Journal of Chromatography, 183 (1980) 295—302
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 604

SEMI-AUTOMATED FLUORIMETRIC METHOD FOR THE ESTIMATION OF URINARY CATECHOLAMINES USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received March 28th, 1979; revised manuscript received February 18th, 1980)

SUMMARY

A high-performance liquid chromatographic method for the quantitation of adrenaline and noradranaline in urine is described, using fluorescence detection. The effluent from the liquid chromatograph is led directly into an analyser to produce the fluorescent trihydroxyindoles from the catecholamines. The method is more reliable and specific than conventional fluorescence techniques. Both catecholamines can be detected at levels of 0.5 ng on the column.

INTRODUCTION

The trihydroxyindole fluorescence method has been widely used for the determination of catecholamines in biological fluids [1—10]. This method, although very sensitive, has the disadvantage that it detects agents other than the catecholamines. L-Dopa, α-methyldopa [11], and labetolol [12] are documented as interfering, but there are other possible interfering agents. Carruthers et al. [13] found that ampicillin, promethazine, protamine, sulphadimidine, vitamin B complex, as well as coffee, tea and cocoa produced considerable interference in vitro. Whether these drugs and dietary components interfere in vivo is not known. Very high blanks are often associated with urine specimens, and quantitation becomes difficult in such cases.

Using high-performance liquid chromatography (HPLC) it is possible to separate the catecholamines, but there is a problem in measuring the physiological levels. Ultraviolet (UV) absorption is a commonly used detection system in HPLC, but is not really sensitive enough — Mell and Gustafson [14]

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0378-4347/80/0000-0000/$02.25

had to use 200 ml of urine in order to detect noradrenaline (NA) and dopamine (DA) in urine by UV absorption. They were not able to measure adrenaline (A). Similarly, Knox and Jurand [15], using a UV detector, were unable to quantify normal levels of the catecholamines.

Since the fluorimetric methods are so sensitive, it seemed reasonable to use such a detection technique for the catecholamines. Davies et al. [16] prepared fluorescent *o*-phthalaldehyde derivatives of NA and DA and separated these by HPLC. The derivatisation only works for primary amines so A cannot be analysed by this technique. Similarly, in the post-column *o*-phthaladehyde derivatisation method of Froehlich and Cunningham [17], no A can be measured. Schwedt and Bussemas [18] describe 5-dimethylamino-1-naphthalinesulphonyl (dansyl) derivatives of NA, A, and DA, but they do not describe the detection of physiological levels nor any urine analyses.

Ueda et al. [19] describe an HPLC method using an automated trihydroxyindole reaction for the detection of NA, A and DA. The autoanalyser is extremely complicated, using five pumps, and although the authors quantify DA, in fact the system is relatively insensitive to this amine. Schwedt [20] described an HPLC trihydroxyindole method for NA and A using isoprenaline (IP) as an internal standard. In 1978 [21] he described its application to urinary catecholamines. The chromatograms shown in the paper are, however, rather noisy. The work described in this paper is similar but uses ascorbic acid rather than sodium sulphite as the fluorophore stabiliser, and produces clearer chromatograms. Using the method described in this paper, we are able to combine the high sensitivity of the trihydroxyindole detection with the separating power of HPLC. This permits A and NA to be resolved on a liquid chromatography column and introduced separately into the fluorimetric analyser.

METHODS

Sample collection

Twenty-four-hour urine specimens were collected over 25 ml of 6 *M* hydrochloric acid. The volume of each was recorded and an aliquot stored at −20°C until assayed.

Extraction procedure

A 5-ml sample of urine was added to a test-tube containing 5 ml of 0.2 *M* sodium acetate buffer (pH 8.4), 1 ml of 5% ethylenediamine tetraacetate, three drops of thymol blue (1% in ethanol), 0.2 g of activated alumina, and, as an internal standard, 0.5 μg of IP. The contents were taken to pH 8.4—8.6 with 0.5 *M* sodium carbonate. At this point the indicator has turned blue. The test-tube was stoppered and the contents shaken for 5 min. After allowing the alumina to settle, the supernatant was aspirated and the alumina washed with distilled water. The wash water was removed and the catecholamines were eluted by shaking the alumina for 5 min with 0.5 ml of 0.25 *M* acetic acid.

