Of particular significance is the fact that diazoketone IV*b* is more active than ketol V*b* which is derived from it. This strongly supports the assumption that the Clauberg activity measured for the diazoketones is a measure of their inherent activity and not simply a measure of the activity of their hydrolysis products.

It was also of interest to observe that the carboxylic acids III*a*–III*d* showed a low, but real, Clauberg activity. The limited data available suggest that although these acids may not elicit a high level of response in this assay, up to a response level of 2.0+, their activity is equal to, or slightly greater than, that of the isomeric methyl esters. The calculated $\pi$-values (5) are also slightly greater for the acids than for the isomeric methyl esters.

None of the diazoketones has an activity so abnormal, either in comparison with the other members of this series or with that of the analogous methyl esters, as to suggest that it has alkylated the Clauberg receptor. However, the activity of diazoketones IV*a*, IV*b*, and, to a lesser extent, IV*c* seems surprisingly high, and it may warrant a reinvestigation with an assay based on intrauterine application of these compounds.

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# Spectrophotofluorometric Determination of Salicylamide in Blood Serum and Urine


**STEPHEN A. VERESH, FOO SONG HOM, and JOHN J. MISKEL**



**Abstract** □ A spectrophotofluorometric method is reported for the determination of salicylamide in blood serum and urine. The procedure involves the simultaneous determination of salicylamide and salicylic acid at pH 11 after the acid hydrolysis of the salicylamide metabolites. The precision and accuracy of the method are comparable to the regular ferric-ion complex procedure. The present method has the sensitivity and specificity desired in the assay of blood serum and urinary salicylamide concentrations after the oral ingestion of a 325-mg. dose of the drug. The method may be used in monitoring bioavailability of salicylamide provided by different dosage forms.



**Keyphrases** □ Salicylamide—spectrophotofluorometric determination, in blood serum, urine □ Bioavailability evaluation—application of spectrophotofluorometric salicylamide determination □ Spectrophotofluorometry—salicylamide determination, blood serum, urine


The absorption and pharmacokinetics of salicylamide in humans were found to be both dose and dosage form dependent (1–3). However, most of the salicylamide blood level results reported were elicited with larger than normal doses of the drug. Therefore, a sensitive yet specific analytical method is needed to study the blood serum levels of salicylamide in humans after the ingestion of as little as 325 mg. of the drug. The ferric-ion complex colorimetric method is not sufficiently sensitive for this purpose.

In basic solution, salicylamide is fluorescent. Based on this property, several methods were reported for the assay of free salicylamide in biological fluids. Lange *et al.* (4) employed a fluorometric technique to determine salicylamide and other salicylates following a gel filtration separation. Barr and Riegelman (3) detailed a spectrophotofluorometric procedure for the determination of salicylamide and its metabolites in plasma and other biological fluids. Unfortunately, the body metabolizes salicylamide; hence, very little exists as free salicylamide in the blood or urine (1, 5, 6). For this reason, total salicylamide is determined as free salicylamide after acid hydrolysis. Barr and Riegelman (3)

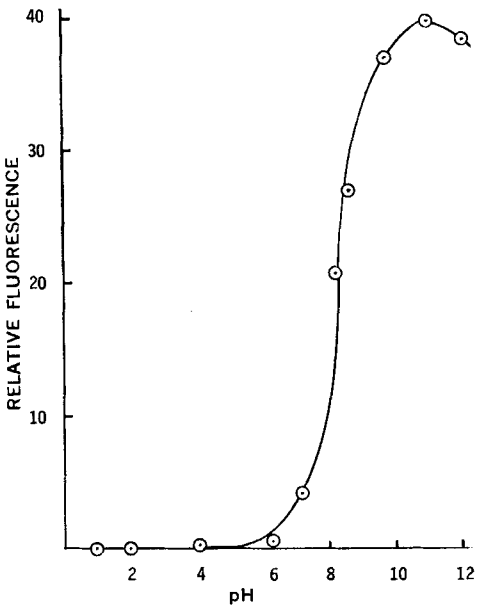


**Figure 1**—*Relative fluorescence of 0.1 mcg./ml. of salicylamide as a function of pH at the activation and emission wavelengths of 340 and 435 nm., respectively.*

