Rapid and Specific High-Pressure Liquid Chromatographic Assay for Folic Acid in Multivitamin–Mineral Pharmaceutical Preparations

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Abstract □ A high-pressure liquid chromatographic assay for folic acid in multivitamin–mineral pharmaceutical formulations was developed. The internal standard solution used for sample extraction contained a chelating agent, pentetic acid, for prevention of metal ion-catalyzed degradation of folic acid in the prepared samples. Samples were chromatographed using a paired-ion mobile phase (water–methanol, ~76:24; 0.015 *M* phosphate buffer, pH 7.0; and 0.3% tetrabutylammonium hydroxide) on a column packed with octadecylsilane bonded to microparticulate silica gel. Sample preparation was rapid, and total chromatographic time was ~20 min. The method was accurate, precise, and highly specific. Folic acid and the internal standard, methylparaben, were separated from other tablet components and a number of potential impurities and degradation products of folic acid.

Keyphrases □ Folic acid—high-pressure liquid chromatography, multivitamin–mineral pharmaceutical preparations □ High-pressure liquid chromatography—folic acid, multivitamin–mineral pharmaceutical preparations □ Multivitamin–mineral formulations—high-pressure liquid chromatography, folic acid

Previous work described the use of various organic ammonium salts as ion-pairing reagents for the separation of ascorbic acid and folic acid on columns packed with octadecylsilane bonded to 5–10-μm silica gel (1–4). A high-pressure liquid chromatographic (HPLC) method for the quantitation of folic acid bulk drug is now available for common use (5, 6). However, the successful separation and quantitation of folic acid in mineral-containing multivitamin pharmaceutical formulations have not been reported.

This report describes a stability-indicating and highly specific HPLC assay for folic acid, which was applied to mineral-containing multivitamin formulations and their mineral-free counterparts.

EXPERIMENTAL

Reagents and Materials—Dihydrofolic acid[1], *dl*-L-tetrahydrofolic acid[1], *dl*-*N*-5-methyltetrahydrofolic acid[1] (sodium salt), *p*-aminobenzoic acid[1], pterine[1], pterin-6-carboxylic acid[1], *N*-(*p*-aminobenzoyl)-L-glutamic acid[1], and pteroic acid[2] were used as received. Reagents used were analytical reagent grade or better. The pharmaceutical preparations analyzed are commercially available, and their compositions are described in Table I.

Instrumentation—The liquid chromatograph consisted of a high-pressure pump[3], a valve injector[4] equipped with a 10-μl loop, heating tape[5] and associated temperature control unit[6], and a 280-nm fixed-wavelength UV detector[7] operated at a sensitivity of 0.01 aufs. Separations were performed on a 30-cm × 4-mm i.d. column containing 10-μm silica gel with bonded octadecylsilane[8]. Chromatograms were produced on a strip-chart recorder[9]. Peak responses were measured using an electronic integrator[10].

Table I—Compositions of Representative Pharmaceutical Preparations

Coated Compressed Tablet with Minerals[a]	Compressed Tablet[b] and Soft Elastic Capsule[c]
Vitamin A, IU 5000	5000
Vitamin D, IU 400	400
Vitamin E, IU 15	15
Vitamin C, mg 300	60
Folic acid, mg 0.4	0.4
Thiamine, mg 10	1.5
Riboflavin, mg 10	1.7
Niacin, mg 100	20
Vitamin B_6, mg 6	2
Vitamin B_{12}, μg 18	6
Pantothenic acid, mg 10	
Iodine, μg 150	
Iron, mg 18	
Copper, mg 2	
Zinc, mg 15	
Manganese, mg 1	
Potassium, mg 5	

[a] Unicap T tablets, The Upjohn Co., Kalamazoo, Mich. [b] Unicap Chewable tablets, The Upjohn Co., Kalamazoo, Mich. [c] Unicap capsules, The Upjohn Co., Kalamazoo, Mich.

Mobile Phase—The mobile phase was prepared by adding 650 ml of water, 7.5 ml of a 40% aqueous solution of tetrabutylammonium hydroxide[11], 2.04 g of monobasic potassium phosphate, 7.0 ml of 3.0 *N* phosphoric acid, and 240 ml of methanol to a 1-liter volumetric flask. This mixture was diluted to volume with water, adjusted to pH 7.0 with phosphoric acid or ammonium hydroxide, membrane filtered[12], and degassed prior to use. (Column variations may require alterations in the methanol and phosphate concentrations.) A flow rate of 1.5 ml/min and a pressure of ~1300 psig were used. Column temperature was maintained at 35°.

