# Simultaneous Determination of Tegafur and 5-Fluorouracil in Serum by GLC Using Nitrogen-Sensitive Detection

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**Abstract** □ A sensitive assay of both tegafur (I) and 5-fluorouracil (5-FU) using GLC with a nitrogen–phosphorus-sensitive detector is described. The drugs were extracted from rabbit serum with ethyl acetate and methylated with diazomethane. Linearity was obtained over the concentration ranges of 3.13–200 μg/ml for I and 0.0313–2 μg/ml and 10–50 ng/ml for 5-FU. The detection limits of I and 5-FU in serum were 50 and 8 ng/ml, respectively. The serum concentrations of the drugs determined by the present method closely agreed with those obtained by spectrophotometry for I and microbial assay for 5-FU.

**Keyphrases** □ Tegafur—simultaneous determination with 5-fluorouracil, rabbit serum, GLC using nitrogen-sensitive detection □ 5-Fluorouracil—simultaneous determination with tegafur, rabbit serum, GLC using nitrogen-sensitive detection □ GLC—nitrogen-sensitive detection, tegafur and 5-fluorouracil, simultaneous determination in rabbit serum

Tegafur [5-fluoro-1-(tetrahydro-2-furyl)uracil (I)], a prodrug of 5-fluorouracil (5-FU), is widely used as an antitumor agent (1–3). Various assays for I and/or 5-FU in biological fluids, such as spectrophotometry (4), microbial (4, 5), GLC (6–15), GLC–mass spectrometry (6, 16–20), and high-performance liquid chromatography (HPLC) (20–30), have been reported. All of these methods either lack adequate sensitivity and specificity and/or involve complicated and lengthy procedures. This paper describes a highly sensitive GLC method using a nitrogen–phosphorus-sensitive detector for simultaneously determining I and 5-FU.

## EXPERIMENTAL

**Materials**—Tegafur (I)[1], 5-fluorouracil (5-FU)[2], and orotic acid[2] were used as received. Ethereal diazomethane solution was prepared according to the method of Arndt (31). All other chemicals and solvents were analytical reagent grade.

**GLC Determination**—The serum sample (1 ml) was adjusted to pH 6.3 with 0.1 ml of 0.5 *M* $NaH_2PO_4$ and extracted with 8 ml of ethyl acetate. After centrifugation at 2500 rpm for 10 min, the organic layer was removed and then evaporated to dryness under vacuum at room temperature. (With the lower concentration of 5-FU, <30 ng/ml, it was necessary to extract the serum sample with 8 ml of ethyl acetate twice.) The residue was dissolved in 0.1 ml of 30-μg/ml methanolic orotic acid and then 0.1 ml of ethereal diazomethane was added. The mixture was allowed to stand at room temperature for 30 min and then was evaporated to dryness under vacuum at room temperature. The residue was dissolved in 100 μl of acetone, and 1–2 μl of this solution was injected into a gas chromatograph[3] equipped with a nitrogen–phosphorus-sensitive detector for the measurement of I and 5-FU.

A 2 m × 3-mm i.d. glass column packed with 1% PEG-HT on 60–80 mesh Uniport HP[4] was used for the chromatography. The temperatures of the injection port, column, and detector were 300°, 175°, and 300°, respectively. Helium, the carrier gas, was maintained at a flow rate of 20 ml/min. The flow rates of air and hydrogen were optimized at 60 and 3 ml/min, respectively.

The sensitivities for the measurement of 5-FU and I were set at 1 × 16 and 10 × 32, respectively. The serum concentrations of I were ~100 times higher than those of 5-FU, so the sensitivity of the instrument was lowered to 1/20 for I after 5-FU and orotic acid were detected. Quantitation was carried out by studying the calibration curves obtained by plotting the ratio of the peak area of the methylated derivative of I to that of orotic acid, or the ratio of the peak height of the methylated derivative of 5-FU to that of orotic acid against the concentrations of these two compounds.

