Journal of Chromatography, 342 (1985) 135—143
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2607

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION AND DETERMINATION OF 2-(DIETHYLAMINO)ETHYL DIPHENYLPROPYLACETATE AND ITS METABOLITES FROM BIOLOGICAL SAMPLES

ZHIQIANG LIU and MICHAEL R. FRANKLIN*

Department of Biochemical Pharmacology and Toxicology, University of Utah, Salt Lake City, UT 84112 (U.S.A.)

(First received December 6th, 1984; revised manuscript received February 22nd, 1985)

SUMMARY

A high-performance liquid chromatographic method for the determination of 2-(diethylamino)ethyl diphenylpropylacetate (SKF 525-A; Proadifen), its two deethylated metabolites and a hydrolytic metabolite in biological samples has been developed. From many solvent systems investigated the compounds are best separated on an ODS/Sil-X column using an acetonitrile—phosphate buffer system containing 0.2% decylamine. The use of acetonitrile overcomes limitations associated with previously published methods for separating basic compounds where decylamine was used in a methanolic eluent. With the sampling procedures used, overall recoveries from biological tissues were around 80%. The chromatographic system also separates three tricyclic antidepressants which can be used as internal standards.

INTRODUCTION

2-(Diethylamino)ethyl diphenylpropylacetate (SKF 525-A; Proadifen; I) has been used as an inhibitor of cytochrome P-450 dependent oxidative drug metabolism for several decades [1]. As an alternative substrate it is a competitive inhibitor but because of its ability to form a cytochrome P-450 metabolic-intermediate (MI) complex, it also shows non-competitive inhibition characteristics [2]. Which of these two mechansims is operating when I is used in vivo has remained unclear due in part to the lack of pharmacokinetic data on I. In addition, information on the pharmacokinetics of its deethylated metabolites, which are also inhibitory [3], has been lacking.

0378-4347/85/$03.30

Despite its widespread use, very few investigations have been reported for assaying I and its metabolites in biological samples [3—6]. The reported methods only measured I [4], its deethylated metabolite (SKF 8742; II) [5], or both [3], and were either of low recovery [4] or lacked recovery data and quantitative information [3, 5, 6]. In addition, the gas chromatography used in those methods would generally be considered less ideal than high-performance liquid chromatography (HPLC) for the separation of any hydrolytic metabolites of I.

The research presented in this manuscript establishes an HPLC method, which separates I and its two deethylated metabolites, i.e. II and SKF 26754 (III) using an ODS stationary phase and a mobile phase of acetonitrile—phosphate buffer containing a competitive amine. In addition, an ion-suppression HPLC method for the measurement of the hydrolytic metabolite of I (SKF 2314; IV) and sampling procedures which show good recovery and suitability to the HPLC assays are described.

EXPERIMENTAL

Apparatus

The HPLC was performed using a Perkin-Elmer Series 3 liquid chromatograph with a Rheodyne 7105 sample injector and a Perkin-Elmer LC-75 variable-wavelength UV detector set at 220 nm. The column used was a 10-μm HC ODS/Sil-X reversed-phase column (300 × 2.6 mm, Perkin-Elmer, Norwalk, CT, U.S.A.). It was used at room temperature, 22°C. Retention volumes throughout are corrected for the dead volume of the system.

Materials

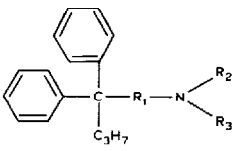
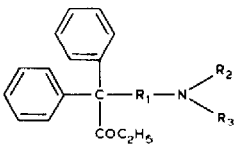
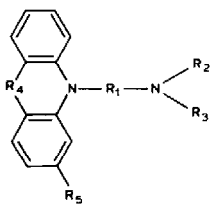
HPLC-grade organic solvents purchased from Fisher Chemical and water, double-distilled in glass, were used to make the eluents. Decylamine was purchased from Sigma. I, II, III, IV, chlorpromazine and trimeprazine were gifts from Smith Kline and French Labs. Imipramine was obtained from Ciba-Geigy. Methadone was supplied by Eli Lilly. The structures of all the compounds are shown in Table I.