Internal Standard Solution—The internal standard solution was prepared by mixing together 40 mg of methylparaben, 240 ml of methanol, 650 ml of water, 12 ml of a 40% aqueous solution of tetrabutylammonium hydroxide, 2.04 g of monobasic potassium phosphate, and 30 ml of a solution containing 100 mg/ml of pentetic acid (I) in 0.75 *N* ammonium hydroxide. This preparation was diluted to 1000 ml with water.

Standard Preparation—Approximately 32 mg of folic acid USP reference standard was accurately weighed into a 100-ml volumetric flask, diluted to volume with the internal standard solution, and mixed well until all of the folic acid reference standard was dissolved. Then 4.00 ml of this solution was pipetted into a second 100-ml volumetric flask, diluted to volume with the internal standard solution, and mixed well. This solution was subsequently protected from prolonged exposure to light.

Sample Preparation—*Folic Acid Bulk Drug*—Samples of folic acid bulk drug were prepared as described for the standard preparation.

Tablets—Ten tablets were accurately weighed and then finely powdered. A quantity of the powder equivalent to 0.3 mg of anhydrous folic acid was accurately weighed and transferred to a 35-ml centrifuge tube. The internal standard solution (25 ml) was pipetted into the centrifuge tube, and the headspace was immediately flushed with nitrogen and then stoppered tightly. This preparation was shaken vigorously for ~5 min,

[1] Sigma Chemical Co., St. Louis, Mo.
[2] Gillies International Inc., La Jolla, Calif.
[3] Mini-Pump model, Milton Roy Co., Riviera Beach, Fla.
[4] Model 70-10, Rheodyne Inc., Berkeley, Calif.
[5] Catalog No. BS-6 1/2, Briscoe Mfg. Co., Columbus, Ohio.
[6] Model ITC-J10, Valco Instruments Co., Houston, Tex.
[7] Model 1203, Milton Roy Co., Riviera Beach, Fla.
[8] μBondapak C_{18}, Waters Associates, Milford, Mass.
[9] Honeywell, Philadelphia, Pa.
[10] Model 23000-011, Spectra-Physics, Santa Clara, Calif.
[11] Catalog No. 17,878-0, Aldrich Chemical Co., Milwaukee, Wis.
[12] Type BD (0.6-μm), Millipore Corp., Bedford, Mass.

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Table II—Folic Acid Bulk Drug Assay Results

Lot	This Work, %	USP HPLC Assay, %
A	88.8	89.4
B	91.9	91.6
C	90.9	90.4
D	91.5	91.3
E	92.3	90.3
F	91.0	93.8
Mean	91.1	91.1

after which the resulting solution was membrane filtered[13]. The headspace of the sample container was again flushed with nitrogen and capped tightly until injected.

Soft Elastic Capsules—Ten capsules were accurately weighed and then cut in half. The capsule filling was transferred to a glass container, and the empty capsule shells were washed with two 25-ml portions of chloroform, which were discarded. The washed capsule shells were dried and weighed to determine the average capsule fill weight. A quantity of the capsule filling equivalent to 0.3 mg of anhydrous folic acid was accurately weighed and transferred to a 35-ml centrifuge tube, and ~30 ml of hexane then was added. The contents were shaken vigorously for ~5 min and then centrifuged at ~1070×*g* for 15 min.

The hexane was carefully aspirated, and the residue was dried at 60°. The internal standard solution (25 ml) was pipetted into the centrifuge tube containing the dried residue, and the headspace was immediately flushed with nitrogen and then stoppered tightly. This preparation was shaken vigorously for ~5 min, after which the resulting solution was membrane filtered. The headspace of the sample container was again flushed with nitrogen and capped tightly until injected.

Calculations—The folic acid content was calculated by the following equations.

