Journal of Chromatography, 145 (1978) 11—27
Biomedical Applications

CHROMBIO. 075

RAPID PROCEDURE FOR CHROMATOGRAPHIC ISOLATION OF DOPA, DOPAC, EPINEPHRINE, NOREPINEPHRINE AND DOPAMINE FROM A SINGLE URINARY SAMPLE AT ENDOGENOUS LEVELS

Y. DALMAZ and L. PEYRIN

Laboratoire de Physiologie, UER Médicale Grange-Blanche, Université Claude Bernard Lyon I, 8 Avenue Rockefeller, 69373 Lyon Cedex 2 (France)

(First received December 9th, 1976; revised manuscript received March 17th, 1977)

SUMMARY

A three-step procedure has been investigated to extract 3,4-dihydroxyphenylalanine (DOPA), 3,4-dihydroxyphenylacetic acid (DOPAC), epinephrine (E), norepinephrine (NE) and dopamine (DA) from a single urinary sample with the object of obtaining extracts pure enough for specific fluorimetric assay. The procedure described in this paper results from the combination of urine purification on an aluminum oxide column, separation by ion-exchange chromatography of the DOPA—DOPAC fraction from catecholamines, and ether isolation of DOPAC from DOPA. The whole procedure is rapid and easily performed in one work day. Extraction recoveries were 72.4 ± 3.5%, 76 ± 2%, 85.7 ± 3.3%, 85.6 ± 1.4% and 92.4 ± 5.5% for DOPA, DOPAC, E, NE and DA respectively (n= 6).

The lowest amounts of the five catechols that could be detected in urinary samples by a combination of this extraction procedure and the methods of assay used in our laboratory were 15 ng for DOPA, 40 ng for NE, 20 ng for E, 152 ng for DA and 2.95 μg for DOPAC. Urinary volumes convenient for accurate estimation of each compound were 20 ml for healthy human subjects. For pathological or pharmacological purposes, 5 ml of human urine were sufficient.

The daily excretion of DOPA, DOPAC, E, NE and DA found by this procedure agrees with data obtained by other authors in healthy subjects. In pathological samples, our three-step procedure led to lower amounts than methods using alumina purification only. The discrepancies between the two methods are discussed in terms of development of internal standards, relative specificity of fluorimetric assays, values of blank eluates, and the possibility of interference from unknown abnormal body metabolites or pharmacological drugs not eliminated by a single-step alumina purification.

INTRODUCTION

A number of procedures have been previously reported for determination of catecholamines, epinephrine (E), norepinephrine (NE), dopamine (DA), (3,4-dihydroxyphenylalanine (DOPA) and 3,4-dihydroxyphenylacetic acid (DOPAC) in urine or tissues. Most of them enable isolation of one, two, three or four catechols [1—26].

None of these methods can be used for specific extraction and assay of DOPA, DOPAC, E, NE and DA from a single urinary sample. The only procedures suitable for this purpose are those of Sroka et al. [27], Sourkes et al. [28] and Routh et al. [29], but they lack sensitivity for accurate estimation of the five catechols and are suitable only for pathological studies.

Specific isolation of DOPA, DOPAC and catecholamines from one another is necessary because of the spectral similarities of these compounds and the small amounts to be detected at endogenous levels. Thus, DOPA interferes in the fluorimetric assay of DA [30] and NE [31] and in the colorimetric assay of DOPAC [32] ; on the other hand, DOPAC must be separated from catecholamines before its colorimetric determination [32].

In this paper, a procedure for isolating DOPA, DOPAC, E, NE and DA from a single urinary sample is described. Great care has been taken to obtain each compound in conditions convenient for its subsequent specific assay without any interference of DOPA or DOPAC on one another or on catecholamines. Furthermore, the final extracts may be used for flow diagram analysis. Our method involves initial purification of urine on aluminum oxide followed by the fractionation of DOPA, DOPAC and catecholamines on Amberlite CG-50 (NH_4^+). Complete separation of DOPA and DOPAC is achieved by diethyl ether extraction.

MATERIALS

The following materials were used:

Pyrex glass columns (1 cm diameter), with long-fibre glass wool (Corning, Corning, N.Y., U.S.A) for alumina adsorption, and a porous glass plate for Amberlite use, at the bottom of the stem; A pH meter Metrohm E 510; a rotating vertical mechanical shaker (home-made); and a Technicon AutoAnalyzer (for fluorimetric and colorimetric assays).

Reagents were: 0.2 and 0.5 *M* sodium acetate buffer containing 0.01% of disodium EDTA (buffer A and buffer B, pH 8.40); 0.2 *M* ammonium acetate buffer, pH 6.10 (buffer C); 0.4 *M* ammonium acetate buffer, pH 5.0 (buffer D). Ascorbic acid (0.3 and 2%) in water, 0.01 *M* citric acid, hydrochloric acid (R.P. $d = 1.19$), diethyl ether (peroxide free), 10% (w/v) EDTA (disodium salt) in water, 20% (w/v) EDTA (disodium salt) adjusted to pH 8.6 with 1 *M* sodium hydroxide, and 0.1 *M* Tris buffer (pH 5).

All chemicals were analytical grade and purchased from Prolabo(Rhône-Poulenc, France) or Merck, (Darmstadt, G.F.R.).

Aluminum oxide (activity II—III), standardized for Brockmann chromatographic adsorption, from Merck, and activated as previously described [33] .

Amberlite CG-50 (H^+; 200—400 mesh) from BDH (Poole, Great Britain) is used in NH_4^+ form as described by Hirs et al [34] and Kirshner and Goodall [22] . The pH of the resin was stabilized to 6.10 by five successive washes (30 min each) in buffer C. At the end of urinary analysis, Amberlite may be reused after numerous washings with buffer D and buffer C.

Standard solutions of DOPA (Fluka, Buchs, Switzerland), DOPAC (Fluka), DA hydrochloride (Fluka), L-(NE) bitartrate (Fluka), L-(E) (Sigma, St. Louis, Mo., U.S.A.); stock solutions of catechols (100 μg/ml) were prepared by dis-

solution in 50 ml of 0.01 *M* hydrochloric acid containing 5 mg of DOPA, 5 mg of DOPAC, 6.2 mg of DA, 9.95 mg of NE and 5 mg of E respectively; these solutions were stored at 4° and kept for no more than three weeks. For specificity studies, the following compounds were used: epinine hydrochloride (Regis, Morton Grove, Ill., U.S.A.), 3-O-methyldopamine (3-MT) hydrochloride (Sigma), 3,4-dihydroxymandelic acid (DOMA) (Sigma), 3,4-dihydroxybenzoic acid (DOBA) (Sigma), 3,4-dihydroxycinnamic acid (DOCI) (Merck), 3,4-dihydroxyphenylglycol (DHPG) (Regis), tyrosine hydrochloride (Sigma).

