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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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Table I—Selective Ion Chemical-Ionization Mass Spectrometric Assessment of the Purity of Fentanyl-d_3

Compound	Relative Ion Intensities[a]			
	$MH^+ - 3$	$MH^+ - 2$	$MH^+ - 1$	MH^+
Fentanyl	0.50	10.01	3.93	100
Fentanyl-d_3	1.72	12.96	9.40	100
Differences	1.21	2.95	5.47	

[a] Relative ion intensities were calculated by taking the ion intensity of MH^+ as 100.

Table II—Plasma Fentanyl Levels[a] (Nanograms per Milliliter) in Dogs after 50-μg/kg iv Dose

Minutes	Dog 1	Dog 2
1	26.50 ± 0.62 (4)	24.72 ± 0.52 (4)
2	19.04 (2)	15.14 ± 0.18 (4)
4	13.74 (2)	12.81 ± 0.38 (4)
6	10.33 ± 0.41 (4)	8.17 ± 0.06 (3)
8	8.47 ± 0.29 (6)	5.84 (2)
10	6.78 ± 0.16 (4)	4.13 ± 0.16 (3)
15	4.83 (2)	2.04 ± 0.04 (4)
30	2.61 (2)	1.25 (2)
45	1.58 ± 0.04 (5)	1.16 ± 0.07 (5)
62	1.22 (2)	0.66 ± 0.03 (3)
90	1.11 (2)	0.60 ± 0.07 (4)
120	1.02 ± 0.03 (4)	0.50 ± 0.05 (3)
150	0.83 ± 0.02 (3)	0.48 ± 0.03 (3)
180	0.57 ± 0.06 (5)	

[a] Mean ± *SD*; the number of determinations is shown in parentheses.

was allowed to stand for 5 min before saturation with ammonium carbonate. Extractions were performed twice with 3 ml of ethyl acetate. The organic layers were pooled and dried by a nitrogen stream. To this residue, 30 μl of ethyl acetate was added and 10 μl was used for analysis. Ions selectively monitored were *m/z* 340, 337, 281, and 233 corresponding to the pseudomolecular ions of fentanyl-d_3, fentanyl, despropionyl, and norfentanyl, respectively. Quantitation was performed by comparison of peak area with respect to that of the internal standard.

RESULTS AND DISCUSSION

In addition to the pseudomolecular ion, MH^+, which appeared as the most abundant species, the chemical-ionization mass spectrum of fentanyl showed four fragment ions at mass units greater than 100, *i.e.*, *m/z* 245, 188, 186, and 150. The mass spectrum of fentanyl-d_3 showed that, in addition to the pseudomolecular ion, the ion species at 248 and 153 were isotopically labeled, with *m/z* 153 resulting from cleavage of the propionyl amide bond and *m/z* 248 resulting from cleavage of the benzyl bond. Both ion species contain the phenyl ring of the propionanilide moiety of the molecule.

To obtain deuterated fentanyl, fentanyl was first hydrolyzed by 20% DCl in deuterium oxide solution to produce despropionyl fentanyl and propionic acid. Deuteration appeared to occur at this stage. After extraction with ethyl acetate, analysis of an aliquot of the reaction mixture revealed that deuterated despropionyl fentanyl was the major component. The electron-impact mass spectrum of this compound revealed a weak but detectable *m/z* 283 ion, which corresponded to the molecular ion. Three major fragment ions were observed, at *m/z* 192, 149, and 96. The mass spectrum was similar to that of the nondeuterated despropionyl fentanyl recorded previously (6), except that *m/z* 283, 192, and 149 ions contained isotopic labels. The chemical-ionization mass spectrum of the component showed a pseudomolecular ion at *m/z* 284, which was also three mass units higher than that of the nondeuterated compound. Reesterization of deuterated despropionyl fentanyl was carried out by incubating the residue with propionyl chloride. Fentanyl-d_3 was then purified by extraction into ethyl acetate.

