[silica gel, chloroform–methanol (4:1)]. Compound III*b*, 960 mg (22%), was obtained as tan flakes from ether–petroleum ether, mp 212–215° dec.; mass spectrum: *m/e* >150, 439 (M + H, 18.9), 438 (M^+, 1.8), 406 (3.8), 365 (74.8), 352 (27.9), 309 (15.3), 268 (100), 266 (12.8), 178 (33.5), and 154 (27.3); methane chemical-ionization mass spectrum: *m/e* >250, 440 (M + 2H, 100), 439 (M + H, 71.1), 424 (27.1), 422 (22.6), 365 (8.3), and 268 (24.0). The electron spin resonance spectrum of III*b* exhibited the usual three-line pattern for a nitroxide derived from tetramethylpiperidine.

Anal.—Calc. for $C_{26}H_{37}N_3O_3$: C, 71.20; H, 8.27; N, 9.58. Found; C, 71.12; H, 8.11; N, 9.81.

A small portion of this material was treated with excess ethereal diazomethane. Evaporation of solvent and excess diazomethane afforded a crystalline residue identical to III*a* in all respects (melting point, IR, TLC, and mass spectral data).

Also isolated from column chromatography of this reaction mixture was a component having an R_f similar to that of IV*b*. This substance was identified as II*c* by its melting point and IR and mass spectra. No IV*b* was eluted from the column.

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ACKNOWLEDGMENTS AND ADDRESSES

Received February 5, 1976, from the *Division of Biochemistry, Walter Reed Army Institute of Research, Washington, DC 20012.*

Accepted for publication September 2, 1976.

The authors are grateful to Dr. Kenner C. Rice, Dr. Werner Klee, and Mrs. Louise Atwell of the National Institutes of Health, Bethesda, Md., for the biological activity tests, and Dr. Edmund S. Copeland of Walter Reed for determination of the electron spin resonance spectra.

[x] To whom inquiries should be directed. Present address: Corporate Research and Development Center, General Electric Co., Schenectady, NY 12301.

Radiochemical GLC Assay for Nortriptyline in Human Plasma

ARTHUR LOH, FRANCIS R. ZULESKI, and FREDERICK J. Di CARLO [x]

Abstract □ A novel method was developed for the assay of nortriptyline in plasma. After nortriptyline was extracted, it was acetylated with ^{3}H-acetic anhydride; the quantity of ^{3}H-acetylnortriptyline in the extract was determined by radiochemical GLC. The method is capable of assaying 5 ng of nortriptyline/ml of plasma. The instrumentation was assembled from commercially available components.

Keyphrases □ Nortriptyline—radiochemical GLC analysis, human plasma □ Radiochemical GLC- -analysis, nortriptyline in human plasma □ GLC, radiochemical—analysis, nortriptyline in human plasma □ Antidepressants—nortriptyline, radiochemical GLC analysis in human plasma

The assay of nortriptyline in plasma is important because its clinical activity is related to plasma levels (1, 2), which vary remarkably among individuals even at steady state (3, 4). GLC methods for nortriptyline assay (5–7), although capable of detecting 10–20 ng of compound/ml of plasma, are frequently inadequately sensitive for measuring the levels attained after a single dose. A recently published TLC assay is considerably less sensitive, having a lower limit of 50 ng/ml (8).

Zuleski *et al.* (9) developed a nortriptyline assay method which involved extracting the compound from plasma by a modification of the method of Hammer and Brodie (10), acetylating it with ^{14}C-acetic anhydride, separating acetylnortriptyline by TLC, locating this derivative by radiochromatogram scanning, removing it from the plate, and counting it by scintillation spectrometry. The present approach was more direct. Radiochemical GLC was performed directly following derivatization with ^{3}H-acetic anhydride. Thus, ^{3}H-acetylnortriptyline was resolved and counted simultaneously. Despite the wide applicability of radiochemical GLC to drug metabolism studies, this method has been developed only in a few laboratories (11–13). The present investigation shows that the method can be developed using commercially available components.