**Analysis of Methylated Derivative Structures by GLC-Mass Spectrometry (MS)**—A 0.1-ml aliquot of I, 5-FU, or orotic acid in acetone solution (1000 μg/ml) was added to 0.1 ml of ethereal diazomethane. After standing for 30 min at room temperature, the mixture was evaporated to dryness under vacuum at room temperature. The residue was dissolved in 100 μl of acetone, and 1 μl of this solution was injected into a gas chromatograph–mass spectrometer[5].

A 1 m × 3-mm i.d. glass column packed with 3% OV-17 on 80–100 mesh Chromosorb W HP[4] was used. The temperatures of the injection port, column, and separator were 260°, 200°, and 220°, respectively. The flow rate of helium, the carrier gas, was 30 ml/min. Electron-impact mass spectra were measured with 70-eV ionization energy, 60-μA ionization current, and 3500-V acceleration voltage.

**Spectrophotometric and Microbial Determination**—The assays were conducted according to the method of Yasuda *et al.* (4). Concentrations of I were measured by UV absorption at 270 nm after extraction from serum samples with chloroform at pH 2.0. The aqueous phase was assayed microbiologically after the pH was adjusted to 7.0.

**Drug Administration**—Male albino rabbits weighing 2.0–2.5 kg were used. Animals were administered I in a 1 *M* sodium carbonate solution (pH 9.0) at a dose level of 50 mg/kg iv. Blood samples were taken from the ear vein at appropriate time intervals up to 8 hr after drug administration and were centrifuged after coagulation. The serum obtained was frozen at −20° for subsequent analysis.

## RESULTS

**Identification of Methylated Derivatives of I, 5-FU, and Orotic Acid by GLC–MS**—The MS data for the methylated derivatives of I, 5-FU, and orotic acid are shown in Table I. The structures of these methylated derivatives were shown to be 1-(tetrahydro-2-furyl)-3-*N*-methyl-5-fluorouracil (II), 1,3-dimethyl-5-fluorouracil (III), and methyl 1,3-dimethylorotate (IV), respectively.

**Calibration Curves**—For the construction of calibration curves, blank rabbit serum was spiked with known amounts of I and 5-FU and subjected to the GLC determination procedure described above. The calibration curves obtained from serum were $y = 0.0251x + 0.0462$ ($r = 0.9988$) for I, $y = 0.8615x + 0.0082$ ($r = 0.9991$) for 5-FU, and $y = 0.0036x + 0.0013$ ($r = 0.9981$) for the lower concentration of 5-FU over the concentration ranges of 3.13–200 μg/ml, 0.0313–2 μg/ml, and 10–50 ng/ml, respectively.

These calibration curves were compared with those obtained with the samples of I or 5-FU dissolved in acetone without the extraction step. Linear relationships were obtained in samples both from the serum and those dissolved in acetone. The mean recoveries of I and 5-FU from the serum were 99 and 75%, respectively. The detection limits of I and 5-FU

[1] Aldrich Chemical Co., Milwaukee, Wis.
[2] Sigma Chemical Co., St. Louis, Mo.
[3] Model 5710A, Hewlett-Packard, Avondale, Pa.
[4] Gaschro Kogyo, Tokyo, Japan.
[5] Shimadzu-LKB 9000B, Kyoto, Japan.



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**Table I—Mass Spectral Data for Methylated Derivatives of I, 5-Fluorouracil, and Orotic Acid**

| Methylated Derivative | *m/z* (Relative Intensity) | Probable Assignment |
|---|---|---|
| 1-(Tetrahydro-2-furyl)-3-*N*-methyl-5-fluorouracil | 214 (9.8) | $M^+$ |
| | 144 (13.0) | $(M—\overline{CHCH_2CH_2CH_2O}+H)$ |
| | 71 (100.0) | $\overline{CH_2CH_2CH_2CH=O}^+$ |
| | 43 (48.5) | $C_2F^+$ |
| 1,3-Dimethyl-5-fluorouracil | 158 (100.0) | $M^+$ |
| | 101 (34.4) | $(M—CH_3NCO)^+$ |
| | 73 (38.4) | $(M—CH_3NCO—CO)^+$ |
| | 42 (46.1) | $CH_3—\dot{N}\equiv CH$ |
| Methyl 1,3-dimethyl-orotate | 198 (92.3) | $M^+$ |
| | 82 (100.0) | $(M—COOCH_3—CH_3NCO)^+$ |

