RESEARCH PAPERS

THE FLUORIMETRIC DETERMINATION OF NORADRENALINE

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SINCE Euler[1] in 1946 demonstrated the important role played by (—)-noradrenaline (I) as a transmitter of adrenergic nerve impulses and later isolated it in pure form from the adrenal medulla[2], this substance has found a wide clinical application. Exerting an overall vasoconstrictor effect, it is used, for instance, to maintain the blood pressure in surgical and traumatic shock and other acute hypotensive states. Because of its greater stability to oxidation it is progressively supplanting adrenaline in many instances, e.g. as a vasoconstrictor in injectable local anæsthetics, as a hæmostatic and so on[3]. Tullar and co-workers[4,5] have discussed the substance from different points of view.

Noradrenaline is used mainly as the lævo-isomer, usually as the hydrochloride or the bitartrate, this isomer being about twice as active as the racemic form. Several solutions containing the equivalent of 0·1 per cent. of (—)-noradrenaline are commercially available, but there is contemplated for inclusion in the Swedish Pharmacopœia a solution containing only 0·001 per cent. of noradrenaline as the bitartrate.

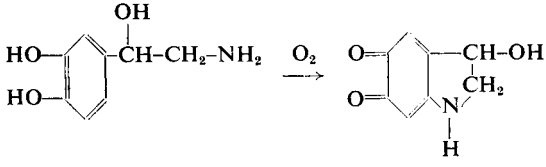
For the chemical estimation of noradrenaline in solution one may utilise, with slight modifications, several methods originally intended for the determination of adrenaline, e.g. the adrenochrome method[6] and our fluorescence method with strong alkali. Moreover the absorption peak at 279 mμ in the ultra-violet spectrum offers a possibility for the quantitative determination of noradrenaline, $E_{1\,\text{cm.}}^{1\,\text{per cent.}}$ being 85·0 for a solution of 0·01 N hydrochloric acid in this laboratory. The condensation of the primary aliphatic amine moiety with a carbonyl compound implies a selective method[7], being capable of determining noradrenaline even in adrenaline.

The solutions containing 0·1 per cent. of noradrenaline may be conveniently assayed by the methods mentioned, but in the case of the 0·001 per cent. solution only the fluorescence method seems to have a sufficient sensitivity. As noted by Ehrlén[8] and others, a drawback of the fluorescence method for adrenaline is the instability of the fluorescence. A great improvement was made, however, in 1949, when Natelson *et al.*[9] introduced a method for the determination of adrenergic amines in blood involving oxidation and condensation in alkaline medium with a primary aliphatic amine. As proposed by Weil-Malherbe and Bone[10] ethylene diamine may be employed as the amine, thus by cyclisation producing a fairly stable fluorescent compound (formula III, below). However, this method does not work in the presence of pyrosulphite, a serious limitation to its usefulness, because noradrenaline solutions generally are stabilised with pyrosulphite.

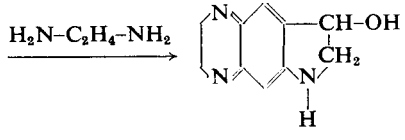
It is the purpose of the present investigation, therefore, to work out a method for the quantitative determination of noradrenaline in solutions as weak as 0·001 per cent. and containing pyrosulphite.

EXPERIMENTAL

The principle of the Weil-Malherbe and Bone method[10] was selected as a working basis for the investigation. The essential feature of this method is an oxidation of the catecholamine in alkaline solution and a condensation with ethylenediamine. The reaction may, probably, be formulated as follows:



I II



III

After preliminary experiments we adopted the following modification of the method:

To 10 μg. of noradrenaline in 10·0 ml. of water add 0·5 ml. of 2 M ethylenediamine dihydrochloride and 0·7 ml. of ethylenediamine base (anhydrous). Heat in a water bath at 50° C. for 20 minutes, after cooling saturate with solid sodium chloride (3 to 4 g.) and extract with 30·00 ml. of *iso*butanol for 4 minutes. Separate the butanol layer by centrifuging, filter into a cuvette and measure the fluorescence using as light source a mercury lamp with a blue primary filter (transmitting radiation up to 500 mμ) and yellow secondary filters (cutting off all radiation below 500 mμ) between sample cell and photocells. A standard containing 10 μg. of noradrenaline in 10 ml. and a reagent blank are also carried through the procedure. As a fluorescence standard a fluorescein sodium solution may be used.