The following radioactive compounds were obtained from Commissariat Energie Atomique, CEA, Saclay, France: DOPA-3-^{14}C (specific activity 58 mCi/mmole), DOPA-2-^{14}C (25 mCi/mmole), DA-2-^{14}C (58 mCi/mmole), DOMA-2-^{14}C (47 mCi/mmole), DHPG-7-^{14}C (45 mCi/mmole), DL-NE-7-^{14}C (48 mCi/mmole), L-tyrosine-3-5-^{3}H (54 Ci/mmole), E-^{14}C (38.6 mCi/mmole).

METHODS

Urines were collected, stored and hydrolyzed as described elsewhere [33]. The successive steps of the method are explained in Fig. 1.

Alumina adsorption

Hydrolyzed human urine (25 ml) or rat urine (2 ml) were diluted to 50 or 20 ml, respectively, with distilled water; 1 ml of 10% EDTA and 0.5 ml of 0.3% ascorbic acid were added, and the pH was adjusted to 8.4 by successive use of 5 *M*, 2.5 *M*, 0.2 *M* sodium hydroxide. Activated aluminum oxide (2 g) was poured into the glass columns and washed just before use with 20 ml of buffer A. After the urinary sample had slowly percolated (10 drops per min) through the column, the alumina was washed with 10 ml of buffer B and 100 ml of 0.01% aqueous disodium EDTA. The elution of all catecholic compounds was performed at a very slow rate (5 drops per min) by 7 ml of 1 *M* hydrochloric acid.

Fractionation of alumina extract on Amberlite CG-50

Amberlite CG-50 columns (10 × 1 cm) were prepared in buffer C and washed with 20 ml of this buffer just before use. 100 µl of 20% EDTA and 100 µl of 2% ascorbic acid were added to the alumina eluate, whose pH was then brought to 6.10. The volume of the extract was made up to 10 ml with buffer C, centrifuged at 6000 *g* at 20° for 10 min and poured carefully on the top of the Amberlite column. As soon as it had been completely adsorbed, buffer D was poured on to the column as eluant. Five fractionated eluates were collected (Fig. 2): fractions A (containing urinary pigment) and C (following neutral and acidic catechols) were discarded; fraction B contained DOPA and DOPAC and fractions D1 and D2 contained E, NE and DA. Eluates D1 and D2 were used without further treatment for the specific assay of E, NE and DA.

Separation of DOPA from DOPAC

In eluate B, a further separation of DOPA and DOPAC was achieved by ether extraction: 2.5 ml of fraction B were brought to pH 2 and extracted for

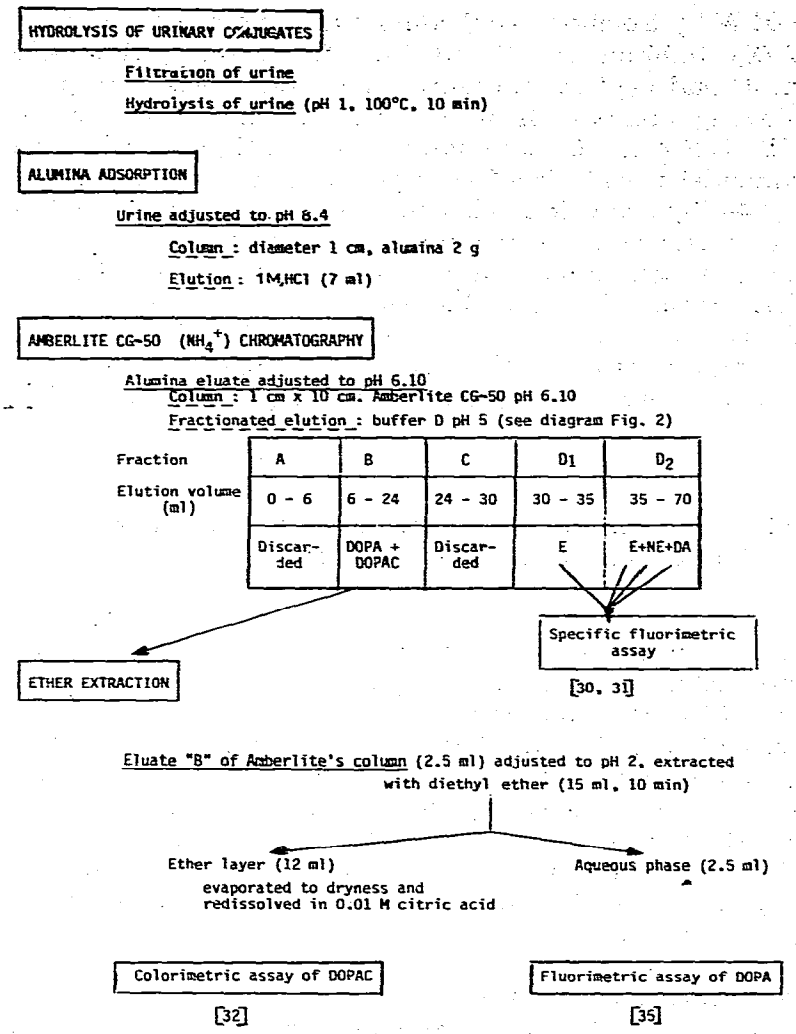

Fig. 1. Main steps of the general procedure used for the extraction of DOPA, DOPAC, E, NE and DA from a single urinary sample. For details, see text.

10 min with 6 volumes of diethyl ether (15 ml) on a rotating vertical shaker. 12 ml of the ether layer were evaporated to dryness at 40° under atmospheric pressure. The dry residue was dissolved by vigorous shaking (5 min) in 5 ml of 0.01 *M* citric acid. DOPAC was estimated in this "final DOPAC extract" and DOPA in the aqueous phase saved after ether treatment of fraction 3 ("final DOPA extract").

