Improved, Rapid Spectrophotofluorometric Method for Assay of Epinephrine: A Comparative Study

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Abstract □ A new and improved two-step spectrophotofluorometric method for the determination of epinephrine in decomposed formulations is described. The method depends on the initial oxidation of epinephrine with iodine to "adrenochrome," which is subsequently cyclized with alkali to "adrenolutin," which is responsible for the fluorescence. The fluorescence is stabilized with alkaline ascorbate. The method is unique in that it is simple and accurate, and it has additional advantages over the official (USP XVIII and NF XIII) methods. Several samples can be accurately analyzed within a short time. Extremely dilute epinephrine solutions (1:100,000) can be analyzed, which is not possible with current official methods. The influence of the various reagents on the final fluorescence and the stability of the fluorescence as a function of time are described. The absence of interference from epinephrine decomposition products on the recovery of epinephrine from simulated formulations is also described.

Keyphrases □ Epinephrine solutions—spectrophotofluorometric analysis, compared to compendial methods □ Spectrophotofluorometry—analysis, epinephrine solutions

Numerous methods have been described for the determination of epinephrine, norepinephrine, and related catecholamines in biological material (1, 2). USP XVIII describes an assay procedure (3) for epinephrine injection which measures the optical rotation of the triacetylepinephrine derivative. This method cannot be utilized directly without modification for the estimation of very dilute solutions of epinephrine (1:10,000 or 1:100,000) or very small volumes of these extremely dilute solutions. To assay these very dilute solutions according to the official method, a large number of samples is required and quantitative extraction of a large volume of solution may lead to large errors. NF XIII describes a colorimetric method (4) for the assay of epinephrine. This method does not distinguish between the biologically active epinephrine from the inactive decomposition products. Thus, both of these methods have disadvantages in that they cannot be used directly for the assay of large numbers of samples of extremely dilute solutions or do not distinguish the active species from the inactive decomposition products.

In view of these disadvantages, the preferred assay method is the spectrophotofluorometric method, which has been extensively employed to determine catecholamine derivatives in biological fluids (5, 6). Backe-Hansen *et al.* (7) reported the application of Hellberg's (8) method for the estimation of epinephrine in injections and verified the specificity of the fluorometric method by a comparison with the biological method of elevation in the blood pressure of rats and the rat uterus method. However, neither experimental details nor effects of catecholamine decomposition products on the fluorometric method were reported.

To date the possible application of the fluorometric method to routine assay of epinephrine samples in parenteral formulation has not been investigated in depth. The various parameters including the choice of the oxidizing agent, the period of oxidation, the development and stabilization of the fluorescence, and the influence of various reagents on fluorescence in parenteral formulations have not been reported. Also, the influence of the decomposition products of epinephrine injections, stabilized with the commonly used antioxidants, on the development of fluorescence has not been investigated.

This report describes a simple, accurate, and rapid fluorometric method for the routine assay of epinephrine parenteral formulations, which is also applicable for the assay of very dilute solutions. Statistical evaluation of the fluorometric and USP XVIII methods for the assay of dilute epinephrine solutions is reported, and the advantages of the new procedure are discussed.

EXPERIMENTAL[1]

Reagents—*Potassium Ferricyanide (0.25%)*—Dissolve 250 mg. of potassium ferricyanide (Fisher certified, ACS reagent) in sufficient distilled water to make 100 ml.

Zinc Sulfate (0.25%)—Dissolve 250 mg. of zinc sulfate heptahydrate (Fisher certified, ACS reagent) in sufficient distilled water to make 100 ml.

Ascorbic Acid (2%)—Dissolve 2.0 g. of USP grade ascorbic acid in sufficient distilled water to make 100 ml. This solution must be prepared fresh daily.

Sodium Hydroxide Solution (5 N)—Dissolve 200 g. of sodium hydroxide (Fisher certified, ACS reagent) in sufficient distilled water to make 1 l.

Phosphate Buffer (0.2 N), pH 6.5—Dissolve 2.8 g. $NaH_2PO_4 \cdot H_2O$ in distilled water to make 100 ml. Dissolve 5.4 g. $Na_2HPO_4 \cdot 7H_2O$ in sufficient distilled water to make 100 ml. Titrate NaH_2PO_4 solution with Na_2HPO_4 to a pH of 6.50 using an expanded scale pH meter[2].

Citrate-Phosphate Buffer (McIlvaine's Buffer), pH 4.0—Prepare 0.1 *M* citric acid solution by weighing 21.0 g. of citric acid monohydrate (ACS certified) into a 1-l. volumetric flask and diluting to volume with distilled water. Prepare 0.2 *M* dibasic sodium phosphate solution by weighing 28.4 g. of anhydrous dibasic sodium phosphate (ACS certified) into a 1-l. volumetric flask and diluting to volume with distilled water. Prepare pH 4.0 buffer by adding

[1] An Aminco-Bowman spectrophotofluorometer (American Instrument Co., Silver Spring, Md.) was used. The excitation wavelength was 410 nm., and the emission wavelength was 530 nm. For the epinephrine concentrations described in the procedures, the Meter Multiplier was set at 0.03, with a slit width of 1.0 and sensitivity at 30.