The values obtained by this method, however, were poor, showing a considerable standard deviation, and this prompted a systematic investigation of every stage in the procedure.

(*a*) *Stability of fluorescence*

Cells with the fluorescent *iso*butanol solution were kept for varying times: (i) in the dark, (ii) in light from incandescent lamps, and (iii) in the filtered ultra-violet light from the mercury lamp. The results are collected in Table I.

TABLE I

	Fluorescence (scale readings)			
	0 min.	30 min.	60 min.	90 min.
Dark	100	105	99	98
Incandescent lamp	100	99	98	99
Mercury lamp	100	53	47	44

One may conclude that work in light from a mercury source is undesirable.

(b) Time and temperature of reaction

In other respects following the procedure given above the heating time was varied (curve marked "B", Fig. 1). As seen, the fluorescence intensity increases with the heating time, a stable value being attained in about 2 hours. The same time-fluorescence-relationship was studied at lower and higher temperatures (see Fig. 1).

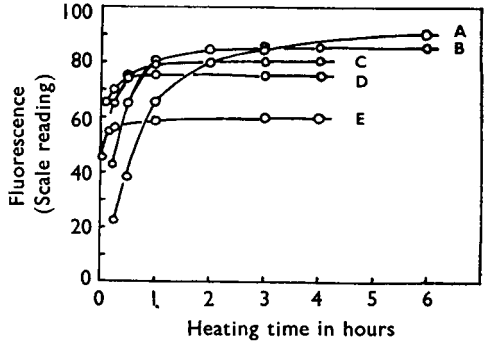


FIG. 1. Influence of heating time on the fluorescence at different temperatures.
A. 40° C., B. 50° C., C. 60° C., D. 70° C., E. 100° C.

The results demonstrate the inconvenience of the 50° C.–20 minutes combination, the curve having a great slope in this region. To minimise the errors one ought to utilise the horizontal part of the curve, i.e. when working at 50° C. one must heat for 2 hours or at 100° C. for 10 minutes.

The form and the position of the curves are interesting. The maximum fluorescence intensity is attained more quickly the higher the temperature, but the height of the plateau varies *inversely* as the temperature. A reasonable explanation of this phenomenon is the interaction of competing reactions. The fluorescent condensation product itself, being quite stable even at 100° C. (Fig. 1 E), is not directly involved, while on the other hand a precursor, probably noradrenochrome (formula II, page 249), seems to be subject to a synthesising as well as to a degrading reaction.

(c) Composition of condensation reagent and amount of reagent

The sum of the amounts of ethylenediamine dihydrochloride and base was fixed at a given value (say "a" ml.), while the proportion of the components was varied. When "a" had the values 0·5 to 8 ml. the curves shown in Figure 2 were obtained.

From these curves one may conclude: (i) that at a reagent composition of about equal volumes of base and of a 2 molar solution of the dihydrochloride the fluorescence is independent of variations in reagent volume

(down to about 1 ml.), and (ii) that at reagent volumes below 1 ml. moderate changes in reagent composition do not affect the fluorescence considerably.

It follows that equal volumes of base and 2 M dihydrochloride constitute an optimal reagent composition. On a molar basis this is equal to a buffer solution containing ethylenediamine and its conjugate monovalent acid in the ratio 3·5 : 1. The *p*H in this solution is 12·0. It is obvious that such a solution may be obtained in 3 different ways: (i) by mixing appropriate amounts of ethylenediamine base and dihydrochloride, (ii) by partially neutralising ethylenediamine base with acid, and (iii) by partially neutralising the dihydrochloride with alkali. The reagent was prepared as follows:

