(6) T. Higuchi and L. C. Schroeter, *J. Am. Chem. Soc.*, **82**, 1904 (1960).
(7) B. R. Hajratwala, *J. Pharm. Sci.*, **64**, 45 (1975).
(8) L. H. Welsh and O. R. Sammul, *J. Assoc. Offic. Anal. Chem.*, **51**, 176 (1968).
(9) "The United States Pharmacopeia," 19th rev., Mack Publishing Co., Easton, Pa., 1975 pp. 169–172, 274–276, 377–379.
(10) "The British Pharmacopoeia 1973," Her Majesty's Stationery Office, London, England, 1973, pp. 14, 15, 257, 365, 366.
(11) "The National Formulary," 14th ed., Mack Publishing Co., Easton, Pa., 1975, pp. 253, 368–371.
(12) V. K. Prasad, R. A. Ricci, B. C. Nunning, and A. P. Granatek, *J. Pharm. Sci.*, **62**, 1130 (1973).
(13) *Ibid.*, **62**, 1135 (1973).
(14) T. James, *J. Pharm. Sci.*, **62**, 669 (1973).
(15) K. K. Kaistha, *ibid.*, **59**, 241 (1970).
(16) J. Levine and T. D. Doyle, *ibid.*, **56**, 619 (1967).
(17) E. Shotton and D. J. Priaulx, *J. Pharm. Pharmacol.*, **26**, 197 (1974).
(18) J. Doulakas, *Pharm. Acta Helv.*, **50**, 66 (1975).
(19) R. B. Salama and S. K. W. Khalil, *J. Pharm. Sci.*, **63**, 1301 (1974).
(20) N. H. Choulis, *ibid.*, **56**, 196 (1967).
(21) D. Cantin, J. Alary, and A. Coeur, *Analusis*, **2**, 654 (1974).
(22) *Ibid.*, **3**, 5 (1975).
(23) S. Kawai and Z. Tamura, *J. Chromatogr.*, **25**, 471 (1966).
(24) G. M. Anthony, C. J. W. Brooks, I. Maclean, and I. Sangster, *J. Chromatogr. Sci.*, **7**, 623 (1969).
(25) P. F. G. Boon and A. W. Mace, *J. Pharm. Pharmacol., Suppl.*, **21**, 49S (1969).
(26) L. J. Dombrowski, P. M. Comi, and E. L. Pratt, *J. Pharm. Sci.*, **62**, 1761 (1973).
(27) H. G. Lovelady and L. L. Foster, *J. Chromatogr.*, **108**, 43 (1975).
(28) E. Gelpi, E. Peralta, and J. Segura, *J. Chromatogr. Sci.*, **12**, 701 (1974).
(29) K. P. Wong, C. R. J. Ruthven, and M. Sandler, *Clin. Chim. Acta*, **47**, 215 (1973).
(30) L. Bertilsson, *J. Chromatogr.*, **87**, 147 (1973).
(31) J. R. Watson, *J. Pharm. Sci.*, **63**, 96 (1974).
(32) J. R. Watson and R. C. Lawrence, *J. Chromatogr.*, **103**, 63 (1975).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 5, 1976, from the *Drug Research Laboratories, Health Protection Branch, Health and Welfare Canada, Ottawa, Canada.*
Accepted for publication June 11, 1976.
[x] To whom inquiries should be directed.

High-Performance Liquid Chromatographic Analysis of Isoniazid and Its Dosage Forms

LEONARD C. BAILEY [x] and HAMED ABDOU

Abstract □ A high-performance liquid chromatographic analysis is described for isoniazid as a drug entity and in its tablet and injectable dosage forms. After incorporation of the drug or dosage form in a solvent mixture and addition of an internal standard, tribenzylamine, an aliquot is chromatographed using a pellicular silica gel medium followed by UV spectrophotometric detection at 254 nm. The response of the chromatographic system was linear over a concentration range corresponding to 20–200% of the labeled amount of isoniazid. Comparison of the results with those obtained by the official USP XIX method indicates similar accuracy and precision. The advantages of the proposed method are its simplicity and rapidity, its potential for automation, and its specificity. The specificity was demonstrated in the presence of potential degradation products of isoniazid, other drugs used with isoniazid in combination dosage forms, and an adduct formed by the reaction of isoniazid with lactose in the tablet.

