Determination of an Anti-Inflammatory Methanesulfonanilide in Plasma by High-Speed Liquid Chromatography

SHAW F. CHANG ˣ, ALDORA M. MILLER, and ROBERT E. OBER

Abstract □ A sensitive and chemically specific high-speed liquid chromatographic method was developed for the determination of 4-nitro-2-phenoxymethanesulfonanilide in plasma. The method includes selective extraction of the anti-inflammatory drug and an internal standard, 2-(4′-chlorophenoxy)-4-nitromethanesulfonanilide, into benzene from acidified plasma followed by reextraction into 0.2 *N* NaOH. The aqueous layer is acidified, and the drug is reextracted into benzene. The benzene is evaporated, and the residue is dissolved in a small volume of acetonitrile. A 10-μl aliquot is analyzed on a reversed-phase column. The mean overall extraction recovery, after correction for aliquot factors, is 99%. The accuracy, expressed as the relative error, is 4, 0.3, and −3% at 0.60, 1.50, and 3.00 μg/ml, respectively. Repeated analysis of reference standards indicates that the precision, expressed as the relative standard deviation, is 3% or less. The lower sensitivity limit is 0.2 μg/ml with a 2-ml plasma sample. The method was applied successfully to the determination of plasma levels of 4-nitro-2-phenoxymethanesulfonanilide in humans and rats in metabolic experiments at pharmacological doses.

Keyphrases □ 4-Nitro-2-phenoxymethanesulfonanilide—high-speed liquid chromatographic analysis, plasma □ High-speed liquid chromatography—analysis, 4-nitro-2-phenoxymethanesulfonanilide in plasma □ Anti-inflammatory agents—4-nitro-2-phenoxymethanesulfonanilide, high-speed liquid chromatographic analysis in plasma

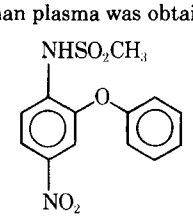
The anti-inflammatory properties of methanesulfonanilides in animal screening models (1–3) led to the synthesis of various compounds containing the methanesulfonanilide group (4). 4-Nitro-2-phenoxymethanesulfonanilide (I) has anti-inflammatory activity in animals (3).

Since the pharmacologically effective dose for animals and the therapeutic dose projected for humans are relatively low, a sensitive and specific plasma level method is needed to follow the pharmacokinetics of I. An electron-capture GLC method was developed and used; however, the procedure was quite time consuming and sometimes erratic. Recent advances in high-speed liquid chromatography (HSLC) and many examples of the determination of drugs in biological fluids by this technique (5–10) prompted the development of a sensitive and specific HSLC method using a reversed-phase column, capable of quantitating the plasma I levels in rats and humans following a single, oral, pharmacological dose. This method was applied successfully to the measurement of plasma pharmacokinetic parameters and used in dosage form comparison studies.

EXPERIMENTAL

Reagents—All solvents were nanograde or pesticide grade, and all reagents were analytical grade. The following aqueous solutions were made in distilled water: 1 *N* HCl, 0.2 *N* NaOH, and 2 *N* HCl.

Blank Plasma—Human plasma was obtained from volunteers who had fasted overnight and had not taken any medication for the previous week. Blank rat plasma samples were obtained from male Holtzman rats *via* cardiac puncture.



I

HSLC—A liquid chromatograph[1] equipped with a constant-flow pump, a fixed-wavelength UV detector (254 nm), and a septumless injector[2] was used. A 0.64 × 30-cm reversed-phase column[3] was preconditioned with the elution solvent, 50% (v/v) acetonitrile in distilled water, at a flow rate of 2.7 ml/min. The solvent was degassed by stirring for 2 min under slight vacuum prior to use.

The liquid chromatograph was operated at room temperature. The range was set at 0.02 absorption unit full scale. Less sensitive settings were used, if necessary, to contain the peak on scale. The chart speed was 1.27 cm/min.

Standard Solutions—All stock solutions of I and the internal standard were made in methanol. Standard solutions containing 20, 10, 5, 1, and 0.2 μg of I/0.1 ml were made by diluting a 1-mg/ml primary standard solution. The concentrated stock solution of 2-(4′-chlorophenoxy)-4-nitromethanesulfonanilide was diluted 20-fold to give an internal standard solution of 5 μg/0.1 ml.

