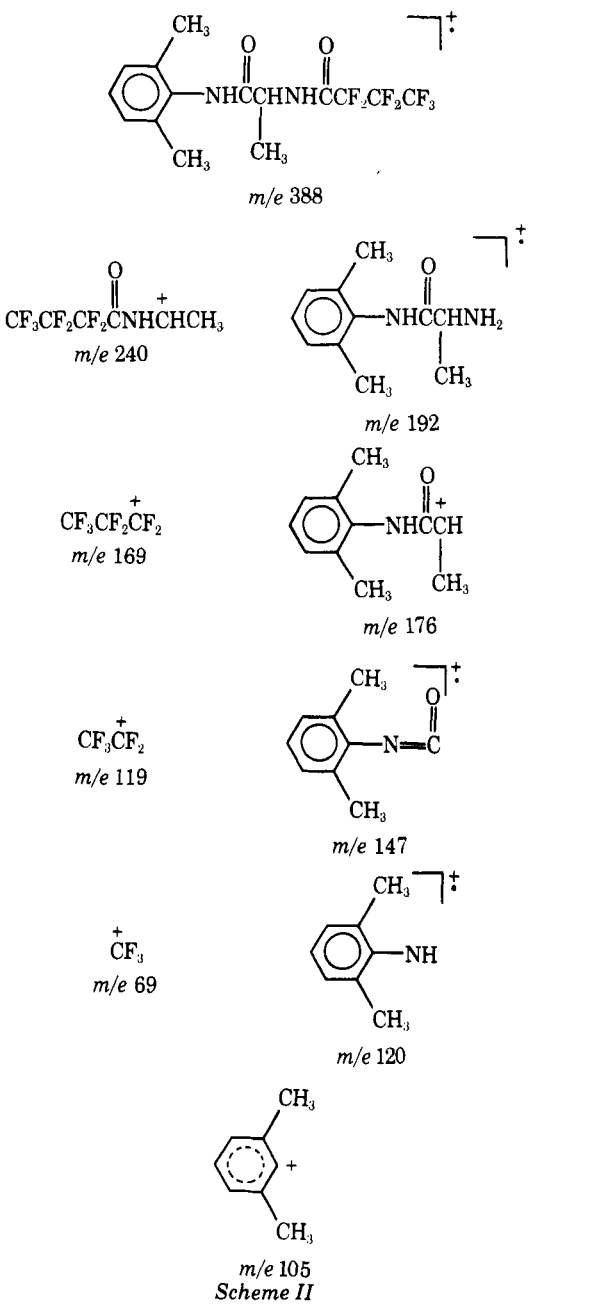
Confirmation of Derivative Structure—Plasma samples from rats administered tocainide were pooled, extracted, and analyzed by GLC–mass spectrometry after derivatization. Mass spectra corresponding to tocainide were compared to spectra obtained from the analysis of pure derivatized compound and were identical. As shown in Scheme I, heptafluorobutyric anhydride (III) reacts with II to form a monoheptafluorobutyryl derivative (IV) and heptafluorobutyric acid (V).

The postulated fragmentation pattern is shown in Scheme II and was consistent with the formation of IV. The peak at *m/e* 388 corresponds to the molecular ion peak; other abundant ions were observed at *m/e* 240, 192, 176, 169, 147, 120, 119, 105, and 69 (Fig. 3). These observations confirm that intact II, devoid of any plasma interference, was measured in the assay.

Removal of Excess Reagent—The presence of even trace amounts of the strongly electron-withdrawing heptafluorobutyric anhydride in the sample gave rise to severe disturbances in the chromatogram. Therefore, it was essential to remove the excess reagent as well as the by-product of the reaction, heptafluorobutyric acid. Walle and Ehrsson (11) suggested a method of water addition to hydrolyze the anhydride, followed by the addition of ammonium hydroxide to remove the acid into



Scheme I



Scheme II

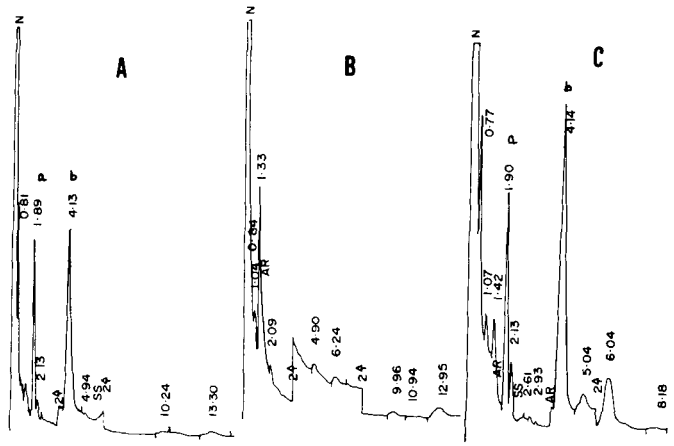


Figure 4—*Gas–liquid chromatograms of extracts by using an electron-capture detector. Key: A, extraction from an aqueous solution; B, blank plasma extract; C, extraction from rat plasma spiked with II; a, 1-bromonaphthalene; and b, derivative of tocainide. All other peaks present in the chromatograms were due to endogenous substances present in plasma or to solvents used for the analysis.*

the aqueous layer. However, after such a treatment, the derivative deteriorated rapidly. Evaporation of the mixture to remove the excess reagent resulted in a derivative that was stable over 4 hr, so this latter method was employed in all other studies.