Keyphrases □ Isoniazid—high-performance liquid chromatographic analysis, tablets and injectables □ High-performance liquid chromatography—analysis, isoniazid, tablets and injectables □ Tuberculostatic antibacterials—isoniazid, high-performance liquid chromatographic analysis, tablets and injectables

Because isoniazid (isonicotinic acid hydrazide) is the drug of choice for the treatment of tuberculosis, many attempts have been made to develop methods for its quantitation in dosage formulations. The procedures rely primarily on the redox reactivity of the hydrazide group or upon color formation with an appropriate chromogen.

One analysis is based on the addition of excess standard iodine followed by back-titration with standard thiosulfate (1). This procedure was official in four USP revisions but was supplanted in the current USP XIX (2) by a nitrous acid titration similar to that used for the determination of sulfa drugs. The method official in the BP (3) is similar to the earlier USP method, except that excess bromine is used instead of iodine. These oxidimetric methods and a nonaqueous titration procedure were reviewed and compared previously (4).

Another method (5) involves the determination of isoniazid and other hydrazine-derived drugs by potentiometric titration in an acid solution, using chloramine-T as the titrant. However, its applicability to dosage forms was not explored. A colorimetric method was proposed based on the reaction of isoniazid with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (6). A colorimetric procedure uses 9-chloracridine as the chromogenic reagent (7).

The purpose of this study was to investigate the utility of high-performance liquid chromatography (HPLC) in the determination of isoniazid in its dosage forms. The speed and accuracy of this technique and the availability of relatively inexpensive modular instrumentation should make such analysis particularly applicable in quality assurance situations.

The proposed method is simple, involving only the dissolution of the sample, addition of the internal standard, and introduction of an aliquot of the resulting mixture onto a liquid chromatograph with an adsorption column and a

UV detector operated at 254 nm. The accuracy and precision of the method are comparable to those of the current USP XIX analysis, but the time required for a determination is significantly less. In addition, the HPLC method is more specific in that it is insensitive to isonicotinic acid, a possible hydrolysis product of isoniazid, and 1-isonicotinoyl-2-lactosylhydrazine, the hydrazone formed by the reaction of isoniazid with lactose, which may be present as a diluent in the formulation.

EXPERIMENTAL

Reagents and Chemicals—High purity isoniazid[1] was recrystallized twice from 95% ethanol, dried at 105° for 4 hr, and stored in a vacuum desiccator, mp 171°. Isonicotinic acid[1] was recrystallized twice from distilled water. Tribenzylamine[2] was recrystallized twice from 95% ethanol and dried *in vacuo*, mp 93–93.5°.

The lactose hydrazone of isoniazid, 1-isonicotinoyl-2-lactosylhydrazine, was prepared as follows. Isoniazid, 6.85 g (0.05 mole), was mixed with 300 ml of 95% ethanol in a 500-ml round-bottom flask and warmed on a steam bath until solution was complete. In a 100-ml beaker, 17.1 g (0.05 mole) of lactose was mixed with 40 ml of distilled water and warmed on a hot plate until the lactose was dissolved. The aqueous lactose solution was added slowly, with continuous stirring, to the isoniazid solution and refluxed for 8 hr. The mixture was concentrated under vacuum until a pasty mass was obtained.

After mixing the crude product with 20 ml of methanol and refrigerating overnight, the resulting slurry was filtered and the residue was dried in a desiccator under vacuum. Recrystallization from the minimum amount of 95% ethanol–water (3:1) yielded white needles, mp 199° (uncorr.) [lit. (8) mp 202°]; λ_{max} (ethanol, 95%): 261.5 nm.