EXPERIMENTAL

Reagents—^{3}H-Acetic anhydride (50 mCi/4.36 mg in 5 ml of dry benzene) was received[1] in a sealed ampul. A 0.05-ml aliquot of this so-

[1] New England Nuclear, Boston, Mass.

lution was transferred quantitatively to a 50-ml volumetric flask with benzene, which had been dried over metallic sodium and then diluted to volume with the same solvent. Air in the neck of the flask was displaced gently with dry nitrogen; the flask was stoppered, sealed with tape, and stored under refrigeration in a bottle containing desiccant (calcium sulfate). The bottle and its contents were allowed to equilibrate to room temperature before opening. After using a portion of the ^{3}H-acetic anhydride solution, the flask was prepared for storage as described.

Nortriptyline hydrochloride was used as received[2]. Reagent grade solvents were used in this study.

Radiochemical GLC—An oven and proportioning temperature controller[3] were connected to a gas proportional counter[4] by a heated glass-lined metal tube. The gas chromatograph was fitted with a 1.83-m × 2-mm i.d. coiled-glass column packed with 20% SE-30 on 80–100-mesh Gas Chrom Q. Oven and injector temperatures were set at 215 and 245°, respectively.

Helium was used as carrier gas at a flow rate of 50 ml/min. The transfer line to the gas proportional counter was maintained at 237°. The hydrogen and propane (quench gas) flow rates were 10 and 2.5 ml/min, respectively. The temperature of the oxidizer and reduction furnace was 750°.

Standard Nortriptyline Solutions—Nortriptyline hydrochloride (11.35 mg) was dissolved in 10 ml of distilled water to produce a free base concentration of 1.0 mg/ml. From this solution, others were prepared in duplicate concentrations of 100, 50, 25, 12.5, and 6.25 ng/ml.

Nortriptyline Extracts and Derivatization—Twelve 2.0-ml aliquots of normal human plasma were placed into 15-ml polyethylene-stoppered conical test tubes. Duplicate standard nortriptyline solutions (1.0 ml) were added to 10 of these tubes, and water (1.0 ml) was added to the other two tubes to serve as blanks. Then 0.5 ml of 1 *N* NaOH was mixed with the contents of each tube.

Each plasma specimen was extracted twice with 5.0 ml of *n*-hexane. Any emulsion was broken by placing the tube into a dry ice–acetone mixture followed by thawing and recentrifugation. The hexane layer was transferred to another tube with an isopentyl alcohol-pretreated pipet (10). The combined hexane extracts were evaporated to dryness with nitrogen, and the residue was dissolved in 0.65 ml of dry benzene. ^{3}H-Acetic anhydride solution, 100 μl, and 0.25 ml of pyridine were added to the benzene solution, which was then mixed thoroughly and incubated at 60° for 1 hr.

After the solvent was evaporated in a water bath at 90°, 2.0 ml of 0.1 *N* NaOH was added and the tube was shaken for a few seconds. Then the solution was placed in a water bath at 95° for 15 min. The solution was cooled to room temperature, and acetylnortriptyline was extracted with two 5.0-ml aliquots of heptane. The combined heptane extracts were evaporated under nitrogen. Then the inner wall of the tube was washed down with heptane, and the solvent was evaporated to dryness. Each residue was dissolved in 50 μl of heptane.

Measurement of ^{3}H-Acetylnortriptyline—Aliquots (10 μl) of each plasma blank and ^{3}H-acetylnortriptyline solution in heptane were injected into the gas chromatograph. The area under each radioactive peak on the chromatograms was measured by planimetry. A standard curve was constructed by plotting mean areas against quantities of nortriptyline (expressed as free base) per milliliter of human plasma.

RESULTS

No peak appeared on chromatograms of "blanks" consisting of plasma and ^{3}H-acetic anhydride. Under the described operating conditions, radiochemical GLC yielded a single peak representing ^{3}H-acetylnortriptyline (Fig. 1). The retention time of this compound was 31.8 min from each plasma extract. A linear standard curve resulted from plotting peak areas against human plasma concentrations of nortriptyline ranging from 3.125 to 50.0 ng/ml. This curve passed through the intercept of the ordinate and abscissa; the peak area per nanogram of nortriptyline was 0.658 cm^2. By preparing the extracted acetylnortriptyline concentrate as described and using only one-fifth of it for assay, the radiochemical GLC method was reliable for levels of nortriptyline down to 5 ng/ml of plasma.

