Table II—Plasma Concentrations of Radioactivity in a Monkey Given 2 mg/kg ip of ^{3}H-I

Hours	Plasma Radioactivity[a], μg/ml		Distillable / Total × 100
	Total	Distillable	
0.5	2.48	0.060	2.4
1	2.11	0.076	3.6
2	1.34	0.043	3.2
4	0.61	0.102	16.7
7	0.19	0.057	29.9
24	0.11	0.093	84.5
48	0.08	0.076	94.7

[a] Each value is the amount of administered drug that would give rise to the observed radioactivity.

reabsorption processes of the kidney could not distinguish tritiated water from unlabeled water. Therefore, tritiated water would have comparable concentrations in urine and plasma and would be excreted at the same rate as unlabeled water. The exact value for the half-life of water in the monkey is not known, but corresponding values of 3.5, 3.9, and 5.1 days have been reported for the rat, rabbit, and dog, respectively (5).

In contrast, I and/or its metabolites were rapidly excreted and their plasma levels fell below their concentration in urine. The result of these differences in the excretion rates of tritiated water and I was a greater ratio of distillable to total radioactivity in plasma than in urine.

The observed *in vivo* loss of the tritium label might be attributed to a tritium–hydrogen exchange, perhaps facilitated by some biological mechanism, or to a specific metabolic alteration of the molecule at the site of tritium substitution. To distinguish between these two mechanisms of *in vivo* instability, one must isolate the unchanged drug from urine or plasma and compare its specific activity with that of the administered drug. Comparable specific activities would suggest that the loss of label occurred in conjunction with metabolism at the site of tritium substitution. In contrast, a decrease of specific activity would indicate that the loss of label was caused partly or entirely by exchange.

The findings reported in this communication may have general applicability and serve as a warning to the uninitiated user of tritium. One must be careful in drawing conclusions from urinary data regarding the *in vivo* stability of the tritium label in a compound. Furthermore, our results strengthen the argument that when a substantial fraction of the label is lost, tritium should not be used as a tracer for the parent molecule.

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GLC Determination of Pilocarpine

Keyphrases □ Pilocarpine—GLC analysis, rabbit aqueous humor □ GLC—analysis, pilocarpine, rabbit aqueous humor □ Ophthalmic cholinergic agents—pilocarpine, GLC analysis, rabbit aqueous humor

To the Editor:

Measurement of the transport of pilocarpine into the internal eye as well as information concerning its distribution and inactivation within individual intraocular structures depends on accurate determination of small quantities of this drug in biological fluids. Although many procedures have been used for this purpose, including those involving polarimetric, polarographic, volumetric, colorimetric, and kinetic techniques (1–6), all have been associated with problems of complexity, lack of specificity, or lack of sensitivity.

GLC coupled with electron-capture detection has been employed during the past 10 years for the determination of nanogram amounts of organic compounds exhibiting certain similarities to pilocarpine (7–9). A similar technique provided an ideal system for a sensitive new procedure for the reliable assay of pilocarpine.

Heptafluorobutyric anhydride[1], trimethylamine, and pesticide grade benzene[2] were utilized in the formation of the derivative of pilocarpine[3]. Heptafluorobutyric anhydride (10 μl) and 0.1 ml of trimethylamine (0.05 *M* in benzene) were added to 500 μg of pilocarpine hydrochloride in benzene (10). The reaction was performed in screw-capped vials[4]. After the sample was heated for 15 min at 50°, 1 ml of water was added and the vial was shaken for 1 min. One milliliter of aqueous ammonia (5%) was added, and the reaction mixture was shaken for an additional 5 min.

After centrifugation, the benzene layer was diluted to give a derivative concentration of 100 ng/ml. Then 1 μl (equal to 0.1 ng) was injected into a 1.2-m × 4-mm i.d. U-shaped column packed with 2% XF-1105[5] on Gas Chrom Q[6] (80–100 mesh) for GLC analysis. Separation of the derivative was achieved using a gas chromatograph[7] equipped with two electron-capture detectors, each containing a 150-mCi tritium foil.

The oven temperature was kept at 190°; the detector, inlet, and outlet temperatures were maintained at 200°.

[1] Pierce Chemical Co., Rockford, Ill.
[2] Fisher Scientific Co.
[3] Mallinckrodt Chemical Co.
[4] Lined with Teflon (du Pont).
[5] Now called OV-105.
[6] Ohio Valley Specialty Chemical, Marietta, Ohio.
[7] Packard model 7400.

