REFERENCES

(1) S. E. Unger, R. G. Cooks, R. Mata, and J. L. McLaughlin, *J. Am. Chem. Soc.*, in press.

(2) J. Strombom and J. G. Bruhn, *Acta Pharm. Suec.*, **15**, 127 (1978).

(3) R. Mata and J. L. McLaughlin, *Phytochemistry*, in press.

(4) J. E. Lindgren and J. G. Bruhn, *Lloydia*, **39**, 464 (1976).

(5) J. L. McLaughlin and A. G. Paul, *ibid.*, **29**, 315 (1966).

(6) R. Mata, M.S. thesis, Purdue University, West Lafayette, Ind., Dec. 1976, pp. 107–122.

(7) P. W. Doetsch, J. M. Cassady, and J. L. McLaughlin, *J. Chromatogr.*, in press.

(8) B. N. Meyer, Y. A. H. Mohamed, and J. L. McLaughlin, *Phytochemistry*, in press.

(9) A. G. Paul, *Lloydia*, **36**, 36 (1973).

(10) A. A. Chachoyan, B. T. Garibdzhanyan, and E. Z. Markaryan, *Biol. Zh. Arm.*, **25**, 102 (1972) (Russ.); through *Chem. Abstr.*, **78**, 52538m (1973).

(11) L. E. Hollister and A. J. Friedhoff, *Nature*, **210**, 1377 (1966).

(12) C. Backeberg, "Cactus Lexicon," English ed., Blandford, Poole, Dorset, England, 1977, p. 80.

(13) A. C. Gibson and K. E. Horak, *Ann. Mo. Bot. Gard.*, **65**, 999 (1978).

(14) R. L. Ranieri and J. L. McLaughlin, *J. Org. Chem.*, **41**, 319 (1976).

(15) R. L. Ranieri and J. L. McLaughlin, *J. Chromatogr.*, **111**, 234 (1975).

ACKNOWLEDGMENTS

Supported in part by BRSG RR-05586 from the National Institutes of Health and a grant from the Cactus and Succulent Society of America. R. Mata acknowledges fellowship support from the Departmento de Becas del Consejo de Desarrollo Cientifico y Humanistico de la Universidad Central de Venezuela.

Thanks are given to Dr. Arthur C. Gibson for suggesting this project and for supplying the plant material.

Determination of Two Fenamates in Plasma by High-Performance Liquid Chromatography

CHIEN K. LIN, CHING S. LEE ˣ, and JOHN H. PERRIN

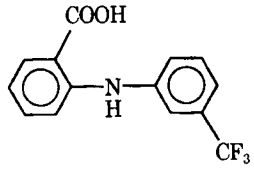
Received March 6, 1979, from the *Department of Pharmacy, College of Pharmacy, University of Florida, Gainesville, FL 32610.* Accepted for publication July 2, 1979.

Abstract □ A high-performance liquid chromatographic determination of two fenamates in human plasma is described. Plasma samples, 1.0 ml, to which 4 μg of internal standard had been added, were extracted with carbon tetrachloride under acidic conditions. Portions of the organic layer were transferred and evaporated to dryness under nitrogen. Residues were dissolved in methanol, and an aliquot was injected into the liquid chromatograph. An intermediate polarity, bonded cyanopropylsilane column was used with a mobile phase of water–acetonitrile–acetic acid (60:30:10 v/v/v). The flow rate was 1 ml/min, and the effluent was monitored at 254 nm. Flufenamic acid and mefenamic acid had retention times of 10.4 and 9.2 min, respectively. In the 1–10-μg range, the mean flufenamic acid recovery from control plasma was 100.7 ± 3.4% (n = 18). A typical calibration curve had a regression equation of $y = 0.132x - 0.04$ with $\gamma^2 = 0.99$. Preliminary stability tests showed that flufenamic acid is stable for at least 2 weeks in plasma after freezing.