Anal.—Calc. for $C_{18}H_{27}N_3O_{12}$: C, 46.85; H, 5.90; N, 9.10; O, 38.14. Found: C, 46.24; H, 6.04; N, 9.14; O, 35.58.

TLC of the hydrazone, using silica gel G plates[3] and a mobile phase of methanol–chloroform (60:65) (9), showed a single spot with an R_f of 0.17 (R_f of isoniazid under these conditions is 0.60). Analysis of the product using the USP XIX procedure specified for isoniazid (2) indicated an apparent purity of 97%.

Solvents—Reagent grade methanol[4] was redistilled from an all-glass apparatus. Reagent grade methylene chloride[4] and spectranalyzed chloroform[4] and 2-propanol[4] were used as received.

Apparatus—HPLC was performed with an apparatus consisting of a pump[5] with a pulse dampener[6] and a UV monitor[6] operated at 254 nm. Samples were introduced through a septum injector[6] using a 10-μl syringe[7]. The output of the detector was displayed on a recorder[8] having a full-scale range of 10 mv. The output signal was integrated, and results were calculated using an electronic integrator[9]. A 1-m × 2.1-mm i.d. stainless steel column was packed with a pellicular silica gel adsorbent[10] according to the manufacturer's instructions. The mobile phase was pumped at a pressure of 300 psig, which resulted in a flow rate of 1.20 ml/min.

USP analyses for isoniazid were performed using standard 10.00- and 50.00-ml burets[11] and an expanded scale pH meter[12].

Mobile Phase—The mobile phase was a mixture of either methylene chloride or chloroform with methanol, 2-propanol, and water in the following proportions: 85:5:10:0.5.

Standard Solutions—*Solvent Mixture* —The solvent mixture used was methylene chloride (or chloroform, if the mobile phase was chloroform based)–methanol–2-propanol (85:5:10).

Isoniazid Standard Solution—Accurately weigh 0.2000 g of isoniazid, transfer to a 100-ml volumetric flask, and dilute to the mark with the solvent mixture.

Internal Standard Solution—Accurately weigh 3.0000 g of tribenzylamine, transfer to a 100-ml volumetric flask, and dilute to the mark with the solvent mixture.

Standard Preparation—Into a 50-ml volumetric flask, pipet 25.0 ml of isoniazid standard solution, 5.0 ml of internal standard solution, and 0.5 ml of water. Mix well to ensure incorporation of the water into the solution. Dilute to volume with the solvent mixture and shake until a homogeneous mixture is obtained. Introduce 5 μl into the liquid chromatograph. Measure the areas under the two peaks and calculate the ratio, R_s, by dividing the area of the isoniazid peak by the area of the internal standard peak.

Isoniazid—*Assay Preparation*—Dissolve 100 mg of isoniazid, accurately weighed, in 50 ml of the solvent mixture in a 100-ml volumetric flask. Then add 1.0 ml of water and 10.0 ml of internal standard solution and shake until the water is incorporated. Dilute to the mark with the solvent mixture and mix until homogeneous.

Assay Procedure—Introduce a 5-μl portion into the liquid chromatograph. Measure the areas under the two peaks and calculate the ratio, R_u, by dividing the area of the isoniazid peak by the area of the internal standard peak. Calculate the quantity, in milligrams, of isoniazid present in the sample taken by the formula:

$$\text{isoniazid} = W_s(R_u/R_s) \qquad \text{(Eq. 1)}$$

where R_u and R_s are as defined previously, and W_s is the weight, in milligrams, of isoniazid in 100.0 ml of the standard preparation.

Isoniazid Tablets—*Assay Preparation*—Weigh and finely powder not less than 20 isoniazid tablets. Weigh accurately a portion of the powder, equivalent to 100 mg of isoniazid, and transfer it to a 100-ml volumetric flask using about 50 ml of the solvent mixture. Add 1.0 ml of water and 10.0 ml of internal standard solution. Mix well for 1 min to ensure extraction of the isoniazid from the powder. Dilute to volume with the solvent mixture and mix until homogeneous.