Procedures

Fresh samples (2 g of liver or an adequate volume of blood) from a rat receiving I were homogenized with 4—5 ml of 0.15 *M* potassium chloride solution. An internal standard (5—25 μg of trimeprazine) and 1 ml of 1 *M* sodium hydroxide solution were then added to the homogenate. The mixture was shaken vigorously for 3—5 min with 3 ml of ethyl acetate and 1 g of anhydrous sodium sulfate, and then centrifuged for 3 min at 300 *g*. The ethyl acetate layer was aspirated away and the aqueous layer re-extracted twice with additional 3-ml aliquots of ethyl acetate. The ethyl acetate extracts were combined, and evaporated to dryness at 60°C under nitrogen and redissolved in 0.5 ml of heptane. This heptane solution was back-extracted twice with 0.25 of 0.1 *M* hydrochloric acid. The acid extract was made basic with a quarter volume of 2 *M* sodium hydroxide and mixed with an organic solvent so that the final sample solution (termed basic extract) was similar in composition to the

TABLE I

STRUCTURES OF I AND RELATED COMPOUNDS

Chemical structure	Compound	Code	R_1	R_2	R_3	R_4	R_5
	I	Et_2	$COOC_2H_4$	C_2H_5	C_2H_5	—	—
	II	HEt	$COOC_2H_4$	C_2H_5	H	—	—
	III	H_2	$COOC_2H_4$	H	H	—	—
	IV	COOH	COOH	—	—	—	—
	Methadone	Me	$CH_2CH(CH_3)$	CH_3	CH_3	—	—
	Imipramine	Im	$CH_2CH_2CH_2$	CH_3	CH_3	CH_2CH_2	H
	Trimeprazine	Tri	$CH_2CH(CH_3)CH_2$	CH_3	CH_3	S	H
	Chlorpromazine	Chlo	$CH_2CH_2CH_2$	CH_3	CH_3	S	Cl

eluent to be used in the reversed-phase HPLC separation. An aliquot (normally 50 μl) of this basic extract was injected into the HPLC column. After ten to twelve injections of the basic extract, the ODS/Sil-X column was washed with acetonitrile—water (9:1) for about 20 min and then re-equilibrated with about 20 ml of eluent to maintain high efficiency.

The procedure for the measurement of the hydrolytic metabolite of I was similar to that mentioned above except that *n*-heptane—ethyl acetate solution (9:1) was used to extract the acidic metabolite from the homogenate, which had been adjusted to a pH of 3—4 with 1 *M* phosphoric acid—phosphate solution, pH 2.1 and the back-extraction of the heptane solution of the residue was performed with 0.2 *M* sodium hydroxide solution. The alkaline extract was made acidic with a quarter volume of 2 *M* hydrochloric acid and mixed with organic solvent to make the sample solution (termed acidic extract) similar in composition to the eluting solvent. In addition, an external standard method was used to quantitate the acidic metabolite (IV).

RESULTS AND DISCUSSION

To determine the pharmacokinetics and in vivo concentrations of I and its metabolites an HPLC method with good separation, short assay period and high sensitivity was required. Of several reported methods for separating and assaying basic drugs, the most attractive appeared to be ion-pair HPLC. With this approach, many basic drugs including orphenadrine, catecholamines and chlorpromazine can be easily separated [7—13]. In addition, this system enables acidic components to be separated in the same chromatographic process using the principle of ion-suppression reversed-phase HPLC [14]. Preliminary experiments showed that using a solvent system of *n*-propanol—

acetonitrile—25 m*M* phosphoric acid—phosphate solution, pH 2.1 (1:1:2) containing 2.5 m*M* sodium dodecyl sulfate (SDS), the chromatographic peaks of I and its deethylated metabolite were sharp and symmetrical but their retention volumes were very similar (Table II, eluent E). Increasing the concentration of the counter-ion in the eluent, which improved the separation of orphenandrine, diphenhydramine and *o*-methylbenzhydrol, a neutral metabolite of orphenandrine [7], was not useful in separating I from its deethylated metabolites, and was detrimental to the peak shape. To investigate the possibility that the ion-pair formed from a large-molecular-weight counter-ion would mask the fine difference of chromatographic behavior of basic components, eluents containing simple acids as ion-pairing reagents [10, 12, 13, 16] were tested. It was found that simple acids such as acetic acid, phosphoric acid, trichloroacetic acid and perchloric acid were not helpful in the separation of I and its deethylated metabolites with the non-silylated ODS column.

