To determine whether conjugates were present in the bile, samples obtained from Dog Q7-147, which had received fluphenazine-^{14}C enanthate intravenously, were analyzed. Bile from this animal was treated with alkali in order to remove the heptanoic acid group. A chromatogram of this sample (Fig. 7A) shows that all of the radioactivity remained at the origin. Figure 7B shows the same result for a sample of bile from Dog Q8-262, which had received fluphenazine-^{14}C (10 mg./kg. i.v.) (1). Similar results are also obtained with these two samples of bile if the chromatograms are developed in Solvent System 2. Figure 7C reveals that a second peak, which corresponds in R_f value to Metabolite C of fluphenazine-^{14}C, is formed after bile from Dog Q7-147 has been treated with β-glucuronidase. Metabolite C was shown in a companion paper (3) to be identical with 7-hydroxyfluphenazine.

Confirmatory evidence that Metabolite C can be derived from the bile of a dog that had received fluphenazine-^{14}C enanthate was obtained by the following series of experiments. Bile obtained from Dog Q7-147 was given one of two sequential treatments. Either the bile was treated first with β-glucuronidase, followed by alkaline hydrolysis, or the bile was subjected first to alkaline hydrolysis and then treated with β-glucuronidase. In both cases, the samples were chromatographed after the second treatment in Solvent Systems 1, 2, and 3. The results obtained with Solvent System 3 are shown in Fig. 8. Regardless of the sequence of treatments, the same results are obtained in each solvent system; two peaks are observed, one of which corresponds to a brownish zone on the chromatogram and also to the R_f value of Metabolite C. These results are explainable only by the conversion of fluphenazine-^{14}C enanthate to the glucuronide of 7-hydroxyfluphenazine. Although the formation of the glucuronide of 7-hydroxyfluphenazine enanthate is a theoretical possibility for the metabolism of this compound, no 7-hydroxyfluphenazine enanthate was available as a chromatographic reference standard. Such a material would be expected to have an R_f value in Solvent System 3 intermediate between that of fluphenazine and fluphenazine enanthate. In fact, none of the radioactivity seen in Fig. 7C, for example, was observed to move with such an R_f value.

In conclusion, fluphenazine-^{14}C enanthate was shown to be hydrolyzed to fluphenazine-^{14}C by plasma esterases of the dog *in vitro*. Radioactivity present in the bile of a dog that had received fluphenazine-^{14}C enanthate was excreted as the glucuronide conjugate of 7-hydroxyfluphenazine. From the present studies, the authors are unable to determine whether fluphenazine-^{14}C enanthate is first hydroxylated and then has the ester bond cleaved, or whether the ester bond is first cleaved and the compound is then metabolized in a manner essentially like that previously described for fluphenazine-^{14}C (1).

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Separation and Spectrofluorometric Assay of the β-Adrenergic Blocker Sotalol from Blood and Urine

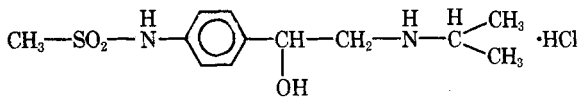
EDWARD R. GARRETT and KURT SCHNELLE

Abstract □ A sensitive spectrofluorometric assay of sotalol·HCl (*d,l*-4-(2-isopropylamino-1-hydroxyethyl)methanesulfonanilide hydrochloride; MJ 1999), a potent and specific β-adrenergic blocking agent, was developed. The compound fluoresces in alkali at 250/350 nm. and in acid at 235/309 nm. The maximum extractability was found at a pH of 9, where the substance is primarily the zwitterion with potentiometric pKa′ values of 8.30 and 9.80, where the former has been spectrophotometrically assigned to the dissociation of the conjugated sulfanilino group. A mixture of *n*-amyl alcohol and chloroform (1 : 3, v/v) at a volume ratio of organic solvent and pH 9 buffer solution of 8 : 1 extracted 85% of the compound, which was completely reextracted into 5.2 *N* HCl. The fluorescence of the monocharged molecule was measured at 235/309 nm. in the acid solution. The sensitivity of the assay was 0.1 mcg./ml. in plasma and 2.5 mcg./ml. in urine. Statistical analyses of assays of sotalol-spiked plasmas, urines, and acidic extraction blanks showed no dose × day interactions and no significant variations of the slopes of calibration curves within 3–4 days of assay; no significant assay differences when the drug was stored under refrigeration in water, acidic extraction blanks, whole blood, plasma, serum, and urine for 2–6 days; and no significant effects from blood and urine obtained from different dogs. The recovery of the drug was 69.4% from plasma and 81% from urine. The sensitivity of 0.1 mcg./ml. in plasma and 2.5 mcg./ml. in urine and the reproducibility of 2% at a midrange concentration of 0.5 mcg./ml. in plasma and of 2.2% at a midrange concentration of 10 mcg./ml. in urine allow the assay to be applicable for monitoring blood levels and urinary excretion of the drug when administered in therapeutic amounts.

Keyphrases □ Sotalol determination—blood, urine □ Partition coefficients—sotalol·HCl □ pKa determination—sotalol·HCl □ Variability parameters—sotalol·HCl fluorometric analysis □ Fluorometry—analysis, biological fluids

Sotalol·HCl, *d,l*-4-(2-isopropylamino-1-hydroxyethyl)methanesulfonanilide hydrochloride (MJ 1999), is a specific and potent β-adrenergic blocking agent in animals (1, 2) and man (2–4), with a structure (I) similar to isoproterenol.

The plasma levels of tritium-labeled drug were shown to be proportional to its pharmacological activity in dogs (2). However, the study of distribution and excretion of drugs in animals and man to establish opti-



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mum dose regimens demands nonradioactive analytical methods. This paper describes the chemistry, extraction, and spectrofluorometric assay for sotalol·HCl in blood and urine.