[2] Beckman.

600 ml. of 0.1 *M* citric acid solution to 400 ml. of 0.2 *M* dibasic sodium phosphate solution.

Potassium Carbonate Solution (0.2 N)—Dissolve 1.38 g. of potassium carbonate (ACS certified reagent) in sufficient distilled water to make 100 ml.

Sodium Ascorbate Solution—Pipet 5.0 ml. of ascorbic acid solution into a 50-ml. volumetric flask and dilute to volume with 5 *N* sodium hydroxide. This solution must be prepared immediately before use. Unless this reagent is clear and colorless, it should not be used.

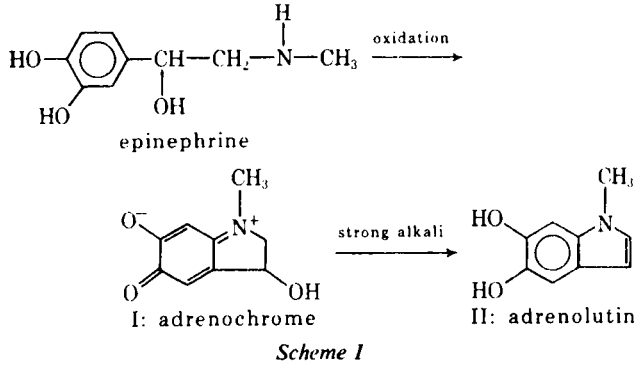
Iodine Solution (0.005 N *in 1.25% w/v Sodium Iodide)*—Weigh 5.0 g. of sodium iodide (Fisher certified, ACS reagent) into a 100-ml. volumetric flask containing 30 ml. of distilled water. Pipet 20 ml. of 0.1 *N* iodine solution and dilute to 100 ml. with distilled water. Pipet 25.0 ml. of this solution into a 100-ml. volumetric flask and dilute to volume with distilled water.

Epinephrine Standard—Accurately weigh 100 mg. of epinephrine base[3] or 182 mg. of epinephrine bitartrate[3] into a 1-l. flask. Pipet 10 ml. of 0.1 *N* HCl, swirl flask to dissolve the material in the acid, and dilute to volume with nitrogen-purged distilled water. When stored in the refrigerator, this solution is stable for several weeks.

Procedures—*Potassium Ferricyanide Method*—Dilute the unknown epinephrine solution with pH 6.5 phosphate buffer to result in approximately 10 mcg./ml. solution. Pipet 2 ml. of this solution into two test tubes, and add 1.0 ml. of distilled water. Adjust the pH of this solution to 6.50 by the addition of 0.2 *N* potassium carbonate solution. To one test tube, add 0.1 ml. of 0.25% zinc sulfate followed by 0.1 ml. of 0.25% potassium ferricyanide reagent. Mix them thoroughly and let stand for exactly 3 min. To both tubes, add 1.0 ml. of sodium ascorbate reagent (freshly made), mix well, and allow to react for 10–20 min. To the tube that did not contain any oxidant, add 0.1 ml. of 0.25% zinc sulfate and 0.1 ml. of 0.25% potassium ferricyanide reagents, in that order. Mix thoroughly and use this solution as the blank. Readings are to be taken at the wavelengths described (*Footnote 1*) on the spectrophotofluorometer 10–20 min. after the addition of the alkaline ascorbate reagent.

Prepare a standard curve using the same procedure as that described for the unknown sample. Use concentrations of 2.5, 5.0, 10.0, 15.0, and 20.0 mcg./ml. for the standard curve. Read the unknown sample from the graph.

Iodine Method—The following procedure was used to assay epinephrine solutions initially containing 100 mcg./ml. of the base. Dilute 4.0 ml. of the unknown sample solution to 100 ml. with the pH 4.0 citrate–phosphate buffer. To each of two different 100-ml. volumetric flasks, labeled Samples A and B, add 10.0 and 15.0 ml., respectively, of this diluted solution and make up to volume with the same buffer. Pipet 5.0 ml. of solution from each of these flasks (containing about 0.4 and 0.6 mcg./ml. of epinephrine base, respectively) into separately labeled 10-ml. volumetric flasks, add 1.0 ml. of 0.005 *N* iodine reagent, and shake well. After exactly 3 min., dilute to 10 ml. with freshly prepared sodium ascorbate reagent. Shake thoroughly and read the fluorescence on the spectrophotofluorometer at the specified wavelengths and settings. The readings should be taken between 10–25 min. after the addition of the sodium ascorbate reagent. The standard solution of epinephrine containing 100 mcg./ml. is also diluted in an analogous manner. Each sample and standard are diluted to two different levels and assayed simultaneously. At least two blanks are always determined, consisting of 5.0 ml. of pH 4.0 citrate–phosphate buffer to which is added 1.0 ml. of the 0.005 *N* iodine solution, and the entire procedure is carried through in the same manner as described for samples and standards.