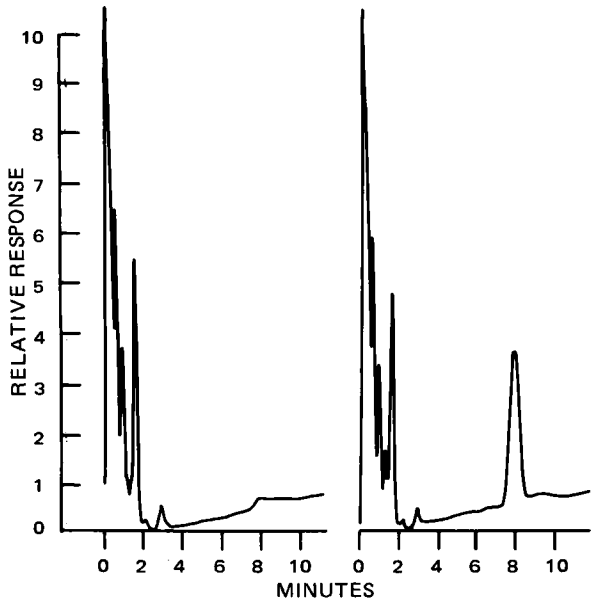

Figure 1—*Chromatogram of rabbit aqueous humor as the control and after the addition of pilocarpine. Chart speed was 0.5 cm/min.*

The flow rate of prepurified nitrogen was 70 ml/min, and the high voltage was set at 20 v. The full-scale deflection used on the 1-mv recorder was 1×10^{-9} amp.

The determination of retention time and linearity was done on dilutions of the standard. The standard curve was obtained by adding known amounts of pilocarpine hydrochloride to rabbit aqueous humor and isolating the drug as follows. Twenty-five microliters of rabbit aqueous humor was added to a 5-ml ground-glass centrifuge tube and diluted to 1 ml with a saturated solution of sodium chloride. The sample was acidified with 6 *N* hydrochloric acid to pH <1 and extracted with 3 ml of ethyl acetate by shaking for 1 min.

The ethyl acetate layer was discarded, and the aqueous layer was made basic (pH > 8) with 2 *M* potassium carbonate and reextracted with ethyl acetate (3, 3, and 2 ml) by shaking for 1 min. Then the ethyl acetate extracts were combined and evaporated in a 40° water bath. The sample was then transferred to a 3-ml screw-capped vial[4] with 0.5 ml of methanol followed by 0.5 ml of ethyl acetate. The specimen was again evaporated under a stream of dry nitrogen and derivatized as previously described.

The heptafluorobutyramide derivative of pilocarpine gave a single, sensitive, symmetrical peak with a retention time of 8 min. A linear response with respect to peak height was demonstrated by injection of 10–150 pg. The derivatized samples were stable for more than 1 week, and the recovery of pilocarpine added to samples was quantitative (95 ± 2%). The standard curve, obtained by addition of 25–200 ng of pilocarpine to 25 μl of rabbit aqueous humor, was described by the equation $y = 0.29x - 0.18$ ($r = 0.998$). No substances interfering with pilocarpine were noted in physiological saline, Ringer's solution, or rabbit aqueous fluid (Fig. 1).

Addition of serum to aqueous fluid caused no significant changes in the standard curve. The method thus obviates the analytical problem of serum binding of pilocarpine (11). The use of tritiated pilocarpine to test the reproducibility of the method was unsuitable because of the instability of the label in acidic and basic solutions. The minimum detectable quantity of this substance under the conditions of this study is 1 pg.

A comparably sensitive and specific method for the assay of small amounts of pilocarpine has not been described previously. Use of an apparently similar technique was alluded to recently (12), but details of the methodology are not available.

Assays in the past have been sensitive only to microgram quantities. Those employing tritiation have been limited by problems of purification (13), uncertainty of labeling position in the molecule, and tritium exchange when in solution. Those involving colorimetry required extreme care in excluding interfering compounds. The many potential pitfalls of UV spectrophotometry rendered such analyses complex and time consuming because of the requirement for elaborate controls (14, 15). Preparation of ^{14}C-pilocarpine, currently unavailable commercially, is complex and expensive (16).

The GLC procedure described here is reliable, easily performed, and more than three orders more sensitive than previously used methods. It is ideally suited to determinations of small quantities of pilocarpine (1 ng) in small amounts of biological fluid (such as 25 μl of aqueous humor).

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Effect of Plasma Protein Binding of Drugs on Duration and Intensity of Pharmacological Activity

Keyphrases □ Plasma protein binding—effect on duration and intensity of pharmacological activity of drugs □ Binding, plasma protein—effect on duration and intensity of pharmacological activity of drugs □ Pharmacological activity—duration and intensity, effect of plasma protein binding of drugs

To the Editor:

There is increasing evidence that the pharmacological activity of drugs is a function of their free (not protein bound) concentration (1–4). It has also become apparent that the total clearance of drugs by the body is affected by the extent to which they are plasma protein bound (5–7). It is informative, therefore, to consider the effect of a change in plasma protein binding of a drug on the duration and intensity of its pharmacological activity, particularly since these indicators have been used to study drug interactions involving competitive displacement from plasma protein binding sites (8, 9).