EXPERIMENTAL

Equipment—A Farrand MK-1 spectrofluorometer was used in the analysis of sotalol·HCl[1]. The pKa′ values were determined with a Radiometer recording titrator, type TTT 1c/SBR 2c, equipped with a combination electrode, miniature (Sargent); a recording UV spectrophotometer (Cary model 15) was also used. All solutions used in the analytical procedure were prepared with all-glass-distilled water. The glassware was cleaned with Hemosol, rinsed repeatedly with tap water and deionized water, and soaked in 1:3 concentrated HNO_3–HCl mixture. After a final rinse with deionized water and distilled water, the glassware was dried and ready for use.

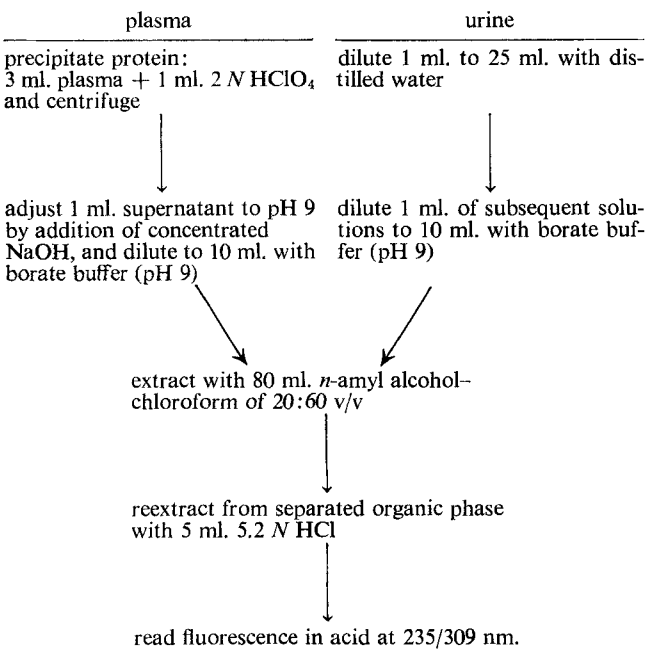
Reagents—The following were used: 5.2 *N* HCl (1:1 dilution of HCl reagent ACS), 2 *N* $HClO_4$ (analytical reagent), Na_2SO_4 (analytical reagent), and 0.5 *M* borate buffer pH 9 (5). All organic solvents used were of analytical reagent grade. The sotalol·HCl was used as the powder[2].

pKa′ Determinations—Sotalol·HCl (0.0054 *M*) was dissolved in 0.0050 *N* HCl, and the ionic strength was adjusted to 0.20 with added KCl. The solution was titrated with 0.3000 *N* NaOH. An equal volume of 0.0050 *N* HCl was also titrated with 0.3000 *N* NaOH in a blank titration.

The spectra of 6.7×10^{-5} *M* sotalol·HCl were recorded at various pH values in the range 5.5–11.0. Phosphate buffers (0.05 *M*) were used within the pH range of 5.5–8.2, borate buffers (0.05 *M*) within 8.4–9.6, and carbonate buffers (0.05 *M*) within 10.0–11.0 (5). All buffers were adjusted to a constant ionic strength of 0.20 with KCl.

Partition Coefficients—Sotalol·HCl (1.07×10^{-4} *M*) was analyzed spectrophotometrically in buffers of various pH values before and after extraction with buffer-saturated chloroform. Other organic solvents which were screened to obtain the solvent system chosen for maximum extraction from aqueous buffer solutions were cyclohexane, ethyl ether, ethyl acetate, *o*-dichlorobenzene, ethylene dichloride, *n*-amyl alcohol, and various mixtures of chloroform with ethyl acetate and *n*-amyl alcohol.

Analytical Procedure (Scheme I): Plasma Analysis—Heparinized dog blood was centrifuged at 3000 r.p.m. for 15 min. to separate the red blood cells from plasma. A stock plasma solution of 1 mcg./ml. sotalol·HCl was prepared by adding a concentrated aqueous solution of sotalol·HCl to this plasma. This stock plasma solution was diluted with plasma to prepare solutions that were 0.1, 0.3, and 0.6 mcg./ml. of sotalol·HCl. One milliliter of 2 *N* $HClO_4$ was added to 3 ml. of these plasma solutions to precipitate the protein. The samples were centrifuged for 5 min. at 3000 r.p.m. One milliliter of the clear supernatant was adjusted to pH 9 with concentrated NaOH and diluted to 10 ml. with 0.5 *M* borate buffer. The solutions were again centrifuged to remove traces of precipitate and were then transferred into 125-ml. separators containing 80 ml. of a H_2O–saturated *n*-amyl alcohol–chloroform mixture (20:60, v/v). The samples were shaken for 30 min. on an automatic shaker. After 30 min. of separation, the solvent was drained into conical flasks containing 3.5 g. anhydrous Na_2SO_4 to remove traces of water in the solvent. The aqueous phase was discarded. The dried solution was then decanted into separators containing 5 ml. 5.2 *N* HCl and shaken for 30 min. After 30 min. of separation, the solvent was drained and discarded. The aqueous phase was centrifuged for 2 min. to remove residual solvent. The samples were then analyzed on the spectrofluorometer at 309 nm. with excitation at 235 nm., using a slit width of 10 nm. for excitation and 20 nm. for fluorescence measurement at a meter sensitivity setting of 0.03.



Scheme I—Analysis of Sotalol·HCl in Plasma and Urine

[1] Mead Johnson & Co.
[2] Obtained from Mead Johnson & Co.

