Semi-automated method for the differential determination of plasma catecholamines

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SYNOPSIS A method is described for the estimation of adrenaline and noradrenaline in a 5 to 10 ml plasma sample.

A batch technique of adsorption and elution from alumina is used and the final estimation is carried out using an automated fluorimetric technique based on the trihydroxyindole method.

Fluorescence is stabilized first by thioglycolic acid to give noradrenaline concentrations only and then by ascorbic acid to give the total catecholamine concentration.

The results of a number of catecholamine analyses are given.

The trihydroxyindole (THI) procedure for the fluorimetric determination of catecholamines involves the oxidation of the catecholamines to adrenochrome and noradrenochrome at a slightly acid pH; the addition of a strong alkali causes rearrangement to the fluorescent lutines (3,5,6-trihydroxyindoles). The alkaline reagent used is concentrated sodium hydroxide containing a reducing agent, generally ascorbic acid, which stabilizes the fluorescence of the lutines. Merrills (1962) has recently shown that if thioglycolic acid is used as the reducing agent only the fluorescent product of noradrenaline is stabilized. This observation forms the basis of an automated method for the differential determination of adrenaline and noradrenaline described by Merrills (1963). Robinson and Watts (1965) have modified this method and used it to determine adrenaline and noradrenaline in urine, rat hearts, adrenals, and adrenal vein blood. Fiorica (1965) increased the sensitivity of the automated method in order to measure the total catecholamine concentration in human plasma, but did not attempt to differentiate the two catecholamines.

The present paper describes a method for the determination of adrenaline and noradrenaline in human plasma, in which a simple batch technique for the isolation of the catecholamines on alumina has been introduced to replace the more time consuming process involving columns. The automated method of determination is based on the technique described by Fiorica (1965) with differentiation of the catecholamines according to Merrills (1963).

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REAGENTS

All chemicals used were analytical grade. Water used throughout the method was deionized.

1 ALUMINA (B.D.H. REAGENT GRADE) Alumina, 250 g, in a 500 ml stoppered container, was washed with 5 × 200 ml 0.3N acetic acid, and 5 × 200 ml deionized water. Each wash was shaken gently for five minutes using a mechanical shaker and the alumina was allowed to settle for approximately one minute before aspirating.

All fine alumina particles should be removed during this procedure.

The final water wash was neutralized with NaHCO₃/Na₂CO₃ buffer (3 g each/100 ml). Water washes of 200 ml were continued until no change in pH of the wash was detected before and after shaking. The alumina was dried in an oven at 105°C and the recovery of catecholamines from each new batch of alumina was checked before use.

2 SODIUM ACETATE BUFFER, 0.2M The buffer was adjusted to pH 8.4 with 0.5N sodium carbonate.

3 PERCHLORIC ACID, 4N.

4 ETHYLENEDIAMINE TETRA-ACETIC ACID (EDTA), 5%

5 SODIUM CARBONATE, 5N

6 ACETIC ACID, 0.3N

7 SODIUM HYDROXIDE, 2.5N

8 SODIUM ACETATE, 1.5M This contained 0.01% w/v potassium ferricyanide, pH 9.0.

9 ASCORBIC ACID, 0.15% This solution was prepared immediately before use.

10 THIOLYGLYCOLLIC ACID, 1% This solution was prepared and adjusted to pH 3.27 with 4N NaOH immediately before use.

11 HYDROCHLORIC ACID, 0.01N

12 SODIUM METABISULPHITE, 10 MG/ML Of this reagent 0.5 ml was added to heparinized tubes and dried.

STANDARDS

STOCK STANDARDS These were prepared monthly and stored at -4°C .

ADRENALINE, 1 MG/ML L-Adrenaline bitartrate monohydrate, 18.2 mg, as L-epinephrine bitartrate (Sigma), in 10 ml 0.01N HCl.