DISCUSSION

The radiochemical GLC assay has advantages over other methodology for nortriptyline in plasma. It is more sensitive than the TLC (8) and GLC (5–7) methods that do not employ radioactivity. Although the radiochemical GLC procedure is no more sensitive than the radiochemical TLC assay (9), it is shorter and more direct. As presently developed, both radioassays can measure nortriptyline levels as low as 5 ng/ml. However, the sensitivity of these and other radioacetylation methods probably can be increased by using acetic anhydride of higher specific activity. The major expansion of radiochemical derivatization assays probably will be in the direction of broadening their application by employing other radiolabeled reagents.

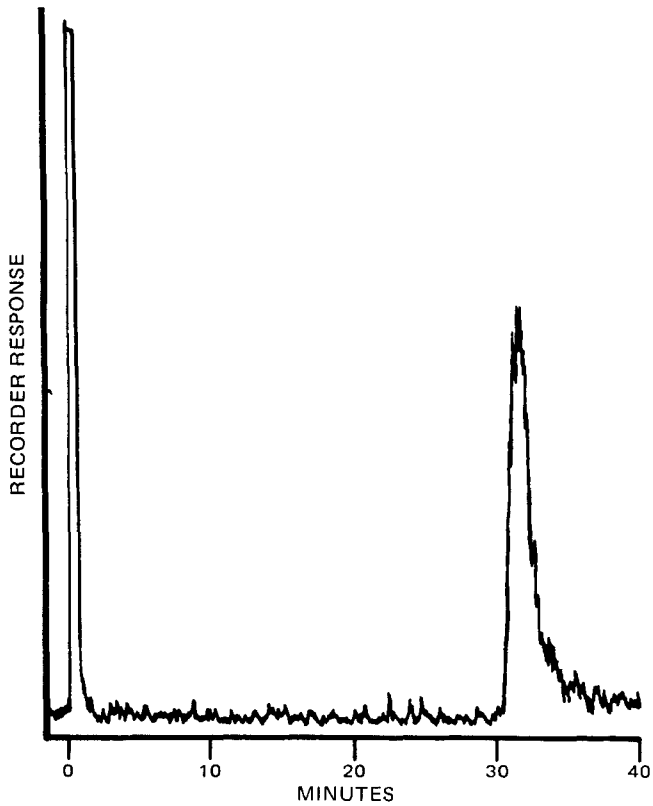

Figure 1—*Radiochemical gas chromatogram of ^{3}H-acetylnortriptyline representing 10 ng of nortriptyline extracted from human blood plasma.*

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ACKNOWLEDGMENTS AND ADDRESSES

Received July 6, 1976, from the *Department of Drug Metabolism,*

[2] Eli Lilly, Indianapolis, Ind.
[3] Warner-Chilcott, Morris Plains, N.J.
[4] Model 894, Packard Instrument Co., Downers Grove, Ill.

Warner–Lambert Research Institute, Morris Plains, NJ 07950.

Accepted for publication August 30, 1976.

The authors are indebted to Mr. E. J. Merrill of this Institute for furnishing a stock solution of ^{3}H-acetic anhydride in dry benzene and for advice on the storage of this material. They also thank Mr. Roger Warner, Packard Instrument Co., for technical assistance in setting up the gas proportional counter.

[x] To whom inquiries should be directed.

Pharmacokinetic Evidence for Improved Ophthalmic Drug Delivery by Reduction of Instilled Volume

THOMAS F. PATTON

Abstract □ The bioavailability of topically applied pilocarpine nitrate was studied as a function of instilled volume. As the instilled volume decreased, the fraction of dose absorbed increased. The relationship between fraction absorbed and instilled volume was not direct, but appropriate adjustment of instilled volume and concentration should permit substantial dosage reductions without sacrifice of drug concentration in the eye. The implications of these findings from both a therapeutic and toxicity standpoint are discussed.