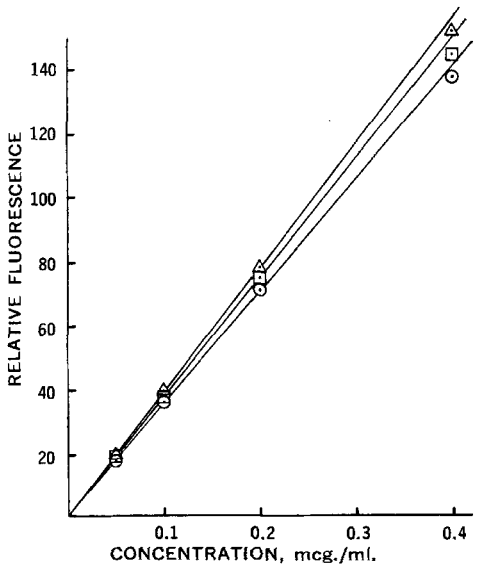


**Figure 2**—*Standard curves of the sum of relative fluorescences at 435 nm. from activations at 310 and 340 nm.* versus *total amounts of salicylamide and/or salicylic acid. Key:* ⊙, *salicylic acid;* △, *salicylamide; and* ⊡, *1:1 mixture of salicylic acid and salicylamide.*

employed hydrolysis methods similar to the one described by Levy and Matsuzawa (1). Total salicylamide was determined by hydrolyzing all metabolites to salicylic acid. The fluorescence of salicylamide in the aqueous basic solution was determined directly at the maximum activation and emission wavelengths of 350 and 430 nm., respectively. However, the use of strongly basic aqueous solutions, such as 0.2 *N* NaOH, may lead to erroneous results due to the hydrolysis of salicylamide, particularly when under irradiation at the activation wavelength. Under such conditions, it is possible for a reference standard solution to change its value during an analysis.

The present method is based on the simultaneous spectrophotofluorometric determination of salicylamide and salicylic acid at pH 11. It is only necessary to hydrolyze the salicylamide metabolites to a resultant mixture of salicylamide and salicylic acid.

## EXPERIMENTAL

**Reagents and Chemicals**—The chemicals used were: salicylamide powder[1], salicylic acid crystals USP[2], gentisamide[3], and gentisic acid[4]. The pH 11 buffer solution was made by dissolving 1.0 g. of sodium bicarbonate and 4.0 g. of sodium carbonate in water to make 100 ml. of solution.

**Instrumentation**—The fluorescence was determined by use of an Aminco-Bowman spectrophotofluorometer[5]. The Teflon-lined, screw-cap, 15-ml. capacity centrifuge tubes[6] were hand ground and polished to fit tightly.

**Human Blood and Urine Samples**—Blood samples were withdrawn into sterile, 7-ml. "Vacutainers"[7] at 0, 1, 2, 3, and 6 hr. after the ingestion of 312 mg. of salicylamide. Blood samples were stored in a refrigerator pending assay. The urine samples were collected into bottles at 0, 1, 2, 3, 4, 5, and 7 hr. and stored in a refrigerator pending assay.

[1] New York Quinine and Chemical Works.
[2] S. B. Penick and Co.
[3] USV Pharmaceutical Corp.
[4] Eastman Kodak Co.
[5] American Instrument Co.
[6] Matheson Scientific.
[7] Becton-Dickinson.

**Table I**—Determination of Total Salicylamide from Known Mixtures of Salicylamide and Salicylic Acid, Using a 1:1 Mixture as Reference

| Concentration, mcg./ml. | | Relative Fluorescence | | | | |
|---|---|---|---|---|---|---|
| Salicylamide | Salicylic Acid | 310–435 nm. | 340–435 nm. | Sum | Found, mcg./ml. | Percent of Theory |
| 0.00 | 0.20 | 69.0 | 7.8 | 76.8 | 0.196 | 98.1 |
| 0.04 | 0.16 | 57.5 | 19.8 | 77.3 | 0.197 | 98.7 |
| 0.08 | 0.12 | 46.7 | 31.1 | 77.8 | 0.199 | 99.4 |
| 0.12 | 0.08 | 36.2 | 42.6 | 78.8 | 0.201 | 100.6 |
| 0.16 | 0.04 | 24.4 | 56.7 | 81.1 | 0.207 | 103.6 |
| 0.20 | 0.00 | 12.9 | 68.9 | 81.8 | 0.209 | 104.5 |

**Procedure**—Pipet 1.0 ml. of blood serum or an appropriately diluted urine sample (0–12 mcg./ml. of salicylamide) into a Teflon-lined screw-cap centrifuge tube. Add 1.0 ml. of a 2 *N* HCl solution. Secure cap tightly, and heat sample in a boiling water bath for 2.5 hr. At the end of the heating period, cool to room temperature with cold water. Add 5.0 ml. of chloroform and shake well. Centrifuge and then carefully aspirate off the upper aqueous phase. Extract a 1.0-ml. aliquot of the chloroform phase with 4.0 ml. of 0.1 *N* NaOH. Dilute a 1.0-ml. aliquot of the aqueous phase with 4.0 ml. of the pH 11 buffer solution. Determine the fluorescence of the resulting solution directly with the spectrophotofluorometer at the activation and emission wavelengths of 340 and 435 nm. and then at 310 and 435 nm., respectively. Add the two corresponding relative fluorescence readings together, and then refer to a standard curve treated in the same manner as the samples. Estimate the amount of salicylamide present in the sample from the standard curve.