NORADRENALINE, 1 MG/ML L-noradrenaline bitartrate monohydrate, 19.9 mg, as L-arterenol (Sigma), in 10 ml 0.01N HCl.

WORKING STANDARDS

These were prepared immediately before use. (1) 10 $\mu\text{g}/\text{ml}$, 1 ml stock in 100 ml water; (2) 0.1 $\mu\text{g}/\text{ml}$, 1 ml (1) in 100 ml water; (3) 5 $\mu\text{g}/\text{l}$. 5 ml (2) in 100 ml water. Five ml sodium metabisulphite 10 mg/ml was used in making up each standard. Then 0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 ml (0 to 3 $\mu\text{g}/\text{l}$) working standards were pipetted into buckets containing 1 ml 4N HClO_4 .

APPARATUS

All glassware was very carefully washed to prevent contamination with fluorescent compounds, particularly those present in tap water and detergents. No detergent was used, and after thorough washing with tap water, all glassware was rinsed twice with deionized water.

Adsorption tubes were washed in batches with 2×20 ml deionized water before use.

The following are required: (1) 12 ml tubes containing 1 ml perchloric acid stored at 4°C ; (2) 30 ml plastic buckets with lids; (3) 30 ml glass stoppered centrifuge tubes, internal diameter 25 mm; (4) mechanical shaker and shaking racks; (5) pH meter and glass electrode; (6) automatic burette for 2 ml delivery of 0.3N acetic acid.

The autoanalyser was used with a Locarte fluorimeter using a hydrogen lamp as the ultraviolet source. The primary filter was a Locarte LF2 (360 to 380 $\text{m}\mu$), and the secondary filter, an LF7 which passes all wavelengths longer than 510 $\text{m}\mu$.

The flow system is shown in Fig. 1 and was used in conjunction with a type II sampler to ensure a constant air:fluid ratio within the system.

PROCEDURE

SAMPLE COLLECTION AND STORAGE Blood was collected in two 10 ml heparinized tubes each containing 5 ml sodium metabisulphite as antioxidant, and chilled in ice until centrifuged. The plasma was separated and 5 to 10 ml sample pipetted into tubes containing 1 ml 4N perchloric acid. After thorough mixing the tubes were centrifuged at $30,000 \times g$ for 15 minutes, and the supernatant was transferred to 30 ml buckets with 1 ml of acetate buffer.

The deproteinized acid extracts were stored at -4°C until required for analysis which was generally carried out within a week although the solutions were stable for up to one month.

ADSORPTION Batches of 12 tubes could be conveniently handled at one time and two or three batches could be prepared for analysis in one day. EDTA, 1 ml, and 5 ml acetate buffer were added to each bucket, the standards, tests, and blank being treated in the same way.

The pH of the solution was adjusted to 8.4 ± 0.1 by dropwise addition of 5N sodium carbonate, with continuous mixing until a constant pH was maintained. The

FIG. 1. Flow system for the differential determination of plasma catecholamines.

contents of the bucket were transferred with 1 ml of acetate buffer to 30 ml tubes containing 500 mg alumina. The tubes were stoppered and shaken for five minutes.

The supernatant was aspirated without loss of alumina and the contents washed with 2×20 ml water. Provided that all fine particles of alumina had been removed during the original purification, it was not necessary to centrifuge the tubes. The final wash was removed as completely as possible and the tubes inverted on clean tissue. Excess water was aspirated from the sides of the tube.

ELUTION Acetic acid, 2 ml 0.3N, was added to each tube which was then stoppered and shaken vigorously for four minutes. The supernatant was transferred to auto-analyser cups which were capped and stored overnight at -4°C . These eluates were stable at this temperature for several days but were generally analysed within 24 hours.

ANALYSIS The tests were processed through the auto-analyser (Fig. 1) at the rate of 60 samples per hour, with alternate water washes between each sample.

Standards were inserted at intervals between the tests to correct for the slight decrease in sensitivity which occurs during the analysis.