It will be assumed as a matter of convenience that the free fraction of drug in plasma (f) is essentially constant over a wide concentration range (5, 6, 10), that the elimination of the drug is by apparent first-order kinetics and not affected by organ perfusion rate, that the drug is distributed in the body so rapidly as to justify the use of a one-compartment pharmacokinetic model, and that the only *direct* perturbation of the biological system is a change in f. Under these conditions:

$$\text{total clearance} = k''f \qquad \text{(Eq. 1)}$$

where k'' is the intrinsic clearance of the drug (5, 6). Also:

$$\text{total clearance} = V_d k_{app} \qquad \text{(Eq. 2)}$$

where V_d is the apparent volume of distribution of total (free and bound) drug, and k_{app} is the apparent first-order elimination rate constant. It is evident that an increase in f results in a corresponding increase in total clearance or $V_d k_{app}$. The quantitative effect of an increase in f on V_d is difficult to predict, but it appears to be smaller than the effect on k_{app} (6).

Figure 1 shows the effect of an increase in f from 0.01 to 0.03 on the time course of free and total drug concentrations after intravenous injection of 100 mg/kg of a drug with V_d = 0.20 liter/kg and k_{app} = 0.05776 hr^{-1} (equivalent to $t_{1/2}$ = 12 hr) under conditions where: (*a*) V_d increases to 0.25 liter/kg or (*b*) V_d is unaffected. In either case, it is evident that the increase in f causes an

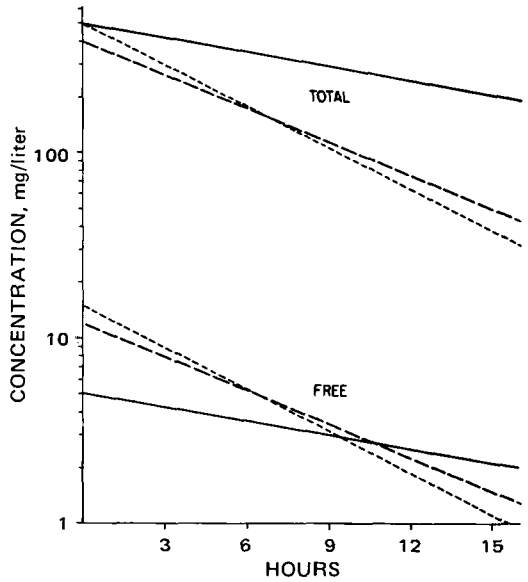

Figure 1—*Effect of a change in free fraction in plasma (f) on the time course of total and free drug concentrations in plasma after intravenous injection of 100 mg/kg. Key: continuous line, V_d = 0.20 liter/kg, $t_{1/2}$ = 12 hr, and f = 0.01; short stippled line, f increased to 0.03, and V_d unchanged; and long stippled line, f increased to 0.03, and V_d increased to 0.25 liter/kg. These simulations are based on the assumption that the total clearance increases proportionally with f.*

increase in the initial concentration of free drug but also a more rapid decline so that the free concentration when f = 0.03 will eventually be lower than when f = 0.01. This means that an increase in f will cause an increase in the intensity of the initial (maximum) pharmacological effect but that the duration of action may be increased *or* decreased, depending on the dose and the minimum effective concentration of free drug. For any one drug, an increase in f under the stated conditions may be expected to prolong the duration of pharmacological activity of small doses and to shorten the duration of action of large single doses.

It has been observed that the incidence of adverse

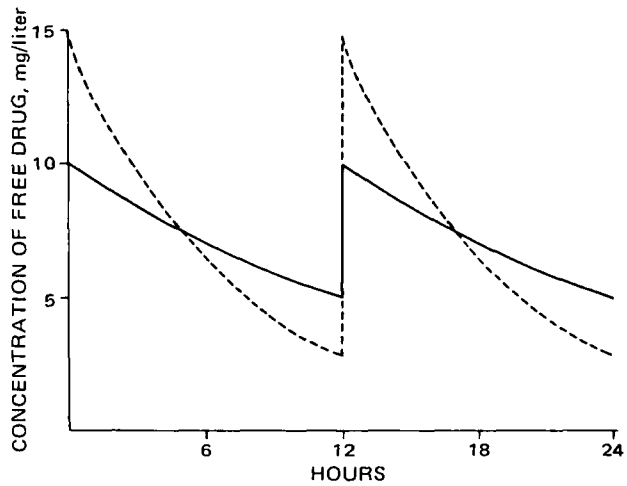

Figure 2—*Effect of a change in f on the time course of free drug concentrations in plasma at the steady state when 100 mg of drug/kg is administered intravenously every 12 hr. Key: continuous line, V_d = 0.20 liter/kg, $t_{1/2}$ = 12 hr, and f = 0.01; and stippled line, f increased to 0.03, V_d increased to 0.25 liter/kg, and $t_{1/2}$ decreased, therefore, to 5 hr.*