in the serum were 50 and 30 ng/ml, respectively. When the extraction procedure was performed twice (the lower concentration of 5-FU), the mean recovery increased to 94% with a detection limit of 8 ng/ml. The limits per injection of I and 5-FU on the column were 50 and 8 pg, respectively. In the triplicate determinations of the serum samples containing various concentrations of I or 5-FU, the coefficients of variation (*CV*) indicated high accuracy and good reproducibility (Table II).

**GLC Chromatogram**—A typical chromatogram, obtained with the serum sample taken at 2 hr after intravenous administration of I at a dose level of 50 mg/kg, is shown in Fig. 1. The retention times of III, IV, and II were 7.2, 12.4, and 24.0 min, respectively. An unknown endogenous component was detected in the serum with a retention time of 6.0 min, but this peak was well separated from the analyzing compounds. In addition to this peak originating from the endogenous component, another peak with a retention time of 16.4 min was detected in the serum after the administration of I, which might be one of the metabolites of I.

**Serum Concentrations of I and 5-FU**—The mean serum concentrations of I and 5-FU as a function of time obtained by the present GLC nitrogen–phosphorus-detector method, the spectrophotometric assay method, and the microbial assay method after administration of I at a dose level of 50 mg/kg iv are shown in Fig. 2. Slightly higher concentrations of 5-FU were obtained by the microbial assay method, especially at low levels, than by the present method. The mean serum concentration of I obtained by the present method declined gradually with an elimination half-life of 2.8 hr (Table III). For 5-FU, the peak of serum concentration was at 2 hr after the administration of I, and the elimination half-life was 2.3 hr (Table III).

## DISCUSSION

Compound I has been widely used in cancer chemotherapy as a prodrug of 5-FU and is less toxic than 5-FU (32, 33). Compound I is slowly metabolized primarily in the liver to 5-FU. The hepatic microsomal drug-metabolizing enzymes such as cytochrome $P_{450}$ may play a very significant role in this activation (1).

As reported previously (16, 34), when 5-FU is administered, it is rapidly eliminated from the blood with an elimination half-life of 10–30 min. But in the case of the administration of I, 5-FU is released little by little from I for a fairly long period *in vivo*. Various sensitive assay methods for blood

**Table II—Accuracy and Reproducibility of GLC Nitrogen–Phosphorus-Detector Analysis of I and 5-Fluorouracil Added to Rabbit Serum**

| | Amount Added, μg/ml | Amount Found, μg/ml[a] | *CV*, % |
|---|---|---|---|
| I | 3 | 3.1 ± 0.3 | 9.7 |
| | 50 | 51.3 ± 1.2 | 2.3 |
| | 200 | 200.0 ± 11.2 | 5.6 |
| 5-Fluorouracil | 0.01 | 0.0100 ± 0.0001[b] | 1.0 |
| | 0.03 | 0.0279 ± 0.0003[b] | 1.1 |
| | 0.05 | 0.047 ± 0.001[b] | 2.1 |
| | 0.50 | 0.51 ± 0.02 | 3.9 |
| | 2.00 | 2.00 ± 0.03 | 1.5 |

[a] Mean ± *SD* of three determinations. [b] Extracted twice.