Scheme I

Calculations—

$$\text{mcg./ml. epinephrine in Sample A} = \frac{4\,(\%\text{ transmission of sample 1} - \%\text{ transmission of blank})}{(\%\text{ transmission of Standard A} - \%\text{ transmission of blank})} \quad \text{(Eq. 1)}$$

$$\text{mcg./ml. epinephrine in Sample B} = \frac{4\,(\%\text{ transmission of sample 2} - \%\text{ transmission of blank})}{(\%\text{ transmission of Standard B} - \%\text{ transmission of blank})} \quad \text{(Eq. 2)}$$

$$\text{mcg./ml. epinephrine in unknown sample} = 12.5\,(\text{mcg./ml. epinephrine in Sample A} + \text{mcg./ml. epinephrine in Sample B}) \quad \text{(Eq. 3)}$$

Recovery of Epinephrine in Presence of Its Oxidation Products—A solution of epinephrine hydrochloride, 115 mcg./ml., was made in distilled water and the pH was adjusted to 2.50 with hydrochloric acid. This solution was kept at 56°, with no precaution taken to prevent oxidation of epinephrine, and was analyzed by the fluorometric method at the end of 6 months. A fresh solution of epinephrine, 1.0 mg./ml., containing 10 mg./ml. of sodium bisulfite was made up in 0.1 *N* hydrochloric acid. To 5.0, 5.0, 5.0, 5.0, and 10.0 ml. of the partially decomposed solution of epinephrine at 56° were added 2.0, 1.5, 1.0, 0.5, and 0 ml., respectively, of the freshly made solution of epinephrine–sodium bisulfite solution already described. The solutions were then diluted to 10 ml. with distilled water and were subsequently analyzed by the fluorometric method.

TLC—Thin-layer plates (5 × 20 cm.) coated with silica gel F_{254}[4] were spotted with 20 μl. of the following solutions: (*a*) freshly made epinephrine solution (100 mcg./ml.), (*b*) epinephrine (100 mcg./ml.) containing sodium bisulfite (10 mg./ml.) which was previously heated at 65° for 2 weeks, and (*c*) epinephrine (100 mcg./ml.) heated in the presence of air for 9 weeks. The plates were developed with a solvent system consisting of 1-butanol–100% ethanol–acetic acid–water (50:10:10:15). With the sample of epinephrine heated in the presence of air, no clearcut separation could be achieved. However, the epinephrine heated with excess sodium bisulfite resulted in another product (R_f 0.26) with excellent separation. In this system, pure epinephrine had R_f 0.45.

RESULTS AND DISCUSSION

By using epinephrine as an example of a substituted catecholamine, the principle involved in the fluorometric method is illustrated in Scheme I. With a mild oxidizing agent, epinephrine is oxidized to adrenochrome (I), which in strong alkaline solution is cyclized to the relatively unstable adrenolutin (II), which is responsible for the fluorescence (9).

Catecholamines such as epinephrine and norepinephrine that occur in biological fluids have been successfully assayed by the fluorometric method (5, 6). However, this method is lengthy and involves an initial extraction with butanol, followed by alumina adsorption to remove noncatechol fluorescent substances (5). This method also requires the addition of reagents at precise intervals, and the final fluorescence is extremely unstable in the acidic medium in which it is determined. Moreover, this method has the serious drawback of a critical oxidation time period followed by an exacting time interval between the addition of the reagents and the actual determination of the fluorescence. Due to the number of steps involved during assay, along with the very specific time intervals, it is very difficult to analyze a large number of samples at a time without seriously affecting the accuracy.

Hellberg (8) reported a method for the estimation of epinephrine and norepinephrine in solutions containing local anesthetics and sulfite as the antioxidant. This method separates the epinephrine from the local anesthetic by column chromatography, with subsequent oxidation of the sulfite with iodine in acidic solution. The excess iodine is oxidized with arsenite, and the epinephrine is then oxidized at pH 6.0 with potassium ferricyanide to adrenochrome,

[3] K & K Laboratories, Plainview, N. Y.

[4] Brinkmann Instruments Inc., Westbury, NY 11590

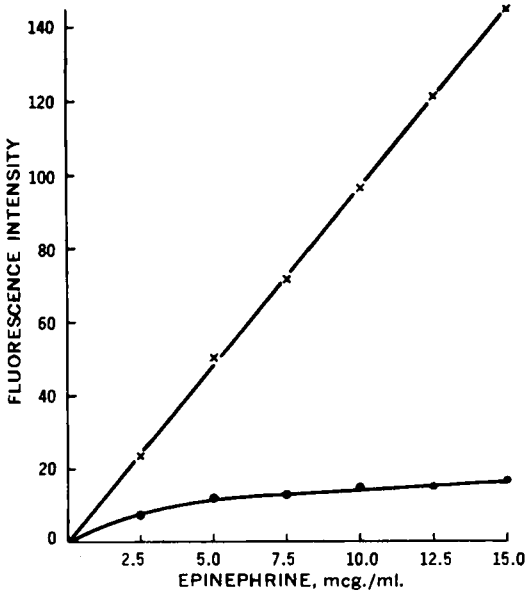


Figure 1—*Fluorometric assay of epinephrine hydrochloride by the potassium ferricyanide–zinc sulfate method. Key: ×, sodium bisulfite absent; and ●, 10-fold excess of sodium bisulfite present over that of epinephrine concentration.*