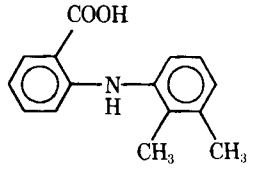
Keyphrases □ Flufenamic acid—analysis, high-performance liquid chromatography, plasma, rats, humans □ Mefenamic acid—analysis, high-performance liquid chromatography, plasma, rats, humans □ High-performance liquid chromatography—analysis, flufenamic acid, mefenamic acid, plasma, rats, humans

Flufenamic acid[1] (I) and mefenamic acid[1] (II) are potent nonsteroidal analgesic and anti-inflammatory agents used in the management of rheumatoid arthritis. Spectrophotometric (1, 2), colorimetric (3), and fluorometric (2, 4, 5) methods have been applied for fenamate analysis in aqueous solution and in biological samples such as plasma, urine, and milk. A fluorometric method for the determination of I in the nanogram range used a chamber paper analysis apparatus (6). A convenient TLC technique was reported for screening three fenamates and their metabolites (7).

Only one GLC method (8) utilizing electron-capture detection has been reported. Although it was described for II, it could be adapted to the analysis of I in blood and urine. However, details of the assay were not provided (8).



I



II

This article describes the high-performance liquid chromatographic (HPLC) determination of plasma fenamate levels. A single extraction step is followed by reversed-phase chromatography, eliminating the tedious and time-consuming procedures required by the previously reported methods (3, 7). Flufenamic acid and mefenamic acid can be internal standards for each other during either assay. The use of an internal standard improves both the precision and the accuracy of plasma level determination.

EXPERIMENTAL

Apparatus—Fenamate analyses were carried out on a liquid chromatograph[2] equipped with dual-delivery pumps[3], a single injector[4], and a single-chamber UV absorbance detector[5].

Reagents—Carbon tetrachloride[6], acetic acid[6], and sulfuric acid[7] were analytical reagent grade. Methanol[8] and acetonitrile[8] were distilled in glass. Solvents including distilled, deionized water were filtered routinely through 0.45-μm filters[9] prior to use in the liquid chromatograph.

[1] Provided by Parke-Davis and Co., Detroit, Mich.

[2] Model 204, Waters Associates, Milford, Mass.
[3] Model 6000A, Waters Associates, Milford, Mass.
[4] U6K, Waters Associates, Milford, Mass.
[5] Model 440, Waters Associates, Milford, Mass.
[6] Mallinckrodt, St. Louis, Mo.
[7] Eastman Kodak, Rochester, N.Y.
[8] Burdick & Jackson, Muskegon, Mich.
[9] Millipore Corp., Bedford, Mass.

0022-3549/80/0100-0095$01.00/0

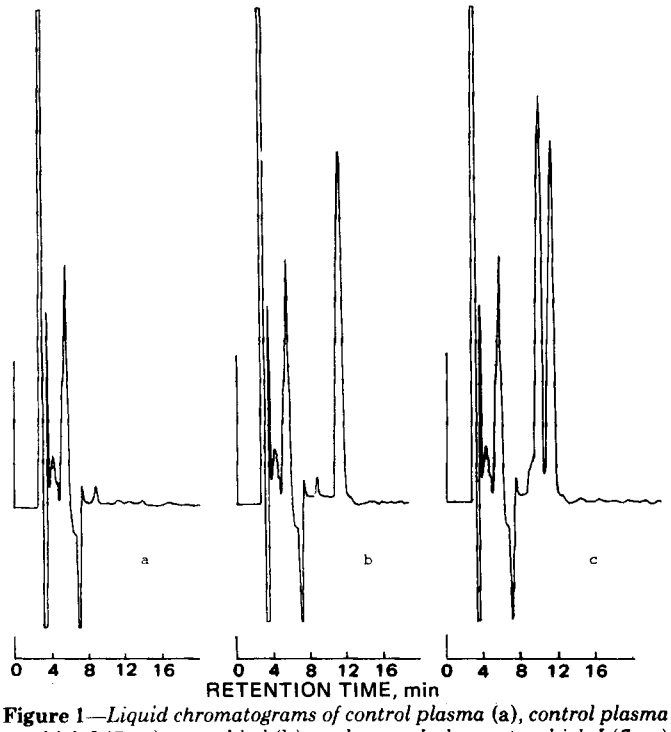


Figure 1—*Liquid chromatograms of control plasma* (a), *control plasma to which I* (7 μg) *was added* (b), *and control plasma to which I* (7 μg) *and II* (4 μg) *were added* (c).