Ion-suppression liquid chromatographic systems have been used to separate basic drugs [15, 17]. Using ion-suppression reversed-phase HPLC with an aqueous methanol eluent [15], I and II could be separated (Table II, eluent D) but the retention times were too short for the quantitation of I and its deethylated metabolites from biological samples. Reducing the proportion of methanol in the eluent in an attempt to increase retention times resulted in tailing peaks. When tetrahydrofuran was used as the organic reagent in the eluent for ion-suppression HPLC, good retention and well separated chromatographic peaks were obtained (Table II, eluent C), but ion-exchange interactions between sample and packing material were still evident as shown in Fig. 1 (i.e. the chromatographic behavior of eluted compounds except I was influenced by the concentration of salt in the eluent). This type of interaction

TABLE II

CHROMATOGRAPHY OF I AND ITS METABOLITES, AND OTHER BASIC DRUGS IN VARIOUS MOBILE PHASES

The mobile phases used were: (A) 53% acetonitrile and 0.20% decylamine in 40 m*M* phosphate buffer, pH 7.2; (B) 68% methanol and 0.24% decylamine in 25 m*M* phosphate buffer, pH 7.2; (C) 42% tetrahydrofuran in 25 m*M* phosphate buffer, pH 7.6; (D) methanol—2 *M* ammonium hydroxide—1 *M* ammonium nitrate (27:2:1) [15]; (E) propanol—acetonitrile—25 m*M* phosphate buffer, pH 2.1 (1:1:2) with 2.5 m*M* sodium dodecyl sulfate.

Compound	Retention volume (ml)				
	A	B	C	D	E
IV	0.90	1.22*			
III	1.80	1.70	1.74	0.48	
II	3.30	3.10	2.96	0.71	4.35
Methadone	3.90	3.12	2.72		
Imipramine	4.06	4.01	5.30	1.61	
Trimepramine	5.50	5.10			
Chlorpromazine	6.86	6.72	7.30	2.09	
I	8.94	6.91	7.72	1.07	4.50

*The retention volume of IV was unstable (see Fig. 2).

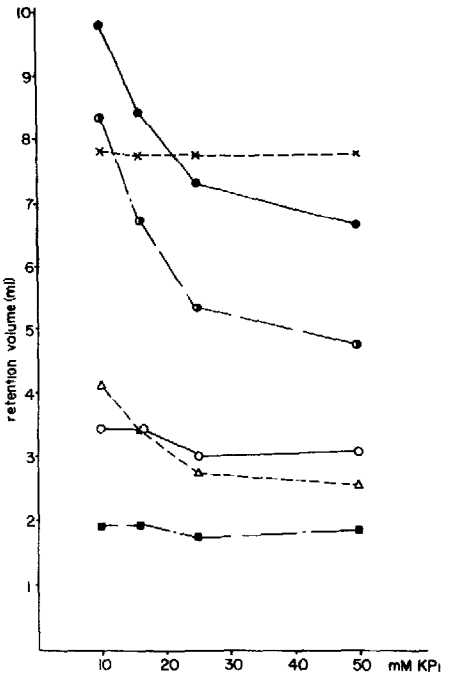


Fig. 1. The influence of mobile phase buffer molarity on the retention volume of I, its deethylated metabolites, and other basic drugs. The mobile phase was 42% tetrahydrofuran in 25 m*M* phosphate buffer, pH 7.6. The compounds investigated were: • = chlorpromazine, ◑ = imipramine, × = I, △ = methadone, ○ = II, and ■ = III.

caused tailing peaks and variable retention volumes. In addition, the high viscosity, the strong absorbance in the UV region and the higher expense made tetrahydrofuran inappropriate for routine assays.

An aqueous methanol solvent system containing decylamine as the competitive amine [18] showed suitable retention characteristics and symmetrical peaks for I and its deethylated metabolites (Table II, eluent B). However, when the basic extracts were assayed with this solvent system, a peak showing variable retention volume interfered with the quantitation of II. This interfering peak was found to be IV, the hydrolytic metabolite of I which was partly coextracted during sampling. While the retention volume increased in a normal manner with lowered pH in the absence of decylamine, in its presence, the increase in retention volume was extremely abrupt (Fig. 2). The increase in the retention volume of IV increased more rapidly than either of the deethylated metabolites (Fig. 2), causing a shift in the order in which peaks were eluted. When acetonitrile was investigated as a substitute for the methanol, IV did not show the rapid rise in retention volume with lowered pH (Fig. 2). Using acetonitrile, the presence of decylamine in the eluent was still necessary to maintain the shape of the chromatographic peaks (Fig. 3). When the apparent pH (pH meter reading) of the mobile phase was above 10, the retention volume of IV was maintained relatively constant. In addition to IV, I showed greater constancy in retention volume with pH changes in the acetonitrile eluent compared to the methanol eluent. Other advantages to the use