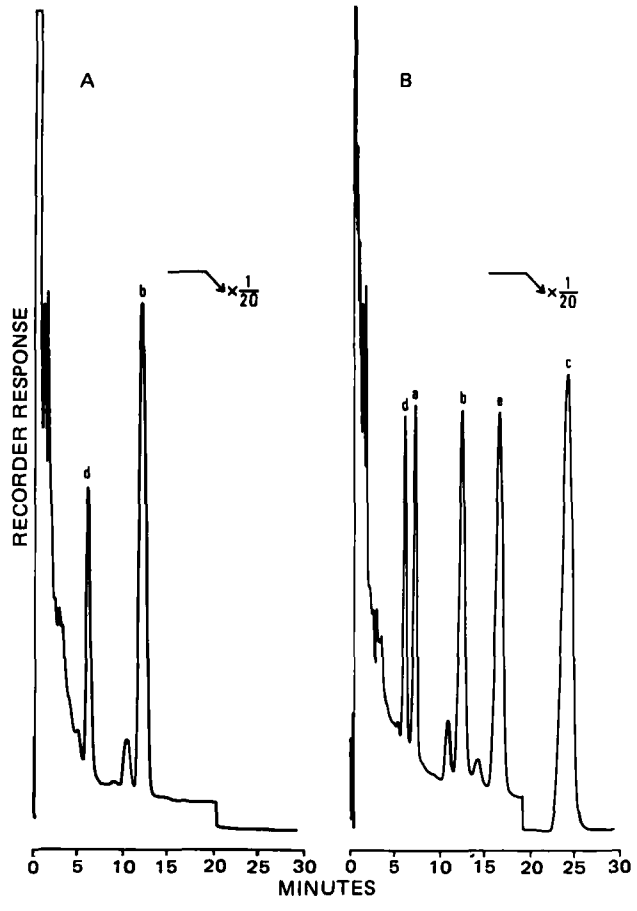


**Figure 1**—*Typical chromatogram of blank rabbit serum (A) and rabbit serum collected at 2 hr after intravenous administration of I at a dose level of 50 mg/kg (B). Key: (a) 5-FU, 0.5 μg/ml; (b) orotic acid; (c) I, 50 μg/ml; (d) endogenous component; (e) unknown metabolite.*

concentrations of I and 5-FU have been investigated. However, there have been no reports showing simultaneous measurement of the amount of I and 5-FU in biological fluids, except for an HPLC method (24, 27) which resulted in poor separation of 5-FU from the endogenous components. In the present study, I and 5-FU could be detected simultaneously in

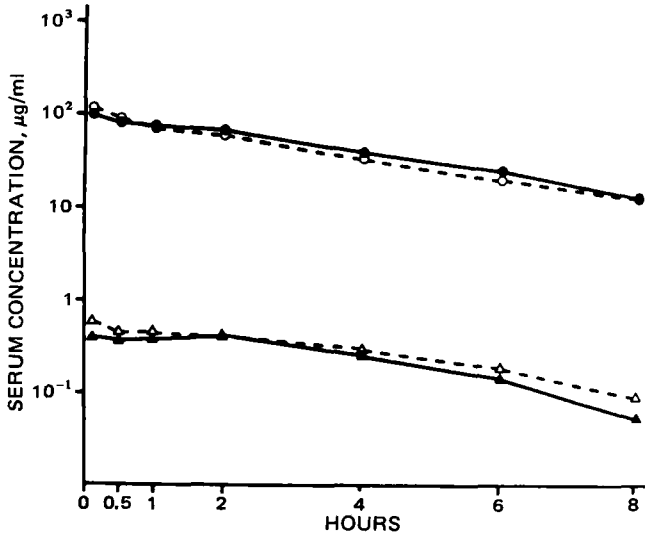


**Figure 2**—*Mean serum concentrations of I and 5-FU obtained from five rabbits after intravenous administration of I at a dose level of 50 mg/kg. Key: (●), I by the GLC nitrogen–phosphorus-detector method; (○), I by the spectrophotometric method; (▲), 5-FU by the GLC nitrogen–phosphorus-detector method; (△), 5-FU by the microbial assay method.*