During the preparation of deuterated fentanyl, two extraction processes were performed to remove the unreacted intact drug from the product. The effectiveness of the extraction is demonstrated by the results of two experiments designed to assess the purity of fentanyl-d_3. Table I shows the relative ion intensities of MH^+, $MH^+ - 1$, $MH^+ - 2$, and $MH^+ - 3$ from 70 ng of fentanyl-d_3 and 100 ng of fentanyl by selective ion chemical-ionization mass spectrometry. Essentially, the differences in the relative ion intensities for $MH^+ - 3$, $MH^+ - 2$, and $MH^+ - 1$ between those of fentanyl and fentanyl-d_3 can be regarded as the relative ion intensities contributed by the molecular ions of fentanyl, fentanyl-d_1, and fentanyl-d_2, respectively. When taking the ion intensity of MH^+ of fentanyl-d_3 as 100, it appeared that the deuterated product was a composite of 1.1% fentanyl, 2.69% fentanyl-d_1, 4.99% fentanyl-d_2, and 91.2% fentanyl-d_3.

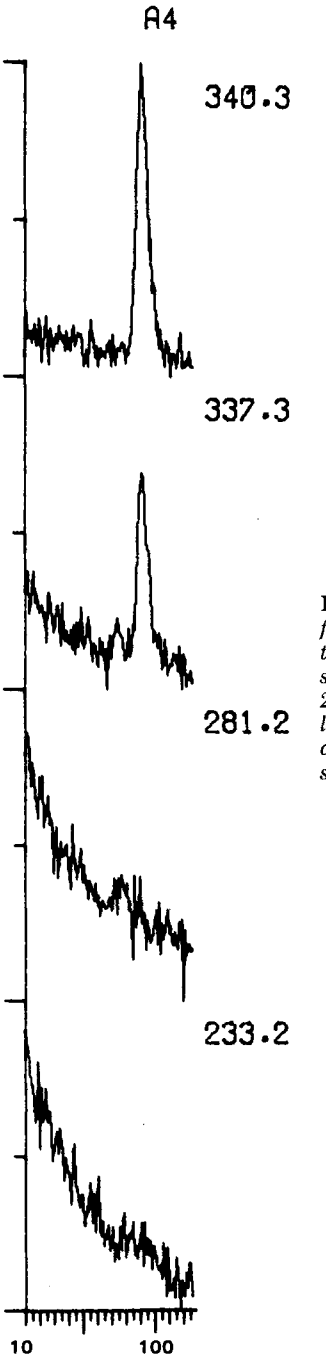

Figure 1—*Mass chromatogram of fentanyl in dog plasma using deuterated fentanyl-*d_3 *as an internal standard;* m/z *340.3, 337.3, 281.2, and 233.2 correspond to the pseudomolecular ions of fentanyl-* d_3, *fentanyl, despropionyl, and norfentanyl, respectively.*

To determine the percent yield of fentanyl-d_3 from the precursor, three separate ethyl acetate extracts corresponding to these reaction mixtures in which 25.0, 37.5, and 50.0 ng of fentanyl were used as the initial starting materials, were added with known amounts of fentanyl (5 ng/10 μl of ethyl acetate) as an internal standard. The unknown amounts of fentanyl-d_3 in the mixtures were then measured by chemical-ionization mass spectrometry, selectively monitoring MH^+ of fentanyl and fentanyl-d_3. The results showed that 23.2, 33.7, and 42.8 ng of the fentanyl were recovered as fentanyl-d_3; therefore, the percent yield of the chemical processes to obtain the deuterated compound was 89.4 ± 3.6%. The standard solution containing fentanyl-d_3 in ethyl acetate (1.75 μg/ml) was stored in a refrigerator at 4° and was stable after 3 months under these conditions. During this time, experiments to check the purity of fentanyl-d_3 were performed prior to each measurement of every batch of dog plasma.