Assay Procedure—Proceed as directed in the assay procedure for isoniazid.

Isoniazid Injection—*Assay Preparation*—Transfer 1.0 ml of isoniazid injection to a 100-ml volumetric flask and add about 75 ml of the solvent mixture. Mix well to ensure incorporation of the aqueous injection vehicle with the solvent mixture and add 10.0 ml of internal standard. Dilute to volume with the solvent mixture and mix until homogeneous.

Assay Procedure—Proceed as directed in the assay procedure for isoniazid.

RESULTS AND DISCUSSION

Mobile Phase—The mobile phase may contain either methylene chloride or chloroform as its main constituent. The initial separations were accomplished using methylene chloride, but lot-to-lot variations with this solvent were more pronounced than with chloroform.

Some batches of methylene chloride contained impurities which deactivated the surface of the column packing after 2–3 liters of mobile

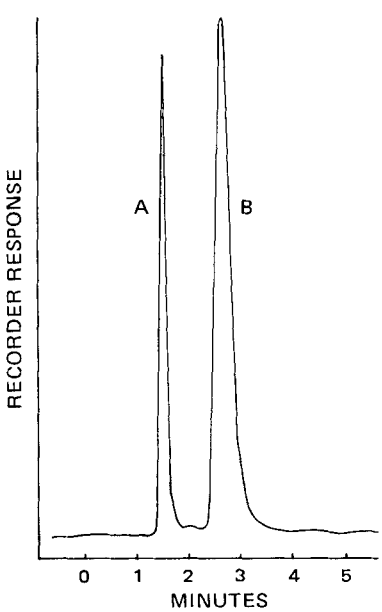

Figure 1—*Typical chromatogram obtained from the analysis of powdered tablet material. Key: A, internal standard (3.0 μg/liter); and B, isoniazid (1.0 μg/liter).*

[1] Eastman Kodak Co., Rochester, N.Y.
[2] Matheson, Coleman and Bell, Norwood, Ohio.
[3] Eastman No. 6061.
[4] Fisher Scientific Co., Fairlawn, N.J.
[5] LDC Minipump, Milton Roy Co., Riviera Beach, Fla.
[6] Laboratory Data Control, Riviera Beach, Fla.
[7] Hamilton model No. 701.
[8] Omniscribe model 5211-151, Houston Instruments, Austin, Tex.
[9] Model 3380A, Hewlett-Packard, Avondale, Pa.
[10] HC Pellosil, Whatman, Inc., Clifton, N.J.
[11] Scientific Glass Apparatus, Bloomfield, N.J.
[12] Model 7413, Leeds and Northrop, North Wales, Pa.

Table I—Determination of Isoniazid by the HPLC and USP XIX Methods

Drug Entity	Manufacturer	Label Claim	Method of Analysis			
			HPLC	n^a	USP XIX	n
Powder	C	—	99.24 ± 0.41[b] 98.75 ± 0.42	4 12	98.14 ± 0.18	6
Tablet	B	100 mg/tablet	98.45 ± 0.44 98.36 ± 0.69	5 20	97.68 ± 0.28	8
Tablet	A (Lot 1)	100 mg/tablet	85.96 ± 0.63[c]	8	95.53 ± 0.24	6
Tablet	A (Lot 2)	100 mg/tablet	93.23 ± 0.96[c]	3	98.41 ± 0.23	6
Injection	A	100 mg/ml	104.31 ± 0.41 104.23 ± 0.43	6 19	103.13 ± 0.48	5

[a] Number of samples analyzed. [b] Percent of label claim ± *SD*. [c] Tablets were shown to contain isoniazid as the lactose hydrazone (see text).

phase passed over it. Moreover, the compound(s) responsible for this effect could not be removed by distillation or by passing the solvent through a column packed with 6–16-mesh silica gel. For batches of solvent containing these deactivating impurities, a suitable level of purity may be attained by passing the solvent twice through 2.8 × 25-cm columns of small particle (76–230 mesh) silica gel 60.