Assay of the various compounds

DOPAC. The DOPAC estimation was performed in the "final DOPAC extract" as described by Peyrin et al. [32] by using the automated colorimetric assay based on the formation of a red compound under effect of nitromolyb-

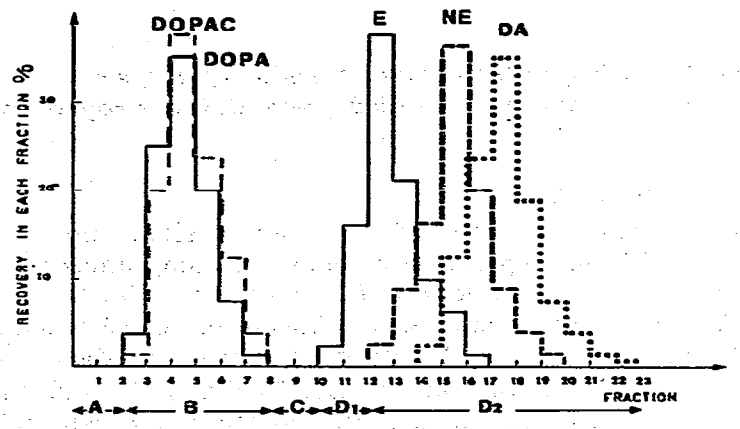

Fig. 2. Elution pattern of exogenous DOPA, DOPAC, E, NE and DA on an Amberlite CG-50 NH_4^+ column (10 × 1 cm) at pH 6.10. The solution applied was the acidic alumina eluate obtained from urine (20 ml). In this experiment stable DOPA (10 μg), E (10 μg), NE (10 μg), DA (10 μg) and radioactive ^{14}C DOPAC (0.25 μCi i.e. 1.6 μg) were added to the alumina eluate just before ion-exchange chromatography. The eluant was buffer D (pH 5.0). Fractions of 3 ml were collected.

dic reagent and sodium hydroxide on DOPAC. External standards of DOPAC (1 μg/ml) were prepared in 0.01 *M* citric acid.

DOPA. The acidic aqueous phase (2.5 ml) saved after DOPAC extraction was adjusted to pH 6 by the addition of an equal volume of 0.38 *M* ammonia. DOPA was then estimated as described by Cottet-Emard and Peyrin [35] using automated fluorimetric assay based on the formation of a 5,6-dihydroxyindole derivative after ferricyanide oxidation of DOPA. Internal standards of DOPA were prepared by mixing equal parts of "DOPA eluate" with standard DOPA solutions (100 ng/ml for human urines, 50 ng/ml for rat urines) in 0.2 *M* ammonium acetate of pH 6.

Epinephrine. E was estimated in fractions D1 and D2 as described by Peyrin and Cottet-Emard [31] using the automated fluorimetric assay of 3,5,6-trihydroxyindole derivative, resulting from specific ferricyanide oxidation of E. Internal standards of E were prepared by mixing equal parts of eluates D1 or D2 with standard E solutions (50 ng/ml for human urines, 10 ng/ml for rat urines) in 0.2 *M* ammonium acetate of pH 5.2.

Norepinephrine. NE was estimated in Amberlite fraction D2 by applying the automated specific fluorimetric assay of NE trihydroxyindole derivative [31]. Internal standards of NE were prepared by mixing equal parts of eluate D2 with standard NE solutions (200 ng/ml for human urines, 50 ng/ml for rat urines) in 0.2 *M* ammonium acetate of pH 5.2.

*Dopamine.*The determination of DA was made in Amberlite fraction D2 by fluorimetric assay of the 5,6-dihydroxyindole derivative resulting from iodine oxidation of DA. The procedure was the same as for 3-MT [36] except that 0.2 *M* ammonium acetate buffer (pH 5.2) was used for preparation of standards. Internal standards of DA were prepared by mixing equal parts of eluate D2 with standard DA solutions (100 ng/ml for human urines, 50 ng/ml for rat urines) in 0.2 *M* ammonium acetate of pH 5.2.

RESULTS

Stability of each compound to heating in the course of acidic hydrolysis

Table I shows that the five compounds studied were not destroyed by boiling at pH 1 for 10 min.

Pattern of elution from amberlite CG-50

The ion-exchange chromatographic analysis of the alumina eluate on Amberlite CG-50 (NH_4^+) led to efficient separation of catechol compounds into acidic or neutral (fraction B) and basic fractions (D1 and D2) (Fig. 2). 5—10% of E were present in fraction D1, the remainder being eluted together with NE and DA in fraction D2. Fraction D1 may be useful to control any displacement of the elution pattern; however, for routine use, fractions D1 and D2 were mixed for E assay. The methods used for E, NE and DA assay were sufficiently specific so that a more effective separation of these amines is not necessary.

After the column has been prepared, the resin has to be washed again with 20 ml of buffer C to avoid the release in the eluates of disturbing fluorescent materials from Amberlite CG-50 resin [7, 10, 12]. Fluorescence blanks from resin were then very low (Table IV).

Extraction recoveries for each compound

Satisfactory recoveries ranging from about 72 to 92% were obtained throughout the whole procedure for the five catechols (Table II).

Reproducibility of the procedure

Results obtained from a urinary sample were satisfactorily reproducible, whatever its catechol content (Table III).

Sensitivity of the method for each catechol

Taking into account, for each compound, extraction recoveries, elution or recuperation volumes and assay sensitivity, the least amounts of catechols which must be present in the sample to be adsorbed on alumina are 15 ng for DOPA, 40 ng for NE, 20 ng for E, 152 ng for DA and 2.95 μg for DOPAC. The smallest volume of urine to be extracted is calculated from the sensitivity

TABLE I

STABILITY OF CATECHOLS TO THE ACIDIC HYDROLYSIS

Exogenous ^{14}C compound added to urinary sample (0.25 μCi)	Recovery from aluminum oxide	
	Compound boiled with urine 10 min at 100° (%)	Compound added at the end of acidic hydrolysis of urine (%)
DOPA ^{14}C	86	83
DOPAC ^{14}C	78.8	79
E ^{14}C	92	90
NE ^{14}C	90	93
DA ^{14}C	92.5	94

TABLE II

EXTRACTION RECOVERIES FOR EACH CATECHOL AFTER ALUMINA ADSORPTION, ION-EXCHANGE CHROMATOGRAPHY ON AMBERLITE CG-50 OR ETHER EXTRACTION

Exogenous ^{14}C compound (0.2 μCi)	Extraction recoveries after			Whole extraction recovery (%)
	Alumina adsorption* (%) (n = 6)	Amberlite CG-50** chromatography (%) (n = 6)	Ether extraction*** (%) (n = 6)	
DOPA	94 ± 6	80 ± 2	0 ± 0.9	72.4 ± 3.5
DOPAC	86.4 ± 4	90 ± 2	98 ± 1.9	76 ± 2
E	91 ± 1.8	96 ± 2		85.7 ± 3.3
NE	95 ± 1	93 ± 2		85.6 ± 1.4
DA	94 ± 2	98 ± 3		92.4 ± 5.5

*Urinary samples were added with a single radioactive compound and adsorbed on alumina as described in Methods. Recoveries were calculated on the 1 *M* HCl eluate (7 ml).
**Alumina eluates free of radioactive compound were added with one radioactive catechol and chromatographed on Amberlite CG-50 as described in Methods. Recoveries were calculated in the corresponding Amberlite eluates.
***Amberlite eluates free of radioactive traces were added with radioactive DOPA or DOPAC just before ether extraction as described in Methods. Recoveries were calculated in the aqueous and ether layers.