Figure 1 shows the quantitative GC–mass spectrometric analysis of fentanyl in dog plasma, with pseudomolecular ions of fentanyl, *m/z* 337,

Table III—Pharmacokinetic Parameters of Plasma Fentanyl [a]

Dose, μg/kg	Dog Number	A, ng/ml	$T_{1/2}\,\alpha$, min	B, ng/ml	$T_{1/2}\,\beta$, min	C, ng/ml	$T_{1/2}\,\gamma$, min
25	005	57.41	1.61	6.33	25.68	2.51	57.54
	006	73.05	1.78	25.08	6.93	4.97	133.19
50	001	22.30	0.97	15.10	6.93	1.74	134.37
	002	19.90	3.73	1.22	6.97	1.14	110.75
	010	100.00	1.10	13.00	16.89	5.27	93.17
100	003	36.72	4.18	20.56	4.57	7.59	116.91
	004	99.99	1.29	24.29	9.11	9.87	95.23
	009	293.58	1.39	36.22	8.88	16.70	251.55
Average ± SD			2.01 ± 1.24		10.75 ± 7.06		124.09 ± 57.17

[a] Nonlinear estimation of triphasic pharmacokinetics of plasma fentanyl employed the NONLIN computer program from The Upjohn Co.

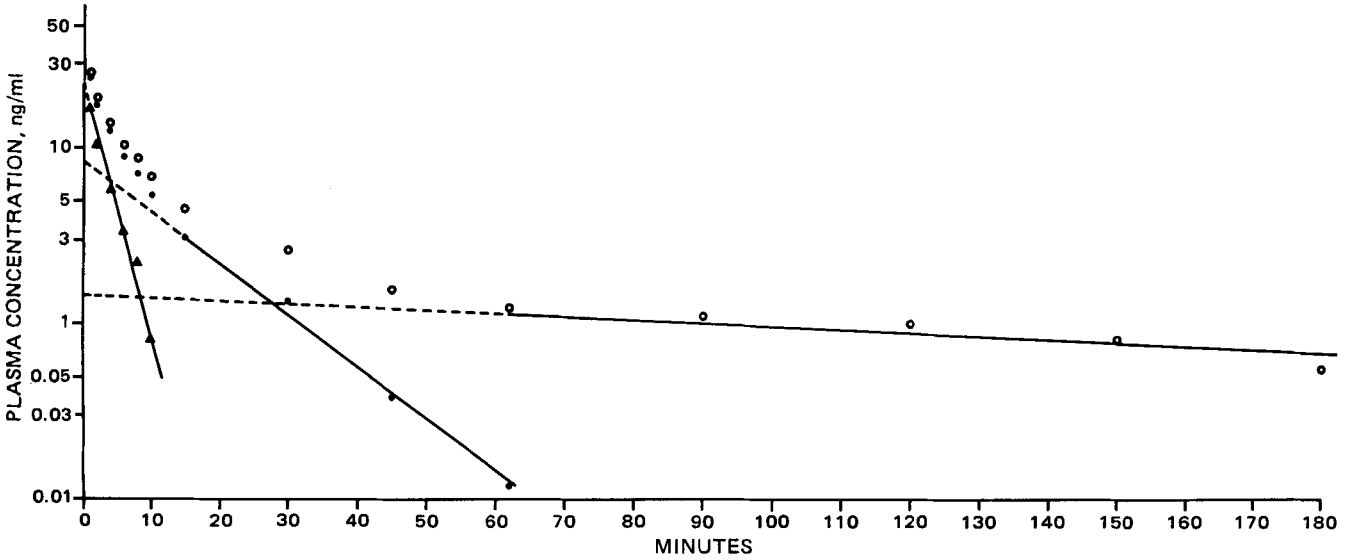

Figure 2—*Application of the method of residuals for nonlinear pharmacokinetic data (8) to the semilogarithmic plot of plasma fentanyl levels with respect to time. Three linear regions of first-order kinetics are shown. Key:* ○, *plasma fentanyl levels in a normal dog after 50 μg/kg iv dose; and* ▲ *and* ●, *data obtained by curve stripping.*

and fentanyl-d_3, m/z 340, used for quantitative measurements. Since despropionyl fentanyl and norfentanyl are proposed major metabolites in the rat urine (6, 7), their MH^+ ions, m/z 281 and 233, respectively, were also selectively monitored. In the first 28 plasma samples analyzed, these two metabolites were not found. The linear range of the calibration curve was determined by adding known amounts of fentanyl and fentanyl-d_3 to 2 ml of water. For standard solutions containing <5 ng of fentanyl/ml, 3.17 ng of internal standard was used; otherwise, 35.7 ng was used. Quantitative measurements were made by comparing the peak areas of fentanyl to that of the internal standard. The results from eight samples, ranging from 500 pg to 100 ng/ml of fentanyl, revealed a linear relationship between the actual and measured values determined by GC–mass spectrometry.