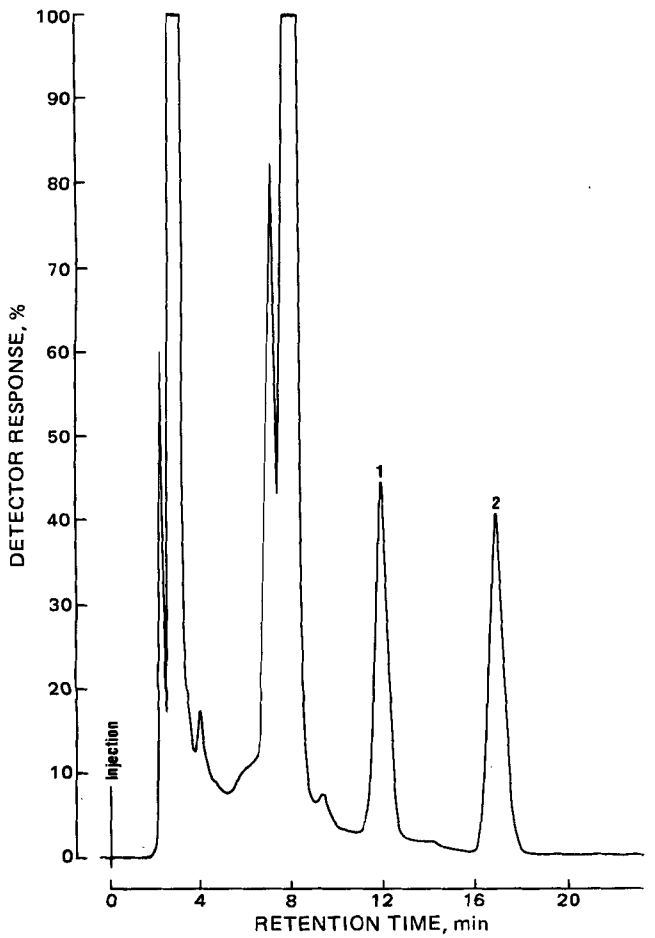

Figure 1—*Chromatogram of multivitamin–mineral pharmaceutical tablet preparation.*

[13] Type HA (0.45 mm), Millipore Corp., Bedford, Mass.

Table III—Stability of Sample Preparation Solutions

Sample	Atmosphere	Folic Acid Remaining after 24 hrs in solution, %
Folic acid plus other vitamins and minerals[a] extracted in dilute sodium hydroxide without I and injected immediately	Air	5–10[b]
Folic acid only in internal standard solution	Air	100.8
Folic acid plus minerals (copper, iron, potassium, manganese, and zinc) in internal standard solution	Air	99.2
Folic acid plus other vitamins and minerals[a] in internal standard solution	Air	82.9
	Oxygen enriched	51.9
	Nitrogen	99.0

[a] See Table I. [b] Range of several trials. Results based on peak height approximations.

For bulk drug:

$$\frac{R_{sam}}{R_{std}} \times \frac{W_{std}}{W_{sam}} \times 100 = \% \text{ purity} \qquad \text{(Eq. 1)}$$

For tablets:

$$\frac{R_{sam}}{R_{std}} \times \frac{C_{std}}{W_{sam}} \times W_{tab} \times 25 = \text{mg/tablet} \qquad \text{(Eq. 2)}$$

For soft elastic capsules:

$$\frac{R_{sam}}{R_{std}} \times \frac{C_{std}}{W_{sam}} \times W_{sec} \times 25 = \text{mg/capsule} \qquad \text{(Eq. 3)}$$

where:

R_{sam} = folic acid peak response divided by internal standard peak response of the sample preparation
R_{std} = folic acid peak response divided by internal standard peak response of the reference standard preparation
W_{std} = weight of folic acid (corrected for moisture) in milligrams in the reference standard preparation
W_{sam} = weight of sample in milligrams
C_{std} = folic acid concentration of the reference standard preparation in milligrams per milliliter (corrected for moisture)
W_{tab} = average tablet weight in milligrams per tablet
W_{sec} = average soft elastic capsule (sec) weight in milligram per capsule
25 = dilution volume in milliliters

RESULTS AND DISCUSSION

Selection of Ion-Pairing Reagent and Chromatographic Conditions—Due to the known suitability of organic ammonium salts as ion-pairing reagents for acidic compounds, efforts were directed to the development of such a system for folic acid in multivitamin–mineral pharmaceutical formulations. Since the method was intended for routine use in a high-volume analytical laboratory, a readily available ion-pairing reagent was desirable. Possible choices included the series of organic ammonium hydroxide compounds, including tetramethylammonium hydroxide through tetrabutylammonium hydroxide.