Table I—Results[a] of Repetitive Duplicate Assays of Drug Spiked in Blanks and Extracted on Different Days from Different Waters, Plasmas, and Urines Spiked on Different Days

Dose, mcg./ml.	Day 1		Day 2		Day 3		Day 4	
	1	2	1	2	1	2	1	2
Drug Added to Acidic Aqueous Extraction Blanks								
0	0.30	0.30	0.28	0.30	0.28	0.40	0.29	0.29
0.01	0.50	0.44	0.48	0.42	0.49	0.46	0.42	0.45
0.03	0.68	0.74	0.70	0.75	0.80	0.80	0.80	0.80
0.06	1.00	1.00	1.00	0.97	1.04	1.04	1.10	1.12
0.10	1.75	2.18	1.67	2.14	1.72	2.04	2.09	2.10
Drug Extracted from Water								
0	0.28	—	0.28	—	0.20	—		
0.1	0.38	0.45	0.38	0.38	0.38	0.34		
0.3	0.59	0.64	0.60	0.64	0.60	0.69		
0.6	0.98	0.98	0.99	1.02	0.99	0.94		
1.0	1.52	—	1.57	1.48	1.28	1.40		
Drug Extracted from Plasma								
0	0.24	—	0.27	0.24	0.23	0.24		
0.1	0.34	0.35	0.33	0.30	0.34	0.32		
0.3	0.50	0.55	0.50	0.51	0.54	0.54		
0.6	0.83	0.90	0.87	0.91	0.85	0.90		
1.0	1.40	1.34	1.36	1.30	1.29	1.30		
Drug Extracted from Urine								
0	0.32	—	0.34	0.34	0.33	0.35		
2.5	0.48	0.47	0.45	0.47	0.45	0.45		
5.0	0.62	0.63	0.56	0.56	0.54	0.54		
10.0	0.86	0.84	0.80	0.82	0.84	0.82		
15.0	1.17	1.10	1.14	1.18	1.15	1.12		
20.0	1.39	1.30	1.36	1.35	1.28	1.36		

[a] Results are given in the arbitrary fluorescence units of the Farrand MK-1 spectrofluorometer at a meter sensitivity setting of 0.03 and measured at 235/309 nm.

Urine Analysis—Dog urine was obtained by catheterization and centrifuged to spin down the sediment. A urine stock solution of 25 mcg./ml. sotalol·HCl was prepared by adding concentrated solutions of sotalol·HCl to this urine. This stock solution of urine was diluted with urine to prepare solutions that were 2.5, 5, 10, 15, and 20 mcg./ml. of sotalol·HCl. Aliquots (1.00 ml.) of these solutions were first diluted up to 25 ml. with distilled H_2O. Aliquots (1.00 ml.) of these subsequent solutions were then diluted up to 10 ml. with 0.5 *M* borate buffer of pH 9. The buffer capacity was sufficient to maintain this pH. The subsequent centrifugation, extraction into *n*-amyl alcohol–chloroform, drying with anhydrous Na_2SO_4, decanting and extraction into concentrated HCl, and spectrophotofluorometric analysis were carried out in the same manner as specified for plasma.

Sources of Variability in Analysis: Instrument Variability—Prior to starting a series of readings, the spectrofluorometer was adjusted to maximum sensitivity and the reading of a quinine bisulfate standard solution (10 mcg./ml. in 0.1 *N* H_2SO_4) was adjusted to a constant meter reading of 2.0 at range 0.03 at 235/309 nm., the

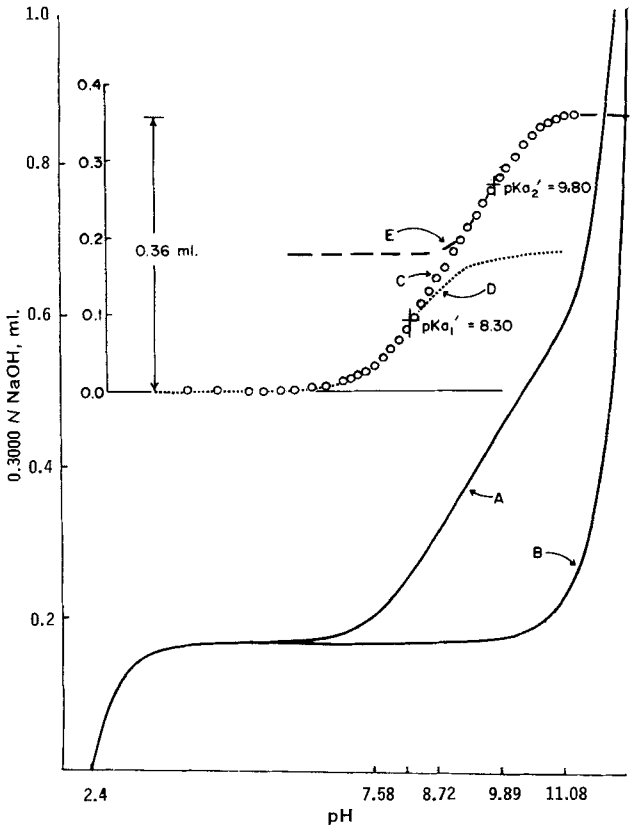


Figure 1—*Potentiometric titrations of 10 ml. 5.4 × 10^{-3} M sotalol·HCl, μ = 0.2 (Curve A), and 5 × 10^{-3} N HCl, μ = 0.2, as a blank (Curve B). The open circles (Curve C) mark the differential titration curve, i.e., the difference between the milliliters of 0.3000 N NaOH necessary to reach a given pH for sotalol (A) and blank (B). The dashed lines are the constructed titration curves (D and E) for the two acid equivalents of sotalol. The midpoints between two asymptotes serve to estimate the pKa_1' and pKa_2' values.*

wavelengths used for the analysis of sotalol·HCl. This reading was checked before and after each sample measurement.

Variability Due to Extraction Procedure—Drug was added to water (0.1, 0.3, 0.6, and 1.0 mcg./ml.), and duplicate extractions were carried out for each concentration on 3 subsequent days. The samples were stored at 4° and assayed on the 4th day; the yield of extraction was determined from direct analysis of aqueous acidic solutions of 0.01, 0.03, 0.06, and 0.1 mcg./ml., which encompass the range of concentrations after the dilution processes of the extraction procedure. These spiked aqueous acidic solutions were prepared from solutions run through the extraction procedures starting with water without drug. The data are given in Table I.