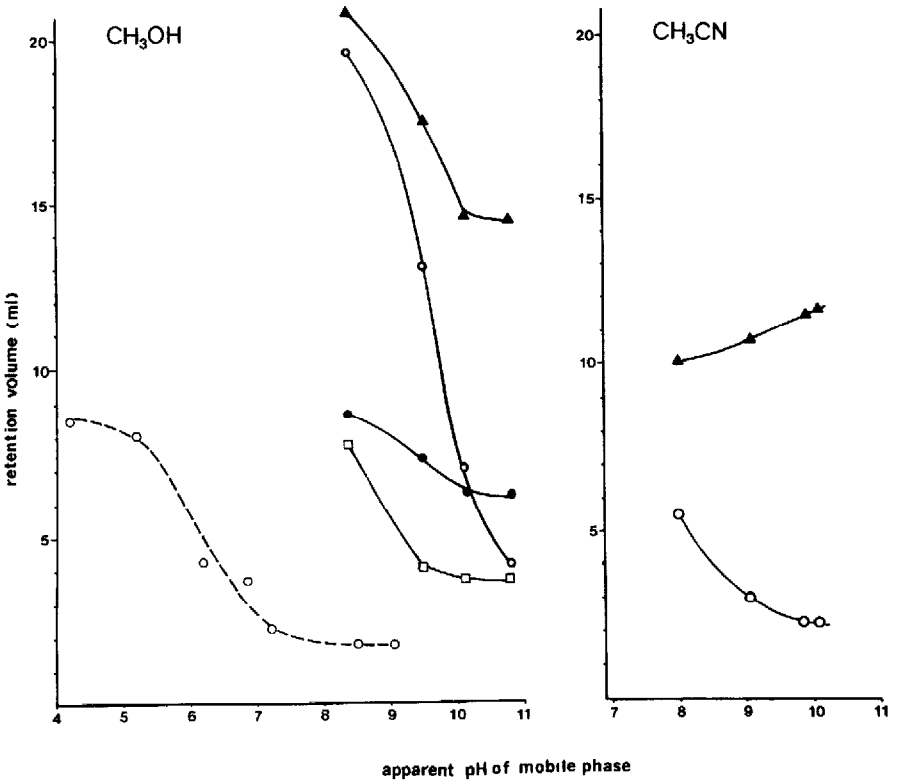


Fig. 2. The influence of composition and pH of the mobile phase on the retention volume of I and its metabolites. Eluents based on methanol (left) and acetonitrile (right) containing 25 m*M* phosphate buffer were investigated. Solid lines indicate the presence of 0.2% decylamine, broken lines its absence. The compounds investigated were: ▲ = I, ● = II, □ = III and ○ = IV.

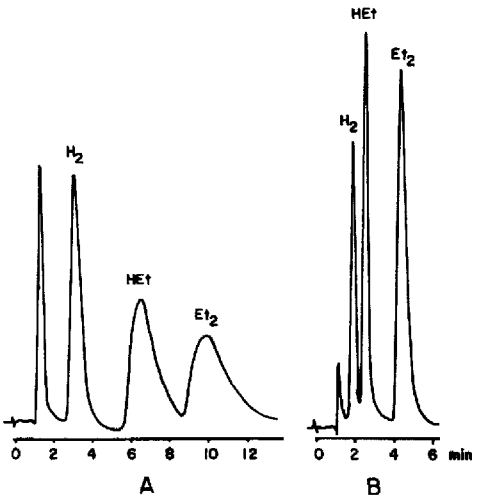
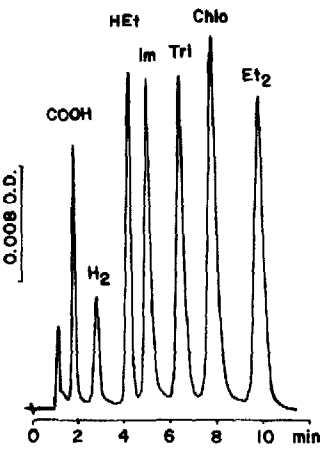


Fig. 3. The chromatographic traces of I, II and III obtained without (A) or with (B) 0.1% decylamine in the mobile phase (65% acetonitrile in 25 m*M* phosphate buffer, pH 7.5). The flow-rate was 1 ml/min and the eluent was monitored for absorbance at 220 nm. For peak identification, see Table I.