Table II shows plasma fentanyl levels in dogs after an intravenous bolus of 50 μg/kg. For plasma samples containing >1 ng of fentanyl/ml, the reproducibility of the analysis was within 6%; for those containing <1 ng/ml, it was ~12%. With its high sensitivity, the GC–mass spectrometric procedures allow the time course of the plasma fentanyl concentration to be followed for up to 3 hr. The lowest measured plasma concentration was 500 pg/ml. The lower limit of analysis by these methods using a signal to noise ratio of 2 was ~200 pg of fentanyl.

The plasma fentanyl concentration profile in a dog is illustrated in Fig. 2. Figure 2 also shows the results from application of the method of residuals to yield the second and third linear segments in addition to the easily recognizable terminal linear segment (8). The time course of the plasma fentanyl level appeared to follow a triphasic phenomenon. The first phase may have represented the uptake of [^{3}H]fentanyl by organs with extensive blood supplies, *e.g.*, lungs, kidneys, heart, and brain (2). The next phase was one of redistribution, especially from the lungs to the blood. Finally, the last phase represented that of metabolism and excretion. The three-compartmental pharmacokinetics of plasma fentanyl agreed with observations made by radioimmunoassay (4, 5) and radioisotope counting techniques (3).

Table III shows the pharmacokinetic parameters of plasma fentanyl of eight dogs by curve fitting with the NONLIN computer program (9). The plasma profile of fentanyl in dogs can be described by the equation $C(t) = Ae^{-\alpha t} + B^{-\beta t} + Ce^{-\gamma t}$, where $C(t)$ represents the fentanyl concentration at any time, t. The half-life, $T_{1/2}$, is calculated by using $0.693/\alpha$, β, or γ. The results shown in Table III indicate that the pharmacokinetic parameters vary widely among dogs. The same variability in the pharmacokinetic parameters of plasma fentanyl in humans was reported by Hull (10). Table III reveals that the half-lives of each compartment are more or less independent of the dose levels of fentanyl administered, despite the wide variation in the data. However, in contrast to other data (11), the intercepts (A, B, and C) are not dose dependent.

Finally, the average half-lives of the terminal linear phase (124.1 ± 57.2 min) and the middle phase (10.74 ± 7.05 min) shown in Table III are shorter than the values reported in the literature (3, 5, 10). These differences may be due to the different specificities of the techniques adapted for measuring the extremely low plasma levels of fentanyl in these two regions. The described GC–mass spectrometric technique is highly specific for several reasons. First, since selective ion monitoring measures only the pseudomolecular ions of fentanyl and fentanyl-d_3, the quantitative determination of plasma fentanyl was free from the interference of its own metabolites. Second, fentanyl in biological samples was repeatedly purified, first by extraction with ethyl acetate and then by GC separation. The sensitivity of measurements was extended from 500 pg/ml to 100 ng/ml, and the use of stable isotopically-labeled fentanyl as an internal standard greatly enhanced the accuracy and precision of the quantitative measurements.

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NOTES

Plasma Protein Binding Interaction between Valproic and Salicylic Acids in Rhesus Monkeys

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Abstract □ The effects of three levels of salicylic acid on the steady-state plasma concentrations of free and total valproic acid were examined in catheterized rhesus monkeys. Valproate was infused intravenously for a total of 41 hr, and salicylate was added after the first 8 hr. The three salicylate infusions were randomly assigned to each monkey. Valproate free fraction was determined by equilibrium dialysis. Statistically significant increases in valproate free fraction and total body clearance were observed after addition of salicylic acid. The increase in valproate clearance was positively correlated with the molar ratio of salicylate to valproate steady-state plasma concentrations. There was no significant change in valproate free concentration after salicylate treatment. The proposed mechanism of this *in vivo* interaction includes plasma protein binding displacement with no change in valproate intrinsic clearance.