Variability Due to Differences among Animals—Blood and urine were obtained from three dogs, each on a separate day. Duplicate extractions were made from each plasma containing 0.1, 0.3, 0.6, and 1.0 mcg./ml. and each urine containing 2.5, 5, 10, 15, and 20 mcg./ml. A different plasma and urine were used on each of the 3 subsequent days. The resultant extracts in 5.2 *N* HCl were stored at 4° and assayed on the 4th day. The data are given in Table I.

Effect of Storage at 4° in Whole Blood, Plasma, and Serum—Sotalol·HCl was added to heparinized whole blood, plasma, and serum (0, 0.1, 1.0, and 10.0 mcg./ml.) and stored at 4°. Whole blood was centrifuged on 2 subsequent days, and the plasma was run through extraction and analyzed. Plasma and serum, containing the drug in the same concentrations, were extracted and assayed on 2 subsequent days and on the 6th day after adding the drug.

Effect of Storage at 4° in Water and in Urine—Drug was added to water (0, 0.3, and 0.6 mcg./ml.) and urine (0, 2, and 6 mcg./ml.). The samples were stored at 4° while carrying out extractions on 3 subsequent days. The samples were assayed on the 4th day.

Statistical Analysis—The calibration curves for sotalol·HCl were statistically evaluated by stepwise regression analysis, using a digital computer program (6), and by analysis of variance (7).

RESULTS AND DISCUSSION

Estimation of Dissociation Constants: Potentiometric pKa' Values—There are two dissociable acidic groups in sotalol·HCl (I): the neutral sulfonated aniline and the protonated amino group in the isopropylamino side chain. The differential potentiometric titration method of Parke and Davis (8, 9) was applied. The difference curve was constructed (open circles in Fig. 1) from the subtraction of the milliliters of standard alkali consumed in the blank titration from that consumed by sotalol·HCl to obtain the same pH value. The distance (0.36 ml.) between the two asymptotic values of the difference curve (inset, Fig. 1) represents the base in milliliters used to neutralize both of the hydrogen ions of sotalol·HCl since 0.18 ml. of 0.3000 *N* NaOH was necessary for one acid equivalent of the titrated 10 ml. of 5.4 × 10^{-3} *M* sotalol·HCl. The constructed titration curves (dashed lines in Fig. 1) for each acid equivalent were used to estimate pKa' values at the pH values of half-neutralization for each equivalent. The pKa_1' value of 8.30 and the pKa_2' value of 9.80 were so estimated.

Spectrophotometric pKa' Determination—Sotalol·HCl has two UV absorption peaks; the $\lambda_{max.}$ 227 nm. is favored below a pH of 8.5, and the $\lambda_{max.}$ 248 is favored above this pH value (Fig. 2). Two isosbestic points were observed, at 234 nm. and approximately at 215 nm. The absorbances at the maximum wavelengths were plotted as a function of pH (Fig. 3). The pKa_1' of 8.35 was calculated from the appropriate plot (Fig. 4) of the Henderson–Hasselbalch equation:

$$\text{pKa} - \text{pH} = \log \frac{|A - A_{(^-MJ)}|}{|A_{(HMJH^+)} - A|} \qquad \text{(Eq. 1)}$$

where A is the absorbance value at the pH of each buffer solution, $A_{(^-MJ)}$ is the asymptotic absorbance value obtained in alkaline solution, and $A_{(HMJH^+)}$ is the asymptotic value in acidic solution. This value can only be related to the spectrally affected dissociation of the anilino hydrogen in sotalol·HCl (I). The pKa_2' of 9.80 can now be assigned to the protonated amine group in the side chain.

The presence of four species of sotalol·HCl in solution can be based on these assignments: a positively charged ($HMJH^+$), a neg-

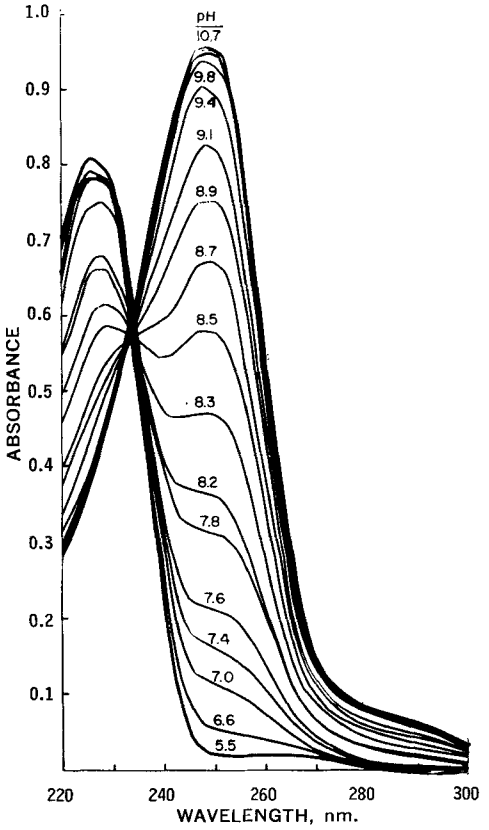


Figure 2—*UV absorption spectra of 6.7 × 10^{-5} M sotalol in buffers of μ = 0.2 as a function of pH.*