Table IV—Recovery of Folic Acid Added to Placebo Preparations

	Coated Compressed Tablet with Minerals	Compressed Tablet	Soft Elastic Capsule
Average recovery, %	99.1	100.3	99.3
Number of determinations	8	5	7
Range studied, mg/dosage unit	0.3026–0.6972	0.3112–0.7130	0.2980–0.7178
RSD, %	1.5	0.9	1.7
Correlation coefficient	0.998	1.000	1.000

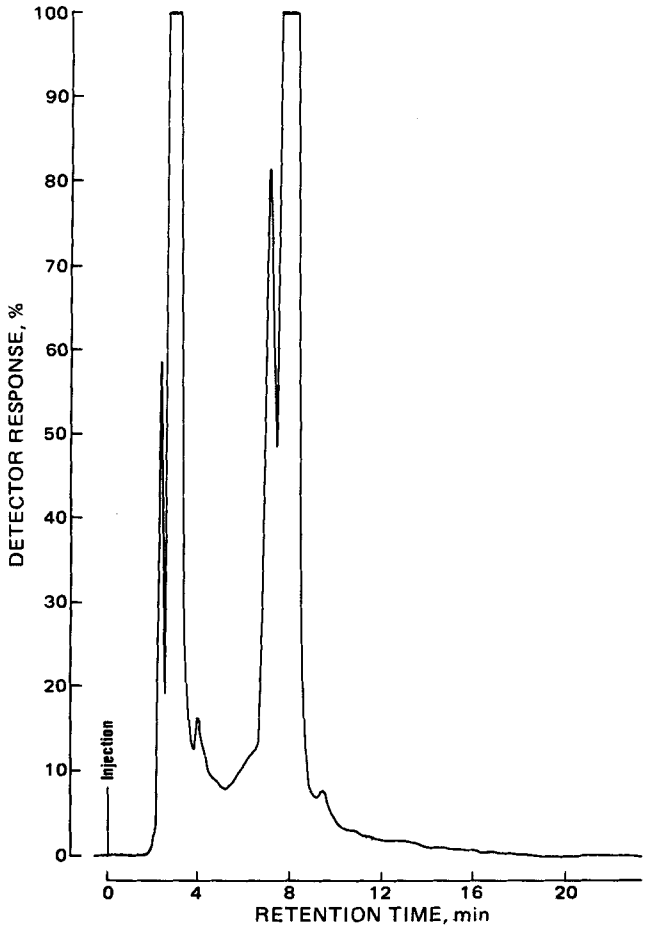

Figure 2—*Chromatogram of a placebo preparation containing all vitamins and minerals except folic acid.*

Tetramethylammonium hydroxide and tetraethylammonium hydroxide failed to separate folic acid from other vitamins and tablet excipients. Tetrapropylammonium hydroxide did produce adequate separation but resulted in unacceptably long chromatographic times due to retention of excipients.

The use of tetrabutylammonium hydroxide as a pairing ion provided a desirable separation and acceptable chromatographic times. Tablet excipients and other vitamins eluted rapidly, with folic acid being retained. The addition of a small quantity of monobasic potassium phosphate increased the separation of folic acid from the other vitamins and tablet excipients and provided improved efficiency. Optimum separations were usually achieved with a monobasic potassium phosphate concentration of ~0.015 *M*. The pH of the mobile phase also influenced the chromatography. Low pH values produced longer retention times for folic acid than the recommended pH 7.0, but broader peaks resulted.

Some variation of the potassium phosphate monobasic content or mobile phase pH may be required due to the chromatographic column being used. However, the chromatographic conditions described provided the best peak shapes for folic acid and the internal standard while adequate separation from other tablet constituents was maintained. The slightly elevated column temperature of 35° was employed to avoid varying the mobile phase composition to compensate for fluctuations in ambient temperature. Under these conditions, column life was comparable to that of other reversed-phase HPLC methods used for the analysis of pharmaceuticals.

Bulk Drug Assays—The method was tested for its comparability to the USP HPLC assay for folic acid bulk drug (4). The data presented in Table II indicate that the method yielded results for bulk drug lots that were equivalent to the official USP assay.

Extraction of Folic Acid—The extraction conditions used are extremely important for the successful quantitation of folic acid in mineral-containing multivitamin formulations. Under acidic conditions, folic acid has limited solubility and tends to be unstable (7). Other vitamins such as cyanocobalamin, pantothenate, riboflavin, thiamine, and ascorbic acid are unstable at high pH values (8). Furthermore, metal ion-catalyzed (particularly Cu^{2+} and Fe^{2+}) oxidative degradation of ascorbic acid is known to occur in the presence of oxygen (9, 10). The oxidative degradation products of ascorbic acid (dehydroascorbic acid, diketogulonic acid, and oxalic acid) are powerful reducing agents. The use of ascorbic acid as a folic acid reductant is known (11).

