lution in 4 *N* sulfuric acid. With increasing sulfuric acid concentration, from 4 to 16 *N*, no effect on the titration was observed; the rate of reaction decreased progressively toward the end-point as the sulfuric acid concentration was decreased from 4 to 0.5 *N*.

Hydrazine sulfate has been mentioned as a standard substance by several investigators (16); it is available in a highly purified form and its solutions are stable even if heated (17). The precision and accuracy of its potentiometric titration with chloramine-T under the proposed conditions suggested that it is suitable as a valuable primary standard substance in the standardization of chloramine-T solution. The possibility of adapting chloramine-T to the titrimetric analysis should not be overlooked. The stability of 0.05 *M* chloramine-T solution, stored in brown glass, was examined over 2 months and there was no detectable change in titer, in agreement with previous papers (11, 18).

The present method appears to offer a convenient alternative to the existing and more expensive oxidometric methods for the determination of the drugs listed in Table I.

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Spectrophotofluorometric Analysis of Procainamide and Sulfadiazine in Presence of Primary Aliphatic Amines Based on Reaction with Fluorescamine

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Abstract □ The potential of 4-phenylspiro[furan-2(3*H*),1′-phthalan]-3,3′-dione (fluorescamine) for use as a reagent for determination of drugs containing the primary aromatic amino substituent in the presence of drugs containing primary aliphatic amino substituents was evaluated. Procainamide and sulfadiazine were used as test drugs in the evaluation. The selective determination is based on a difference in the extent of reaction of aromatic and aliphatic amines with fluorescamine at pH 5.5, as well as small differences in spectral characteristics of the two groups. Use of the reagent for microdetermination of aromatic primary amines was compared with that of the Bratton–Marshall and 9-chloroacridine techniques.

Keyphrases □ Procainamide—spectrophotofluorometric analysis with fluorescamine in presence of primary aliphatic amines □ Sulfadiazine—spectrophotofluorometric analysis with fluorescamine in presence of primary aliphatic amines □ Fluorescamine—reagent in spectrophotofluorometric analysis of procainamide and sulfadiazine in presence of primary aliphatic amines □ Spectrophotofluorometry—analysis, procainamide and sulfadiazine using fluorescamine in presence of primary aliphatic amines

Recently, Weigele *et al.* (1, 2) reported the synthesis and characterization of 4-phenylspiro[furan-2(3*H*),1′-phthalan]-3,3′-dione (fluorescamine) as a reagent for the rapid preparation of highly fluorescent derivatives of primary amines. Udenfriend and coworkers (3, 4) have since shown the applicability of this reagent to the analysis of proteins, peptides, and amino acids in various biological systems. The reaction upon which the technique is based proceeds with a half-time in the range of milliseconds, giving fluorescent derivatives that can be determined in subnanomole per milliliter concentrations and that are stable for at least 24 hr.

Because of the rapid, quantitative nature of this reaction and the apparent stability of the reaction products, it was decided to evaluate fluorescamine as a reagent for use in drug microanalysis. This report presents its use as a reagent for the selective spectrophotofluorometric analysis of drugs containing the primary aromatic amino substituent (with procainamide and sulfadiazine as representative drugs) in the presence of primary aliphatic amines. In addition, the technique is compared with the Bratton–Marshall (5) and 9-chloroacridine (6) analyses for drugs with primary aromatic amino substituents.

Table I—Analysis of Procainamide and Sulfadiazine Solutions

Sample	Component[a]	Amount Added, mole × 10^{-8}/ml	Percent of Theory Found (*SD* of Mean, %[b])		
			Fluorescamine	Bratton–Marshall	9-Chloroacridine
1	Procainamide	4.00	99.47 (0.52)	99.65 (0.46)	100.32 (0.64)
2	Sulfadiazine	4.00	100.43 (0.32)	99.94 (0.31)	99.41 (0.25)
3	Procainamide	1.00	99.25 (0.37)	100.86 (0.84)	99.12 (0.65)
4	Sulfadiazine	1.00	99.47 (0.26)	98.76 (0.94)	99.06 (0.76)
5	Procainamide	0.10	101.02 (0.98)	93.21 (5.24)	104.29 (4.65)
6	Sulfadiazine	0.10	99.08 (0.74)	92.61 (7.34)	105.71 (5.00)
7	Procainamide	0.08	98.76 (1.25)	— —	— —
8	Sulfadiazine	0.08	102.14 (1.98)	— —	— —

[a] Solutions analyzed by the fluorescamine procedure contained 4.0 × 10^{-8} mole/ml of dopa, dopamine, amphetamine, and histamine. [b] $n = 12$.