## RESULTS AND DISCUSSION

The activation and emission wavelengths of salicylic acid at the optimum pH of 11 are listed as 310 and 435 nm., respectively (7). The relative fluorescence as a function of pH for salicylamide is shown in Fig. 1. It can be seen that salicylamide at activation and emission wavelengths of 340 and 435 nm., respectively, also has maximum fluorescence at pH 11. The standard curves of relative fluorescence *versus* concentration of each component for salicylamide, salicylic acid, and a 1:1 mixture of the two are shown in Fig. 2. A study of these preliminary data indicates that it is possible to determine salicylamide and salicylic acid at pH 11 and calculate a value for total salicylamide. This may be accomplished since both compounds have maximum fluorescence (435 nm.) at pH 11 of nearly equal intensity at their respective activation and similar but lesser fluorescence (435 nm.) at each other's activation. Hence, in a mixture of both compounds, the sum of the fluorescence (435 nm.) at the two activation wavelengths will be directly proportionate to the sum of their concentrations and independent of their ratios. This postulation was tested by running known mixtures of salicylamide and salicylic acid in several different proportions. Table I shows the magnitude of the actual deviation in a series of mixtures, using a 1:1 mixture as the reference.

Upon examination of the Percent of Theory column in Table I, one observes the greatest accuracy when the ratios of salicylamide to

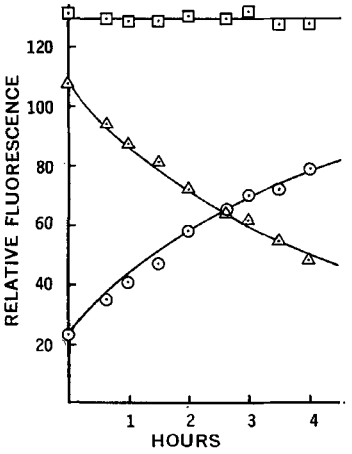


**Figure 3**—*Plot of relative fluorescence* versus *time in hours for the hydrolysis of salicylamide in 1 N HCl at 100°. Key:* △, *Curve A, 340-nm. activation;* ⊙, *Curve B, 310-nm. activation; and* ⊡, *Curve C, sum of Curves A and B.*

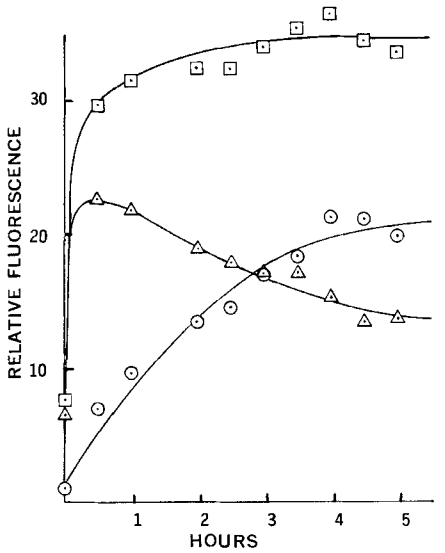


**Figure 4**—*Plot of relative fluorescence at 435 nm.* versus *time in hours for the hydrolysis of a blood serum sample of salicylamide metabolites in 1* N *HCl at 100°. Key:* △, *Curve A, 340-nm. activation;* ⊙, *Curve B, 310-nm. activation; and* ⊡, *Curve C, sum of Curves A and B.*

salicylic acid are close to the reference 1:1 mixture. In actual practice, the sample and standard are subjected to the same hydrolysis conditions so the resultant mixtures are of nearly equal proportions. Under the conditions of the present method, gentisic acid and gentisamide have relatively low fluorescences and they do not interfere. Since gentisic acid, gentisamide, and its glucuronide are present in small amounts in both the blood serum and urine (1), they are not considered in the present study.

**Hydrolysis of Salicylamide**—Figure 3 shows the hydrolysis of salicylamide in 1 *N* HCl at the boiling water bath temperature as a function of time. The relative fluorescence of Curve A (340–435 nm.) drops with time, whereas Curve B (310–435 nm.) rises with time. Curve C is the sum of Curves A and B. It can be seen that Curve A intersects Curve B at about the 2.5-hr. point. This point corresponds to approximately the optimum time for this set of hydrolysis conditions.