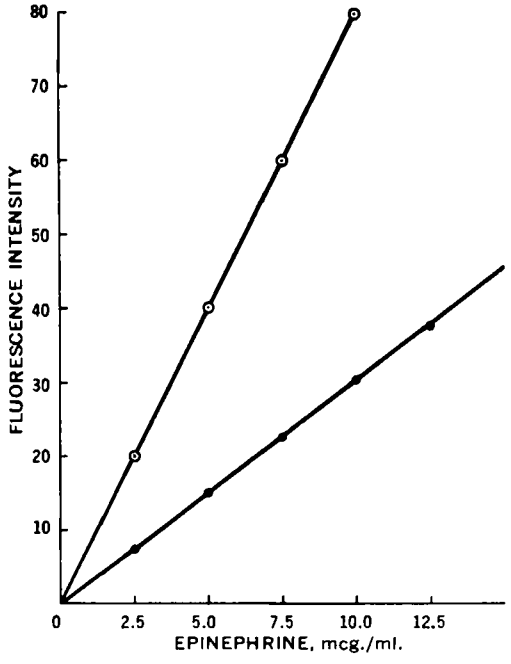


Figure 2—*Assay of epinephrine hydrochloride in the presence and absence of excess sodium bisulfite employing excess potassium ferricyanide (0.58%)–zinc sulfate (0.58%) reagent. Key: ⊙, absence of sodium bisulfite; and ●, presence of sodium bisulfite.*

which is then cyclized to adrenolutin with strong alkali. The fluorescence of these solutions is determined between 15 and 30 min. after the alkali treatment. Since this paper did not mention the wavelength at which the fluorescence was measured, the excitation and emission maxima for epinephrine were determined and found to be 410 and 530 nm., respectively.

Various oxidizing agents have been used for the initial oxidation of epinephrine to adrenochrome. The initial oxidation could be carried out with iodine (5), manganese dioxide (10), or a mixture of potassium ferricyanide and zinc sulfate (9). Manganese dioxide was eliminated from consideration as an oxidizing agent because of its insolubility and the problem of cumbersome removal from solution by centrifugal action. The use of a mixture of potassium ferricyanide–zinc sulfate and iodine solution was investigated in detail. Adrenolutin, which is responsible for the fluorescence, is quite susceptible to further oxidation, resulting in a decrease of the fluorescence. This can be prevented by the addition of a solution of an antioxidant. Of the various antioxidants suggested, the most commonly used are sodium sulfite (5) and sodium ascorbate (10). The antioxidant used not only prevents the further oxidation of adrenolutin but also reduces any excess oxidizing agent that may have been used to convert epinephrine to adrenochrome.

Potassium Ferricyanide–Zinc Sulfate Method—The majority of the commercial parenteral formulations of epinephrine contain sodium bisulfite or sodium metabisulfite as the antioxidant. Hellberg (8) recommended the initial oxidation of the antioxidant with iodine in acidic solution. However, if an excess of iodine is used, an interference with the fluorescence results. The exact amount of the antioxidant left in commercially available products is not known and, therefore, it is difficult to avoid using an excess iodine solution. Von Euler and Floding (9) used a mixture of potassium ferricyanide and zinc sulfate for the oxidation of epinephrine. A modification of this procedure was used to estimate epinephrine where the fluorescence was stabilized with alkaline sodium ascorbate. Excellent correlation between epinephrine concentration and fluorescence was observed with pure epinephrine solutions. However, for solutions containing sodium bisulfite, there was no linearity of response of fluorescence to concentration changes of epinephrine in solution (Fig. 1).

Since sodium bisulfite interferes with the development of fluorescence, the removal of sodium bisulfite by oxidation with excess potassium ferricyanide (0.58%) and zinc sulfate (0.58%) was attempted. The results show (Fig. 2) that the interference of bisulfite cannot be completely eliminated with potassium ferricyanide oxidation, as indicated by the differences in the slopes of the line in Figs. 1 and 2. In the presence of sodium bisulfite, with a lower concentration of the potassium ferricyanide–zinc sulfate reagent, epinephrine oxidation seems to be incomplete (Fig. 1). However, when the concentration of the oxidizing agent is increased, the slope of the standard curve decreases (Fig. 2) with respect to the slope of the curve obtained when a lower concentration of oxidizing agent was used (Fig. 1). This means that the fluorescence is affected by the amount of the oxidizing agent and also the presence of sodium bisulfite. These results indicate that the interference due to the presence of bisulfite cannot be eliminated by increasing the potassium ferricyanide–zinc sulfate concentration. Therefore, iodine solution containing potassium iodide was considered as an oxidizing agent.