Figure 4A shows a typical chromatogram obtained by extraction of an aqueous solution of tocainide. The chromatogram obtained after extraction of blank plasma is shown in Fig. 4B. The extraneous peaks at about 10 and 13 min in Figs. 4A and 4B resulted from concentration of the solvent used for extraction and were not readily removed by normal purification procedures. These peaks, as well as the peak at 1.3 min in Fig. 4B, clearly do not interfere with the analysis of II and the internal standard.

Figure 4C shows a chromatogram obtained when the extraction procedure described under *Experimental* was applied to rat plasma spiked with II. This chromatogram was identical to that observed after extraction of rat plasma in animals receiving II by intravenous administration. The peak at 4.14 min corresponds to the monoheptafluorobutyryl derivative of II, and that at 1.90 min is consistent with the internal standard, 1-bromonaphthalene.

A typical chromatogram as obtained from the flame-ionization detector

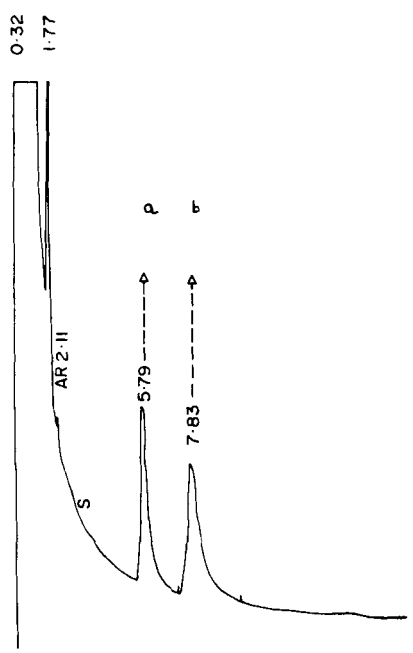


Figure 5—*Typical chromatogram of II (74 ng) and the internal standard (glycinexylidide) (80 ng) using the flame-ionization detector (attenuation 4).*

Table III—Estimation of II Added to Plasma by Flame-Ionization Detector GLC Analysis

Amount Added, ng	Mean Area Ratio[a]	*SE*
2.637	0.048	0.0133
5.273	0.098	0.0046
10.546	0.216	0.0029
15.313	0.313	0.0067
21.092	0.437	0.0031
52.731	1.188	0.0518
105.462	2.250	0.0187
158.193	3.850	0.0980
210.924	4.960	0.2571
527.343	11.969	0.1577
791.015	15.413	0.0824
1054.812	19.484	0.3786

[a] Mean of three values; $y = mx + c$, where $m = 0.229$ and $c = -0.0056$.

Table IV—Estimation of II Added to Plasma by Electron-Capture Detector GLC Analysis

Amount Added, ng	*n*	Mean Area Ratio[a]	*SE*
0.1751	1	0.0336	—
0.3501	6	0.0720	0.0020
0.7002	5	0.1611	0.0024
1.0503	6	0.2633	0.0049
1.4004	6	0.3316	0.0020
1.7505	3	0.3731	0.0015
2.1006	2	0.4899	0.0085
2.4507	6	0.6938	0.0028
2.8008	4	0.7155	0.0019

[a] With $y = mx + c$, where $m = 0.264$ and $c = -0.027$.

is shown in Fig. 5. The peak at 5.79 min corresponds to II, and that at 7.83 min corresponds to the internal standard, glycinexylidide. The area ratio of the derivative of II and the internal standard was used as the index of detector performance and the overall efficiency of the analytical procedure developed. The total recovery of II by the extraction procedure followed was 67.08 ± 5.43% (Table I).

Comparison of Electron-Capture and Flame-Ionization Detectors—Meaningful pharmacokinetic analysis necessitates frequent and extensive blood sampling to characterize drug disposition adequately. The precision of the GLC assay for II using a flame-ionization detector is illustrated in Table III. The results are based on at least three determinations at each II concentration, ranging from 0.35 to 82 μg/ml. The calibration curve obtained by plotting the area ratio of II to the internal standard was linear over a range of 2.6–600 ng (corresponding to 0.35–82 μg/ml). When the equation $y = mx + c$ was used for curve fitting, the slope value obtained was 0.0229 ($r^2 = 0.999$) while the intercept was $c = -0.0056$. This analysis, however, required 1–2 ml of plasma (approximately 2–4 ml of blood). In spite of this extensive linear range, this method could not be applied to animal experimentation using the rat because of the limitation in the frequency and volume of blood samples required.