TABLE III

REPRODUCIBILITY OF THE PROCEDURE EXPRESSED AS VALUES (MEAN ± S.E.) OF TOTAL URINARY DOPA, DOPAC, E, NE AND DA OBTAINED FROM THREE DETERMINATIONS ON THE SAME URINARY SAMPLE

Urinary sample	Volume of urine extracted	Concentration (μg/l)				
		DOPA	DOPAC	E	NE	DA
Adults	25 ml	31±3.2	1547±74	18.2±1.1	138±9.5	701±8.4
Children	25 ml	44.3±2.6	1290±65	3.7±0.34	29.8±3.4	1016±8.3
Children with neuroblastoma	5 ml	93.3±12.9	1643±3.3	16.5±1.67	96.5±6.3	1788±73.5
Mean standard error %		9.9±2.3	3.9±0.96	8.4±1.2	8.2±1.6	2.0±1

procedure. Accordingly, urinary volumes of 25 ml for healthy human subjects or 2 ml for human patients are recommended.

Specificity of the extraction

Related compounds that interfere in the colorimetric or fluorimetric assay were tested as to their extraction properties, recoveries and occurrence in the final eluates (Fig. 3). Over-all interference is summarized as follows.

Monophenols (octopamine, tyramine), acidic or alcoholic phenols (homovanillic acid, vanillomandelic acid, 3-methoxy-4-hydroxyphenylglycol and methoxylated amines (3-MT, normetanephrine, metanephrine) are removed in the alumina step. Thus, the interference of 3-MT on DA iodine assay is completely eliminated.

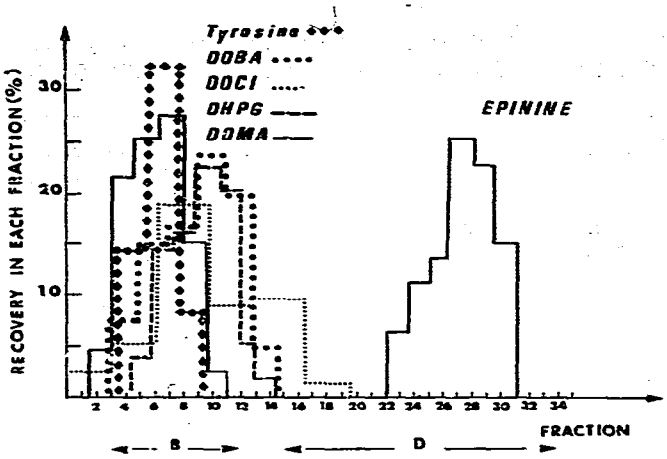

Fig. 3. Elution pattern of other related compounds on an Amberlite CG-50 NH_4^+ column (10 × 1 cm) at pH 6.10.
Compounds were dissolved in 0.9% NaCl (7 ml), mixed with 20% EDTA (pH 8.60) (100 µl) and 2% ascorbic acid (50 µl), adjusted to pH 6.10 and brought up to 10 ml before ion-exchange chromatography. The eluant was buffer D (pH 5.0). Fractions of 2 ml were collected. The positions of the fractions usually collected are noted under the figure.
The whole interference (per cent) of related compounds in the determination of DOPA, DOPAC, E, NE and DA is calculated on the basis of three parameters: final recoveries after alumina, Amberlite and diethyl ether extractions; position in the elution pattern of Amberlite CG-50 (i.e. occurrence in the same eluate as the studied catechols).
Interference in the fluorimetric assay of DOPA [35], E, NE [31], DA [30] and colorimetric assay of DOPAC [32]. Each compound was dissolved in 25 ml of 0.9% NaCl. Alumina adsorption, Amberlite CG-50 chromatography and ether extraction are described in Methods. Radioactive ^{14}C DHPG was used for recoveries.

Compound	Relative interference in assay procedure of		Extraction recoveries			Final recoveries in fractions of		Whole interference on	
(a)	DOPA	DOPAC	Alumina	Amberlite	Ether	DOPA	DOPAC	DOPA	DOPAC
DOMA	0	15.7	76	96.7	27	53.4	20	0	3
DOBA	0.11	83	— 88	—	39	38.7	25	0.04	20
DOCI	1.2	41	— 64	—	54	20	22	0.24	9
DHPG	1	78	70	85.6	12	49	6.7	0.49	5

(b)	E	NE	DA				E	NE	DA
Epinine	0	3.8	0.5	62	96	—	59.5	0	2.2	0.30

Most catechols are extracted by the double-step procedure (alumina, Amberlite); their location in the elution pattern of Amberlite CG-50 is shown in Fig. 3. Epinine was extracted in the same fraction as catecholamines (E, NE, DA): its final interference was 2.2, 0.3 and 0% on NE, DA and E assays respectively. The catechol compounds DOBA, DOMA, DOCI, DHPG were eluted together with DOPA and DOPAC; their final interference was respectively 21, 3, 9 and 5% on DOPAC determination, and 0.04, 0, 0.24 and 0.49% on DOPA assay. These interferences are acceptable for our purpose. Epinine, DOBA and DOCI have never been found in urinary samples [32];

the amounts of DOMA present in urine are low compared with DOPAC concentrations and their interference may be neglected. The possible interference of tyrosine has been investigated in more detail because the high concentration of this monophenol in urine (the range varied for total tyrosine from 41–111 mg per day) [37, 38] and because of the possibility of hydroxylation of tyrosine to DOPA during the alumina step as suggested by Lindqvist et al. [39].