Because the solvent properties of chloroform and methylene chloride are very similar, there were only slight differences in the separations achieved when either of them was in the mobile phase. As a measure of the efficiency of the separation of tribenzylamine and isoniazid, the resolution factor, R, was calculated as described in USP XIX (10). For the methylene chloride-based mobile phase, R was 4.12; for the chloroform-based system, it was 3.14. Either of these results is indicative of a separation greater than 99%.

The overlap between the isoniazid and internal standard peaks was, therefore, slightly greater when chloroform was used, but no effect on the quantitative results was noted whether peak heights or areas were used. No significant difference (t-test) was found between samples of the same dosage forms when analyzed using chloroform or methylene chloride.

Chromatographic Response—The typical response of the internal standard and isoniazid to the chromatographic system is shown in Fig. 1. This chromatogram was obtained from the analysis of a quantity of powdered tablet material containing 100 mg of isoniazid. A methylene chloride-based mobile phase was used.

To determine the linearity of the chromatographic response, a calibration curve was run in which the concentration of the internal standard was maintained constant while that of isoniazid was varied. The curve was linear (r = 0.999) over a 10-fold range, corresponding to quantities of isoniazid that would be obtained in the analysis of dosage forms containing 20–200% of the label claim. The intercept was essentially zero.

The integration method used to measure the response of each component was not critical, since both peaks were nearly symmetrical and almost completely resolved. Peak heights, peak areas obtained by manual methods (height times width at half-height), and electronic integration were used, and similar results were obtained.

Analysis of Dosage Forms—Table I gives the results obtained from the analysis of a number of commercially available dosage forms containing isoniazid. To verify the reliability of the method, portions of the same material taken for analysis by HPLC were also assayed using the method official in USP XIX. The results obtained by the HPLC method were comparable in accuracy and precision to those given by the USP method, with the exception of the two different lots of tablets from Manufacturer A (Table I). These lots gave results consistently 5–10% lower in isoniazid content than indicated by the USP method. Since it was assumed initially that the cause of the lower results was incomplete extraction of the isoniazid from the powdered tablet mass, various techniques, including heating, longer extraction times, and the use of an ultrasonic generator, were employed to improve recoveries. However, none of these procedures resulted in an increase in the quantity of isoniazid found.

To determine the cause of the anomalously low results, a study was initiated based on the observations (9) that isoniazid was capable of interacting with dosage form ingredients, particularly reducing sugars, to form hydrazones. These derivatizations were reported to occur even in the solid state. A portion of powdered material from the tablets that assayed at 85.90% isoniazid by HPLC was extracted by shaking with methanol. The same was done to the powdered mass of a tablet that had been shown to contain 99.24% isoniazid by HPLC.

Aliquots of the filtered extracts were applied to a TLC sheet along with methanol solutions of isoniazid and its lactose hydrazone. After development in methanol–chloroform (60:65), the lane containing the extract of the anomalous tablet showed two spots under UV light. The R_f of one spot corresponded to that of isoniazid; the other was the same as that of its lactose hydrazone. The lane containing the extract of the normal tablet had only one spot, that of pure isoniazid.

It was apparent, therefore, that the low results were due to the derivatization of a portion of the isoniazid by the lactose used as a diluent in the tablet formulation. Blake *et al.* (4) reported, and this work has verified, that the USP analysis does not distinguish between free isoniazid and the hydrazone it forms with a reducing sugar. The fact that HPLC is stability indicating in this regard is considered advantageous in view of the observations of Rao *et al.* (11), which indicated that the absorption of isoniazid from syrups containing reducing sugars was seriously impaired. Attempts to elute the lactose hydrazone of isoniazid using the conditions specified for the analysis of isoniazid were unsuccessful, with no peaks being observed even after 1 hr. Since the hydrazones formed by isoniazid with other reducing sugars would have similar structures and polarities, it is assumed that they too will not interfere with the HPLC method.