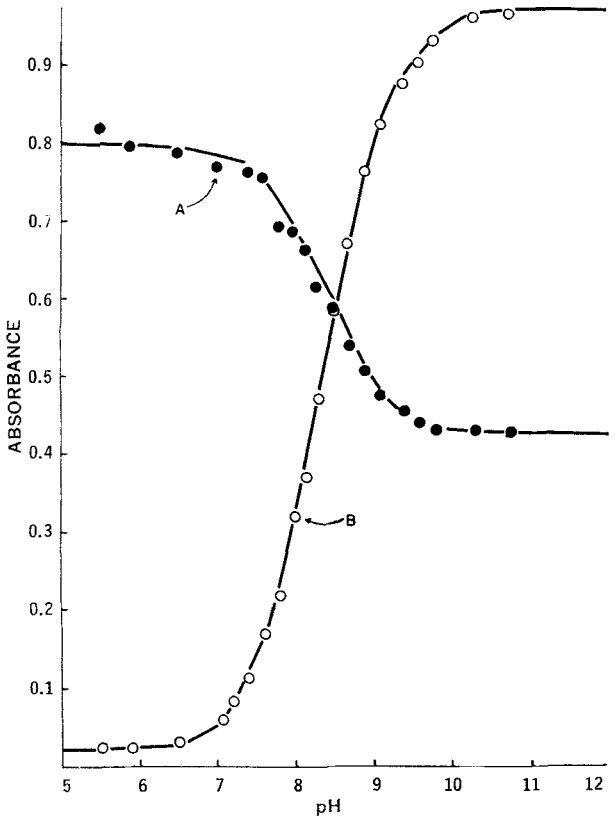


Figure 3—*Absorbance, A, of 6.7 × 10⁻⁵ M sotalol at λ_{max}. 227 nm. (Curve A) and λ_{max}. 248 nm. (Curve B). The lines drawn through these points were calculated on the basis of*

$$pKa' - pH = \log \frac{[A - A_{(^-MJ)}]}{[A_{(HMJH^+)} - A]}$$

for a pKa′ = 8.35, where $A_{(^-MJ)}$ is the absorbance reached in the most alkaline solution, and $A_{(HMJH^+)}$ is the absorbance reached in the most acidic solution.

atively charged ($^-$MJ), a neutral form (HMJ), and a zwitterion ($^-$MJH$^+$) (Scheme II). At high acidities, only the protonated species (HMJH$^+$) should exist; in strong alkaline solution, only the negatively charged form ($^-$MJ) should exist. All four species should be present at the intermediate pH values, and the dissociation con-

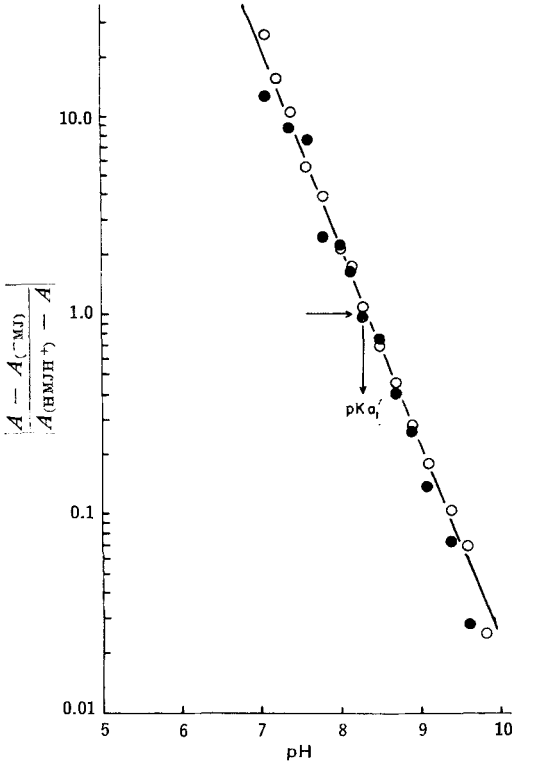


Figure 4—*Semilogarithmic plot for 6.7 × 10⁻⁵ M sotalol, μ = 0.2, in accordance with*

$$pKa' - pH = \log \frac{[A - A_{(^-MJ)}]}{[A_{(HMJH^+)} - A]}$$

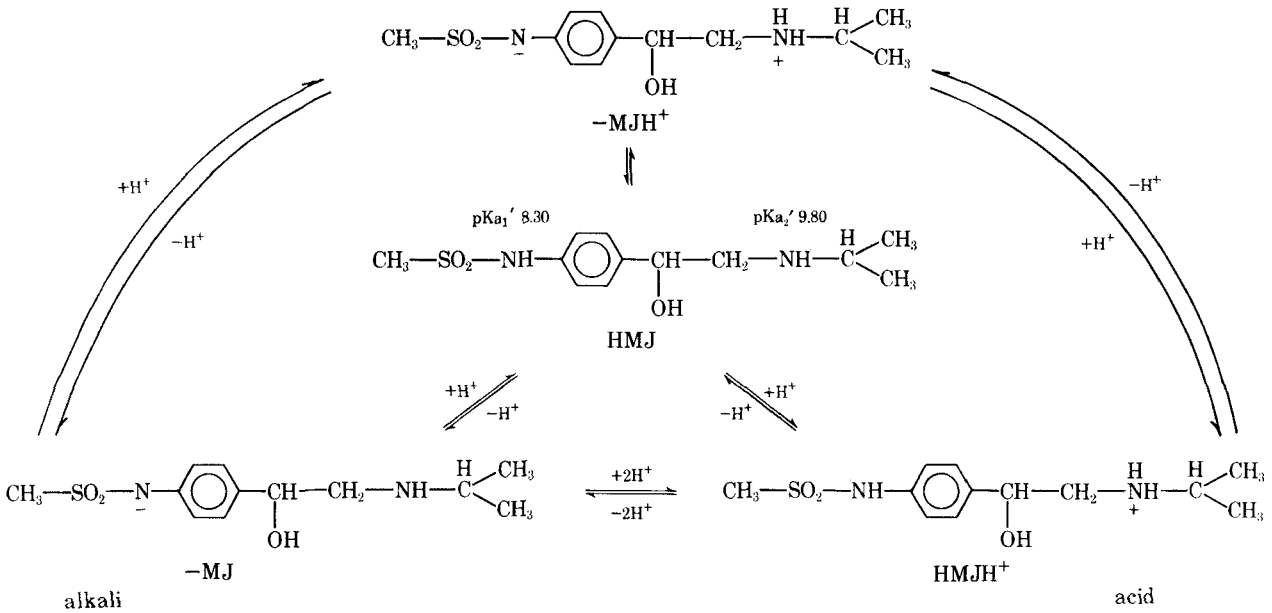
Key: ●, from values obtained at λ_{max}. 227 nm.; and ○, from values obtained at λ_{max}. 248 nm.