New and Improved One-Step Method—A solution of iodine in potassium iodide was described as the oxidizing agent in the estimation of catecholamines in tissues (11). However, the initial oxidation step, the subsequent cyclization of the adrenochrome to adrenolutin, and the measurement of the fluorescence were reported to be extremely sensitive and very time dependent. Attempts to assay several samples at a time by this method failed to yield consistent results. In this method (11) the fluorescence is determined in acidic solution (pH 5.4) immediately after its development. The author also recommended the reduction of excess iodine with alkaline sodium sulfite, with the concurrent cyclization of adrenochrome to adrenolutin. The entire process should be completed within 2 min.

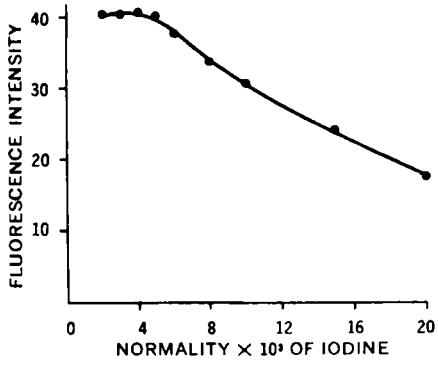


Figure 3—*Effect of iodine concentration on fluorescence of 0.6 mcg./ml. of epinephrine hydrochloride in the presence of 6 mcg./ml. of sodium bisulfite.*

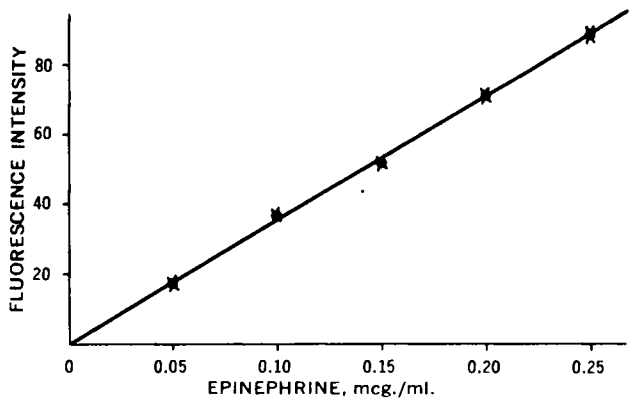


Figure 4—*Assay of epinephrine hydrochloride by fluorometric method using iodine–potassium iodide as the oxidizing agent. Key: ×, epinephrine hydrochloride alone; and ○, epinephrine hydrochloride with 20-fold excess of sodium bisulfite.*

and, subsequently, the solution is acidified with 5 *N* acetic acid to stabilize the fluorescence which must be determined immediately on the fluorometer.

To circumvent these problems and to analyze several samples at a time, the efficiency was investigated of iodine–potassium iodide solution to oxidize not only epinephrine in pH 4.0 buffer solution but also sodium bisulfite and metabisulfite, the most common antioxidants used in parenteral formulations. In the new and improved method, described in the *Experimental* section, alkaline sodium ascorbate was used to reduce the excess iodine and to stabilize the fluorescence. Various parameters such as the concentration of the oxidizing agent, the duration of the oxidation step, the fluorescence stabilizing agent and its concentration, and the duration within which the fluorescence should be determined were all studied in detail.

Effect of Oxidizing Agent on Fluorescence—The effect of iodine–potassium iodide concentration on the fluorescence of the final solution is shown in Fig. 3. Epinephrine solution was spiked with a 10-fold excess of sodium bisulfite. Iodine solution of various normalities was added such that the volumes of the final solutions were held constant, and the resulting fluorescence was measured. From Fig. 3 it is evident that the concentration of the oxidizing agent, iodine, on freshly made solutions of epinephrine containing sodium bisulfite is quite critical. The optimum concentration of the iodine reagent is 0.005 *N*, beyond which the final fluorescence is markedly affected. This concentration of iodine is sufficient to oxidize the epinephrine and the excess sodium bisulfite present in the formulation. Since formulations aged at different temperatures for various times would contain different amounts of sodium bisulfite and epinephrine, the adequacy of iodine solution as the oxidizing agent was checked by keeping the ratio of epinephrine to sodium bisulfite constant (Fig. 4). Since an excess amount of iodine quenches the fluorescence (Fig. 3), this study also indicated that the volume of iodine solution used does not affect the fluorescence in the presence or absence of excess sodium bisulfite. Concentrations of sodium bisulfite as high as 5 mcg./ml. do not interfere with the fluorometric assay of epinephrine. Many of the marketed parenteral formulations contain sodium bisulfite as much as 10 times the weight of epinephrine. Even in the presence of such high concentrations of the antioxidant, the amount of iodine described in the *Experimental* section is found to be adequate, since sodium bisulfite concentration as high as 25 times that of epinephrine does not interfere with the final fluorescence.