Extraction of I from Plasma—To a 15 × 125-mm culture tube with a screw cap, add 2 ml of plasma and 0.1 ml of methanol. Along with the samples, prepare six standards in blank plasma by adding 0, 0.2, 1, 5, 10, and 20 μg of I in 0.1 ml of methanol to 2 ml of blank plasma (for the 0.2-μg standard, I was added in 0.2 ml of methanol). Add 0.1 ml of internal standard solution, 0.4 ml of 1 *N* HCl, and 4 ml of benzene to all tubes. Shake the tubes on a equipoise shaker[4] for 30 min and centrifuge at 1000×*g* for 5 min.

Transfer 3.5 ml of the benzene layer into a tube containing 2 ml of 0.2 *N* NaOH, shake for 15 min, and centrifuge for 5 min. Then transfer 1.8 ml of the aqueous phase into a tube containing 0.5 ml of 2 *N* HCl. Add 2 ml of benzene, shake for 15 min, and centrifuge for 5 min. Evaporate the benzene layer (1.7 ml) to dryness at 60° with a nitrogen stream, redissolve the residue in 40–100 μl of acetonitrile, and mix with a mixer[5] for 60 sec. Then inject a 10-μl aliquot into the liquid chromatograph.

Calculation—Peak height ratios between I standards and the internal standard were plotted against I concentration. A straight line was fitted by the least-squares method, and its slope and intercept at the peak height ratio axis were determined. A mathematical expression of the standard curve is:

$$\text{peak height ratio} = A(\text{I concentration}) + B \qquad \text{(Eq. 1)}$$

where A is the slope of the line, and B is the intercept of the line at the peak height ratio axis.

Unknown samples are calculated from the following equation:

$$\text{I concentration in unknown sample} = \frac{\text{peak height ratio} - B}{A} \qquad \text{(Eq. 2)}$$

RESULTS

Separation—Baseline separation between I and the internal standard was achieved, and there was practically no interference with I or the internal standard by endogenous materials from human plasma or by metabolites of I (Fig. 1). Under the experimental conditions indicated, the elution volumes for I and the internal standard were 12.4 ml (4.6 min) and 15.7 ml (5.8 min), respectively. The 2.5-min peak was due to a metabolite of I.

Similar results were obtained from rat plasma (Fig. 2), but there was minor interference from endogenous material in the vicinity of the I peak. This small interference represented 0.02 μg/ml and was routinely subtracted from the calculation of I concentrations in the rat samples.

[1] Model 202, Waters Associates.
[2] Model U6K, Waters Associates.
[3] μBondapak C_{18}, Waters Associates.
[4] Precision Scientific Co.
[5] Deluxe mixer model S8220, Scientific Products.

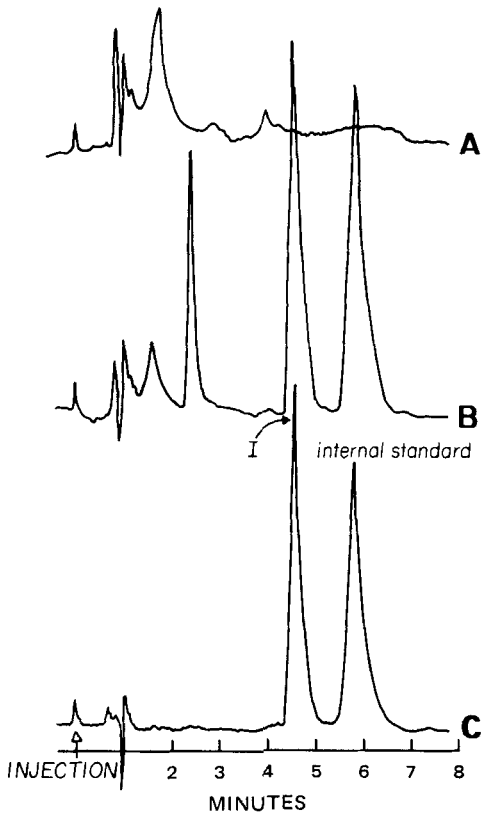


Figure 1—*Chromatogram from human plasma. Key: A, blank plasma; B, plasma of human dosed with I with the internal standard added; and C, mixture of authentic I and the internal standard.*

Extraction Recovery Check—The extraction recovery of I from plasma was determined by a slightly modified extraction procedure in which the internal standard was added after the extraction was completed. The standard curve for this particular experiment was obtained by directly analyzing various mixtures of I and the internal standard without extraction. The extraction recovery check from human plasma was made in duplicate at five concentrations: 0.2, 0.5, 2.5, 5.0, and 10.0 μg/ml. Excellent recovery (Table I) was obtained over this concentration range.