**Hydrolysis of Salicylamide Metabolites**—The blood serum and urine samples, after appropriate dilutions with water, were hydrolyzed in the manner described previously. Figure 4 shows the hydrolysis of serum salicylamide metabolites, and Fig. 5 shows the hydrolysis of a urine sample as a function of time. The reason the

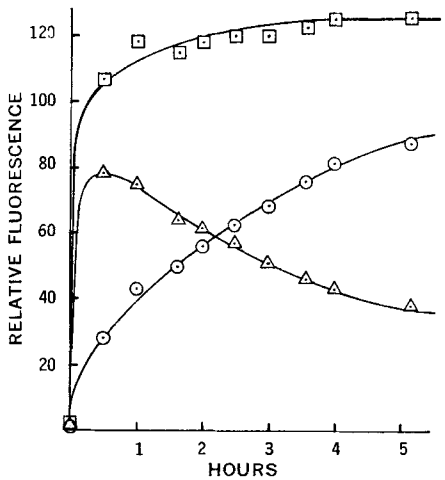


**Figure 5**—*Plot of relative fluorescence at 435 nm.* versus *time in hours for the hydrolysis of a sample of urinary salicylamide metabolites in 1* N *HCl at 100°. Key:* △, *Curve A, 340-nm. activation;* ⊙, *Curve B, 310-nm. activation; and* ⊡, *Curve C, sum of Curves A and B.*

**Table II**—Repetitive Assays of Two Urine Samples as Compared to Data Obtained with Ferric-Ion Complex Colorimetric Method

| Sample | Present Method, mcg./ml. | Colorimetric Method, mcg./ml. |
|---|---|---|
| Urine A | 243 | |
| | 240 | 248 |
| | 244 | 259 |
| | 239 | 249 |
| Average | 242 ± 2.5 or ± 1.0% | 252 ± 6.1 or ± 2.4% |
| Urine B | 217 | 219 |
| | 209 | 216 |
| Average | 213 ± 4.0 or ± 1.9% | 218 ± 2.0 or ± 0.9% |

**Table III**—Repetitive Assays of Pooled Blood Serum Sample and Recoveries of 10 mcg. of Salicylamide Added per Milliliter of Urine Blank Sample

| Salicylamide Found in Serum, mcg./ml. | Salicylamide Found in Urine, mcg./ml. |
|---|---|
| 3.02 | |
| 3.03 | 10.3 |
| 3.02 | 10.2 |
| 3.02 | 10.7 |
| 3.21 | 10.2 |
| Average 3.07 ± 0.08 | 10.35 ± 0.25 |

lines curved at the beginning of hydrolysis is because the salicylamide metabolites have relatively low fluorescence. Curves A and B intersect between the 2.25–2.85-hr. marks. The metabolite curves, C, in Figs. 4 and 5 level off slowly as compared to a pure salicylamide sample. This apparently does not negate the method in any way as shown by comparison recovery studies with the usual colorimetric method.

**Precision and Accuracy of Method**—Repetitive assays of an appropriately diluted urine sample are listed in Table II along with results from a colorimetric method (1). Table III records repetitive determinations of 10 mcg. of salicylamide added to 1 ml. of urine blank samples (zero-hr. sample), as well as the results from five replicate assays of a blood serum sample. No attempts were made to assay this blood serum sample by the colorimetric method. However, when 8.0 mcg. of salicylamide was added per milliliter of blood serum blank (zero-time sample), 8.19 mcg./ml. was found which is a 102% recovery. This is comparable to recoveries from urine blank samples which have an accuracy of 103.5% with a precision of ±2.4%. The colorimetric assay appeared to give higher results as shown in Table II; however, its precision of ±2.4% is identical to the present method.

## CONCLUSIONS

A spectrophotofluorometric method has been developed to determine salicylamide in blood serum and urine samples. The procedure is more sensitive than previously reported methods. The accuracy and precision of the method are 103.5 ± 2.4%, respectively. These results are comparable to the colorimetric ferric-ion complex method. However, the present method is capable of estimating a concentration of salicylamide and/or its metabolites as low as 0.1 mcg./ml. in blood serum or other biological fluids. The use of Teflon-lined screw-cap centrifuge tubes instead of ampuls facilitated the operating procedures and the use of small sample sizes. The method requires milder hydrolysis conditions and shorter time. The present method has been applied satisfactorily in this laboratory in bioavailability evaluation of various dosage form designs, employing salicylamide as the test drug.