**Table III—Pharmacokinetic Parameters of I and 5-Fluorouracil Obtained in Rabbits after Intravenous Administration of I at a Dose Level of 50 mg/kg**

| Rabbit | $\beta$[a], $hr^{-1}$ | | $t_{1/2}$[b], hr | | $AUC_{0-\infty}$[c], hr · μg/ml | | $V_{CL}$[d], liter/kg/hr | $V_{d\beta}$[e], liter/kg |
|---|---|---|---|---|---|---|---|---|
| | I | 5-FU | I | 5-FU | I | 5-FU | I | I |
| 1 | 0.62 | 0.54 | 1.1 | 1.3 | 279.8 | 1.71 | 0.18 | 0.29 |
| 2 | 0.21 | 0.17 | 3.3 | 4.1 | 431.5 | 1.66 | 0.12 | 0.56 |
| 3 | 0.11 | 0.33 | 6.3 | 2.1 | 802.1 | 1.52 | 0.06 | 0.57 |
| 4 | 0.63 | 0.44 | 1.1 | 1.6 | 234.0 | 3.06 | 0.21 | 0.34 |
| 5 | 0.32 | 0.29 | 2.2 | 2.4 | 430.1 | 2.81 | 0.12 | 0.37 |
| Mean | 0.38 | 0.35 | 2.8 | 2.3 | 435.5 | 2.15 | 0.14 | 0.43 |
| ±*SD* | ±0.24 | ±0.14 | ±2.2 | ±1.1 | ±223.2 | ±0.72 | ±0.06 | ±0.13 |

[a] $\beta$ = first-order elimination rate constant. [b] $t_{1/2}$ = biological half-life calculated by $0.693/\beta$. [c] $AUC_{0-\infty}$ = area under the serum concentration–time curve calculated by the trapezoidal rule. [d] $V_{CL}$ = total body clearance, calculated by dose/AUC. [e] $V_{d\beta}$ = volume of distribution during the elimination phase calculated by $V_{CL}/\beta$.

serum with high sensitivity and well separated from the endogenous components by the GLC nitrogen–phosphorus-detector method.

5-FU was extracted from serum using the method described in a previous paper (6), on the basis of a $pK_a$ of 8.1 to minimize the concomitant extraction of other acidic compounds contained in the serum that might interfere with the chromatographic assay procedure. Ethyl acetate was used for the extraction solvent. The recoveries of I and 5-FU from serum were 99 and 75%, respectively, which are slightly higher than the values reported by previous investigators (27) who obtained 85 and 60% for I and 5-FU, respectively, using HPLC. The detection limits of I and 5-FU in serum were 50 and 30 ng/ml, respectively. In the case of the lower concentration of 5-FU, the recovery rate increased to 94% by extracting twice with a detection limit of 8 ng/ml in serum. The determinations of the serum samples were conducted in triplicate on the same day using various concentrations of I or 5-FU, covering the range expected for *in vivo* experiments. The results obtained here indicated a satisfactory reproducibility as judged by the coefficients of variation (*CV*).

Compound I and 5-FU cannot be detected with high sensitivity by GLC if not converted to appropriate derivatives. Many derivatizing reagents have been reported (8, 11, 14, 15). When these reagents are injected directly into the GLC after the derivatizing reaction is completed, the peaks of the reagents become broad, and the detector may be contaminated, resulting in shortened column life. The separation of the compounds from the reagents used is difficult. It was reported that the trimethylsilylation of I causes the decomposition of I with 10–30% formation of the trimethylsilylated derivative of 5-FU (6, 13). For these reasons, I and 5-FU were subjected to quantitative methylation with diazomethane, which could be removed after the reaction was complete. No decomposition of I to 5-FU was observed during methylation, and the sample was stable at −20° for ~10 days without any decrease in the methylated derivatives of I and 5-FU. Even if decomposition of the methylated derivative of I occurred, the subsequent conversion to the methylated derivative of 5-FU could not occur because of the removal of diazomethane after derivatization. The calibration curves obtained with these methylated derivatives showed good linearity.

Previous investigators (8, 14) used thymine as an internal standard. However, thymine did not separate completely from 5-FU, either then or in the present study. Therefore, orotic acid was used instead of thymine in the present study; orotic acid showed a satisfactory separation from I, 5-FU, and other endogenous components in serum.