For this purpose, tritiated tyrosine (1 μCi in 25 ml of buffer A) was first purified from its radioactive DOPA content by alumina adsorption. Tyrosine present in the alumina filtrate was used immediately. Only 0.13% of the initial tyrosine was present in the DOPA fraction from Amberlite (Fig. 3). When the interference of tyrosine on the DOPA assay (0.02%) is taken into account, the whole interference of tyrosine is of 0.000024%. No detectable interference on DOPA assay may be expected from the great amounts of tyrosine normally present in urine. Furthermore, no added fluorescence in DOPA determination was found when purified tyrosine (20 mg/l) was added to urinary samples before extraction. However, the final interference of tyrosine may be increased to 0.05% when the alumina columns, after urine percolation, are left to stand overnight before being washed. This effect may be due to the hypothetical transformation of tyrosine into DOPA, previously suggested by Lindqvist et al. [39]. A consequence of these observations is that the alumina step must be completed in reasonable time (no longer than 4 h).

Biological applications

Our final method enabled us to estimate DOPA, DOPAC, E, NE and DA in urinary samples from healthy human subjects or from patients with diseases correlated with adrenergic dysfunction and in urine of laboratory animals. Daily excretion in 34 healthy adults was 2.85±0.3 mg for DOPAC, 385±84 μg for DA, 89±11 μg for NE, 81±8 μg for DOPA and 19±3 μg for E. except for NE and E, only few data on total daily excretion from men at physiological levels are available in the literature. Our E, NE, DA and DOPAC values agree with data published earlier by other authors for hydrolyzed urines [14, 15, 40, 41]. No comparison was possible for DOPA results because all the values reported up to now in the literature came from analyses of unhydrolyzed urines [26, 42, 43].

Comparison of the data obtained by our procedure or by the use of alumina purification only will be discussed below.

The method allows separate estimation of the five compounds even in urines of patients with excessive amounts of DOPA and catecholamines, i.e. children with sympathetic tumors, adult subjects with chromaffin tumors or parkinsonian patients under treatment with L-DOPA. Even in these cases, no overlap was observed of DOPA on DOPAC nor from neither of them on catecholamines.

DISCUSSION

Choice of extraction procedure

Whatever the method used to extract and separate the urinary catechols

(neutral, acidic or aminated) specific isolation of DOPA and DOPAC is necessary because of the interference of DOPAC on DOPA, E, NE and DA [32] and of DOPA on the three catecholamines [35]. On the other hand, rigorous separation is not necessary for E, NE, or DA since the last does not interfere in E and NE assay and specific assay of E and NE may be performed [31].

It is now well known that all catechols may be adsorbed on alumina and eluted together by an acidic agent [5, 8, 9, 15–17, 26–28]. To estimate the relative amounts of DOPA, DOPAC, E, NE and DA, an alternative method consists in applying specific assay procedures of each compound to the mixed eluate [9, 17]. However, the spectral properties of these catechols are too similar for complete elimination of interference from one another. Accordingly, the specific estimation of these five compounds may be expected only after their true separation has been achieved by a convenient isolation procedure.

Separation of catechols contained in the alumina eluate has been achieved by paper or thin-layer chromatography [15, 20]. Some authors have also suggested the separation of catecholamines (E, NE, DA) from DOPA or DOPAC by a graduated acidic elution of catechols adsorbed on alumina [19, 24]. Cationic exchange resins have been used to elute the three catecholamines together [6, 14, 29] or to separate them partially [1, 7, 9, 13, 18, 23] or completely from one another [12, 22, 24, 44, 45].

The problem of specific isolation of DOPAC from DOPA has not been satisfactorily resolved by earlier methods based on the use of organic solvents, coupled with chromatographic analysis [2, 3, 41, 46–48].

Most of the published methods either lack specificity [2] or are not convenient for catechol determinations at physiological levels.

The aim of our work has been not only to extract the above five compounds from a single sample, but also to attain: rigorous isolation so as to overcome spectral interference inherent in assay procedures; reliable recoveries for each compound; and short experimental duration for the method to be used in serial analysis.

The method described in this paper results from a combination of the most convenient steps of previously published procedures for either amine [5, 22, 24]. The method is based on the association of adsorption of catechols on alumina, separation of acidic (DOPAC) or neutral (DOPA) catechol compounds from catecholamines on Amberlite CG-50 at pH 6.10 and selective extraction of DOPAC with ether. Several steps have been studied to optimize isolation and recoveries.

Critical study of alumina adsorption

Adsorption pH as a function of the nature of the saline content. pH values lower than 8.5 have been used to adsorb pure catechol compounds on alumina [19, 24]. To investigate the effect of adsorption pH and nature of the saline content on the alumina procedure, NE and DOPA solutions were prepared either in 0.2 *M* sodium acetate or in 0.2 *M* sodium phosphate and adsorbed on alumina columns (as described above) at two different pH values 6.10 and 8.40). When adsorption was carried out at pH 8.40 recoveries were good for both DOPA and NE and higher when 0.2 *M* sodium acetate (96 and 95% respectively) instead of 0.2 *M* sodium phosphate (85% for DOPA, 89% for NE) was

used as a diluent to dissolve these compounds before alumina adsorption. At pH 6.10, NE and DOPA in phosphate buffer were not retained on alumina; when catechols were in acetate buffer at pH 6.10, DOPA was strongly fixed (recovery 93%), whilst NE was found in part in the water washes applied just before acidic elution (recovery in alumina eluate 42%). Owing to the presence of great amounts of phosphate ions in most urinary samples, it seems necessary to perform alumina adsorption at pH 8.40.

Molarity of the washing solutions. To ensure the highest recoveries for NE, it was important to use washing solutions (sodium acetate) with molarities greater than that of the initial mixture placed on the column. On the other hand, the molarity of the washings did not modify DOPA recoveries. The same conclusions were obtained by Drell [19].

Attempts to separate catecholamines, DOPA and DOPAC by a double-step elution of alumina. Fractionated elution of catechols retained on alumina has been applied by some authors [5, 19, 24, 41]. However, none of these procedures was completely satisfactory. We therefore studied the eluting properties of orthophosphoric acid, hydrochloric acid, acetic acid and ammonium acetate buffer at different molarities. All the eluents studied were able to elute catecholamines but recoveries greater than 75% were obtained only for eluants with pH values at or below 4 and molarities of 0.05 *M* or more.