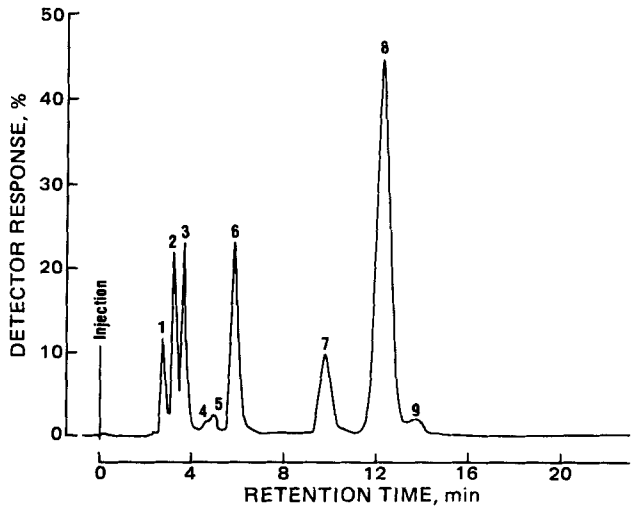

Figure 3—*Chromatogram of a mixture of folic acid and some known degradation products. Key: 1, pterine; 2,* p*-aminobenzoic acid; 3, pterin-6-carboxylic acid; 4 and 5, unknown; 6, pteroic acid and* N-(p*-amino-benzoyl)-*L*-glutamic acid; 7, dihydrofolic acid and tetrahydrofolic acid; 8, folic acid; and 9,* dl-N*-5-methyltetrahydrofolic acid.*

As shown in Table III, folic acid alone or in the presence of minerals did not degrade. However, when other vitamins were present and ascorbic acid was subject to metal ion-catalyzed oxidative degradation, folic acid degradation was observed. Attempts to extract folic acid from mineral-containing multivitamin formulations under high pH conditions using dilute sodium hydroxide or ammonium hydroxide resulted in only ~5–10% recovery of intact folic acid. The reversible enzymatic reduction of folic acid to dihydrofolic acid and tetrahydrofolic acid occurs in biological systems (12). However, it is believed that severely basic extraction conditions result in irreversible degradation of folic acid since these reduced folate species were not observed in chromatography of the above samples.

The internal standard solution used for extracting folic acid was designed to prevent degradation by the described pathways while still providing an efficient extraction. The internal standard solution had a pH of ~9.0–9.5, at which folic acid was readily solubilized. As folic acid and other vitamins and tablet components dissolved, the pH dropped to ~7.0–8.5, depending on the formulation. The lowered pH favors stability of other water-soluble vitamins and thus tends to limit interactions detrimental to folic acid. Metal ion-catalyzed degradation of the water-soluble vitamins was minimized by use of the chelating agent, I. Air oxidation was reduced by flushing the headspace of the extraction container with nitrogen. These extraction conditions produced excellent stability of sample solutions (Table III).

Recovery of Folic Acid from Spiked Placebos—The sample preparation procedure was rapid and simple. As shown in Table IV, average recoveries of 99.1–100.3% were obtained for placebo mixtures containing all of the other tablet vitamins, minerals, and excipients spiked with folic acid over a range of 0.2980–0.7178 mg/dosage unit. The relative standard deviations of the recoveries for these studies varied from 0.9 to 1.7%. Linear regression analysis of folic acid added *versus* peak area yielded correlation coefficients of 0.998–1.000.

Specificity—As shown in Figs. 1 and 2, folic acid and the internal standard, methylparaben, were well separated from the other ingredients contained in multivitamin–mineral formulations. The stability-indicating properties of the method were investigated by chromatographing authentic samples of known folic acid impurities and degradation products. As can be seen in Fig. 3, none of the compounds tested interfered with folic acid or the internal standard.

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Determination of Plasma Fentanyl by GC–Mass Spectrometry and Pharmacokinetic Analysis

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Abstract □ GC–mass spectrometry was used to measure extremely low levels of fentanyl in dog plasma. Deuterated fentanyl was synthesized for use as an internal standard. Fentanyl was hydrolyzed to despropionyl fentanyl by 20% DCl in deuterium oxide. Mass spectrometric analysis of the product revealed that the molecular ion was three mass units higher than that of the authentic despropionyl fentanyl, indicating that the deuterium exchange reactions occurred at this stage. Deuterated despropionyl fentanyl was reesterified by propionyl chloride to fentanyl-d_3. The drug was assayed in biological fluids by extraction into ethyl acetate followed by analysis with GC–chemical-ionization mass spectrometry. The lowest measurable plasma fentanyl level is 500 pg/ml. The method is highly selective and is suitable for monitoring the time course of plasma drug levels. Evaluation of pharmacokinetic data from experiments using nine dogs revealed a triphasic phenomenon. No measurable amounts of the major metabolites, despropionyl fentanyl and norfentanyl, were detected.