Chromatography

A 10-μl aliquot of the acetic acid eluant was injected onto a 25 cm × 5 mm

column of ODS-coated silica (Whatman Partisil PXS 10/25 ODS) with a mobile phase of 1% acetic acid, 0.0001% sodium dodecyl sulphate and about 10% methanol. The flow-rate of 1 ml/min was provided by a Milton Roy pump with a pulse dampener (Phase Separations). The effluent from the column was fed directly into an automated system producing the fluorescent trihydroxindole compounds from NA, A and IP. Fig. 1 shows the arrangement of this auto-analyser system. The autoanalysis equipment was from Technicon Instruments, and the fluorimeter was a Locarte Mk4 fitted with a 0.15-ml flow-cell and an LF2 filter on the excitation side, and LF7 and LF14 filters on the emission side. The recorder was a Linseis.

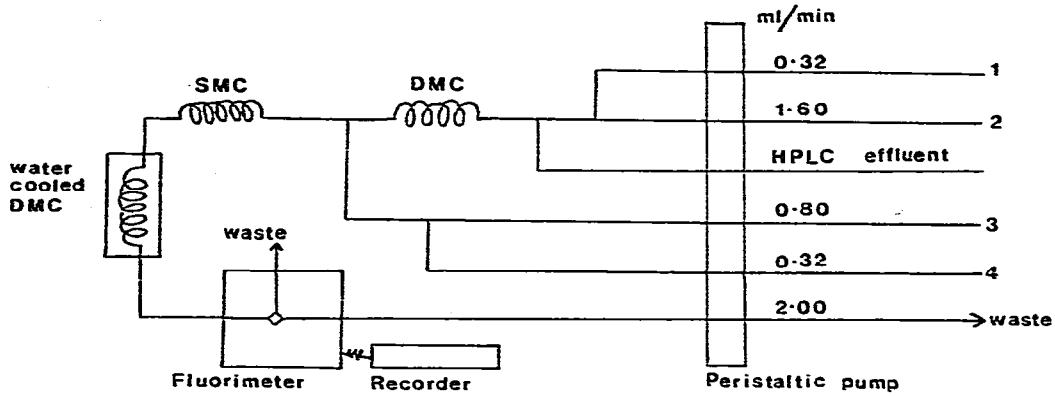

Fig. 1. Diagram of the autoanalyser system used to produce the trihydroxyindoles from NA and A after their separation by HPLC. SMC, single mixing coil; DMC, double mixing coil; 1, air; 2, 1.5 *M* sodium acetate and 0.01% potassium ferricyanide; 3, 2.5 *M* sodium hydroxide; 4, 0.1% ascorbic acid.

Quantitative work

Pure standards of NA, A and IP were made up to 10 μg/ml in 0.1 *M* hydrochloric acid and injected onto the column to determine the retention times. A standard curve was prepared by extracting and analysing solutions containing 0—2 μg of NA and A, and 0.5 μg of IP. Curves were plotted of concentration vs. NA/IP peak height ratios and A/IP peak height ratios. The reliability of the chromatography and the detection was determined by injecting the same specimen six times. The reliability of the whole procedure was determined by extracting six replicate samples of the same urine specimen. The precision was determined by adding known amounts of NA and A to six samples of the same urine taken through the procedure and determining the levels. The results of these tests were compared with equivalent tests on the trihydroxyindole method with prior HPLC.

In order to check that the peaks measured did correspond to those of the three catecholamines, the following standards were injected for possible interference: dopamine, L-dopa, dihydroxyphenylacetic acid, and dihydroxymandelic acid. Any contribution from A in the NA peak, and NA in the A peak, was checked by analysing urine for NA and A. The analysis was repeated twice, first with the addition of 0.2 μg of NA, and second with the addition of

0.2 μg of A. In the former case A was estimated and in the latter NA was estimated. The blank was determined by taking 5 ml of distilled water through the procedure.

The urine from eight children with neuroblastoma, and eight age-matched children in hospital for other complaints were analysed. Urine from three adults suspected of having a phaeochromocytoma were analysed. Urine from twelve of the sixteen children was analysed by the trihydroxyindole procedure without prior HPLC.

RESULTS AND DISCUSSION

The trihydroxyindole reaction involves oxidation of A and NA to adrenochrome and noradrenochrome, respectively, then rearrangement to the respective fluorescent trihydroxyindoles. The fluorescent products are very unstable and have to be protected from oxidation by the presence of a reducing agent. In this laboratory the normal procedure involves stabilisation of both products with ascorbic acid or stabilisation of only the NA product with dithioerythritol. Thus it is necessary to perform two assays — one for total catecholamines and one for NA. A is determined by subtraction, which is liable to compound any errors. An additional "blank" determination, in which the fluorescent products are not stabilised, attempts to compensate for interfering compounds. Thus at least three determinations are necessary to assay NA and A in urine by this conventional method.