On the other hand, ammonium acetate (pH 4) with molarity greater than 0.05 *M* did not elute DOPAC, but did elute 32% of the DOPA. The complete recovery of DOPA and DOPAC needed the use of acids with strong molarities, which, moreover, also eluted catecholamines. An alternative method of separating the two groups of catechols may consist in the use of a calculated volume of 0.05 *M* ammonium acetate (pH 4) to elute catecholamines, followed by a strong acid (0.2 *M* phosphoric acid) to achieve complete elution of DOPA and DOPAC. In these conditions, a satisfactory separation of pure E, NE and DA

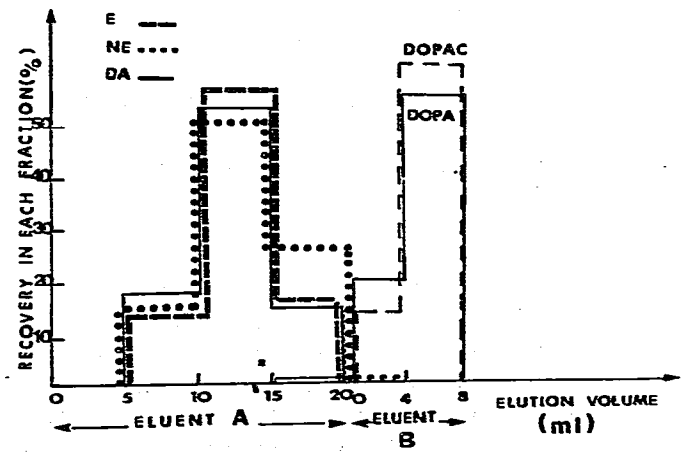

Fig. 4. Separation of catecholamines from DOPA and DOPAC by a single alumina adsorption. Solutions applied were catechols diluted in 0.2 *M* sodium acetate buffer (pH 8.40) and added with 10% EDTA (1 ml) and 0.3% ascorbic acid (0.5 ml). Alumina adsorption was performed as described in Methods but eluant A and B were used instead of 1 *N* HCl. Eluant A: 0.05 *M* ammonium acetate buffer (pH 4.0) (20 ml). Eluant B: 0.2 *M* phosphoric acid (8 ml).

from DOPA and DOPAC was obtained (Fig. 4); however, results from urinary samples were not reproducible and a variable overlap (2—30%) of DOPA and DOPAC was observed on catecholamine fractions. By the use of a similar procedure, Drell [19] reported an overlap of 10% between aminated and acidic catechols. For these reasons, the double-step alumina elution was not further used.

Critical study of Amberlite CG-50 procedure

pH and ionic form of the resin. The pH value of 6.10 previously used by other authors [6, 22, 24] has been found convenient for separation of acidic or neutral catechols from catecholamines. Higher recoveries were obtained when the resin was used in the NH_4^+ form, suggested by Kirshner and Goodall [22] instead of the Na^+ form used by Fleming et al. [6] and Lishajko [13].

Volume of the extract to be deposited and height of the column. Changing the extract volume (from 7 to 10 ml) — but neither the amount of catechol nor the column height (7.5 cm) — delayed the elution of neutral and acidic catechols and increased the eluting volumes of the catecholamine fractions. A column height of 10 cm improved the separation — with a parallel increase of elution volumes by only 20%.

Effect of the urinary saline content. Because of the disturbing effect of salts previously observed on the elution pattern from Amberlite CG-50 [30] the position of peaks and recoveries for each compound were studied on four urinary samples with different initial saline contents (creatinine from 160 to 1500 mg/l). All of them resulted in high recoveries of added catechols and in stability of the fractionation pattern.

Effect of catechol concentration in the alumina eluate. The amounts of catechols to be passed through Amberlite may be fivefold to tenfold the normal human daily excretion without great modifications in recoveries, except for DA whose recovery fell to 80% at very high concentrations (20 mg/l). The eluting pattern was constant over a wide range of concentrations for either compound; however 5% overlap was seen between fractions B and D, when 50 μg of DOPA, E, NE and DA (2 mg/l) or 500 μg of DOPAC (20 mg/l) had been deposited on the column. Such amounts may be found only in highly pathological samples, or in urines from L-DOPA-treated patients. In such cases, it would be advisable to use small volumes of urinary samples (2—5 ml) instead of the 25 ml suggested for normal subjects.

Critical study of the ether separation of DOPAC and DOPA

Diethyl ether [5] or ethyl acetate [2, 3, 9, 41, 46, 47] have been used to extract DOPAC from biological samples. Although similar recoveries were found with both of these solvents, we have preferred diethyl ether for routine use because of easy evaporation at low temperature to avoid oxidative destruction of DOPAC. The prior purification of diethyl ether on alumina, suggested by Spano and Neff [5], has not improved DOPAC recoveries.

Comparative studies with eluates at pH 1, 2 or 3 treated by ether in amounts from 2 to 6 volumes, showed that recoveries were improved from 67 to 98% when an eluate at pH 2 was extracted by 6 volumes of ether. The best recoveries were obtained after evaporation of the ether layer and dissolution of the

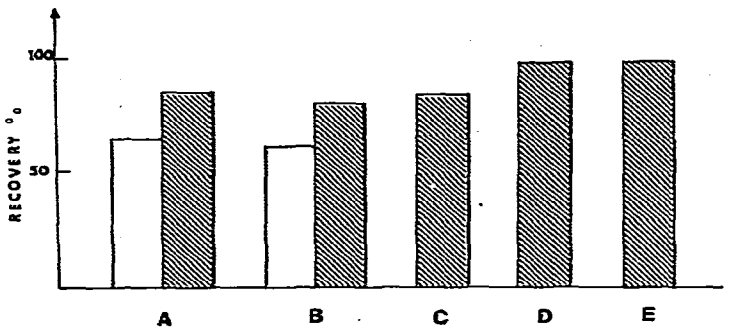

Fig. 5. Transfer of DOPAC from ether to aqueous layer under various buffers.
^{14}C DOPAC was diluted in 2.5 ml of 0.4 *M* ammonium acetate at pH 5.0, adjusted to pH 2.0 and dissolved in 6 volumes of diethyl ether. After shaking two methods were used: open areas, direct transfer of the ether layer in two volumes of buffer; hatched areas, evaporation of the ether layer and redissolution of the dry residue in 5 ml of buffer A, B, C, D, E. A: 0.1 *M* tris (pH 8.0), B: 0.25 *M* disodium phosphate (pH 7.0), C: 0.4 *M* ammonium acetate (pH 5.70), D: 0.2 *M* ammonium acetate (pH 5.70), E: 0.01 *M* citric acid.)

dry residue in 0.01 *M* citric acid (5 ml) (Fig. 5). Furthermore, this diluent is the most convenient for development of color from DOPAC [32].