Keyphrases □ Valproic acid—plasma protein binding interaction with salicylic acid, rhesus monkey □ Salicylic acid—plasma protein binding with valproic acid, rhesus monkey □ Plasma protein binding—interaction between valproic and salicylic acids, rhesus monkey

Valproic and salicylic acids are known to bind extensively to plasma proteins in humans (1–8) and rhesus monkeys (9–11). Three *in vitro* studies showed that salicylic acid decreases valproate binding to plasma or human serum albumin (12–14). However, there is no indication that such an interaction occurs *in vivo*. In addition, these two acids may interact at a metabolic level.

Epileptic patients on valproate treatment occasionally receive salicylates and, depending on its mechanism, this interaction may be of therapeutic significance. The present study was designed to investigate this interaction *in vivo* at the systemic level. Both drugs were administered by constant-rate intravenous infusion to achieve one steady-state concentration of valproic acid and three steady-state salicylic acid levels.

EXPERIMENTAL

Four healthy male rhesus monkeys (*Macaca mulatta*) (mean weight 5.5 kg) were chair-adapted for 1 month prior to implantation of catheters. The jugular and femoral veins of each monkey were chronically catheterized to enable withdrawal of venous blood samples and drug infusion, respectively. The monkeys were maintained in three-level restraining chairs during the studies. Patency of catheters was assured by a slow, continuous saline infusion (1 ml/hr). Daily diet consisted of fresh fruit and monkey food.

Three studies were carried out in each monkey. In each study, valproic

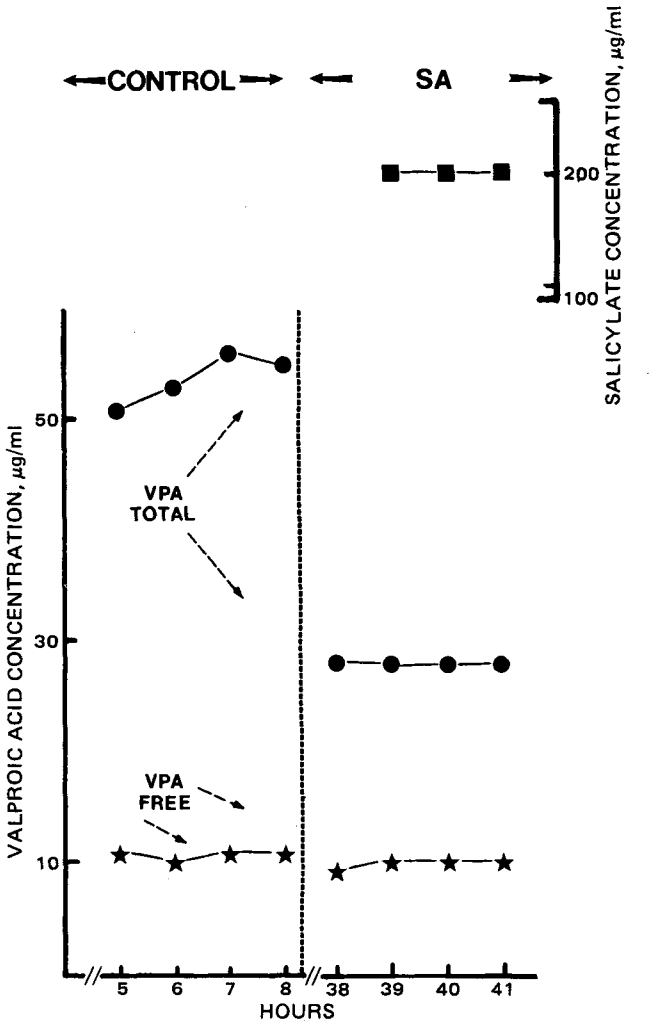

Figure 1—*Steady-state levels of total and free valproate (VPA) before and during salicylate (SA) infusion in Monkey 201.*

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