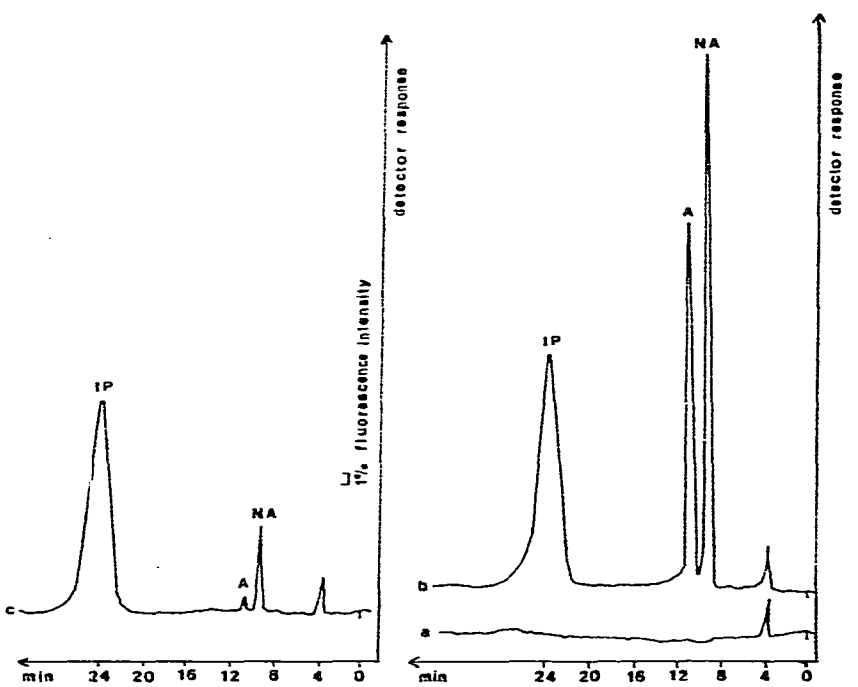

Fig. 2. Typical chromatograms. (a) Blank extraction of 5 ml of distilled water. (b) Standards: 0.2 μg of NA, 0.2 μg of A and 0.5 μg of IP in 500 ml of acetic acid. (c) Urine extract containing 0.05 μg of NA, 0.01 μg of A, and 0.5 μg of IP in 500 μl of acetic acid.

In the method described, both fluorophores can be stabilised by ascorbic acid and a single chromatogram obtained for quantitation of both catecholamines, eliminating the problems associated with blank determinations and, in many cases, interfering compounds. Introduction of an internal standard further improves the reliability of the method.

There is a tendency for the reversed-phase column to change its properties slightly with use [22], and therefore the methanol content of the mobile phase may require slight alterations from time to time in order to maintain the retention times.

The mean recovery of 1 μg of the catecholamines added to urine samples in ten extractions was: NA 72%; A 70%; IP 60%. Since they did not all extract equivalently, it was necessary to prepare an extracted standard curve as described in Methods.

The blank determination shown in Fig. 2 (curve a) shows that NA and A are estimated as 0.0 ng. The chromatograms for pure standards and a urine extract are shown in curves b and c (Fig. 2), respectively. Table I shows the retention times and relative responses of NA, A, IP, and various possible interfering substances. The relative responses show that only α-methyldopa and L-dopa are likely to interfere, and their retention times are well clear of the compounds of interest.

TABLE I

RETENTION TIMES OF NA, A, IP AND POSSIBLE INTERFERING SUBSTANCES

Compound	Retention time (min)	Relative response
Noradrenaline (NA)	9.2	100
Adrenaline (A)	11.0	67
α-Methyldopa	13.2	88
Isoprenaline (IP)	24.0	44
Dopamine (DA)	15.4	1
L-Dopa	10.1	100
Dihydroxyphenylacetic acid	10.4	2
Dihydroxymandelic acid	6.7	0.04

TABLE II

RELIABILITY OF THE METHOD

Aliquots of a single urine specimen were extracted and analysed by HPLC—fluorimetry and aliquots of another urine specimen were extracted and analysed by fluorimetry alone. Replicate injections of a urine extract demonstrated the reliability of the HPLC step. Values are given as the mean ± S.E.M.