Purity of final eluates

The extracts obtained by our procedure are clear and suitable for fluorimetric assay. This was demonstrated by comparing the absorbance at 310 nm, of alumina eluates or Amberlite fractions at 0.44 ± 0.07% (range 0.2 to 0.8) and 0.06 ± 0.01% (range 0 to 0.09) respectively. The high degree of purification of Amberlite extracts was further proved by the low blank values obtained in the fluorimetric assay of each compound (Table IV).

The final DOPAC extract obtained by diethyl ether treatment of Amberlite eluates was completely free from pigments (absorbance at 310 nm = 0.03%) and much clearer than if directly obtained from an alumina eluate as suggested by other authors [5, 9, 40, 41].

TABLE IV

FLUORESCENCE COMING FROM THE ALUMINUM OXIDE AND AMBERLITE CG-50 RESIN

Blank values are expressed as ng of each compound in the whole fraction collected. 20 ml of 0.2 *M* sodium acetate buffer (pH 8.40) were adsorbed on alumina as described in Methods and eluted either by 0.25 *M* acetic acid (10 ml) or by 1 *M* HCl (7 ml). The HCl eluate was chromatographed on Amberlite CG-50, and fractions B and D were collected as described in Methods.

Extraxt	DOPA	E	NE	DA
Alumina eluate 0.25 *M* acetic acid	70	10	0	200
Amberlite CG-50 fractions 0.4 *M* ammonium acetate (pH 5)	10	0	0	10

TABLE V

COMPARISON OF THE ALUMINA EXTRACTION PROCEDURE ALONE AND OF THE DOUBLE–STEP METHOD

Two aliquots of the same urine were extracted simultaneously on alumina only (eluted with 10 ml of 0.25 *M* acetic acid) and alumina + Amberlite CG-50 as described in Methods. Fluorimetric assays were performed in the alumina eluate and Amberlite CG-50 fractions. Results are uncorrected for extraction losses.

	Alumina procedure			Double steps alumina and amberlite CG-50			Ratio alumina/amberlite		
	DA	NE	E	DA	NE	E	DA	NE	E
Healthy subjects μg/24 h	328±56 (*n* = 18)	90±21 (*n*=25)	20±4 (*n*=25)	385±84 (*n* = 18)	89.1±10.8 (*n* = 25)	19.2±0.3 (*n* = 25)	0.85	1.01	1.04
Recovery of added amines (%)* (*n* = 3)	89.3 ±8	80±8.4	89.3±0.6	95.5±1.4	82.2±2	91.6±8			
Patients									
Hypertensive μg/24 h (*n*= 3)	5348±1980	481±33.6	123.2±17.2	1642±834	155.6±82.5	39.3±10.1	3.26	3.09	3.13
Pheochromocytoma μg/24 h (*n* = 1)	18116	4480	224	7644	4340	117.9	2.37	1.03	1.90
Development of internal standard (% of external standard) (*n* = 4)	110±3.5	78±4.5	95±0.7	96.3±3.7	113.7±4.9	103.2±0.3			
Relative interference (%) of each amine in pure solutions									
a/E	1	0	100	0.1	0	100			
b/NE	2	100	3.4	0.7	100	2.2			
c/DA	100	2.9	0	100	2.1	0			

*No differences in recoveries were observed between urinary samples from healthy or ill subjects.

Final recoveries

The final recoveries were satisfactory for the five compounds studied (Table II). Our recoveries obtained for E, NE and DA were in the same range for E and NE as in refs. 9, 12, 24 and for DA as in refs. 9, 12, 19 and 24 or higher for E and NE [15] and for DA [16]. Recoveries of DOPA and DOPAC agreed for DOPA [1, 9] and for DOPAC [5, 48] or were better for DOPA [26] and for DOPAC [5, 48] than those reported by other authors. However, slightly higher recoveries for DOPA were mentioned by some authors [19, 29].

Biological applications

DA, NE and E values obtained by applying our final procedure to urinary samples of healthy subjects correlated satisfactorily with results obtained by the use of alumina extraction alone [31] (Table V).

By contrast, in the same kind of comparison on pathological samples from severe hypertensive patients (n = 3) and from one subject with pheochromocytoma, higher amounts of the three amines were found when alumina purification only was used (Table V).

The discrepancies between the results obtained with the two methods might be explained by differences in the development of internal standard fluorescence, or in the specificity of the amine assay in the two elution buffers (0.25 M acetic acid for alumina and 0.4 M ammonium acetate for the Amberlite procedure). Table V shows that the differences in NE values resulted primarily from higher inhibition of the NE internal standard in alumina extracts. For DA, fluorescence factors (internal standard and assay specificity) accounted for differences averaging 20% between the two methods, but were not sufficient to explain the great variations observed in urinary samples. The fluorescence data are inadequate to account for 40% differences in E amounts obtained after the two methods were applied to pathological samples.

Since the greatest discrepancies between the results were observed in pathological samples, it may be reasonably assumed that interferences arise either from therapeuric drugs or from abnormal metabolites not eliminated by a single-step alumina procedure. The formation of catechols as intermediate metabolites of N-alkyl amphetamines has been recently suggested by Coutts et al. [49]. The possibility of such interference demonstrates the need for high purification. Our final procedure supplies this need.

General comments

No previous published procedure enables one to extract DOPA, DOPAC, E, NE and DA from a single urinary sample, and to obtain these five compounds with a high degree of purification allowing their specific assay.

The extraction procedure described in this paper consists in three steps: (i) purification of hydrolyzed urines on alumina at pH 8.40; (ii) separation of catecholamines from catecholacides on Amberlite CG-50; and (iii) ether separation of DOPA and DOPAC.