All samples, after correcting for aliquot factors, demonstrated 95% or better recovery except one sample each at 0.2 and 0.5 μg/ml, which gave recoveries of 89.5 and 93.8%, respectively. Their duplicate samples gave excellent recoveries (96.0 and 101.4%, respectively). Thus, the relatively poor recovery for one of the 0.2- and 0.5-μg/ml duplicates is likely a random error rather than a concentration-related effect.

The overall mean extraction recovery was 98.8 ± 4.5%. These overall results indicate that quantitative extraction from human plasma is achieved over a concentration range of 0.2–10 μg/ml, which covers the concentrations found in most biological samples obtained from metabolic studies of I.

Accuracy and Precision—The accuracy of this method was checked by carrying samples at three concentration levels, 0.6, 1.5, and 3.0 μg/ml, in replicates of five through the entire method; the detected concentration was calculated from a pooled standard curve. The precision of this method was examined similarly by comparing the results between these five replicate samples at each concentration level (Table II).

The mean detected concentrations were 0.63, 1.50, and 2.91 μg/ml for the 0.60-, 1.50-, and 3.00-μg/ml samples, respectively. The standard deviations were 0.02, 0.04, and 0.05 μg/ml, respectively, and the relative

Table I—Extraction Recovery of I from Human Plasma

Added, μg/ml	Detected[a], μg/ml	Recovery, %
0.2	0.19	96.0
0.2	0.18	89.5
0.5	0.51	101.4
0.5	0.47	93.8
2.5	2.55	102.1
2.5	2.56	102.4
5.0	5.20	104.0
5.0	5.04	100.7
10.0	9.91	99.1
10.0	9.86	98.6
		Mean ± *SD* = 98.8 ± 4.5

[a] After correction for aliquot factors (×1.494).

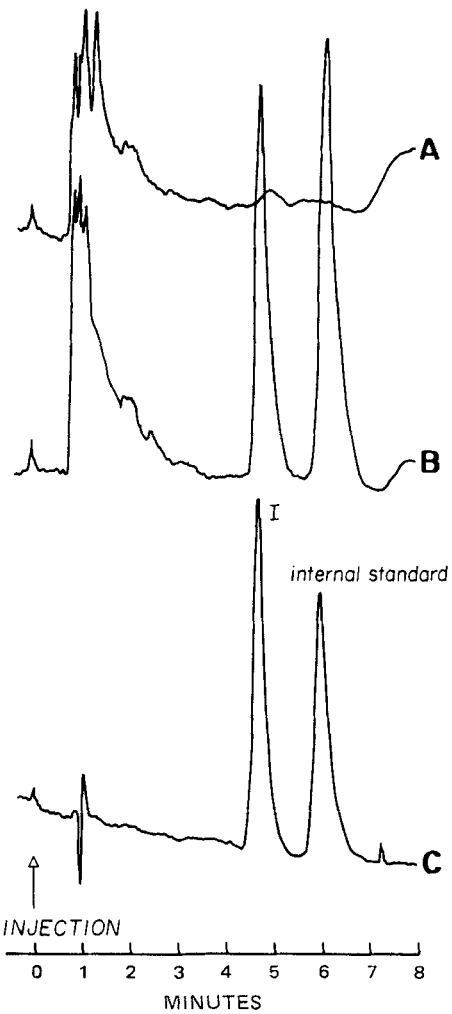


Figure 2—*Chromatogram from rat plasma. Key: A, blank plasma; B, plasma of rat dosed with I with the internal standard added; and C, mixture of authentic I and the internal standard.*

Table II—Accuracy and Precision of I Analysis in Human Plasma by HSLC

I Added to Human Plasma, μg/ml	I Detected, μg/ml	Difference, μg/ml
0.60	0.63	+0.03
0.60	0.64	+0.04
0.60	0.60	0.00
0.60	0.64	+0.04
0.60	0.60	+0.02
	Mean ± *SD* 0.63 ± 0.02	Mean error +0.026
	RSD 3%	Relative error +4%
1.50	1.48	−0.02
1.50	1.48	−0.02
1.50	1.54	+0.04
1.50	1.46	−0.04
1.50	1.56	+0.06
	Mean ± *SD* 1.50 ± 0.04	Mean error +0.004
	RSD 3%	Relative error +0.3%
3.00	2.98	−0.02
3.00	2.90	−0.10
3.00	2.93	−0.07
3.00	2.84	−0.16
3.00	2.90	−0.10
	Mean ± *SD* 2.91 ± 0.05	Mean error −0.09
	RSD 2%	Relative error 3%