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# Sulfur-Containing Derivatives of β-Phenethylamine: Methanesulfonamides

WILLIAM O. FOYE, JON C. ANDERSON, and JAYANT N. SANE

**Abstract** □ *N*-Methanesulfonyl derivatives of β-phenethylamine, (+)-amphetamine, β-4-aminophenethylamine, and 4-(2-trifluoroacetamidoethyl)aniline have been prepared. *N*-Trifluoroacetyl-1-(4-methanesulfonamidophenyl)-2-ethylamine significantly lowered blood pressure in hypertensive rats, and the methanesulfonamide of (+)-amphetamine lowered blood pressure in the dog.

**Keyphrases** □ Methanesulfonamides—synthesis, pharmacological screening as antihypertensives □ β-Phenethylamine, sulfur-containing derivatives—synthesis, pharmacological evaluation □ Antihypertensive agents, potential—synthesis, evaluation of *N*-methanesulfonyl derivatives of β-phenethylamine

In continuation of a study of the effects of sulfur-containing functional groups on the biological activity of β-phenethylamine, it was desired to observe the effects of including the alkanesulfonamide function in the molecule. Larsen and Lish (1) already pointed out that incorporation of the alkanesulfonamide group into the ring of phenethanolamines gives compounds having similar acidities to phenolic structures. Their phenethanolamine sulfonamides either showed the same biological profile or were antagonists to the catecholamines. According to the list of compounds subsequently reported from their laboratory, numerous alkanesulfonamides and arenesulfonamides of the phenethanolamines were synthesized (2, 3), but no alkanesulfonamides of β-phenethylamine itself were prepared. Previously, the *N*-benzenesulfonyl derivatives of β-phenethylamine (4) and β-4-nitrophenethylamine (5), and the 4-methanesulfonamido derivative (6) of 1-phenyl-2-propylamine, were described. 1- and 2-*p*-Methanesulfonylphenethylamines were synthesized and found to have some antibacterial activity *in vitro* (7).

The *N*-methanesulfonyl derivative of β-phenethylamine was readily prepared from the sulfonyl chloride. To introduce the methanesulfonamido group into the 4-position of the ring, β-phenethylamine was first nitrated in the 4-position by the method of Ehrlich and Pistschimuka (8). The amino group was converted to the trifluoroacetamido group, and the nitro group was reduced to give the 4-amino derivative. This was sulfonated by methanesulfonyl chloride, giving *N*-trifluoroacetyl-1-(4-methanesulfonamidophenyl)-2-ethylamine. An attempt to obtain the corresponding molecule having a free aliphatic amino group by lithium aluminum hydride reduction of 4-methanesulfonamidobenzyl cyanide failed to give the desired 1-(4-methanesulfonamidophenyl)-2-ethylamine.

The methanesulfonamide derivatives of (+)-amphetamine and β-4-nitrophenethylamine (9) and the bismethanesulfonamide of β-4-aminophenethylamine were readily obtained. This series of compounds thus provides examples of β-phenethylamines having methanesulfonyl groups on the aliphatic nitrogen, on the 4-amino group of the ring, and on both the aliphatic and aromatic nitrogens. Examples of β-phenethylamines containing methanesulfonamide in addition to dithiocarbamate and thiourea groups were reported in a previous paper (9).

Pharmacological tests for effects on blood pressure in rats and dogs revealed that *N*-trifluoroacetyl-1-(4-methanesulfonamidophenyl)-2-ethylamine caused significant and consistent reductions in the blood pressure when administered orally to rats. The methanesulfonamide of (+)-amphetamine also produced a significant lowering of blood pressure after intravenous injection in a dog. Whereas the previous results of Uloth *et al.* (2) and Larsen *et al.* (3) showed that inclusion of the alkanesulfonamide group in the aromatic ring of phenethanolamines conferred either adrenergic stimulant or adrenergic-blocking activity (the latter generally in conjunction with an isopropyl or larger group on the aliphatic nitrogen), it is apparent from the results reported here that conversion of the aliphatic amino group to an acidic methanesulfonamide or even trifluoroacetamide group can lead to a depressor response from the simpler phenethylamine structure.

## PHARMACOLOGICAL RESULTS

Tests for blood pressure effects in unanesthetized neurogenic hypertensive dogs (10) and in metacorticoid hypertensive rats (11) were performed[1].

[1] At Smith Kline and French Laboratories under the direction of Dr. R. A. McLean. Results were made available through the courtesy of Dr. C. W. French and Dr. J. W. Wilson.