The step sequence is well adapted to urine analysis for two reasons. The aluminum oxide purification eliminates from urine most salts, proteins and pigments that would disturb the subsequent ion-exchange separation of the five compounds and their fluorimetric assay. The Amberlite step leads to high-

ly effective separation of DOPA plus DOPAC and catecholamines. Furthermore, the three steps are necessary to ensure a high extraction specificity for the five compounds. DOPAC is extracted by ether and separated from DOPA which is completely retained in the aqueous phase. This step eliminates the high interference of DOPA (44%) on the colorimetric assay of DOPAC. DOPA, which interferes in the E, NE and DA assay, is always separated from these amines without any overlap. E, NE and DA are removed from Amberlite in the same fraction but the low interference of NE (0.7%) and E (0.1%) on the DA fluorimetric assay [30] and the specificity of E and NE assay [31] do not require a better separation between the three catecholamines. The interference of related compounds is either completely eliminated or greatly reduced by the whole procedure. This point has not been especially studied by most other authors.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Institute of Medical Research (INSERM), France (ATP 71, No. 1.408); from the Centre National de la Recherche Scientifique (E.R. No. 96, "Catecholamines"); from the Foundation of Industry for Pharmaceutical Research, France; from the Department of Biology of CEA (Saclay, France); and from the UER of Human Biology, University Claude Bernard (Lyon, France). We are indebted to Dr. Pichat from CEA (Saclay) for preparing the required labeled compounds studied in this paper, and to Mrs. Jacqueline Pequignot, CNRS, technician, for her technical assistance.

REFERENCES

1 W. Kehr, A. Carlsson and M. Lindqvist, N. Schmied. Archiv. Pharmacol. 274 (1972) 273.
2 D.B. Calne, F. Karoum, C.R.J. Ruthven and M. Sandler, Brit. J. Pharmacol., 37 (1969) 57.
3 A. Vidi and G. Bonardi, Clin. Chim. Acta, 38 (1972) 463.
4 T.A. Hare, B.L. Beasley, S.M. de Simone and W.H. Vogel, Biochem. Med., 11 (1974) 305.
5 P.F. Spano and N.H. Neff, Anal. Biochem., 42 (1971) 113.
6 R.M. Fleming, W.G. Clark, E.D. Fenster and J.C. Towne, Anal. Chem., 37 (1965) 692.
7 F.N. Minard and D.S. Grant, Biochem. Med., 6 (1972) 46.
8 J. Glowinski, L.L. Iversen and J. Axelrod, J. Pharmacol. Exp. Ther., 151 (1966) 385.
9 J.A. Romero, J.P. Chalmers, K. Cottman, L.D. Lytle and R.J. Wurtman, J. Pharmacol. Exp. Ther., 180 (1972) 277.
10 C.V. Atack, Brit. J. Pharmacol., 48 (1973) 499.
11 C.V. Atack and T. Magnusson, J. Pharm. Pharmacol., 22 (1970) 625.
12 J. Haggendal, Scand. J. Clin. Lab. Invest., 14 (1962) 597.
13 F. Lishajko, Acta Physiol. Scand., 72 (1968) 255.
14 G.L. Mattok, D.L. Wilson and R.A. Heacock, Clin. Chim. Acta, 14 (1966) 99.
15 S. Takahashi and L.R. Gjessing, Clin. Chim. Acta, 36 (1972) 369.
16 H. Weil Malherbe, Methods Biochem. Anal., 16 (1970) 294.
17 A.H. Anton and D.F. Sayre, J. Pharmacol., 145 (1964) 326.
18 A. Bertler and E. Rosengren, Acta Physiol. Scand., 47 (1959) 350.
19 W. Drell, Anal. Biochem., 34 (1970) 142.

20 U.S. von Euler and F. Lishajko, Acta Physiol. Pharmacol. Neerl., 8 (1957) 295.
21 H. Hinterberger and C.J. Andrews, Arch. Neurobiol., 26 (1972) 245.
22 N. Kirshner and Mc C. Goodall, J. Biol. Chem., 226 (1957) 207.
23 R. Laverty and D.F. Sharman, Brit. J. Pharmacol., 24 (1965) 538.
24 D.T. Masuoka, W. Drell, H.F. Schott, A.F. Alcaraz and E.C. James, Anal Biochem., 5 (1963) 426.
25 T.L. Sourkes and G.F. Murphy, Methods Med. Res., 9 (1961) 147.
26 K. Türler and H. Käser, Clin. Chim. Acta, 32 (1971) 41.
27 H. Sroka, F. Eichhorn, A. Rutenberg, H. Radwan and B. Bornstein, J. Neurol. Sci., 17 (1972) 61.
28 T.L. Sourkes, R.L. Denton, G.F. Murphy, B. Chavez and S. Saint-Cyr, Pediatrics, 31 (1963) 660.
29 J.I. Routh, R.E. Bannow, R.W. Fincham and J.L. Stoll, Clin. Chem., 17 (1971) 867.
30 Y. Dalmaz, Thesis, Univ. Claude Bernard, Lyon 1972.
31 L. Peyrin and J.M. Cottet-Emard, Anal. Biochem., 56 (1973) 515.
32 L. Peyrin, J.M. Cottet-Emard and B. Claustre, in press.
33 L. Peyrin, Rev. Fr. Et. Clin. Biol., 8 (1964) 1096.
34 C.H.W. Hirs, S. Moore and W.H. Stein, J. Biol. Chem., 200 (1953) 493.
35 J.M. Cottet-Emard and L. Peyrin, J. Neural Transm., 41 (1977).
36 Y. Dalmaz and L. Peyrin, J. Chromatogr., 116 (1976) 379.
37 W.H. Stein, J. Biol. Chem., 201 (1953) 45.
38 H.H. Tallan, S.T. Bella, W.H. Stein and S. Moore, J. Biol. Chem., 217 (1955) 703.
39 M. Lindqvist, W. Kehr and A. Carlsson, J. Neural Transm., 36 (1975) 161.
40 E.R.B. Smith and H. Weil Malherbe, Clin. Chim. Acta, 35 (1971) 505.
41 H. Weil Malherbe and J.M. van Buren, J. Lab. Clin. Med., 74 (1969) 305.
42 A.H. Anton, M. Greer, D.F. Sayre and C.M. Williams, Amer. J. Med., 42 (1967) 469.
43 W. von Studnitz, H. Käser and A. Sjoerdsma, New Engl. J. Med., 269 (1963) 232.
44 Mc C. Goodall and H. Alton, J. Clin. Invest., 48 (1969) 2300.
45 Mc C. Goodall and H. Alton, Biochem. Pharmacol., 21 (1972) 240.
46 B.D. Drujan, N. Alvarez and J. Dias-Borges, Anal. Biochem., 15 (1966) 8.
47 E. Rosengren, Acta Physiol. Scand., 49 (1960) 370.
48 N.E. Arden, B.E. Roos and B. Werdinius, Life Sci., 5 (1963) 319.
49 R.T. Coutts, G.W. Dawson, C.W. Kazakoff and J.Y. Wong, Drug Metab. Disp., 4 (1976) 256.