Potential Interfering Substances—Retention times were determined, using a chloroform-based mobile phase, for a number of compounds that might potentially interfere with the HPLC analysis. The results (Table II) indicate that the method retains its specificity despite the presence of degradation products of isoniazid or of other drugs frequently administered in combination dosage forms with isoniazid.

Two possible degradation products, isonicotinic acid and *N,N'*-bis-(isonicotinoyl)hydrazine, were completely separated. Aminosalicylic acid, an antitubercular drug available in combination with isoniazid, was also completely resolved by HPLC. Pyridoxine hydrochloride, which is administered concurrently with isoniazid to counteract the latter's antagonistic effect on vitamin B_6 activity, also did not interfere.

Table II—Retention Times of Isoniazid, Tribenzylamine, and Potential Interferences

Compound	Retention Time, min
Isoniazid	2.3
Tribenzylamine (internal standard)	1.5
Isonicotinic acid	44.0
Niacin	57.0
N,N'-Bis(isonicotinoyl)hydrazine	8.6
1-Isonicotinoyl-2-lactosylhydrazine	>60.0
Aminosalicylic acid	34.5
Pyridoxine hydrochloride	4.1

REFERENCES

(1) T. Canback, *J. Pharm. Pharmacol.*, **4**, 407 (1952).

(2) "The United States Pharmacopeia," 19th rev., Mack Publishing Co., Easton, Pa., 1975, pp. 272–274, 626, 772.

(3) "The British Pharmacopoeia," Pharmaceutical Press, London, England, 1968, p. 535.

(4) M. I. Blake, D. Bode, and H. J. Rhodes, *J. Pharm. Sci.*, **63**, 1303 (1974).

(5) S. Pinzauti, V. Dal Piaz, and E. La Porta, *ibid.*, **63**, 1446 (1974).

(6) H. S. I. Tan, *ibid.*, **62**, 993 (1973).
(7) J. T. Stewart and D. A. Settle, *ibid.*, **64**, 1403 (1975).
(8) R. Yamamoto and H. Tanaka, *Yakuzaigaku*, **17**, 219 (1957).
(9) W. H. Wu, T. F. Chin, and J. L. Lach, *J. Pharm. Sci.*, **59**, 1234 (1970).
(10) "The United States Pharmacopeia," 19th rev., Mack Publishing Co., Easton, Pa., 1975, p. 640.
(11) K. V. N. Rao, S. Kallasam, V. K. Menon, and L. Radhakrishna, *Indian J. Med. Res.*, **59**, 1343 (1971).

ACKNOWLEDGMENTS AND ADDRESSES

Received March 29, 1976, from the *Department of Pharmaceutical Chemistry, College of Pharmacy, Rutgers—the State University, New Brunswick, NJ 08903.*

Accepted for publication June 9, 1976.

The authors express their appreciation to Carole A. Bailey for technical assistance and to Dr. Thomas Medwick for helpful discussions.

[x] To whom inquiries should be directed.

Comparative Pharmacokinetics of Coumarin Anticoagulants XXI: Effect of Plasma Protein Binding on Distribution Kinetics of Warfarin in Rats

AVRAHAM YACOBI and GERHARD LEVY [x]

Abstract □ The purpose of this investigation was to determine the effect of plasma protein binding on the pharmacokinetic parameters for warfarin that are used conventionally to describe its distribution kinetics on the basis of the time course of plasma warfarin concentrations. Following rapid intravenous injection, warfarin concentrations in the plasma of 14 selected adult male rats declined triexponentially, with the terminal exponential phase starting at about 5 hr. The free fraction, f, of warfarin in the serum of individual animals ranged from 0.303×10^{-2} to 2.89×10^{-2}. The parameters of the equation $C_t = Pe^{-\pi t} + Ae^{-\alpha t} + Be^{-\beta t}$ for plasma concentration C_t at time t were obtained from the experimental data by nonlinear least-squares computer fitting and varied markedly between animals. Strong and highly statistically significant positive correlations with f were obtained for P, B, and β, but no significant correlation was found for A, π, and α. Rate constants and apparent volumes for a three-compartment open mammillary model with elimination from the central compartment were calculated. No apparent correlation was found between f and the intercompartment distribution rate constants. However, strong positive correlations between f and the elimination rate constant, the volume of the central compartment, and the volume of distribution, V_{area}, were observed. There also was a strong linear correlation between f and total clearance. Excellent replication of the experimental data was obtained when the experiments were repeated in some animals after 2 weeks. A detailed analysis of practical pharmacokinetic problems associated with and revealed by such repeated experiments is presented.