Preparation of Standards—Ten milligrams of each fenamate was dissolved in methanol to yield a 100-μg/ml stock solution. Working standard solutions of I containing 1, 2, 3, 5, 7, and 10 μg/ml were prepared by diluting the stock solution with methanol–water (1:1 v/v). Six calibration samples were used in each run to establish the HPLC standard curve and to determine recovery reproducibility. Mefenamic acid solution, 4 μg/ml, served as the internal standard.

Measurement of Plasma I—Plasma samples, 1.0 ml, to which 4 μg of internal standard had been added, were mixed thoroughly by hand shaking and acidified with 0.9 *M* H_2SO_4. After the addition of 8 ml of carbon tetrachloride, the sample tubes were spun on a test tube rotator for 15 min and centrifuged. The aqueous phase was removed by aspiration. Portions of the carbon tetrachloride were transferred to another tube and evaporated to dryness under nitrogen. Residues were dissolved in 0.5 ml of methanol, and a 40-μl aliquot was injected into the liquid chromatograph.

The standard plasma curve was constructed by plotting the peak height ratios (I/II) *versus* the weight content of I. The peak height ratio of an unknown sample was used to determine the amount of I present. The standard samples were run concurrently with the unknown samples as described previously.

Chromatographic Conditions—A stainless steel column (30 cm × 4 mm i.d.) was packed with a stable reversed-phase stationary phase of porous silica beads (mean diameter 10 μm) coated with chemically bonded cyanopropylsilane monolayers[10]. The mobile phase was water–acetonitrile–acetic acid (60:30:10 v/v/v). The operating temperature was ambient, and the flow rate was 1 ml/min with an operating pressure of 1000 psi. The column effluent was monitored continuously at 254 nm with a full-scale deflection of 0.02 aufs. A short wash (acetonitrile for 15 min followed by chloroform for 10 min at 1 ml/min) at the end of each analytical day removed strongly retained solutes.

RESULTS AND DISCUSSION

Acceptable separation of the fenamates in this reversed-phase system was dependent on the amount of organic solvent in the mobile phase and on the effective pH of the aqueous component. A simple acetonitrile–water system (30:70 v/v) with an effective pH of 5.5 did not resolve the solutes. Since the fenamates are moderate acids (pKa 3.9 for I and 4.2 for II), their capacity factors were increased by lowering the effective pH of this solvent, and the water–acetonitrile–acetic acid system (60:30:10 v/v/v, effective pH 2.7) resolved the two solute peaks.

[10] μBondapak CN, Waters Associates, Milford, Mass.

Table I—Flufenamic Acid (I) Recovery from Human Plasma (n = 3)

I Added, μg	I Found, μg	Recovery[a], %
1	1.028 ± 0.031	102.8 ± 3.1
2	2.090 ± 0.430	104.5 ± 4.3
3	2.760 ± 0.079	92.0 ± 7.9
5	5.260 ± 0.051	105.0 ± 5.0
7	7.000 ± 0.000	100.0 ± 0.0
10	10.000 ± 0.000	100.0 ± 0.0
1–10[b]	—	100.7 ± 3.4

[a] Expressed as mean ± *SD*. [b] n = 18.

Table II—Peak Height Ratios for the Calibration Curve

I Added, μg	Peak Height Ratios	*SD*
1	0.099	0.003
2	0.230	0.010
3	0.355	0.026
5	0.605	0.025
7	0.857	0.015
10	1.297	0.021

Figure 1 presents liquid chromatograms of control human plasma, control plasma to which I was added, and control plasma to which both fenamates were added. As indicated in Fig. 1*a*, negligible interfering peaks were found in the control plasma specimens. Under the described chromatographic conditions, I and II had retention times of 10.4 and 9.2 min, respectively. Better resolution for the two fenamates can be obtained at a slower flow rate. However, no real benefits (*e.g.*, precision in the measurement of peak height ratios) were gained using prolonged retention times.