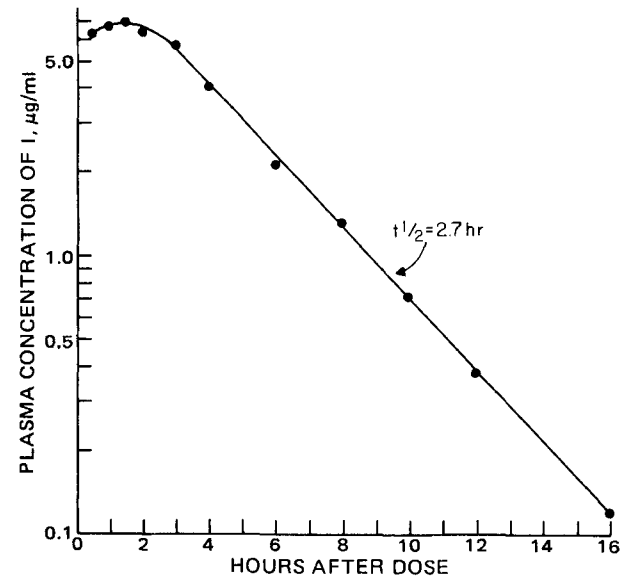


Figure 3—*Plasma I levels in a human subject following a single oral 200-mg dose of I in aqueous suspension.*

standard deviation was 3% or less at all three levels. Thus, excellent precision was demonstrated. The accuracy of this method is indicated by the small mean error between the detected and theoretical values. The mean errors were 0.026, 0.004, and −0.090 μg/ml for the 0.60-, 1.50-, and 3.00-μg/ml samples, respectively. Their corresponding relative errors were 4, 0.3, and −3%.

Specificity—In a metabolic study in humans, the HSLC fraction of I from plasma was collected and purified by repeated chromatography under the analytical conditions described. The purified material (~200 μg) was analyzed by NMR, IR, and high-resolution mass spectrometry in comparison with authentic I. The results unequivocally prove that the HSLC fraction from plasma corresponding to I is in fact I.

Human Plasma Standard Curves—Twenty-two standard curves (0.2–10 μg/ml) were constructed with the same I standard solutions by two analysts over 2 months. The response ratios, I/internal standard for each concentration standard, were pooled, and the mean and standard deviation were calculated. The relative standard deviations were 9, 8, 5, 6, and 5% for the 0.2-, 0.5-, 2.5-, 5.0-, and 10.0-μg/ml standards, respectively. The small relative standard deviations again indicate the precision of this method.

A pooled standard curve of mean response ratios *versus* their corresponding concentrations was constructed by linear regression fitting. The mathematical expression of the pooled standard curve based on all data is $y = 0.540x - 0.016$ (where y = response ratios and x = plasma I concentration in micrograms per milliliter). A correlation coefficient (r) of 0.99991 was obtained, and the excellent linear fit is indicated by the coefficient of determination (r^2) of 0.99982.

The minimum concentration that can be quantitatively determined by this method is 0.2 μg/ml with a 2-ml plasma sample. Lower concentrations can be detected, but quantitation is less accurate.

Rat Plasma Standard Curves—Five standard curves (0.2–10 μg/ml) from blank rat plasma were pooled and treated similarly to those from human plasma. The relative standard deviations were 9, 5, 3, 2, and 1% of their corresponding mean response ratios for the 0.2-, 0.5-, 2.5-, 5.0-, and 10.0-μg/ml standards, respectively. The good precision is indicated by the small relative standard deviation. A straight line was fitted through the mean response ratios and their corresponding concentration standards by linear regression. The mathematical expression of the standard curve based on all data is $y = 0.512x + 0.012$. The correlation coefficient was 0.99999, and the coefficient of determination was 0.99998.