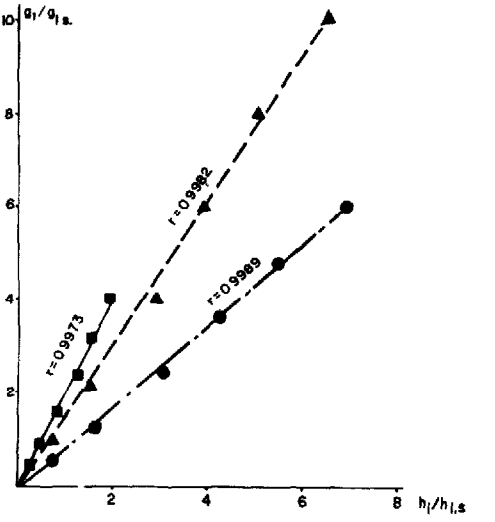


Fig. 4. Chromatogram of amine drugs and I and its metabolites. The mobile phase was 53% acetonitrile and 0.2% decylamine in 40 m*M* phosphate buffer, pH 7.2. The injected sample contained 0.05 μg IV, 0.2 μg III, 0.3 μg II, 0.5 μg each of imipramine · HCl, trimeprazine tartrate and chlorpromazine · HCl and I. The flow-rate was 1 ml/min and the eluent was monitored for absorbance at 220 nm. For peak identification, see Table I.

Fig. 5. The standard curves for quantitation of I and its deethylated metabolites using peak ratio ($h_i/h_{I.S.}$) versus weight ratio ($g_i/g_{I.S.}$). Trimeprazine was used as the internal standard (I.S.) at 5 μg/ml. Correlation coefficients (r) are given for each line. ■ = III, ● = II, ▲ = I.

TABLE III

RECOVERIES OF I, ITS METABOLITES AND THREE TRICYCLIC ANTIDEPRESSANTS FROM RAT LIVER OR BLOOD

Compound	Added (μg/g or μg/ml)	Percentage recovery* (mean ± S.D.)	Coefficient of variation (%)	*n*
Liver				
III	2	70.1 ± 10.8	15.4	5
	20	87.0 ± 4.7	5.4	4
II	3	93.3 ± 7.6	8.1	5
I	5	97.5 ± 7.3	7.5	5
IV	5	93.7 ± 8.2	8.8	5
Imipramine	2	90.3 ± 7.3	8.1	7
Trimeprazine	5	81.5 ± 7.5	9.2	6
Chlorpromazine	2.5	79.4 ± 9.8	12.4	5
Blood				
III	2	50.0 ± 6.8	13.6	4
II	3	93.7 ± 4.3	4.6	4
I	5	89.9 ± 3.5	3.9	4
Trimeprazine	5	85.9 ± 5.3	6.2	4

*The recovery was calculated based on external standard method for both blood and liver.

of acetonitrile were the lower column pressure and the ability to use shorter-wavelength light, and thereby increase the detection sensitivity for I and its metabolites (however, shorter-wavelength light reduces the sensitivity for the detection of the internal standard tricyclic antidepressants). With the aqueous part of the acetonitrile eluting system being 25—40 m*M* phosphate buffer, pH 7.2—7.5 rather than water, decylamine dissolution was facilitated and a more constant pH value was obtained for the eluent. For 40 m*M* phosphate buffer the apparent pH of the eluent was 10.2—10.5, but no deterioration of the ODS/Sil-X column was observed. Using this system, the chromatography of a number of compounds was investigated and showed excellent separation and peakshape (Table II, eluent A, and Fig. 4). In addition, the excellent linearity