The fluorescence of all the catecholamine eluates was determined using thioglycolic acid as the stabilizing reagent. This reagent was then replaced by ascorbic acid and the complete analysis was repeated.

CALCULATION Standard curves were constructed by plotting the peak heights of the standards, in both stabilizing agents, against the initial concentration of catecholamines (0 to 3 $\mu\text{g/l.}$), and the total catecholamine and noradrenaline concentrations of the 10 ml plasma sample were read from the ascorbic and thioglycolic acid graphs (Fig. 2). The concentration of adrenaline was obtained from the difference between the two values.

RESULTS

STANDARD CURVE Over a range of 0 to 25 $\mu\text{g/l.}$, which is equivalent to 0 to 5 $\mu\text{g/l.}$, initial concentration due to the five-fold concentration attained by adsorption on alumina, the fluorescence intensity is related in a linear manner to the concentration of both catecholamines in ascorbic acid and to the concentration of noradrenaline in thioglycolic acid.

Figure 2 shows the standard curves obtained over the range 0 to 3 $\mu\text{g/l.}$ initial concentration after adsorption and elution from alumina.

REPRODUCIBILITY To determine the precision of the method, duplicate samples were analysed for total catecholamine and noradrenaline content over various concentration ranges. Results are summarized in Table I.

RECOVERY Samples, 10 ml of each standard (1 to 5 $\mu\text{g/l.}$), were carried through the entire

FIG. 2. Standard curve of adrenaline and noradrenaline with ascorbic acid and thioglycolic acid stabilization after adsorption and elution.

1 Adrenaline, 2 noradrenaline stabilized with ascorbic acid, 3 noradrenaline, 4 adrenaline stabilized with thioglycolic acid.

TABLE I

PRECISION OF THE METHOD FOR TOTAL CATECHOLAMINES AND NORADRENALINE

	Concentration Range ($\mu\text{g/l.}$)		
	0.0-1.0	1.0-2.0	2.0-4.0
Number of duplicate determinations	6	5	5
Standard deviation for total catecholamines (%)	± 6	± 6	± 5
Standard deviation for noradrenaline (%)	± 9	± 7	± 7

procedure and the eluate fluorescence was compared with that obtained from corresponding standards (5 to 25 $\mu\text{g/l.}$) in 0.3N acetic acid, pH 3.85. Recoveries ranged from 93 to 100% for noradrenaline and from 87 to 95% for adrenaline.

SENSITIVITY It is possible to detect 0.0005 $\mu\text{g/ml}$ of total catecholamine or noradrenaline in the final eluate, which corresponds to an initial concentration of 0.1 $\mu\text{g/l.}$ in a 10 ml plasma sample.

ANALYSIS OF SAMPLES The resting levels of adrenaline and noradrenaline in venous peripheral blood were determined in five normal male subjects aged 20 to 40 years, who had each rested for at least 10 minutes before sampling. Mean resting concentrations and ranges were as follows: total catecholamines 0.63 $\mu\text{g/l.}$ (0.46 to 0.81 $\mu\text{g/l.}$), noradrenaline 0.61 $\mu\text{g/l.}$ (0.46 to 0.79 $\mu\text{g/l.}$), adrenaline 0.02 $\mu\text{g/l.}$ (0.00 to 0.10 $\mu\text{g/l.}$).

Plasma catecholamines were determined in arterial samples from 40 patients under thiopentone/nitrous oxide/oxygen anaesthesia, as preliminary control values in a study of the effects of various anaesthetics on catecholamine concentrations. Mean resting concentrations and ranges were as follows: total catecholamines 0.46 $\mu\text{g/l.}$ (0.10 to 1.06 $\mu\text{g/l.}$), noradrenaline 0.41 $\mu\text{g/l.}$ (0.10 to 1.06 $\mu\text{g/l.}$), adrenaline 0.05 $\mu\text{g/l.}$ (0.00 to 0.32 $\mu\text{g/l.}$).