The results obtained following analyses of various amounts of I in control human plasma are summarized in Table I. In the 1–10-μg range, the mean I recovery from control plasma was 100.7 ± 3.4% (n = 18). Table II contains peak height ratios obtained from calibration curves prepared with human plasma. Each value represents the average of four samples at each concentration. A typical calibration curve prepared by extracting human plasma samples containing different concentrations of I and 4 μg of II had a regression equation of $y = 0.132x - 0.04$ with $\gamma^2 = 0.99$. The UV absorption was linear between 1 and 10 μg, where therapeutic concentrations can be interpolated conveniently. The lower detection sensitivity limit was 1 μg/ml of plasma.

When UV absorption spectra of I and II were obtained in methanolic solutions, the maximum absorption wavelength, λ_{max}, was 290 nm. Since the chromatograph was equipped with a single-wavelength detector at 254 nm, the optimal condition has not yet been achieved in this assay. Higher detecting sensitivity can be obtained by monitoring the absorption at 290 nm with a variable-wavelength UV detector.

Preliminary 2-week stability tests were performed by dissolving 1–10 μg of I in 1 ml of plasma, which was then frozen for 3, 7, or 15 days; I was stable in frozen plasma for at least 2 weeks (Table III).

Table III—Effect of Frozen Storage on the Flufenamic Acid Stability in Human Plasma

I Added, μg	Days			
	0	3	7	15
1	0.98	1.20	1.04	1.00
2	1.91	2.35	2.19	1.93
3	3.21	3.11	3.12	2.72
5	4.79	—	4.96	4.88
7	7.03	6.76	7.07	6.74
10	10.07	9.85	10.57	9.76

Table IV—Plasma Flufenamic Acid Levels in Two Rats at Various Times after Intravenous Sublingual Administration of 5 mg of I/kg in 0.2 *N* NaOH

Minutes	Plasma Levels, μg/ml	
	Rat 1	Rat 2
15	—	120
20	134	—
30	110	128
60	82.3	90.6

Experiments utilizing this procedure in animals are in progress. Plasma flufenamic acid levels in two rats at various times after intravenous sublingual administration of 5 mg of I/kg in 0.2 *N* NaOH are shown in Table IV. Due to the high plasma I concentrations at the dose administered, only 50 μl of the plasma sample was taken for analysis. The assays described here may be used to measure therapeutic levels of I in patients.

REFERENCES

(1) J. B. Carey, *J. Clin. Invest.*, **40**, 1028 (1961).

(2) A. R. Rosenberg and T. R. Bates, *Proc. Soc. Exp. Biol. Med.*, **145**, 93 (1974).

(3) G. Deveaux, P. Mesnard, and A. M. Brisson, *Ann. Pharm. Fr.*, **27**, 239 (1969).

(4) A. J. Glazko, *Ann. Phys. Med. Suppl.*, **9**, 24 (1967).

(5) H. D. Dell and R. Kamp, *Arch. Pharm.*, **303**, 785 (1970).

(6) W. S. Schmollack and U. Wenzel, *Pharmazie*, **29**, 583 (1974).

(7) B. Demetriou and B. G. Osborne, *J. Chromatogr.*, **90**, 405 (1974).

(8) S. A. Bland, J. W. Blake, and R. S. Ray, *J. Chromatogr. Sci.*, **14**, 201 (1976).

ACKNOWLEDGMENTS

Presented at the APhA Academy of Pharmaceutical Sciences, Anaheim meeting, April 1979.

Supported in part by the Division of Sponsored Research, University of Florida.

The authors thank Ms. Judy Fuller for manuscript preparation.

Antitumor Activity of Hydrazones and Adducts between Aromatic Aldehydes and *p*-(3,3-Dimethyl-1-triazeno)benzoic Acid Hydrazide

TULLIO GIRALDI *, PHYLLIS M. GODDARD ‡, CARLO NISI §x, and FABIO SIGON §

Received May 3, 1978, from the **Istituto di Farmacologia, Università di Trieste, I 34100, Trieste, Italy, the* ‡*Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, London SW3 6JB, England,* and the §*Istituto di Chimica Farmaceutica, Università di Trieste, I 34100, Trieste, Italy.* Accepted for publication June 1, 1979.