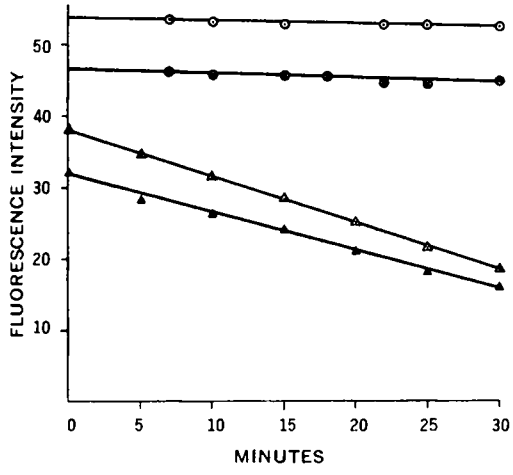


Figure 5—*Stability of fluorescence as a function of time, pH of the final solution, fluorescence stabilizer used, and final epinephrine hydrochloride concentration. Key: sodium sulfite as fluorescence stabilizer with final pH 5.0 and final epinephrine hydrochloride 0.2 mcg./ml. (▲) or 0.3 mcg./ml. (△); and sodium ascorbate as fluorescence stabilizer with final pH greater than 10 and final epinephrine hydrochloride 0.2 mcg./ml. (⊗) or 0.3 mcg./ml. (⊙).*

Table I—Effect of Iodine as Oxidizing Agent and Ascorbic Acid as Fluorescence Stabilizing Agent in the Assay of Epinephrine Hydrochloride Injections by Fluorometric Method

Ascorbic Acid, mg./ml.	Fluorescence Intensity, % Samples[a] 0.001 *N* Iodine 1	2	3	4	0.005 *N* Iodine 5	6	7	8
1.0	52.8	76.8	41.3	61.3	44.0	64.5	33.5	51.2
2.0	49.3	74.4	39.3	58.2	44.3	65.7	34.2	50.2
3.0	46.5	68.8	36.7	55.0	43.5	65.4	35.1	51.5

[a] Sample sets 1,2; 3,4; 5,6; and 7,8 were made by the appropriate dilution of four different samples such that the final concentration of epinephrine hydrochloride was in the ratio of 2:3 for each set. Final epinephrine hydrochloride concentrations were 0.2 and 0.3 mcg./ml., respectively, at these two dilutions.

The optimum pH of the solution to be oxidized was reported (6) to be 4.0. Solutions buffered with citrate–phosphate buffer at pH 4.0 also resulted in maximal fluorescence in the present investigation. Laverty and Taylor (6) recommended 1 min. as the oxidation time; however, in this study, a 3-min. oxidation period was necessary to oxidize the antioxidant normally present in formulations and also to oxidize the epinephrine to adrenochrome.

Choice of Antioxidant to Stabilize Final Fluorescence—The fluorescence of adrenolutin is quite unstable, probably due to easy oxidation, and, therefore, it needs stabilization with an antioxidant. Sodium sulfite and ascorbic acid were most often used to stabilize the fluorescence. Laverty and Taylor (6) described the use of sodium sulfite initially and subsequent acidification with glacial acetic acid, measuring the fluorescence in acidic solution (pH 5.0). Their paper failed to mention the stability of the fluorescence in the assay of epinephrine; however, the statement: "fluorescent derivatives were stable for at least 100 minutes" was made without presentation of any data to substantiate it. The stability of fluorescence in epinephrine assay was investigated in this study, using essentially the procedure of Laverty and Taylor and employing sodium sulfite as antioxidant and glacial acetic acid to make the final solution acidic. Under these conditions, the fluorescence is not as stable as was reported (6). The results according to the literature method (6) are shown in Fig. 5. When the fluorescence is determined under acidic conditions, it should be determined immediately, as was reported by Chang (5). The method reported by Laverty and Taylor (6) is identical with that reported by Chang. The relative instability of the fluorescence at pH 5.0 is evident from Fig. 5. It is also evident that the fluorescence is stable up to 25 min. when stabilized with ascorbic acid under strongly alkaline conditions.

To determine the optimum concentration to stabilize the fluorescence, ascorbic acid concentrations ranging from 1 to 3 mg./ml. were used, with parallel iodine concentrations of 0.001 and 0.005 *N* as the oxidizing agent. The results shown in Table I indicate that the readings were higher when 0.001 *N* iodine solution was used as the oxidizing agent along with 1 mg./ml. ascorbic acid as the fluorescence stabilizing agent. However, the fluorescence was less stable under the above set of conditions than when the 0.005 *N* iodine solution and 2 mg./ml. ascorbic acid combination was used. The use of 0.005 *N* iodine also assures complete oxidation of the antioxidant used in the formulation. It was also found that fluorescence with intensity readings higher than 70 units would quench faster. Fluorescence was stable for longer periods when the final readings were

Table II—Comparison of USP XVIII and Fluorometric Methods for the Assay of 1:1000 Epinephrine Solutions[a] Stored at 65° for Several Weeks

Sampling Time, Weeks	Epinephrine Hydrochloride, mcg./ml. USP XVIII Method	Fluorometric Method
Original	1000	1000
1	360	355
2	264	260
4	175	170
7	085	090

[a] These formulations contained 10 mg./ml. of sodium bisulfite, and the final pH was adjusted to 2.50.