Price, Linde, Jones, Black, and Price (1959) have reported that this anaesthetic does not cause increases in plasma catecholamine levels and these results tend to confirm this observation since they lie within the range reported by Weil-Malherbe (1961) for normal resting plasma catecholamines.

Catecholamine levels were determined in a patient with a phaeochromocytoma of the bladder, which was confirmed by biopsy but appeared to be of a non-secretory nature since the patient's clinical symptoms were minimal. This was confirmed by determination of the catecholamine levels in plasma drawn from various sites. The levels shown in Table II were within the normal range except in the region of the diaphragm. The tumour was subsequently removed and on histological examination it was considered to be a phaeochromocytoma.

TABLE II

PLASMA CATECHOLAMINES IN PHAECHROMOCYTOMA OF URINARY BLADDER

Sample	Total ($\mu\text{g/l.}$)	Noradrenaline ($\mu\text{g/l.}$)	Adrenaline ($\mu\text{g/l.}$)
Right atrium	1.12	1.12	0.00
Diaphragm	2.00	1.50	0.50
Below renal vein	1.18	1.18	0.00
Pelvis	0.33	0.28	0.05
Femoral vein	0.63	0.48	0.15

A 500 mg portion of the tumour was homogenized in 100 ml 4N perchloric acid containing 50 mg sodium metabisulphite. The extract was centrifuged at $30,000 \times g$ for 15 minutes and a 1 ml sample of supernatant was diluted to 500 ml in 4N perchloric acid. Three further dilutions were prepared from this stock solution, 1:10, 1:50 and 1:100 in 4N perchloric acid, and 1 ml portions of each dilution were taken for catecholamine analysis. The tumour was found to contain 5.1 $\mu\text{g/mg}$ noradrenaline and 4.4 $\mu\text{g/mg}$ adrenaline. The figures quoted by von Euler (1956) for the catecholamine content of

tumours ranged from 0.63 to 8.40 $\mu\text{g/mg}$ noradrenaline, 0.05 to 2.30 $\mu\text{g/mg}$ adrenaline, 0.70 to 9.7 $\mu\text{g/mg}$ total catecholamines.

A bioassay of the tumour was carried out independently (Glover and Wallace) using the isolated central artery of the rabbit ear (de la Lande and Harvey, 1965). The result, 11.7 $\mu\text{g/mg}$ of total catecholamine, was in good agreement with the automated trihydroxy indole method (Glover, McCullough, and Wallace, 1967).

DISCUSSION

The introduction of automated chemical analysis has solved many of the problems involved in the fluorimetric estimation of catecholamines by ensuring reproducible addition of reagents and constant reaction time.

When thioglycollic acid is used as the stabilizing agent Merrills (1962) has shown that the fluorescence of the catecholamines increases rapidly to a maximum and then slowly fades, so that reproducible reaction time is particularly important during the differential stabilization.

The automated method is capable of more complete differentiation of the catecholamines than other methods, which depend on the suppression of noradrenaline fluorescence, since the relative fluorescence of the amines in the two stabilizing reagents is very different (Robinson and Watts, 1965).

In the present method several modifications of Merrills' original (1962) method are present. The 0.3% ascorbic acid has been replaced by a 0.15% solution as recommended by Robinson and Watts (1965) since this reagent gives a reduced reagent baseline without decreasing the sensitivity or stability of the fluorescent products.

The pH of the acid eluate (3.85 ± 0.5) is adjusted automatically by the buffered ferricyanide, during the analysis, to give an oxidation mixture, pH 5.8, which is suitable for both catecholamines.

Since this procedure, as described by Fiorica (1965), avoids the use of an internal standard, the incorporation of a dialyser module in the auto-analyser flow line is unnecessary and the sensitivity of the estimation is greatly increased.