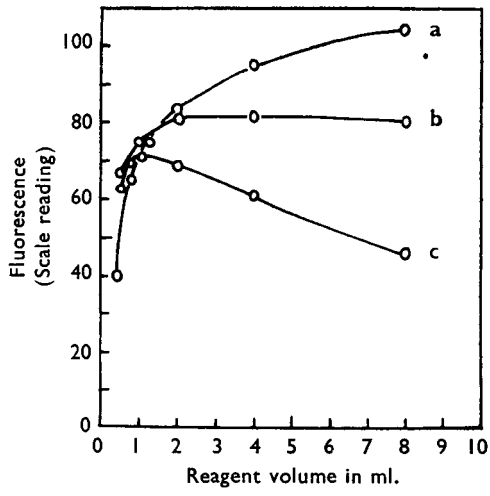


FIG. 2. Influence of reagent volume and composition on the fluorescence. Heating at 50° C. for 2 hours.

a. ethylenediamine base and 2 M dihydrochloride 1 + 3
b. ,, ,, ,, ,, ,, ,, 1 + 1
c. ,, ,, ,, ,, ,, ,, 3 + 1

Ethylenediamine (anhydrous, redistilled) ..	15·0 ml.
Hydrochloric acid 5 M	10·0 ml.

(d) Catalysis

Attempts to retard the (hypothetical) degrading reaction, mentioned under (*b*), by adding reducing agents were unsuccessful. Experiments with oxidants added to promote the formation of the intermediate compound which condenses with ethylenediamine also gave negative results.

While trying catalytically to influence the reaction, ammonium molybdate was found, however, to have a favourable effect on the formation of the fluorescent compound. When heating time is plotted versus fluorescence (*cf.* Fig. 1) with molybdate added to the reaction mixture, curves as shown in Figure 3 are obtained.

It is apparent that the curves for the different temperatures almost coincide in their horizontal course and that every curve has been displaced upwards.

The catalytic influence is within the region 1 to 20 drops of a 10 per cent. solution independent of the amount of molybdate added. Consequently, if some drops of ammonium molybdate solution are added to the sample,

the heating at 50° C. for 2 hours may be replaced by heating in a boiling water bath for a minute or two.

(e) Concentration versus fluorescence

It is clear from Table II that the concentration-fluorescence relationship is not strictly linear.

Thus it is important always to have the concentrations of the standard and the sample as close to one another as possible to obtain good agreement.

TABLE II

Concentration µg./10 ml.	Fluorescence scale readings
0	0
2	8
4	14
8	27
16	55
24	78

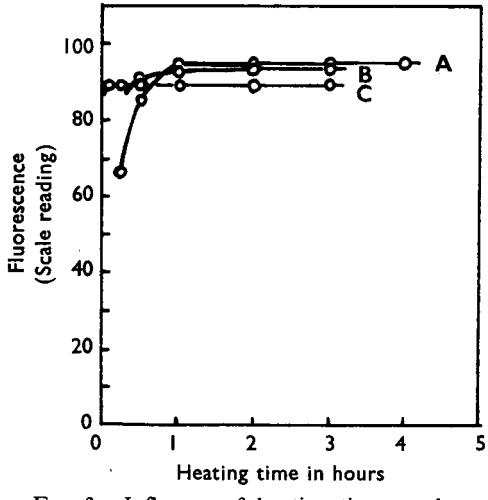


FIG. 3. Influence of heating time on the fluorescence in the presence of molybdate. A. 50° C., B. 70° C., C. 100° C.

(f) Elimination of sulphite

As mentioned above the presence of pyrosulphite ions during the condensation almost completely quenches the fluorescence. In order to eliminate this interference ion exchangers were resorted to. As sulphurous acid is a weak acid it is necessary to use anionic exchangers of the strongly basic type for that purpose. As, however, the base form of the strongly basic resin possibly may cause an alkaline degradation of noradrenaline, the exchanger was converted to its chloride form, which is inert to noradrenaline.

TABLE III

10 µg. OF NORADRENALINE + 10 mg. OF PYROSULPHITE

	Sulphite reaction	Fluorescence
Before ion exchange	+++	35
After ,, ,,	—	78
Noradrenaline standard	—	79

Sulphite was determined qualitatively by the nitroprusside-zinc ferrocyanide spot test according to Feigl[11], the minimum amount detectable being about 5 µg. of pyrosulphite at a concentration limit of 1 : 15,000.