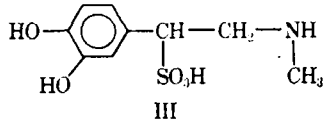
in the range of 30–70. It is also evident from Table I that when 3 mg./ml. of ascorbic acid was used, the value of the fluorometric readings dropped sharply compared to 1 mg./ml. of ascorbic acid.

Comparison of USP XVIII and Fluorometric Methods—To determine the precision of the USP XVIII method for the assay of epinephrine, a 1:1000 stock solution was made in hydrochloric acid and stored in the refrigerator. This solution was analyzed by the USP XVIII method, which involved the conversion of the unchanged epinephrine to triacetylepinephrine, its extraction with chloroform, and subsequent determination of its optical rotation. The amount of unchanged epinephrine is calculated from the given formula. Replicate determinations of the epinephrine assay according to the USP XVIII method resulted in a standard deviation of 0.5% with a standard error of 0.2% and precision of 0.5%. Similar replicate determinations by the USP XVIII method were carried out with 1:10,000 epinephrine solution, which is 10 times more dilute than the USP solution. The solutions were assayed by a slight modification of the extraction procedure with chloroform. The standard deviation is 0.62% with the standard error being 0.28%. Simultaneous determination of epinephrine by the fluorometric method resulted in a standard deviation of 0.48% and a standard error of 0.22%. According to Hellberg's (8) method, the standard deviation of a single determination was about ±2% for epinephrine. Therefore, this new procedure is precise and reproducible.

Assay of Epinephrine Formulations—A comparison of the two analytical procedures was made using 1 mg./ml. epinephrine formulation containing 10 mg./ml. of sodium bisulfite. The final pH was adjusted to 2.50, and the samples were kept at 65° for several weeks. These samples were withdrawn at various intervals and analyzed by the USP XVIII and fluorometric methods (Table II). The excellent agreement between the two methods points to the facts that: (*a*) by the fluorometric method, only the unchanged epinephrine is measured as verified by the acetylation procedure described in USP XVIII; and (*b*) the inactivation product, namely epinephrine sulfonic acid (III), does not interfere in the fluorometric assay. In the presence of a large excess of sodium bisulfite, the only decomposition product observed by TLC is epinephrine sulfonic acid. This was reported earlier by Schroeter *et al.* (12) to be the major route of degradation.

The applicability of the fluorometric procedure to formulations containing other antioxidants was investigated. Parenteral epinephrine formulations containing up to 10 mg./ml. ascorbic acid or up to 4 mg./ml. α-monothioglycerol were made anad analyzed by the new procedure. Since ethylenediaminetetraacetic acid is often used as an antioxidant synergist, formulations containing as high as 1 mg./ml. were made and analyzed. In all instances, no interference from any of the formulation stabilizers was observed. Thus, it can be safely concluded that the new fluorometric method is of great value in analyzing epinephrine formulations containing a variety of stabilizers.

Recovery of Epinephrine from Solutions Containing Its Oxidation Products—To study the interference of oxidation products on the recovery of epinephrine, solutions at a concentration of 1:10,000 and with pH adjusted to 2.5 were allowed to degrade in the absence of antioxidant. When more than 70% of the epinephrine decomposed, a constant volume of the degraded solution was added to



III

Table III—Comparison of Biological and Fluorometric Methods for the Assay of Aged Epinephrine Injection (1:10,000) at Different Temperatures[a]

	Sample	37°, 8 Weeks	37°, 12 Weeks	25°, 8 Weeks	25°, 12 Weeks
Biological assay[b], %	A	91.8	69.7	93.4	87.4
	B	80.4	63.5	81.6	86.8
Fluorometric assay, %	A	85.6	66.7	94.5	92.6
	B	89.3	64.3	97.1	94.5

[a] Epinephrine injection (1:10,000) contained sodium chloride and sodium bisulfite, and the pH of the original solution was 2.50. Results are expressed as percentage of original activity remaining. [b] Standard error, expressed as percent of potency, ranged from 0.98 to 7.03.

known amounts of epinephrine hydrochloride in solution. The resulting solution mixtures were analyzed by the fluorometric method, with concurrent determination of pure epinephrine hydrochloride solutions. The results of such a recovery experiment showed that the fluorometric method only measures the unchanged epinephrine even in formulations containing epinephrine oxidation products. Such recovery experiments carried out according to the USP XVIII method were unsuccessful due to the interference from the oxidation products. Thus, the USP XVIII method cannot successfully estimate epinephrine when its oxidative decomposition products are also present. However, these epinephrine oxidation products do not interfere in the fluorometric method.

Further evidence of the specificity of the fluorometric method to unchanged epinephrine came when completely oxidized epinephrine solutions (as evidenced by TLC) in different amounts were added to a constant amount of epinephrine hydrochloride solution, which was later analyzed by the fluorometric method. The recoveries of epinephrine were quantitative, and the results conclusively proved that the various epinephrine oxidative degradation products, even when present in high concentrations, have no effect on the determination of epinephrine by the fluorometric method.

