A METHOD FOR THE ESTIMATION OF ADRENALINE AND NORADRENALINE IN MIXTURES

By W. O. James and Natalie Kilbey

Oxford Medicinal Plants Scheme

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The method to be described was developed for the estimation of small quantities of adrenaline and noradrenaline by purely chemical means, and does not distinguish between *d*- and *l*-isomers. It depends on holding the bases in weakly acid solution and obtaining a separation by means of a one-way paper chromatogram. After removal from the paper the bases are estimated colorimetrically by the reduction of arsenophosphotungstic acid.

Choice of Acid. Neither of the bases is sufficiently stable in neutral or alkaline solution for quantitative manipulation. A faintly acid *p*H is all that is required for stabilisation and a wide choice of acids is possible. The bases are stable in the presence of phenol, but, while this is most convenient for chromatographic separation[1], it is ruled out by its reduction of the arsenophosphotungstic reagent. N hydrochloric acid is a good stabiliser, but it slows down the chromatographic separation to an inconvenient degree. Good stabilisation with better chromatographic separation was obtained by using an organic acid such as 5 per cent. acetic or 5 per cent. trichloracetic[2]. These have the further advantages that they do not affect the arsenophosphotungstic reagent and are conveniently and commonly used for tissue extraction.

Estimation. It is convenient to consider the method of estimation before the separation. Solutions of the bases in 0·01N hydrochloric acid, into which they are extracted from the paper of the chromatogram, are estimated by a modification of the arsenophosphotungstic method for uric acid[3].

The following reagents are needed:—

(a) 5 per cent. sodium cyanide solution containing 2 ml. of strong solution of ammonia (0·880) per l. This solution should not be kept longer than 4 weeks.

(b) Arsenophosphotungstic acid. This is prepared by dissolving 100 g. of sodium tungstate in 600 ml. of water in a 1-l. flask, adding 50 g. of arsenic pentoxide followed by 25 ml. of 85 per cent. orthophosphoric acid and 20 ml. of concentrated hydrochloric acid (sp. gr. 1·18). The mixture is boiled for 20 minutes, allowed to cool, and made up to 1l. in a measuring flask. The solution thus prepared keeps indefinitely.

To approximately 6 ml. of solution containing adrenaline or noradrenaline in a test tube, are added 2 ml. of sodium cyanide solution (a) followed by 0·4 ml. of solution (b). The mixture is shaken and allowed to stand for at least 15 minutes for the colour to develop. It is then transferred by pouring down a fine glass rod into a 10-ml. measuring flask without the use of a funnel. The tube is rinsed with 1 to 2 ml. of

water, the rinsings are added to the flask, made up to 10 ml. and again shaken. The solution is then transferred to the cell of the absorptiometer and readings taken within the next 15 minutes. On longer standing a white precipitate forms. If necessary this can be removed by filtration and does not involve any loss of the blue colour, which is stable for at least 1 hour. The presence of acid in the solution not only stabilises the adrenaline or noradrenaline, it also delays the formation of the undesirable white precipitate.

Colorimetry. The colour produced is measured in a 1 cm. absorption cell (requiring about 8 ml. of solution) of a Spekker absorptiometer with orange (No. 3) filters. The colour obtained is considerably more intense with adrenaline than with noradrenaline, and a separate calibration curve must therefore be prepared for each. The curves we obtained were linear up to amounts corresponding to 50 μg. of adrenaline (Fig. 1). Above these amounts the colour became too dark for accurate estimation and dilution was necessary. The useful range of the estimation appears to lie between 5 and 50 μg. of adrenaline, but is rather higher with noradrenaline owing to the weaker colour obtained.