Keyphrases □ Fentanyl—determination by GC–mass spectrometry and pharmacokinetic analysis in dog plasma □ GC–mass spectrometry—determination of plasma fentanyl, pharmacokinetic analysis □ Anesthetics—determination of plasma fentanyl by GC–mass spectrometry

Fentanyl[1], *N*-(1-phenethyl-4-piperidyl)propionanilide, is a potent narcotic analgesic widely used in clinical anesthesia. It has a rapid onset of effect and a short duration of action when compared with morphine and most other drugs of this group (1). Direct correlation between pharmacokinetic data and drug effect is difficult due to the lack of an analytical method suitable for measuring extremely small quantities of the drug in plasma (2–4). Only recently was fentanyl measurement in a subnanogram quantity made possible by radioimmunoassay (5). However, preparation of antiserum samples of fentanyl involves lengthy biological procedures. In addition, the possibility of cross interaction due to various biological components presents difficulties in using the radioimmunoassay method for specific analyses.

This report describes a sensitive and specific GC–mass spectrometric method using stable isotopically-labeled fentanyl-d_3 as an internal standard. The method is capable of quantitatively detecting subnanogram levels of fentanyl in plasma. After intravenous administration of 25, 50, or 100 μg of fentanyl/kg to dogs, the plasma level fell rapidly to 2.5 ng/ml within 30 min and then remained at 0.5–2 ng/ml for up to 3 hr.

[1] Sublimaze, McNeil Laboratories.

EXPERIMENTAL

Preparation of Fentanyl-d_3—After 1.2 ml of 20% DCl in deuterium oxide[2] (99 atom % deuterium) was added to 25 μg of fentanyl[3], the solution was incubated overnight at 100°. The acidity of the reaction mixture was partially neutralized by 0.2 ml of 40% NaOD in deuterium oxide[2] (99 atom % deuterium), and the unreacted fentanyl was removed by extraction twice with 2 ml of ethyl acetate. The remaining aliquot of sample was again acidified by 1.2 ml of 20% DCl solution before evaporation to dryness *in vacuo*. To the residue, 20 μl of pyridine and 1 ml of propionyl chloride[2] were added, and the mixture was incubated at 60° for 1 hr. The final product, fentanyl-d_3, was then purified by extraction twice with 3 ml of ethyl acetate. After evaporation to dryness by a nitrogen stream, the residue was dissolved in 0.5 ml of ethyl acetate and stored in a refrigerator at 4°.

Instrumentation—Combined GC–chemical-ionization mass spectrometry[4] was employed with methane as the reagent gas and an electron energy of 150 ev. GC separation was carried out isothermally at 235° with a 0.91-m × 2-mm i.d. U-shaped glass column packed with 3% SE-30 on 80–100-mesh Gas Chrom Q[5]. The temperature of the GC injector and the separator was 250°.

Biological Samples—Two hours before fentanyl administration, the nonselective mongrel dogs, 12–14 kg, were anesthetized with 300 mg of pentobarbital sodium. Fentanyl (25, 50, or 100 μg/kg) was introduced intravenously by 1-min infusion, and blood samples were collected into heparinized vials at 1, 2, 4, 6, 8, 10, 15, 30, 45, 62, 90, 120, 150, and 180 min after infusion was completed. Plasma was separated from red blood cells by centrifugation at 2500 rpm in the cold and stored in a freezer at −40° when necessary.

Extraction and Quantitation—To 2 ml of plasma, a known amount of fentanyl-d_3 (25 ng in 25 μl of ethyl acetate) was added as an internal standard. After rigorous mixing with the aid of an agitator[6], the plasma

[2] Aldrich Chemical Co., Milwaukee, Wis.
[3] McNeil Laboratories, Fort Washington, Pa.
[4] Finnigan 3300 gas chromatograph-mass spectrometer equipped with a 6000 data acquisition.
[5] Applied Science Laboratories, State College, Pa.
[6] Vanlab, Scientific Industries, Bohemia, N.Y.

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