Comparison between Fluorometric Method and Biological Method with Epinephrine Formulations—Epinephrine injections (1:10,000) containing sodium bisulfite as the antioxidant were stored at 25 and 37° and were assayed by the fluorometric method. The rise in the mean blood pressure of dogs upon the intravenous administration of epinephrine hydrochloride injection was measured according to the method described by Noel (13). The results summarized in Table III indicate that there is a remarkable correlation between the two methods. Backe-Hansen *et al.* (7) also established such a correlation between blood pressure measurement and the fluorometric method described by Hellberg (8).

In summary, the unique feature of the described fluorometric method lies in the fact that the stabilization of the fluorescence is amenable to the routine assay of several samples at a time. The USP XVIII method is time consuming and cannot be successfully applied to the assay of a large number of samples at a time. The other major advantages of this new method are:

1. It can be used to assay dilute (even 1:100,000) epinephrine hydrochloride injections with accuracy.
2. The specificity of the new method even in the presence of epinephrine oxidation products has been established, whereas interferences were observed with the USP XVIII method.
3. The method is simple, precise, and timesaving, with the added potential for automation.
4. The fluorometric method requires a small volume of sample, whereas the USP XVIII method requires a sample containing 30 mg. of epinephrine. Thus the latter method requires a large number of samples, resulting in very large volumes to handle.
5. This new method can be used for the assay of epinephrine injections containing antioxidants other than sodium bisulfite.

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Improved, Rapid Spectrophotofluorometric Method for Assay of Isoproterenol Hydrochloride Injections: A Comparative Study

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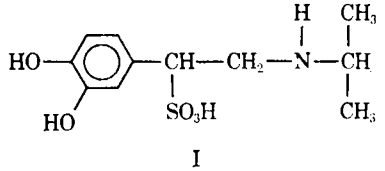
Abstract □ A spectrophotofluorometric method for the determination of isoproterenol hydrochloride in parenteral formulations is described. This method is quite simple, precise, and extremely specific for unchanged isoproterenol in decomposed formulations. The method depends on the initial oxidation of isoproterenol hydrochloride with iodine–potassium iodide solution in pH 4.0 (McIlvaine's citrate–phosphate) buffer. The partially oxidized isoproterenol "chrome" derivative is cyclized to the fluorescent "lutin" derivative with strong alkali. The fluorescence is stabilized with alkaline ascorbate solution, which is subsequently determined. The influence of the various decomposition products of isoproterenol, such as isoproterenol sulfonic acid and isoproterenone, on the fluorometric procedure was investigated with concurrent determination of the same solutions by the USP XVIII method. The usefulness of the fluorometric procedure to analyze fresh as well as partially decomposed isoproterenol solutions, stabilized with other antioxidants, is evaluated. The inapplicability of the existing methods to analyze very dilute (1:50,000) solutions of isoproterenol is also described. The specific advantages of the fluorometric procedure in the routine analysis of a large number of samples with speed and accuracy are discussed. Statistical evaluation of the fluorometric method is also reported.

Keyphrases □ Isoproterenol hydrochloride solutions—spectrophotofluorometric analysis, compared to compendial method □ Spectrophotofluorometry—analysis, isoproterenol hydrochloride solutions

The official USP XVIII method (1) for the analysis of isoproterenol hydrochloride injection in specific formulations and isoproterenol hydrochloride in other formulations involves the column chromatographic separation of unchanged isoproterenol from its degradation products. This is achieved by ion-pair extraction of the intact isoproterenol with bis(2-ethylhexyl)phosphoric acid in ether solution, leaving decomposition products behind on the column (2). The major product of decomposition of isoproterenol injections containing the most commonly used antioxidant sodium bisulfite is isoproterenol sulfonic acid (I). The formation of this derivative is pH dependent, and the reaction is favored at higher pH values. This fact was demonstrated by Higuchi and Schroeter (3). However, when an insufficient quantity of sodium bisulfite is used or when other antioxidants are employed, complications arise due to the presence of other isoproterenol decomposition products such as isoproterenone (II). In such instances, the USP XVIII method may not work satisfactorily. This fact was investigated in depth, and the data are presented in this paper.

Another major disadvantage with the USP XVIII method is that it cannot be used to assay isoproterenol injections in very dilute solutions such as 1:50,000 concentrations. The official method does not work when large volumes of sample have to be eluted from the column. Other disadvantages are: (*a*) the method is lengthy and time consuming; (*b*) the method cannot be successfully used to assay several samples at a time; and (*c*) it is extremely difficult to reproduce results, since the USP XVIII does not describe any of the column chromatographic variables such as tightness of the



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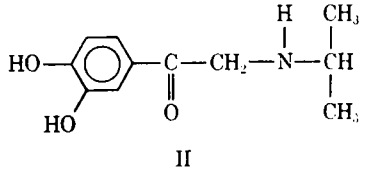

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