Application to I Analysis in Human and Rat Plasma—A normal human volunteer was given a single, oral, 200-mg dose of I in aqueous suspension in a comparative absorption study. Heparinized blood samples were taken before medication and at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 16, and 24 hr. The plasma I levels determined by this HSLC method are plotted on a log–linear scale in Fig. 3. Following an oral 200-mg dose in this human subject, a peak plasma level of 7 μg/ml was reached at 1.5 hr; the decrease in plasma level followed a monoexponential decline with an estimated plasma I $t_{1/2}$ of 2.7 hr. These results indicate that the method is more than adequate to determine plasma I concentrations in humans at this and lower dose levels.

Ten rats were sacrificed at each of the several time periods following a single oral 1.5-mg/kg dose of I. Heparinized blood samples were collected, and the concentration of I in plasma was determined by the HSLC method. The mean plasma concentrations at various sampling times are plotted on a log–linear scale in Fig. 4. The first sample, 3 hr after the dose, had the highest concentration, 3 μg/ml; the decrease in plasma concentrations between 6 and 12 hr followed a monoexponential decline with an estimated plasma $t_{1/2}$ of 4 hr. These results indicate that the method is capable of monitoring plasma I levels in the rat at low doses.

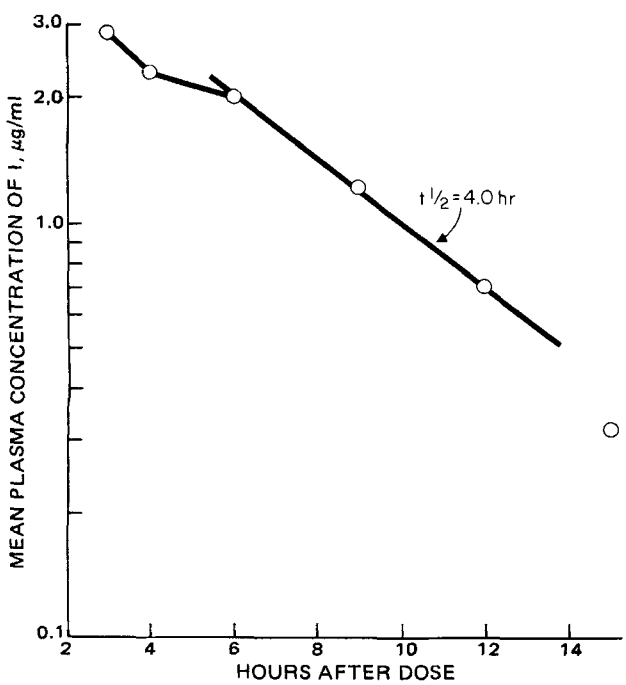


Figure 4—*Mean plasma I levels in 10 rats following a single oral 1.5-mg/kg dose of I in 4% gum acacia.*

DISCUSSION

The results demonstrate that the reversed-phase column is effective in the determination of I, a highly lipophilic compound, in human and rat plasma. The lipophilicity of I provides the strong affinity for the liquid phase, and the drug is eluted with a large elution volume. Most of the endogenous material or metabolites of I carried over in the extraction are relatively less lipophilic than I, so a clean separation is obtained. A minor interference from rat plasma extract (equivalent to 0.02 μg/ml) was present and was routinely corrected.

The low limit of sensitivity is 0.2 μg/ml with a 2-ml plasma sample; if needed, the sensitivity can be improved by measuring I at 300 nm instead of 254 nm. At 300 nm, the response of I is 130% of that at 254 nm. In the present procedure, the residue from the extraction of low concentration samples is dissolved in 40 μl of acetonitrile, and only 10 μl is injected into the chromatograph. Injecting a larger aliquot of the sample should improve the sensitivity.

Both the accuracy and precision of this method are very good. The precision, expressed by the relative standard deviation, is 3% or less at 0.6, 1.5, and 3.0 μg/ml; at the same concentration levels, the accuracy, expressed by the relative error, is 4, 0.3, and −3%, respectively. The precision is further documented by the small relative standard deviation at various concentration levels from the pooled standard curves in humans and rats. Since this analytical method is intended for samples from biological experiments, these levels of precision and accuracy are more than adequate.

For 30 samples, including six standards, the total extraction and analysis time was 10 hr. About 1000 human and rat plasma samples were analyzed by this method over 6 months. About 10% of these samples were subjected to replicate analysis, and duplicate analyses were rarely different by more than 10%.