As is evident from Table III the anion exchanger saturated with chloride easily removes sulphite ions from the solution, the noradrenaline being quantitatively recovered in the eluate.

Proposed Method

Applying the results obtained the final method can be given in the following revised form:

Reagents and materials

Anion exchange resin, IR-A 400 or Dowex 2, particle size 0·4 to 0·8 mm.
Sodium chloride solution 20 per cent.
Ethylenediamine reagent:
Ethylenediamine (anhydrous) 15·0 ml.
Hydrochloric acid 5 M 10·0 ml.
Ammonium molybdate solution $(NH_4)_2MoO_4$, 10 per cent., in 5 M nitric acid
*iso*Butanol
Noradrenaline standard, containing 1·00 μg. of noradrenaline base per 1 ml., in 0·01 N hydrochloric acid.

Procedure

Prepare a column (10 × 100 mm.) of anionic resin. Saturate with sodium chloride solution, 20 per cent., and wash out the excess chloride with water. Add to the column of the unknown solution amounts corresponding to 50 to 100 μg. of noradrenaline in 10 ml., wash with water, and collect 100·0 ml. of eluate. Transfer 10·00 ml. of eluate to a glass stoppered flask, add 2·0 ml. of ethylenediamine reagent and 2 drops of ammonium molybdate solution, and heat for 5 minutes on a boiling water bath. Cool, saturate with solid sodium chloride, and extract for 4 minutes with 30·00 ml. of *iso*butanol. Separate the butanol layer and filter through a dry filter into a cell. Carry 10·00 μg. of noradrenaline and a reagent blank through the condensation in the same way.

TABLE IV

Noradrenaline added	Fluorescence (scale readings)	
	Without ion exchange step	Full procedure
10 μg.	78; 79; 80; 78	77; 79; 78; 80
10 μg. with 10 mg. of pyrosulphite ..	35; 40	79; 79; 78; 79

Measure the fluorescence in an apparatus permitting the excitation with light of about 400 to 450 mμ, while from the light reaching the photocells all radiation below 500 mμ is screened off. The Lumetron photofluorimeter, model 402 EF, provided with primary and secondary B2 filters is convenient for this purpose. As a fluorescence standard a solution of fluorescein sodium, diluted 1 : 10^7, may be used. The concentration of the sample is adjusted so as to match the noradrenaline standard within ± 20 per cent.

This procedure was tested on a series of solutions (Table IV).

It is clear that the recovery of noradrenaline within the experimental error is 100 per cent. The error of the method is ± 2 per cent.

Applications

Some experiments were performed on local anæsthetic solutions containing procaine and lidocaine in addition to noradrenaline and pyrosulphite. (Table V.)

TABLE V

Noradrenaline added	Fluorescence
10 μg.	77; 79
10 μg. + 100 mg. of procaine + 10 mg. of pyrosulphite ..	78; 78
10 μg. + 100 mg. of lidocaine + 10 mg. of pyrosulphite ..	77; 78

It follows that this determination—in contrast to many others—is unaffected by the presence of local anæsthetics, in the concentrations tested.

In passing it is to be noted that adrenaline as well as *iso*propylnoradrenaline (isoprenaline) gives the same fluorescence reaction with ethylenediamine, although in these cases the hue of the fluorescence is somewhat more yellow white. These compounds can therefore be assayed by the same method. Moreover, as has been demonstrated earlier[12], the method is fairly specific for pressor amines of the catechol type.

Summary

1. The present investigation was prompted by the need for analysing weak (0·001 per cent.) solutions of noradrenaline stabilised by sodium pyrosulphite. The common methods are generally too insensitive and fluorescence methods are not applicable in the presence of sulphites.

2. A method has been worked out based on a combination of ion exchange and subsequent fluorimetry. A strongly basic anion exchange resin converted to its chloride form was employed for eliminating the sulphite, and noradrenaline was determined in the eluate by fluorimetry after condensation with ethylenediamine, ammonium molybdate being used as a catalyst.

3. No detectable loss of noradrenaline occurs during the ion exchange process, and the fluorimetric values are reproducible within 2 per cent. of the mean. The results are not affected by the presence of local anæsthetics, such as procaine and lidocaine.

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