Compound I and 5-FU concentrations in rabbit serum after intravenous administration of I at a dose level of 50 mg/kg could be measured as the methylated derivatives by the present method using a column packed with 1% PEG-HT on Uniport HP. The pharmacokinetic parameters are summarized in Table III; the $t_{1/2}$, $V_{CL}$, and $V_{d\beta}$ of I were 2.8 hr, 0.14 liter/kg/hr, and 0.43 liter/kg, respectively. These values are fairly consistent with those reported by Au and Sadée (26) who obtained $t_{1/2}$ = 1.4–2.1 hr, $V_{CL}$ = 0.18–0.20 liter/kg/hr, and $V_{d\beta}$ = 0.40–0.54 liter/kg in rabbit plasma by HPLC after intravenous administration of I at a dose level of 60 mg/kg. The elimination half-life of 5-FU derived from I was 2.3 hr, almost equivalent to the value of 2.6 hr in the dog (34).

Good correlations were obtained between the concentrations of I and 5-FU obtained by the present method and by the spectrophotometric assay method (I) ($y = 1.1243x - 6.4795$, $r = 0.9717$) or by the microbial assay method (5-FU) ($y = 0.9265x + 0.0936$, $r = 0.9081$), approximating a theoretical slope of 45°. The 5-FU concentrations obtained by the microbial assay method were slightly higher than those obtained by the present method, especially in the low concentrations, suggesting that the accuracy of the microbial assay method is not as good at low 5-FU levels. 5-FU released *in vivo* is further metabolized to other antibacterial compounds such as 5-fluorouridine, 5-fluorouridine-5′-monophosphate, 5-fluoro-2′-deoxyuridine, 5-fluoro-2′-deoxyuridine-5′-monophosphate, *etc.*, which may result in the higher concentrations obtained by the microbial assay method than those by the present method.

In the present studies, an unknown compound with a retention time of 16.4 min was detected, probably a dehydrogenated metabolite of I as reported in previous papers (24, 27). Dehydrogenated I is more labile than I and could be decomposed to 5-FU *in vivo* (27). But nonenzymatic decomposition of dehydrogenated I was not detected after incubation in 0.1 *M* phosphate buffer (pH 6.0) at 25° (27). On the other hand, in a previous study (6) I was decomposed 0.03% to 5-FU during analysis employing an evaporation procedure under nitrogen at 50° after extraction. Since I is thermally labile, the analytical procedure should be conducted at as low a temperature as possible. In the present study, the overall procedure was carried out at room temperature, resulting in no decomposition of I to 5-FU. This perhaps prevents decomposition of dehydrogenated I, which causes the increased concentration of 5-FU. Furthermore, no decrease of the peak possibly corresponding to the methylated derivative of dehydrogenated I was observed during the analysis. Investigation of valid evidence of this unknown compound as dehydrogenated I is in progress.

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# Comparative Assays for Doxepin and Desmethyldoxepin Using High-Performance Liquid Chromatography and High-Performance Thin-Layer Chromatography

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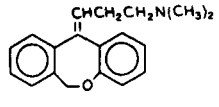
**Abstract** □ Two chromatographic methods, high-performance liquid chromatography (HPLC) and high-performance thin-layer chromatography (HPTLC) were compared for sensitivity and reproducibility in the analysis of the tricyclic antidepressant doxepin and its metabolite, desmethyldoxepin, in plasma. The HPLC procedure yielded a better reproducibility, as reflected by the coefficient of variation, and a higher sensitivity, as reflected by the minimum detectable quantity. The application of these methods for therapeutic and subtherapeutic monitoring of plasma levels of the drug is described.