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Equilibrium Reaction of Pyrazolodiazepinones in Aqueous Solution

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Abstract □ This study of the behavior of some pyrazolodiazepinones in aqueous solution at near ambient temperature indicated that they form stable equilibrium mixtures consisting of ring and opened forms. Under isothermal conditions, mixtures are produced whose composition is dependent on pH and may vary from that corresponding to essentially complete ring opening to complete closure. Ring closure equilibrium constants were calculated, and the influence of methyl substitution was determined. Substitution of a methyl group for an amido hydrogen in the open form results in a fivefold increase in this constant. Methylation of the terminal amino group, however, did not cause a corresponding increase and may not significantly affect equilibrium.

Keyphrases □ Pyrazolodiazepinones, various—equilibrium reaction in aqueous solution, effect of pH and methyl substitution □ Equilibrium—various pyrazolodiazepinones in aqueous solution, effect of pH and methyl substitution □ Structure–activity relationships—various pyrazolodiazepinones, equilibrium reaction in aqueous solution, effect of pH and methyl substitution

1-Ethyl - 4,6 - dihydro-3-methyl-8-phenylpyrazolo[4,3-*e*][1,4]diazepin-5(1*H*)-one (I*a*) (ripazepam) is an investigational new drug of the diazepinone class being evaluated for clinical utility (1–3). This pyrazolodiazepinone, in contrast to 1,4-benzodiazepinones, is easily hydrolyzed to yield the open chain compound 4-(2-aminoacetamido)-5-benzoyl-1-ethyl-3-methylpyrazole (II*a*) (1).

Reports concerning hydrolysis or stability of benzodiazepinones (4–8) indicate that the open form in this series is usually unstable under the conditions required to hydrolyze the diazepinone ring, so it does not permanently accumulate. In addition to the ease of hydrolysis, other characteristics associated with the reaction of the pyrazolodiazepinones in aqueous solution are sufficiently different from those of the benzodiazepinones to warrant further study.

EXPERIMENTAL

Reagents—All chemicals used to prepare 0.1 and 0.05 *M* acetate, citrate, phosphate, and borate buffers of known pH values were reagent grade and were used without further purification. Hydrochloric acid solutions were prepared from prestandardized volumetric solutions[1]. The solvents for equilibrium studies, including hydrochloric acid solutions and various buffers, were adjusted to an ionic strength of 0.3 with potassium chloride.

Compound I*a* was from an experimental batch[2] with a purity of 99.75% *via* differential scanning calorimetric analysis. 4-(2-Aminoacetamido)-5-benzoyl-1-ethyl-3-methylpyrazole (II*a*) dihydrochloride and 1-ethyl-4,6- dihydro -3,4- dimethyl-8-phenylpyrazolo[4,3-*e*][1,4]diazepin-5(1*H*)-one (V*a*) were used as received[3].

Synthesis of 4-[2-(Methylamino)acetamido]-5-benzoyl-1-ethyl-3-methylpyrazole Hydrochloride (VII*c*)—To a solution of I*a*, 10 g in 100 ml of dichloromethane, 8 g of methyl fluorosulfonate was added dropwise over 5 min with stirring. The reaction mixture was allowed to stir for another 2 hr and was then poured into 1 liter of ether with stirring. The yellow precipitate was collected, rinsed with ether, and dissolved in 160 ml of water. The resulting solution was washed with 3 × 30-ml portions of chloroform, and the washings were discarded. The aqueous layer was adjusted to pH 8 to obtain optimal turbidity with sodium hydroxide solution and subsequently extracted with 5 × 30-ml portions of chloroform.

The combined chloroform extract was dried over anhydrous sodium sulfate and filtered. Then dried hydrochloric acid gas was introduced into the chloroform solution until the turbidity was no longer increased. The solvent was removed *in vacuo* using a flash evaporator. The residue thus obtained was recrystallized several times from acetonitrile to afford fine white needles, mp 187–188° dec., in a yield of 6.8 g (54%); UV (methanol): $\epsilon_{258.5} = 1.23 \times 10^4$.

Anal.—Calc. for $C_{16}H_{21}ClN_4O_2$: C, 57.05; H, 6.28; Cl, 10.53; N, 16.64. Found: C, 57.27; H, 6.21; Cl, 10.57; N, 16.65.

Determination of Methylation Site in VII*a*—One gram of VIIc in

[1] Acculute standard volumetric solution, Anachemia Chemicals Ltd.
[2] RxX Lot 41201.
[3] Dr. H. DeWald, Chemistry Department, Parke, Davis & Co.