Isolation of the catecholamines, by adsorption on alumina and subsequent elution, has been accomplished by a batch technique, which has been shown to be accurate and reproducible provided that the alumina is correctly washed before use.

Since the adsorption and elution can be carried out more rapidly by this technique than by one involving columns, loss of catecholamines by oxidation in an alkaline medium is minimized.

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release chuck designed to accept both metal and lint-capped bottles. To overcome the out-of-balance load of the bottle, the chuck is fitted with a flexible coupling allowing the bottle to precess around its dynamic centre while spinning. For loading and unloading the spinners a horizontal bar is fitted above the cabinet on which the spinners can hang.

Details of the design are shown in the sectional diagram (Fig. 2). With this equipment the times for freezing solutions starting at room temperature were as follows: 100 ml of solution in a standard 540 ml transfusion bottle three minutes or, in a 250 ml bottle five minutes; 400 ml of plasma in a 540 ml bottle 15 to 20 minutes.

We wish to thank Mr I. C. Costar for the diagrams.

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CORRECTION

In Figure 1 of the paper by Helen McCullough, 'Semi-Automated method for the differential determination of plasma catecholamines' (*J. clin. Path.*, 21, 759), showing the flow system the waste tube (no. 10) should pass through the pump once only. Reagents for the various tubes and tube diameters in inches should be shown as follows: (2) Sample (0.045); (4) Acetate-Ferricyanide (0.065); (6) Air (0.056); (8) 2.5N NaoH (0.045); (9) Stabilizing reagent (0.030); (10) Waste (0.065).

Letters to the Editor

THYROID FUNCTION TESTS

I was most interested in the paper by Thomson, Boyle, McGirr, Macdonald, Nicol, and Brown (1968)¹ in which they describe difficulties they have experienced in the interpretation of some thyroid function tests. I feel, however, that their conclusions are too dogmatic, and shall appreciate space to reply to some of their statements.

Thyroid function tests fall into two groups, first those which aim to determine 'thyroid status', and secondly those directed at specific thyroid disorders independent of thyroid status. The commonest example of the second group is the diagnosis of Hashimoto's disease by demonstration of high titres of specific antibodies to thyroid components in the peripheral blood. A patient with Hashimoto's disease may be euthyroid, hypothyroid or, rarely, thyrotoxic, but the antibody findings are quite independent of this aspect. It is useful, when considering and comparing tests of thyroid function, to separate the two groups of investigations to avoid confusion.

The authors state in their synopsis that 'an uptake test and estimation of the serum protein-bound radioactive iodine (PB¹³¹I), supplemented as required by the protein-bound iodine (PBI), remain the best routine tests of thyroid function'. This conclusion is not based on a comparison with the variety of newer tests now available, of which they used only the triiodothyronine resin uptake in a few cases. Although their statement might have been valid between 1963 and 1965, when the work was done, it does less than justice to the many authors who have published studies since then.

Recent work on the determination of thyroid status has largely been concerned with the direct measurement of levels of thyroid hormone in blood. Ekins (1960) and Murphy (1965) developed specific thyroxine assay techniques based on the saturation analysis principle and Nauman, Nauman, and Werner (1967) described a method for the determination of serum triiodothyronine also based on this technique. These methods eliminate inaccuracies inherent in PBI measurements by being specific for the hormones themselves. Following the extensive development by Robbins and Rall (1957) of the concept of protein-binding of thyroid hormones in blood, it has been recognized that thyroid function is most closely related to the concentration of unbound hormone. This level is dependent on the concentrations of thyroxine-binding globulin (TBG) and thyroxine-binding prealbumin (TBPA), as well as on the level of total thyroid hormone. Free thyroxine can be measured directly by equilibrium dialysis or ultrafiltration, and indirectly by methods derived from that of Hamolsky, Stein, and Freedberg (1957) which reflect the concentration of thyroxine-binding proteins. These have been well

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