**Keyphrases** □ Doxepin—high-performance thin-layer and liquid chromatographic methods, comparison, human plasma, desmethyldoxepin metabolite □ High-performance liquid chromatography—doxepin and desmethyldoxepin in human plasma, comparison with high-performance thin-layer chromatography □ Thin-layer chromatography—high-performance, doxepin and desmethyldoxepin in human plasma, comparison with high-performance liquid chromatography

Doxepin (I) is a tricyclic antidepressant commonly prescribed for the treatment of endogenous depression. Increasing evidence of a correlation between the total tricyclic plasma level [doxepin plus desmethyldoxepin (II)] and the antidepressant effect suggests that monitoring tricyclic plasma levels may be beneficial in the clinical management of depression.

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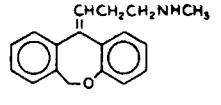


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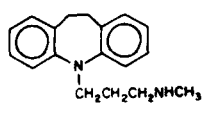


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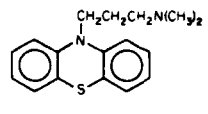


IV

Several assay methods for I and II have been reported, including GLC (1–3), radioimmunoassay (4), GC–mass fragmentography (5), and high-performance liquid chromatography (HPLC) (6–9). Most of these methods either require extensive sample preparation or lack the necessary specificity and sensitivity for pharmacokinetic studies. This paper compares two chromatographic procedures, HPLC and high-performance thin-layer chromatography (HPTLC), for sensitivity and reproducibility in the analysis of I and II in plasma. Desipramine (III) and promazine (IV) were used as internal standards in HPLC and HPTLC, respectively.

## EXPERIMENTAL

**Materials**—A high-performance liquid chromatograph[1] equipped with a variable-wavelength UV detector[2], a sample loop injection valve[3], and a 5-μm octadecylsilane column[4] (150 mm × 4.6-mm i.d.) was used for HPLC analyses. A scanning spectrophotometer–densitometer[5] was used for HPTLC measurements.

Doxepin[6], desmethyldoxepin[6], desipramine[7], and promazine[8] were obtained as hydrochloride salts. Toluene[9], chloroform[9], pentane[9], and 2-propanol[9] were reagent grade and were glass-distilled before use. HPLC-grade acetonitrile[10] and reagent-grade *N*-nonylamine[11] were used as supplied. All glassware used for samples or extracts were silylated with hexamethyldisilazine at elevated temperature and reduced pressure. Working standard solutions were prepared in the following strengths: I, 1 μg/ml; II, 1 μg/ml; III, 2μg/ml; IV, 1 μg/ml.

**HPLC Procedures**—Analyses were performed using a modified procedure of Kabra *et al.* (10). Plasma standards were prepared by spiking known quantities of I and II in 1 ml of plasma containing 0.1 μg of desipramine internal standard (III). To the spiked plasma samples were added 0.25 ml of saturated sodium carbonate and 4.5 ml of pentane. The sample mixture was rotated for 45 min and then centrifuged. The pentane phase was transferred into a silylated conical vial[12] and back-extracted with 0.1 ml of 0.1 *N* HCl. The organic layer was discarded, and aliquots of the aqueous phase were injected into the liquid chromatograph. The mobile phase was 45% acetonitrile in 0.01 *M* phosphate buffer containing 600 ppm of *N*-nonylamine (pH 3.1). The deaerated and fil-

[1] ConstaMetric IIG; Laboratory Data Control, Riviera Beach, Fla.
[2] Spectromonitor III; Laboratory Data Control, Riviera Beach, Fla.
[3] Model SV-7; Glenco Scientific Inc., Houston, Tex.
[4] Spherisorb; Custom LC Inc., Houston, Tex.
[5] Zeiss Instruments, New York, N.Y.
[6] Pennwalt Corp., Rochester, N.Y.
[7] USV Pharmaceutical Corp., Tuckahoe, N.Y.
[8] SKF Laboratories, Philadelphia, Pa.
[9] Fisher Scientific, Fair Lawn, N.J.
[10] MCB Mfg. Chemicals Inc., Cincinnati, Ohio.
[11] Aldrich Chemical Co., Milwaukee, Wis.
[12] Reacti-Vial; Pierce Chemical Co., Rockford, Ill.

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