Individual calibration curves need to be prepared for other instruments;

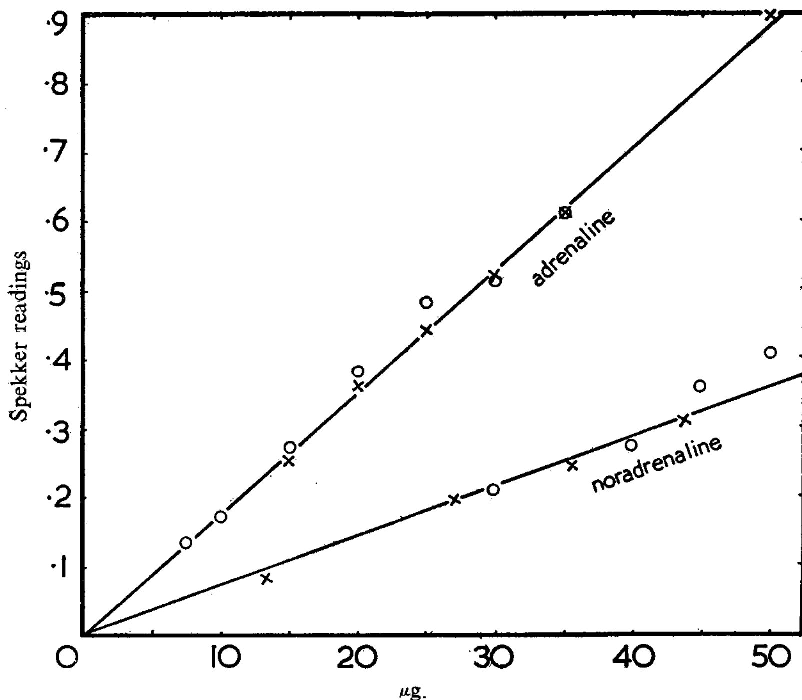

FIG 1. Calibration graphs.

but we did not find it necessary to alter the calibration for new batches of reagent (Fig. 1). In this respect the method seems to be superior to the

somewhat similar arsenomolybdate method[4]. The colour was also independent of the nature of the acid used to dissolve the bases.

Separation. The best separation of adrenaline and noradrenaline on paper chromatograms was obtained by using phenol as carrier[1]; but this had the disadvantage that traces of phenol remaining in the paper after drying caused slight tinting of the reagent used for the colorimetric estimation. These small amounts of phenol could be removed without removing the adrenaline by first treating the paper with ether in a micro-extractor designed for the purpose. The need for this preliminary extraction[1] was avoided when it was found that *n*-butanol did not reduce the reagent even in high concentrations. Butanol did not dissolve the bases appreciably and was useless as a stabiliser; but the latter difficulties were got over by saturating the *n*-butanol with 16·6 per cent. acetic acid. There was then sufficient movement of the bases and separation was complete in 22 hours.

For quantitative experiments 2 strips (a) and (b) of Whatman No. 1 paper 12 cm. wide were cut. A faint pencil line was drawn across each paper at 9 cm. from the upper end. Lines 2 cm. from each edge were also drawn the full length of each paper to mark off two lateral guide strips. Solution was applied to each guide strip at the centre of the cross-line and at 5 equidistant positions where the cross-line traversed the main width of paper between the guide strips. Each of these spots was thus centred 1 cm. from its neighbour and the end ones 2 cm. from those on the guide strips. Solutions were applied by means of an Agla pipette. A drop, 0·01 ml., was delivered to each position in turn and then dried, if necessary, by gentle heating. The process was then repeated until the 5 spots in the main strip contained the desired amount of solution. The glass trough of the chromatograph chamber was filled with butanol-acetic acid, prepared by shaking 4 parts of *n*-butanol with 1 part of glacial acetic acid and 5 parts of water and discarding the lower layer. The atmosphere of the chamber was saturated by means of a free surface of the same mixture. The chromatograms were set up in the usual way and run for 22 hours. The face of the solvent was then marked in pencil and the papers dried by gentle heating over a hot plate. The guide strips were cut off and sprayed with an indicator consisting of 0·44 per cent. potassium ferricyanide solution in 0·2 sodium phosphate (Sörensen salt) solution to give *p*H 8·3. The position of adrenaline on the guide strips was revealed by a pink spot, and that of noradrenaline behind it by a mauve spot. If the colours were faint they could be strengthened by holding over the mouth of a bottle containing strong ammonia solution (0·880). The two guide strips were then placed alongside the main strip in their original positions and the respective adrenaline and noradrenaline zones marked off and cut out. Two pieces of paper (a) and (b) were thus obtained for each base, 8 cm. long and 2·5 to 3·0 cm. wide. These were extracted separately. Each strip was folded into corrugations about 0·5 cm. wide and placed at the bottom of a test-tube and covered with at least 6 ml. of 0·01 N hydrochloric acid. The tube was well shaken to ensure that the paper was completely wetted and allowed to stand for at least half an