Keyphrases □ Warfarin—distribution kinetics, effect of plasma protein binding, rats □ Distribution—pharmacokinetic parameters, effect of plasma protein binding, rats □ Pharmacokinetics—warfarin distribution, effect of plasma protein binding, rats □ Binding, plasma protein—effect on warfarin distribution kinetics, rats □ Protein binding, plasma—effect on warfarin distribution kinetics □ Anticoagulants—warfarin, distribution kinetics, effect of plasma protein binding, rats

Plasma protein binding is a major determinant of the elimination kinetics of warfarin in rats (1) and humans (2). Consistent with theoretical considerations (3), the total clearance, TC, of warfarin by the body was found to be proportional to the free fraction, f, of warfarin in plasma or serum. Wide intersubject differences in f and, consequently, in the TC of warfarin have been observed.

The purpose of this investigation was to determine the relationship between f and the pharmacokinetic constants conventionally used to describe the kinetics of warfarin distribution on the basis of the time course of drug concentrations in plasma after rapid intravenous injection. A detailed analysis of certain practical problems of pharmacokinetic data interpretation was also undertaken.

EXPERIMENTAL

Single 3-ml blood samples were obtained from 63 adult male Sprague–Dawley rats[1] for determination of f, and 14 animals with widely differing f values were selected for further study. Three weeks later, with

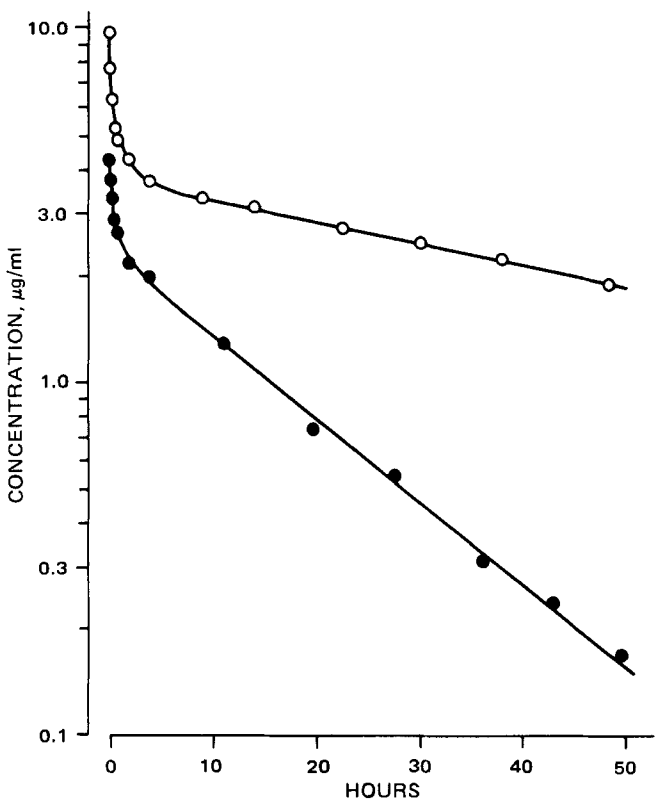

Figure 1—*Warfarin concentration in the plasma of two rats as a function of time after intravenous injection of* ^{14}*C-warfarin, 0.51 mg/kg. Key:* ●, *Rat 1; and* ○, *Rat 14. The curves were fitted to the data by a nonlinear least-squares computer program.*

[1] Blue Spruce Farms, Altamont, N.Y.