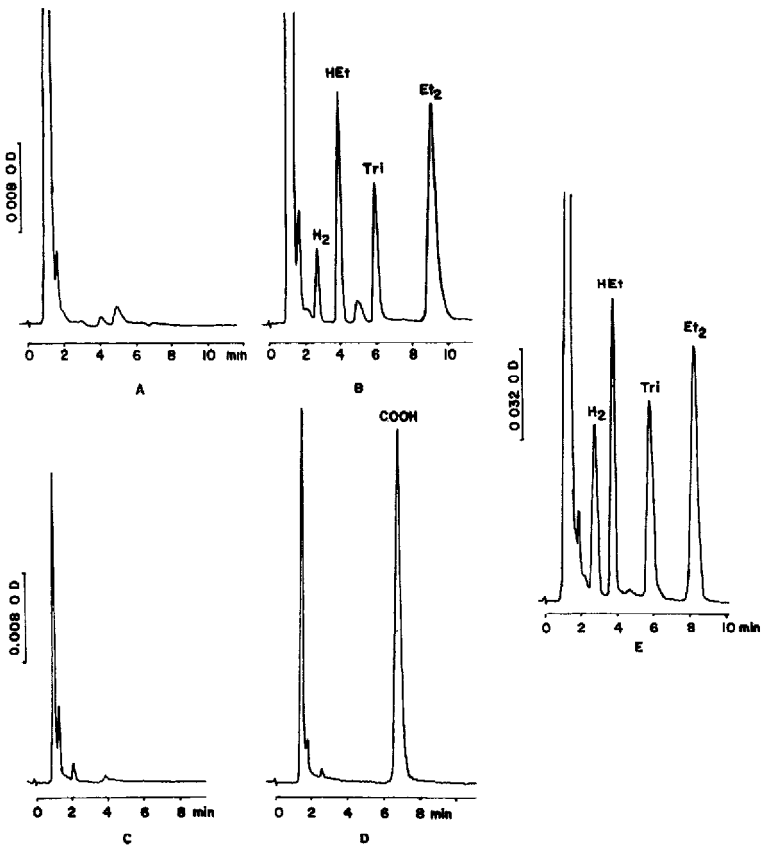


Fig. 6. Chromatograms of rat liver extracts. (A) Basic extract of liver from control rat. (B) Basic extract of control rat liver spiked with 4, 6, 5, and 10 μg/g III, II, trimeprazine internal standard and I, respectively. (C) Acid extract of liver from control rat. (D) Acid extract of liver spiked with 5 μg/g IV. (E) Basic extract of 50 mg liver sampled 1 h after 80 mg/kg I administration (intraperitoneally) to the rat. An internal standard was added at a concentration of 25 μg/g of liver. The concentrations of III, II and I, were 123.5, 81.5, and 141.0 nmol/g of liver, respectively. The mobile phases for the basic and acidic extracts were 53% acetonitrile and 0.2% decylamine in 40 m*M* phosphate buffer, pH 7.2 and 50% acetonitrile in 25 m*M* phosphate buffer, pH 2.1 respectively. The eluent was monitored for absorbance at 220 nm. For peak identification, see Table I.

of the standard curves (correlation coefficients of 0.997 to 0.999) facilitated quantitation (Fig. 5).

The recoveries of I and its metabolites and the three tricyclic antidepressants investigated as internal standards are presented in Table III. With the exception of III at low concentrations, i.e. 2 μg/g of liver (a value 30 times lower than the initial concentration of III present in the liver of rats administered 80 mg/kg I in a pharmacokinetic study [16]), the recoveries were reasonable. Attempts to increase recovery of III using a coextractable amine, as proposed by Fenimore et al. [19] were unsuccessful for this compound.

The elution profiles of freshly prepared basic extracts of whole liver homogenates both with and without the addition of I, its metabolites and internal standards are shown in Fig. 6. There were two small chromatographic peaks in the trace, which were unrelated to the compounds concerned. One was near the tail of the II peak and the other was at the position where imipramine elutes. In the chromatogram of the acid extract of liver, no interfering peak was present at the position of IV. The chromatographic traces of blood samples were similar to those obtained with liver extracts. Fig. 6 also shows a typical chromatogram of a basic extract of 50 mg of liver sampled 1 h after administration of I. The concentrations of III, II and I were 123.5, 81.5, and 141.0 nmol/g of liver, respectively.

ACKNOWLEDGEMENT

This investigation was supported by USPHS Grant No. CA 15760.

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