stants could be written as

$$K_{a_1}' = 10^{-8.30} = \frac{([HMJ] + [^-MJH^+])\,[H^+]}{[HMJH^+]} \qquad \text{(Eq. 2)}$$

and

$$K_{a_2}' = 10^{-9.80} = \frac{[^-MJ]\,[H^+]}{([HMJH^+] + [^-MJH^+])} \qquad \text{(Eq. 3)}$$



Scheme II—Possible Equilibria among Ionic Species Derived from Sotalol·HCl

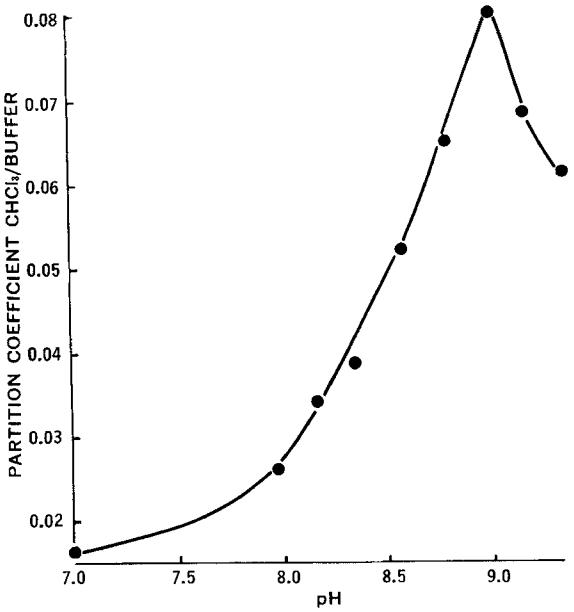


Figure 5—*Plot of apparent partition coefficient between chloroform–buffer for 1.07 × 10⁻⁴ M sotalol in buffer as a function of the pH of the aqueous phase.*

The relative amounts of the neutral species (HMJ) and zwitterion ($^-MJH^+$) present could be estimated from the shape of the plot of UV absorbance *versus* pH (Fig. 3), since it can be assumed that $^-MJH^+$ and ^-MJ have the same spectrum. If considerable amounts of neutral species were formed with increasing pH, the semilogarithmic plot (Fig. 4) of Eq. 1 would not be linear and of unit slope since the asymptotic absorbance only would be reached at much higher pH values, *i.e.*, > 10.8. The reason for this is that significant amounts of the uncharged form, HMJ, would exist through the process that gave the second pKa_2'. Since this is not so, it may be concluded that only very small amounts of the neutral species exist in solution.

Partition Coefficient—Sotalol·HCl must be extracted from body fluids to be assayed. It was anticipated that maximal extraction could be effected into an organic solvent at maximum concentration of the neutral form in equilibrium with the zwitterion, halfway between the two pKa's, *i.e.*, about pH 9. Sotalol·HCl, dissolved in

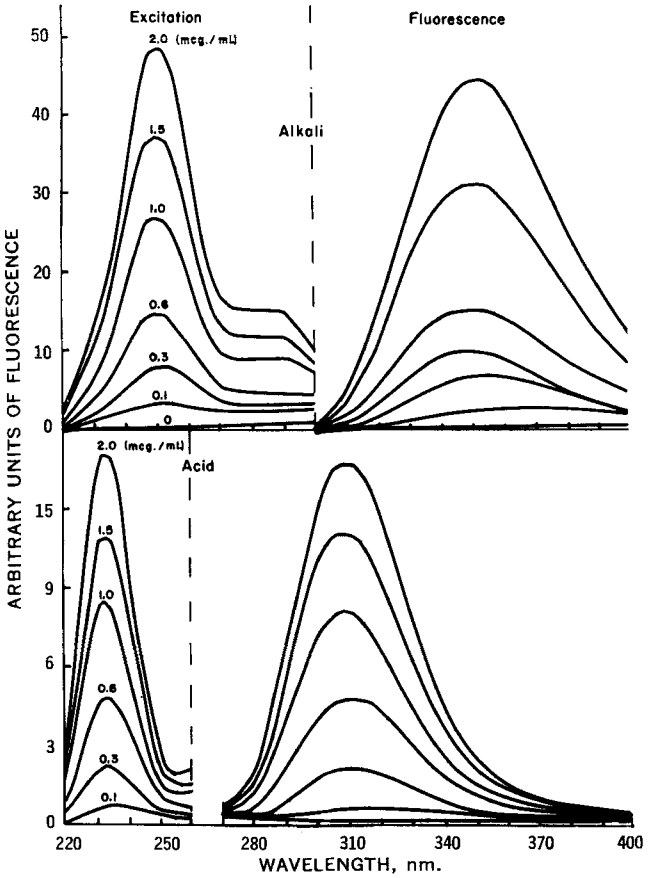


Figure 6—*Excitation and fluorescence spectra of various concentrations of sotalol·HCl labeled in micrograms/milliliter in both acid (5.2 N HCl) and alkali (1.0 N NaOH). The excitation peak was 235 nm. in acid and 250 nm. in alkali; the fluorescence peak was 309 nm. in acid and 350 nm. in alkali.*