EXPERIMENTAL

Apparatus—The spectrophotofluorometer[1] was equipped with a 150-w, 7.5-amp, 17–23-v, dc xenon lamp[2] and a IP 21 photomultiplier tube. Meter multiplier settings varied from 0.1 to 0.005, and the photomultiplier photometer was operated at full sensitivity. The band passes were maintained at 2.0 mm. A spectrophotometer[3] was used for the Bratton–Marshall and 9-chloroacridine determinations.

Materials and Reagents—Histamine dihydrochloride[4], dopamine hydrochloride[4], fluorescamine[5], procainamide[6], sulfadiazine[7], dopa[7], and amphetamine[8] were used. Other materials and reagents were of the best grade commercially available and were used without further purification.

Solutions—Analyzed solutions of each drug were prepared by dilution of stock solutions of the individual drugs, the pH of which had been previously adjusted to 5.0 with 0.1 *N* HCl. Buffer solutions were prepared according to official procedures (7). Solutions necessary for the Bratton–Marshall and 9-chloroacridine analyses were prepared as previously outlined (6). In the initial studies, fluorescamine was used as a solution (5.0 mg/100 ml) in dioxane. Because of stability problems, the solvent was later changed to acetone.

Determination of pH Dependency—Solutions (1.0 ml, 4.0 × 10^{-8} *M*) of each drug were mixed with phosphate buffer (0.5 ml, pH 4.0–9.5). Fluorescamine solution (0.5 ml) was added immediately with shaking, and fluorescence determinations were made after 15 min. Excitation and emission wavelengths were 400 and 498 nm, respectively, for procainamide and sulfadiazine and 388 and 480 nm, respectively, for the remaining drugs.

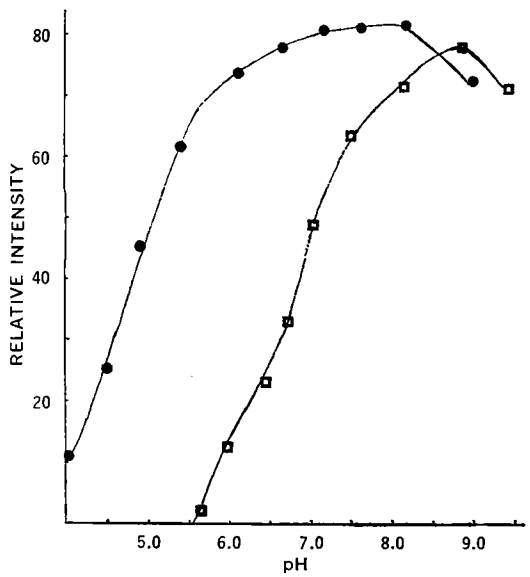


Figure 1—*pH dependency of the reaction of sulfadiazine* (●) *and amphetamine* (□) *with fluorescamine.*

Development Time and Fading Tendencies—Solutions (20.0 ml) of the drugs were prepared as already described in phosphate buffer (pH 5.5) and were mixed with fluorescamine solution (10.0 ml). Aliquots were taken for analysis at 1.5-min intervals for 6 min, at 5-min intervals for 1 hr, and at the end of 24 hr.

Spectrofluorometric Properties—Solutions (2.0 × 10^{-8} mole/ml) of procainamide and sulfadiazine were prepared in phosphate buffer (pH 5.5 and 8.5) for the remaining drugs. Fluorescamine (0.5 ml) was added, with shaking, to each solution. After 6 min, the pH of each solution was adjusted to 5.5 with 0.1 *N* HCl. Absorption and emission spectra were determined.

Analysis of Procainamide and Sulfadiazine—Solutions were prepared in the concentrations listed in Table I. Solutions analyzed by the fluorescamine-based procedure contained dopa, dopamine, amphetamine, and histamine (4.0 × 10^{-8} mole/ml) in addition to procainamide or sulfadiazine. An aliquot (2.0 ml) of each solution was mixed with fluorescamine solution (1.0 ml). After 6 min, the fluorescence of the solution was determined against a reagent blank, and the concentration was calculated by reference to previously determined calibration curves.