In contrast, the electron-capture detector method permits detection of picogram levels of II, thereby permitting the use of substantially smaller sample volumes and more frequent blood collection. The reproducibility of the electron-capture assay technique is demonstrated in Table IV. The observed results are based on four determinations at each concentration of II, ranging from 0.39 to 4.0 μg/ml of plasma. All analyses by the electron-capture detector required only 50–100 μl of rat plasma to quantitate II adequately. The regression line obtained after electron-capture detector GLC analysis of II was $y = mx + c$, where m was 0.264 and $c = -0.027$ ($r^2 = 0.988$).

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Bilirubin Dynamics in the Gunn Rat during Phototherapy

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Abstract □ Bilirubin dynamics were studied in homozygous Gunn rats under normal room lighting conditions and under conditions simulating phototherapy. A kinetic model was developed for the formation, distribution, and elimination of bilirubin. The decrease in plasma bilirubin concentration during illumination with low intensity [300 footcandles (fc)] and high intensity (1000–1100 fc) light was studied. The plasma bilirubin concentration in the rats decreased under phototherapy until a new steady-state concentration was reached, the decline being more rapid under high intensity light conditions. Gunn rats were also injected with a tracer dose of ^{14}C-bilirubin following a period during which the rats were illuminated with low or high intensity light. The distribution and elimination of the labeled bilirubin were followed under continuous illumination. The chosen kinetic model, when adapted to the set of data under investigation, fit all of the data concerning bilirubin kinetics in Gunn rats under continuous illumination.

Keyphrases □ Bilirubin—kinetics of formation, distribution, and elimination in rats, effect of phototherapy □ Kinetics—bilirubin formation, distribution, and elimination in rats, effect of phototherapy □ Phototherapy—effect on kinetics of bilirubin formation, distribution, and elimination, rats

Phototherapy was first described by Cremer *et al.* (1) and has since become a popular procedure for the treatment of neonatal hyperbilirubinemia. The efficacy of phototherapy is well established (2–7), but reservations have been expressed regarding the exposure of infants to blue or visible light intensities of 300–500 footcandles (fc), often continuously for as long as 3–5 days (8–14). It has been suggested that the generalized acceptance of this mode of therapy for neonatal hyperbilirubinemia has occurred without standardization of technique or individualization of application (15). Considerations of effects of variables such as light intensity and duration of exposure have been based primarily on clinical response following empirical selection of such parameters.

The present study was designed to investigate bilirubin kinetics in Gunn rats under normal room lighting conditions and under light exposure simulating phototherapy, with the objective of developing a kinetic model to predict the relative effectiveness of various phototherapy procedures. Gunn rats lack glucuronyltransferase activity for bilirubin conjugation and thus cannot excrete the pigment normally (16). Since Gunn rats show a markedly increased plasma bilirubin level, they are frequently used as a model in bilirubin studies.

Bilirubin kinetics previously were studied in humans (17–23) and in Gunn rats (17, 24–26). The effect of light on the catabolism of labeled bilirubin was studied in Gunn rats (27) and in infants with severe unconjugated hyperbilirubinemia (28), but in no report to date has a kinetic model been described that explains the change in the plasma bilirubin concentration due to phototherapy.

The approach used in the current study was based on the determination of the plasma level–time course of an intravenously injected tracer dose of ^{14}C-bilirubin in Gunn rats maintained under conditions of normal room lighting and under various conditions of illumination simulating phototherapy and on the determination of the rate at which the bilirubin plasma pool approaches a new steady-state level when Gunn rats are exposed to varying conditions of illumination.

EXPERIMENTAL

Reagents—Bilirubin[1] and riboflavin 5′-phosphate monosodium salt[2] were used as received from the supplier. All other solvents and reagents were analytical reagent grade.

Preparation of ^{14}C-Bilirubin Injections—This preparation was carried out in subdued light, as were all assays. Crystalline ^{14}C-bilirubin was prepared by a modification of the biosynthetic method of Barrett *et al.* (29), using Sprague–Dawley rats instead of dogs, and purified by the method of Ostrow *et al.* (30). After recrystallization (twice) from methanol, an aliquot was examined for radiochemical purity by TLC, using chloroform–acetic acid (99:1) on silica gel sheets[3], and by paper chromatography[4] with pyridine–ethyl acetate–water (1:2:1). The chromatograms were cut into 1-cm strips, and the amount of radioactivity on each strip was measured.

Ninety-five percent of the total activity was located in the bilirubin

[1] Lot 9324, Nutritional Biochemicals Corp., Cleveland, OH 44128.
[2] Mann Research, Orangeburg, NY 10962.
[3] ITLC type SG, Gelman Instrument Co., Ann Arbor, Mich.
[4] Whatman Grade 1, Scientific Products, Columbus, OH 43207.