Table II—Analyses of Variances of Table I Data

Source	Degrees of Freedom	Mean Square	Components of Variance[a]	*F*-Value	Significance at 5% Level
		Direct Analysis of Drug Added to Blanks			
Dose	4	3.4277		263.7	
Days	3	0.0102	$\sigma_D^2 = \sigma^2 + 10\,\sigma_1^2$	0.8	N.S.
Dose × days	12	0.0052		0.4	N.S.
Duplicates	20	0.0135	σ^2		
Total	39				
		Drug Extracted from Water and Analyzed			
Dose	4	1.1317		566.0	
Days	2	0.0095	$\sigma_D^2 = \sigma^2 + 7.14\,\sigma_1^2$	4.7	S.
Dose × days	8	0.0034		1.7	N.S.
Duplicates	11	0.0020	σ^2		
Total	25				
		Drug Extracted from Plasma and Analyzed			
Dose	4	1.1240		1605.7	
Days	2	0.0005	$\sigma_D^2 = \sigma^2 + 9.37\,\sigma_1^2$	0.6	N.S.
Dose × days	8	0.0009		1.1	N.S.
Duplicates	14	0.0008	σ^2		
Total	28				
		Drug Extracted from Urine and Analyzed			
Dose	5	0.8992		1284.6	
Days	2	0.0014	$\sigma_D^2 = \sigma^2 + 11.37\,\sigma_1^2$	2.0	N.S.
Dose × days	10	0.0009		1.2	N.S.
Duplicates	17	0.0007	σ^2		
Total	34				

[a] σ^2 = Mean square of error within duplicate determinations. σ_D^2 = Mean square of error within duplicate determinations plus variance component due to differences among days.

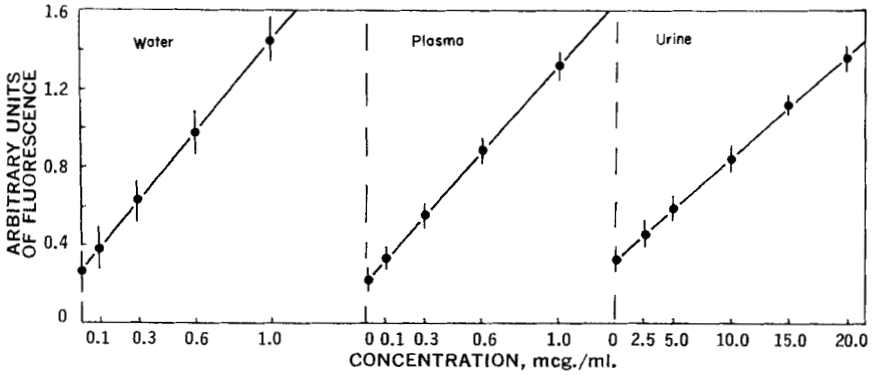


Figure 7—*Calibration curves for sotalol·HCl extracted from spiked water, plasma, and urine. The vertical lines indicate the 95% confidence intervals (twice the standard deviation) for the mean values at various concentrations obtained from duplicate extractions made from replicate solutions, each prepared and run through the analytical procedure on 3 successive days.*

buffers of various pH, was extracted with chloroform. A definite pH dependency of the partition coefficient was observed (Fig. 5), with a maximum partition coefficient of $C_{CHCl_3}/C_{buffer} = 0.08$ at pH 9. This was indicative of the presence of some neutral molecules (HMJ) in equilibrium with the zwitterion ($^-MJH^+$) which, although in low concentration, possessed a sufficiently high coefficient to permit partition into the organic solvent. The mixture of *n*-amyl alcohol–chloroform (1:3, v/v) was the most efficient, with a partition coefficient of 0.68. At a volume ratio of aqueous buffer phase and solvent phase of 1:8, about 85% of the compound was extracted into the solvent mixture. This amount was quantitatively reextractable into acid or alkali where practically all of the molecules were charged.

Spectrofluorometric Assay—After oral administration of a therapeutic dose of 1–2 mg./kg., plasma levels of sotalol·HCl may be expected to be 1 mcg./ml. and below. Since the limit of detection by means of UV spectrophotometry was about 2 mcg./ml., a more sensitive assay method for the drug had to be found. When the excitation and emission spectra of the compound on a spectrofluorometer were scanned, peaks were found in acid medium at 235/309 nm. and in alkali at 250/350 nm. (Fig. 6). The fluorescence was about three times higher in alkali than in acid. The lower limit of detection in both acid and alkali was 0.01 mcg./ml. Since the background reading for extracts from blood and urine in acid was lower and less variable than in alkali, the method of choice was to reextract the compound into 5.2 *N* HCl and to read the fluorescence in that medium.

Calibration Curves for Sotalol—Calibration curves for sotalol·HCl added to and extracted from water, plasma, and urine are given in Fig. 7. Each point represents the mean of three duplicate determinations carried out on each of 3 subsequent days. Each solution was separately prepared on each day, using plasma and urine from different animals. The curves were fitted by the method of least squares, and the 95% confidence intervals ($\pm 2\sigma$) of each point are indicated by the vertical lines in Fig. 7. The slopes of the combined response–dose data are 15.89 (spectrofluorometric response units per microgram/milliliter at 235/309 nm. using a meter sensitivity setting of 0.03) for direct analysis of drug added to the extraction blanks, 1.188 for the procedure when the drug was extracted from water, 1.102 when the drug was extracted from plasma (10-fold dilution compared to direct analysis), and 0.0515 for the procedure when the drug was extracted from urine (250-fold dilution compared to direct analysis). The data on which the calibration curves were based are listed in Table I.

After correction of the slopes for the dilutions made during the analytical procedure, the average recovery of the drug could be determined from the ratio of the corrected slopes to the slope of 15.89 obtained from the drug-spiked extraction blanks. The mean recovery values on extraction and analysis obtained in this manner were 74.8% from water, 69.4% from plasma, and 81.0% from urine. Of course, additional extractions would increase the effectiveness of the separation procedures.

Analysis of Variance—The analysis of variance tables for the four studies (Table I, Fig. 7) are given in Table II and are for concentrations of drug analyzed in extraction blanks and concentrations of drug extracted from water, plasma, and urine and subsequently analyzed. None of the dose × day-interactions was significant. Therefore, within any one study (either drug added to extraction blanks and analyzed or drug extracted from water, plasma, or urine and analyzed), the slopes of the lines on different days (Fig. 7) are the same. The slopes were previously summarized.

A significant difference due to days was found only in the extraction of the drug from water and subsequent analysis. In all other instances, the variation about regression was not significant among days and thus was not significant among the different plasmas and urines obtained from different dogs since plasmas and urines from different dogs were used on the different days. Since there were no significant differences among days for the assay of drug stored in refrigerated acidic extraction blanks for 4 days, it can be concluded that extracted samples may be safely stored for this length of time.