RESULTS AND DISCUSSION

Procainamide and sulfadiazine were chosen as representative drugs containing the primary aromatic amino substituent. Dopa, dopamine, amphetamine, and histamine were included to evaluate the method's potential for selectively determining drugs having a primary aromatic amino substituent in the presence of drugs with a primary aliphatic amino substituent.

Reaction of fluorescamine with primary amines has been shown to be strongly pH dependent (8), proceeding with increased rapidity as the pH is increased up to a maximum at 8.5–9.5. It has been suggested that protonation of the amine prior to reaction retards the reaction. Competing with the reaction that yields the fluorescent derivative is the reaction whereby the reagent is degraded to nonreactive products. Thus, a procedure for selective analysis of primary aromatic amines in the presence of primary aliphatic amines might utilize a pH at which fluorescamine reacts with aromatic amines but degrades before reacting with the more basic aliphatic amines.

On the basis of this rationale, the pH of the reaction medium was varied to observe the relative fluorescence of compounds in the study under these reaction conditions (Fig. 1). Since the fluorescence of fluorescamine derivatives was previously shown to be constant over the pH 4–9.5 range, fluorescent intensity at a particular pH is directly related to the extent of reaction at that pH. Sulfadiazine exhibited relatively high fluorescent intensity at pH 5.5, whereas amphetamine did not. Procainamide exhibited the same pH-dependency properties as sulfadiazine, and dopa and dopamine exhibited properties similar to those of amphetamine. Histamine gave a more intense fluorescence at pH 8.0 but did not fluoresce at pH 5.0. Thus, phosphate buffer of pH 5.5 was tentatively chosen as the reaction medium.

Figure 2 presents data regarding reaction time and stability of the reaction products for procainamide. Sulfadiazine studies gave similar results. On this basis, it appears that any time from 6 min

[1] Aminco-Bowman model 4-8202D.
[2] Hanovia 901 Cl.
[3] Cary model 118.
[4] ICN Nutritional Biochemicals Corp., Cleveland, Ohio.
[5] Roche Diagnostics, Nutley, N.J.
[6] E. R. Squibb, Princeton, N.J.
[7] USP reference standard.
[8] Analabs, North Haven, Conn.

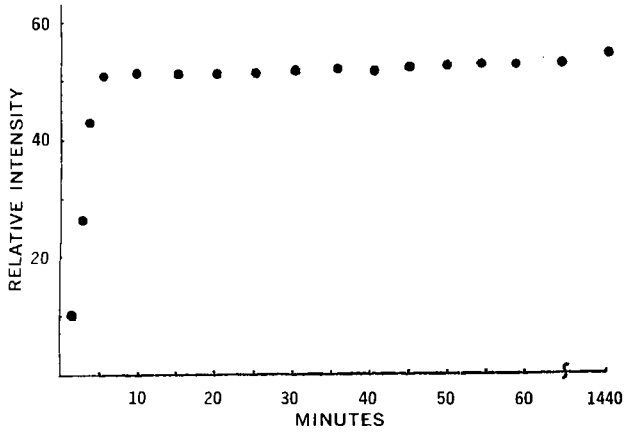


Figure 2—*Developing time and stability of products in the procainamide–fluorescamine reaction.*

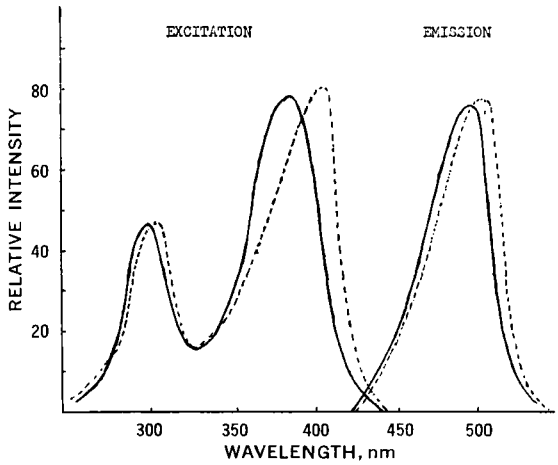


Figure 3—*Spectrofluorometric properties of sulfadiazine (– – –) and amphetamine (——) after fluorescamine reaction.*

after addition of fluorescamine up to 24 hr could be used for the analysis. The development time is somewhat longer than that previously reported for fluorescamine-based determinations, but this result is in accord with the lower pH of the reaction medium. The reaction time compared favorably with that of the 9-chloroacridine and Bratton–Marshall techniques, where the recommended development time is 15 min. In the latter procedure, product instability is severe, making a timed analysis essential.