hour. The paper was then removed by means of a glass rod and pressed again the side of the tube to expel moisture, the last drops being squeezed out between the fingers. Estimations of the two extractions were carried out separately and good agreement was usually obtained; if there was any considerable discrepancy a further pair of strips was examined.

This procedure was tested in two ways. The efficiency of the removal

TABLE I

EXTRACTION OF 30 μG. OF ADRENALINE FROM WHATMAN NO. 1 PAPER

Time of Extraction Hours	(a)	(b)	Mean	Per cent.
0·0 (direct determination)	29·5	30·4	30·0	100
0·5	28·7	28·7	28·7	96
1·0	27·0	28·3	27·7	92
1·0	28·3	28·7	28·5	95
2·0	29·7	—	28·7	96
overnight	29·5	31·3	30·4	101

of the bases from the paper was checked after various periods of extraction and the results are given in Table I.

The recovery after half an hour's soaking, as calculated from the mean, was 96 per cent.; increasing the extraction time to 1 or 2 hours

TABLE II

ESTIMATION OF A MIXTURE CONTAINING 30 μG. OF ADRENALINE AND 30 μG. NORADRENALINE IN 0·6 ML. OF 0·01 N HYDROCHLORIC ACID

	ADRENALINE			NORADRENALINE		
	Direct before Mixing	After Separation and Extraction	Recovery per cent.	Direct before Mixing	After Separation and Extraction	Recovery per cent.
	0·55	0·44		0·20	0·20	
	0·63	0·48		0·19	0·30	
	0·53	0·53		0·19	0·19	
	0·51	0·49		0·20	0·21	
	0·52			0·19	0·16	
	0·50			0·18	0·23	
	0·49			0·21	0·19	
	0·53					
	0·48					
	0·51					
Mean (colour units)	0·515	0·485		0·194	0·197a	
μg.	30·0	28·2	94·0	30·0	30·4	101·0

a Omitting the second reading.

did not improve this. A second washing of the paper did not extract any measurable residue; but prolonged soaking overnight appeared to raise the value. We have usually been satisfied with 1- to 2-hour extrac-

tion periods entailing a systematic error of about 5 per cent.; but this can probably be reduced, if required, by longer periods of extraction.

The presence of the chromatographic solvent and the second base did not appreciably increase this error. Table II lists results obtained in complete runs using a known mixture of the two bases.

Summary of the Method

1. The bases are extracted into 5 per cent. acetic acid or 5 per cent. trichloracetic acid. Best results are obtained with 20 to 30 μg. of base in 0·1 to 0·5 ml.

2. The solution is delivered quantitatively on to a one-way paper chromatogram from an Agla pipette (p. 24).

3. Duplicate chromatograms are run for 22 hours using a butanol-acetic mixture as solvent (p. 24).

4. Lateral guide strips are cut off and the bases located with a ferri-cyanide indicator (p. 24).

5. The adrenaline and noradrenaline spots are cut from the main strips of the chromatograms and extracted separately for at least 30 minutes into 0·01 N hydrochloric acid.

6. Reagents (a) and (b), described on p. 22, are added to the solutions.

7. When the colour is fully developed after 15 minutes, the solution is transferred to an absorptiometer. Instrument readings are converted to μg. of adrenaline and noradrenaline by reference to a calibration curve (p. 23).

References

1. James, *Nature,* 1948, **161,** 851.
2. James and Kilbey, *Nature,* 1950, **166,** 67.
3. Peters and van Slyke, *Quantitative Clinical Chemistry,* London, 1932.
4. Allport, *Colorimetric Analysis,* London, 1945, 330.