If the regression equation is taken as

$$y = a + bx \qquad \text{(Eq. 4)}$$

the variance of a predicted drug concentration x from a given response can be approximated by (10):

$$\text{variation } (\hat{x}) = \frac{\sigma^2}{b^2\sum(x-\bar{x})^2}\left[\frac{\sum x^2}{n} + \frac{2(y-a)\bar{x}^2}{b} + \left(\frac{y-a}{b}\right)^2\right] \qquad \text{(Eq. 5)}$$

Table III—Sotalol·HCl[a] Spiked in Whole Blood, Plasma, and Serum and Stored for Several Days and Analyzed

Dose, mcg./ml.	Whole Blood Days 1	Whole Blood Days 2	Plasma Days 1	Plasma Days 2	Plasma Days 6	Serum Days 1	Serum Days 2	Serum Days 6
0.1	0.11	0.11	0.11	0.11	0.10	0.16	0.14	0.16
1.0	0.44	0.50	0.56	0.58	0.53	0.54	0.58	0.55
10.0	3.95	4.35	4.00	3.75	4.10	4.35	4.50	4.10

[a] Results are given in the arbitrary fluorescence units of the Farrand MK-1 spectrofluorometer at a meter sensitivity of 0.03 and measured at wavelength 242/309 nm.

Table IV—Analysis of Variance of Table III Data

Source	Degrees of Freedom	Mean Square	*F*-Value	Significance at 5% Level
Dose	2	37.8169	2260.42	
Media	2	0.0424	2.53	N.S.
Dose × media	4	0.0301	1.80	N.S.
Error among days	15	0.0167		

Table V—Storage Effects on Sotalol·HCl[a] Spiked in Water and Urine and Analyzed

Dose, mcg./ml.	Water Days 1	Water Days 2	Water Days 3	Dose, mcg./ml.	Urine[a] Days 1	Urine[a] Days 2	Urine[a] Days 3
0.0	0.38	0.32	0.35	0.0	0.67	0.60	0.67
0.3	0.87	0.73	—	2.0	2.65	2.75	2.77
0.6	1.13	1.18	1.14	6.0	8.10	8.50	8.65

[a] Results are given in the arbitrary fluorescence units of the Farrand MK-1 spectrofluorometer at a meter sensitivity of 0.03 and measured at wavelength 235/309 nm. The urines were diluted 10-fold during the analytical procedure.

Table VI—Analysis of Variance of Table V Data

Source	Degrees of Freedom	Mean Square	*F*-Value	Significance at 5% Level
Drug Extracted from Urine				
Dose	2	48.5496		
Days	2	0.0384	1.59	N.S.
Dose × days	4	0.0242		
Drug Extracted from Water				
Dose	2	0.5269		
Days	2	0.0757	1.05	N.S.
Dose × days	4	0.0721		

where a = intercept, b = slope, σ^2 = mean square of error, x = dose, and $\bar{x}$ = mean dose.

The percent error in terms of concentrations for a given response is obtained by

$$\text{percent error} = \frac{\sqrt{\text{variation } (\hat{x})} \cdot 10}{x} \qquad \text{(Eq. 6)}$$

Therefore, for a drug concentration of 0.5 mcg./ml. in plasma, the error would be estimated as 1.99%; and for a drug concentration of 10 mcg./ml. in urine, it would be 2.23%.

Effect of Storage at 4° in Whole Blood, Plasma, and Serum—Sotalol·HCl was extracted and analyzed from the plasma prepared from spiked whole blood on 2 subsequent days. The drug was extracted and analyzed from spiked plasma and serum on 2 subsequent days and on the 6th day after spiking.

The data obtained are listed in Table III. The analysis of variance is given in Table IV.

The results indicate that there is no contribution of the different media to the error among days, *i.e.*, there is no difference in the spectrofluorometric response whether the medium spiked was whole blood, plasma, or serum.

Effect of Storage at 4° in Water and Urine—Sotalol·HCl was extracted from spiked water and urine on 3 subsequent days and analyzed. The data are listed in Table V.

Since there were no duplicate determinations, the dose × day-interaction term was used as the error term in the analysis of variance (Table VI). No difference was found among days when the drug was stored in urine or water.

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Vasomotor and α-Block Negating Actions of a Hydroxamic Acid

HORST KEHL

Abstract □ β-Hydroxylamine cinnamonyl hydroxamic acid produced a rapidly reversible hypotension in the anesthetized dog. The hypotensive action of β-hydroxylamine cinnamonyl hydroxamic acid was not augmented by α-receptor block, nor was it negated by β-receptor block. In the presence of an α-blocking agent, the classical vasodepressor action of epinephrine was clearly reversed by the simultaneous intravenous infusion of β-hydroxylamine cinnamonyl hydroxamic acid. The β-hydroxylamine cinnamonyl hydroxamic acid-induced hypotension and α-block negation could not be explained in terms of any known neurohumoral mediator mechanism; its action appears to be direct and attributable to its unique molecular structure.

Keyphrases □ Hydroxamic acid derivative—vasomotor, α-block negating action □ β-Hydroxylamine cinnamonyl hydroxamic acid—hypotensive, α-block negating action □ α,β-Receptors, blockade effect—β-hydroxylamine cinnamonyl hydroxamic acid action □ Antihistamine effect—β-hydroxylamine cinnamonyl hydroxamic acid action

Hydroxamic acids may be described as *N*-hydroxylated amides occurring naturally in plants and microbes. Neilands (1) described the outstanding biochemical features of hydroxamic acids and reviewed their biological actions. Because phenylethyl hydroxamic acids (Ar—C—C—CO—NH—OH), analogous to vasoactive phenylethylamines, have never been examined for vasomotor activity, a number of hydroxamic acids with two- and three-carbon structures juxtapositioned between a phenyl ring and the hydroxamic acid moiety