Excitation and emission spectra of fluorescamine derivatives of sulfadiazine and amphetamine are presented in Fig. 3. The 10–15-nm increase in wavelengths of maximum absorption and emission is consistent with the data previously derived for aniline (10). This increase may be rationalized by reference to structures of the fluorescent derivatives for an aromatic amine (Structure A) and an aliphatic amine (Structure B).

In Structure A, the aromatic ring derived from the aromatic amine provides additional electron delocalization not present in the structure obtained from the aliphatic amine, shifting activation and emission maxima to longer wavelengths. Similar results were obtained when sulfadiazine was compared to dopa and dopamine. For histamine, both activation and emission maxima were shifted to shorter wavelengths with respect to the other aliphatic amines.

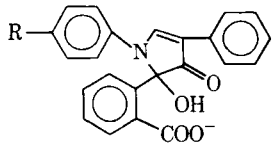
The difference in fluorescence spectra provides an additional procedure for ensuring selective analysis of primary aromatic amines in the presence of primary aliphatic amines. The fluorescent intensity resulting from the reaction of dopamine with fluorescamine in phosphate buffer of pH 8.5 was one-twelfth the value of that of an equimolar solution of procainamide when measured at the wavelength of maximum absorption and emission of procainamide. Comparable results were obtained with the other aliphatic amines. This difference in spectral properties, in combination with the more rapid reaction of aromatic amines at pH 5.5, provides the basis for selectivity of the analytical technique.

Standard curves for procainamide and sulfadiazine were prepared by plotting relative intensity against volumes taken of equimolar concentration of the two drugs. In the range of 0.5–125 nmoles, Beer's law holds for the system. Standard curves were prepared daily for 10 days. In initial studies, a large negative deviation in the slope of the curve was noted after 7 days, but a change of the fluorescamine solvent from dioxane to acetone removed this problem. The standard deviation of the slope of the curve for procainamide was 0.17%; it was 0.21% for sulfadiazine. The average correlation coefficient was 0.996 for procainamide and 0.991 for sulfadiazine. The average y-intercept value was −0.006 for procainamide and −0.009 for sulfadiazine.

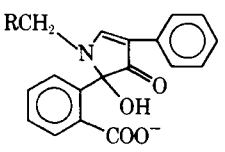
Results comparing the sensitivity and precision of the procedure to that of the Bratton–Marshall and 9-chloroacridine procedures are presented in Table I. With the drugs included in this study, fluorescamine reaction apparently allows analysis of solutions at least an order of magnitude more dilute than the other two procedures.

The potential for selective measurement of aromatic amines in the presence of aliphatic amines was evaluated by determining sulfadiazine and procainamide in solutions containing varying amounts of histamine, dopa, amphetamine, and dopamine. Concentration of the latter drugs 200 times higher than sulfadiazine or procainamide gave no observable effect on accuracy or precision.

Use of fluorescamine as a reagent for analysis of drugs containing primary aromatic amino substituents appears to have several advantages over the Bratton–Marshall or 9-chloroacridine techniques. Reaction proceeds more rapidly, and the fluorescence is stable for longer periods. Only one solution (fluorescamine in acetone) is required, and it has been shown to be stable at room temperature for up to 3 months. No tedious pH adjustments are necessary. Finally, the procedure gives data on solutions (0.08×10^{-8} mole/ml) with standard deviations comparable to those obtained on solutions (1.0×10^{-8} mole/ml) with the two other procedures, indicating that fluorescamine-based analysis gives reliable data on solutions at least 10 times more dilute than the Bratton–Marshall and 9-chloroacridine techniques.



Structure A



Structure B

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