Keyphrases □ Pilocarpine nitrate—bioavailability, effect of instilled volume □ Bioavailability—pilocarpine nitrate, effect of instilled volume □ Ophthalmic cholinergics—pilocarpine nitrate, bioavailability, effect of instilled volume

The drainage of an instilled drug solution away from the eye is considerable and can affect its biological activity (1). This drainage is dependent upon the volume instilled and increases linearly with instilled volume over a large range (1). This fact (2, 3) has been incorporated into experimental design. However, the concept of reducing drop size to improve ophthalmic drug delivery has not become widespread, perhaps because the pharmacokinetic advantages of such an approach have not been emphasized.

A study of the dynamics of instilled fluid drainage (1) showed that the miotic activity of pilocarpine was enhanced by administering the same amount of pilocarpine in decreasing volumes. No actual measurements of drug penetration to the interior of the eye were made. More recent studies showed that the bioavailability of ophthalmic drugs was improved by slowing the rate of drainage of instilled drug solutions (4–8). The measurement of drug concentration in the eye following the reduction of instilled volume and the resultant pharmacokinetic parameters were not reported previously.

In a recent study (9), the aqueous chamber drug distribution volume for ophthalmic drugs was calculated, thereby making it possible to define more precisely the pharmacokinetics of topically applied ophthalmic drugs. Therefore, the purpose of the present study is to show that, as the instilled volume of pilocarpine nitrate is decreased, the fraction of dose absorbed increases. As a result, the parameters of instilled volume and drug concentration can be quantitated to provide a desired level of drug in the eye.

EXPERIMENTAL

Pilocarpine Solutions—Pilocarpine nitrate was used as received[1]. Tritiated pilocarpine[2], specific activity 4.1 Ci/mmole, was purified prior to use by evaporation as recommended previously (10). The concentration of pilocarpine nitrate used was 1×10^{-2} *M* in all studies. Solutions were prepared in isotonic pH 6.24 Sorensen phosphate buffer and were filtered but not sterile. Procedures for the preparation of the tritiated solutions were described previously (10).

Methods—Male, New Zealand, albino rabbits, 60–67 days old, were used. Prior to experimentation, the animals were maintained in standard laboratory animal cages and allowed food and water *ad libitum*. During the experiments, all test animals were kept in restraining boxes in the normal upright position. The head was unencumbered so that all normal eye movements were maintained. The animals were unanesthetized in all cases.

The volumes of instilled solutions administered were 5, 10, 15, and 25 μl and were delivered accurately with a microliter syringe[3]. At various times postinstillation (5, 10, 15, 20, 30, 45, 60, 90, and 120 min), rabbits were sacrificed with an overdose of pentobarbital sodium injected into a marginal ear vein. Eyes were immediately rinsed and blotted, and aqueous humor (at least 100 μl) was aspirated from the anterior chamber.

One hundred microliters of aqueous humor was quantitatively transferred[4] to scintillation counting vials[5] containing 5 ml of prerefrigerated liquid scintillation cocktail[6]. After storage in the dark at room temperature for at least 24 hr, samples were counted[7]. These counts were converted to micrograms of pilocarpine per milliliter of aqueous humor using suitable standard and blank corrections.

RESULTS

Aqueous humor concentration–time profiles of pilocarpine for the four instilled volumes are shown in Table I. These data were plotted semilogarithmically, and the terminal slopes were calculated *via* linear regression analysis. From these slopes, the elimination rate constants were calculated. By using the trapezoidal rule with extrapolation to infinity, the areas under the aqueous humor concentration–time profiles were calculated (Table II). The apparent maximum in area under the curve with an instilled volume of 15 μl is currently unexplained. This maximum also was observed in other experiments and will be discussed in more detail later.

Using rabbits of similar size and age, Conrad and Robinson (9) calculated the aqueous humor distribution volume of pilocarpine to be 0.575

[1] Sigma Chemical Co., St. Louis, Mo.
[2] New England Nuclear, Boston, Mass.
[3] Hamilton Co., Reno, Nev.
[4] Biopette, Schwarz/Mann, Orangeburg, N.Y.
[5] Mini-vial, ICN Isotope and Nuclear Division, Cleveland, Ohio.
[6] Aquasol, New England Nuclear, Boston, Mass.
[7] Model LS-150 liquid scintillation counter, Beckman Instruments, Fullerton, Calif.