Method	*n*	NA	A
HPLC—fluorimetry (μg per 24 h)	6	54.8 ± 3.5	6.2 ± 0.0
Fluorimetry alone (μg per 24 h)	6	37.3 ± 1.5	8.2 ± 1.2
Replicate injections (μg)	5	0.44 ± 0.0	0.10 ± 0.0

TABLE III

PRECISION OF THE METHOD

Method	n	Amount recovered (mean ± S.E.M.)	
		NA	A
HPLC—fluorimetry			
Added: 1 μg of NA, 0.2 μg of A	6	0.99 ± 0.04 μg	0.21 ± 0.01 μg
Fluorimetry alone			
Added: 10 ng of NA, 2 ng of A	7	10.04 ± 0.73 ng	1.80 ± 0.65 ng

Comparison of the reliability and precision of the HPLC method and the trihydroxyindole method without prior HPLC shows that the former is much more reliable, especially for A. Tables II and III show the results.

Additions of NA made no difference to the A determination and the same applied to A in the NA determination. Table IV gives the results of this test.

TABLE IV

CONTRIBUTION OF NA TO A DETERMINATIONS, AND A TO NA DETERMINATIONS

Sample	NA (μg)	A (μg)
Urine 1 alone	0.03	0.01
Urine 1 + 0.2 μg of NA	0.24	0.01
Urine 2 alone	0.05	0.01
Urine 2 + 0.2 μg of A	0.05	0.21

The urine estimations are shown in Table V. The neuroblastoma samples PD, JH, MC, and GN have much lower catecholamine levels when measured by the HPLC technique than when assayed by the less specific fluorimetric method. Other peaks in the chromatogram were observed, separated chromatographically from NA and A; these compounds had obviously interfered in the less specific method. None of the possible interfering compounds tried (Table I) seemed to cause the observed peaks in these samples. Possibly we are looking at drug effects.

The sensitivity of this method is such that 0.5 ng of NA or A on the column can be detected and quantified. This is clearly not sensitive enough for plasma catecholamine determinations except in cases of phaeochromocytoma where the amines are drastically raised. NA and A in a plasma sample from such a patient were measured; the catecholamines were found to be thirty times higher than in normal subjects.

The method is used in this laboratory for the routine measurement of NA and A in urine. It is quick, permitting one analysis in place of the usual three by conventional techniques. Also it is more specific and precise than the trihydroxyindole procedure alone. The method is thus more widely applicable to clinical studies where patients are heavily medicated, for example in open heart surgery, where potentially interfering antibiotics are given. It can be used to determine urinary catecholamines in patients on Aldomet and indeed can be used to estimate the drug.

TABLE V

ANALYSIS OF URINE SAMPLES

Subject	Age	Diagnosis	Concentration* in urine (μg per 24 h)			
			Fluorimetry alone		HPLC—fluorimetry	
			NA	A	NA	A
JL	14	Neuroblastoma	52	74	29	n.d.
RS	11	Neuroblastoma	35	16	60	12
GN	9½	Ganglioneuroblastoma	Interference		70	10
PD	9	Neuroblastoma	214	54	74	5
JH	6½	Abdominal ganglio-neuroblastoma	93	115	85	20
MC	6	Ganglioneuroblastoma	102	6	131	18
MC	4	Ganglioneuroblastoma	59	9	26	4
MY	3	Neuroblastoma	37	8	25	5
GF	14	Neurofibromatosis	32	1	137	11
EW	10¾	Abdominal pain	17	10	14	6
CK	9¾	Vomiting	n.e.	n.e.	79	37
KA	9	Pyrexia of unknown origin	73	0.3	21	17
JH	6	Vomiting	36	n.d.	23	5
JP	6	Enlarged liver	21	4	10.3	n.d.
BB	15	Optic glioma	19	4	8	1
AR	3½	Bobbing eyes	n.e.	n.e.	37	90
MG	?	?Phaeochromocytoma	95	18	49	6
MG	?	?Phaeochromocytoma	79	20	83	10
NF	20	?Phaeochromocytoma	n.e.	n.e.	72	17
HC	18	?Phaeochromocytoma	n.e.	n.e.	128	14
HC	18	?Phaeochromocytoma	n.e.	n.e.	116	22
HC	18	?Phaeochromocytoma	n.e.	n.e.	175	24
IW	?	?Phaeochromocytoma	Interference		107	19
IW	?	?Phaeochromocytoma	Interference		96	24

*n.e. = not estimated; n.d. = not detected.

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