Abstract □ Hydrazones and adducts between aromatic aldehydes and *p*-(3,3-dimethyl-1-triazeno)benzoic acid hydrazide were synthesized and tested for antitumor activity. Two adducts derived from 2,6-dinitro- and 4-cyanobenzaldehyde were active against TLX5 lymphoma in mice. The hydrazone of the latter aldehyde was significantly more active than the adducts.

Keyphrases □ *Antineoplastic agents, potential*—hydrazones and adducts between aromatic aldehydes and *p*-(3,3-dimethyl-1-triazeno)-benzoic acid hydrazide, structure–activity relationships □ *p*-(3,3-Dimethyl-1-triazeno)benzoic acid hydrazide—antineoplastic activity, hydrazones and adducts with aromatic aldehydes, structure–activity relationships

A series of 1-aryl-3,3-dimethyltriazene derivatives, characterized by the presence of a carbonyl group and a triazene function in the *para*-position on the aromatic nucleus, was previously synthesized and examined for antitumor activity against TLX5 lymphoma in mice (1). Among these compounds, the *o*-nitrophenylhydrazone of the *p*-(3,3-dimethyl-1-triazeno)benzoic acid hydrazide and the adduct between this hydrazide and *p*-nitrobenzaldehyde showed considerable activity. Therefore, a further group of adducts and related hydrazones carrying electron-withdrawing substituents was synthesized and tested for their antitumor activity. Their structures and activities are reported in Tables I and II.

EXPERIMENTAL[1]

Adducts I–IV—Adducts I, II, and IV were prepared by the addition of a solution of the aldehyde in hot ethanol to an equimolar solution of *p*-(3,3-dimethyl-1-triazeno)benzoic acid hydrazide (2) in the same solvent. After standing for a few minutes, the products precipitated and were washed with a few milliliters of cold ethanol. Methanol was used as a solvent for preparing adduct III, and this reaction mixture was heated gently for 15 min before allowing the precipitation.

The structures assigned to I–IV are in accordance with their elemental analyses and are supported by the following data:

1. When these substances were heated to 120°, they lost a molecule of water, yielding the corresponding hydrazone, except for III which decomposed.

2. TLC mobilities and UV spectra of these compounds were quite different from those of the relevant hydrazones, so they cannot be simply monohydrates of these substances. Furthermore, all of these compounds exhibited a strong characteristic band at 1040 cm^{-1} attributable to CO stretching, which disappeared after heating. In addition, the UV spectra of I–IV in ethanol corresponded to the sum of the spectra of the starting hydrazide and aldehydes. TLC on silica gel of these substances at very low concentrations showed that they are split, giving two spots corresponding to the starting reagents; the hydrazones, however, gave single spots. The very poor solubilities of these substances did not allow any NMR investigations of their structures.

Hydrazones V–VII—These hydrazones were prepared by heating the corresponding adducts for a few hours at 120°. The completion of the conversion was determined by TLC and by the disappearance of the strong characteristic band at ~1040 cm^{-1}; the yield was quantitative since no further recrystallization was required.

RESULTS AND DISCUSSION

Tests of the triazene derivatives against TLX5 lymphoma[2] in mice are reported in Table III. The activities observed for each compound were compared on the basis of the number of dose levels at which an ILS of ~50% was observed and by considering also the maximum ILS values obtained when >50%. The compounds carrying nitro groups as substit-

[1] Melting points were determined in open glass capillaries using a Büchi apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer model 225 spectrophotometer, and UV spectra were determined on a Hitachi Perkin-Elmer model 124. Kieselgel HF 254 + 366 (Merck) and methanol–ethyl acetate–ligroin (3:2:1) were used for TLC.

[2] TLX5 lymphoma cells (10^5) were injected subcutaneously in the inguinal region of CBA/LAC female mice, 20–25 g, bred in the Chester Beatty Research Institute. The drugs were used as a solution freshly prepared by sonication in acetone–arachis oil (10:90 v/v); the treatment was performed by daily intraperitoneal administration from Day 3 to 7 after tumor inoculation. For each substance, 10 control mice and groups of five mice for each dose level were used. ILS is the percent increase of the mean survival time of each treated group to that of the relevant controls.

0022-3549/80/0100-0097$01.00/0