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SPECIFIC ION-EXCHANGE CHROMATOGRAPHY AND FLUORIMETRIC ASSAY FOR URINARY 3-O-METHYLDOPAMINE

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SUMMARY

A technique for the selective extraction of 3-O-methyldopamine, normetanephrine and metanephrine from a single urine sample has been investigated. After hydrolysis of the conjugates, the diluted mixture is passed through a Dowex 50W-X2 column and the methoxylated amines are eluted by means of concentrated ammonia. The eluate, containing metanephrine, normetanephrine and 3-O-methyldopamine is evaporated, and a solution of the residue in borate buffer is fractionated under strictly controlled conditions on an Amberlite CG-50 column. The three amines so separated are estimated by specific fluorimetric methods. The extraction recovery is $80 \pm 3\%$ for pure solutions and $78 \pm 4\%$ for 3-O-methyldopamine added to urine. The fluorimetric procedure, carried out under well-defined conditions, allows the estimation of 10 ng of 3-O-methyldopamine in the sample, the fluorescence intensity being linear for up to 4 μg of 3-O-methyldopamine. The spectral characteristics of the fluorescent derivative are similar to those obtained with dopamine, so that it can be assumed that iodine oxidation of 3-O-methyldopamine demethylates this compound and oxidises the resulting dopamine to the dopamine fluorophore (5,6-dihydroxyindole). Of the compounds that might interfere in the fluorimetric procedure, dopamine, DOPA and α-methyl-DOPA are destroyed by the ammoniacal elution from the Dowex column and 3-O-methyl-DOPA is eliminated in the effluent from the Amberlite column. The elimination of interfering compounds and the improved separation on Amberlite ensure high specificity for this procedure. We have applied the method to normal urine and to pathological urines from patients with adrenergic tumours or untreated and treated parkinsonian subjects; vital information has been obtained on the prognosis of adrenergic tumours. The presence of large amounts of dopamine, normetanephrine and/or metanephrine does not affect the assay for 3-O-methyldopamine. The method is also applicable to rat and dog urine, and can be applied to tissue extracts with little modification.

INTRODUCTION

3-O-Methyldopamine (or 3-methoxytyramine, 3-MT) an intermediary metab-

TABLE I

MAIN PROCEDURES FOR ISOLATION OF 3-MT

Abbreviations: E = epinephrine; NE = norepinephrine; NMN = normetanephrine; MN = metanephrine; 3-MT = 3-O-methyldopamine; DM = dopamine; GLC = gas–liquid chromatography; FID = flame ionization detector; ECD = electron-capture detector.

Reference	*Sample*	*Extraction*	*Separation*	*Recovery of 3-MT (%)*
Kakimoto and Armstrong[12]	Non-hydrolysed urine	Dowex 50W-X2(H^+). Alkaline elution (1 *M* NH_4OH in ethanol)	Two-dimensional paper chromatography	
Häggendal[10]	Deproteinised brain	Amberlite CG-120(H^+) (pH 6). Continuous elution* (1 *M* HCl)	Fractionated elution; (6 fractions). Separation of E, NE, NMN, NM, DM, 3-MT	
Carlsson and Lindqvist[14]	Deproteinised brain	Dowex 50W-X4 (Na^+) (pH 6.5). Alkaline discontinuous elution** (0.1 *M* carbonate of pH 10, then 0.1 *M* NH_4OH)	Fractionated elution; (2 fractions). Separation of NM and 3-MT	
Masuoka *et al.*[20]	Tissues	Alumina (pH 6.1); Effluent (pH 6.1)	Amberlite CG-50 (pH 6.5). Continuous fractionated elution pH 5; (3 fractions). Separation of MN, NMN, 3-MT	96
Carlsson and Waldeck[4]	Deproteinised brain	Dowex 50W-X8 (H^+) (pH 6.5). Acid discontinuous elution (1 *M* then 2 *M* HCl)	Fractionated elution; (2 fractions). Separation of (E + NE + DM) and 3-MT	
Rutledge and Jonason[15]	Brain	Alumina (pH 6.5); effluent	Dowex 50W-X4 (Na^+) pH 6.5. Acid-fractionated discontinuous elution (*M* then 2 *M* HCl) (2 fractions). Separation of NMN and 3-MT	52.7
Rutledge and Weiner[16]	Heart	Dowex 50W-X8 (Na^+) (pH 6.5). Acid discontinuous elution (1 *M* then 2.5 *M* HCl)	Fractionated elution; (2 fractions). Separation of (NE + MN) and (DM + 3-MT); DM and 3-MT are not sepated.	
Greer *et al.*[3]	Urine of tumour subjects	Ether	GLC–FID on 12% EGA column at 200°, trifluoroacetate derivatives of MN, NMN and 3-MT	Not mentioned

Goodall and Alton[17]	Urine	Amberlite CG-50 (NH_4^+) (pH 6.2). Elution at pH 5	Fractionated elution (5 fractions). Separation of NM, (NMN + E), NE, DM, 3-MT	
Jonas and Scheel-Krüger[19]	Deproteinised brain	Amberlite CG-120 (Na^+) (pH 6.5). Acid discontinuous elution (1 *M* then 4 *M* HCl)	Fractionated elution (2 fractions). Separation of (NE + NMN + DM) and 3-MT	83
Anggard and Sedvall[11]	Standard solution		GLC–ECD (^{63}Ni) on 3% XE-60 column at 17.0°, trifluoroacetate or pentafluoropropionate derivatives of MN, NMN and 3-MT	Not mentioned
Crawford and Yates[6]	Deproteinised brain	Ethyl acetate acetylation of amines, extraction of acetylated derivatives by dichloromethane	Paper chromatography and subsequent elution of spots	Not mentioned
Tagliamonte *et al.*[7]	Deproteinised brain	Alumina (pH 8.3); effluent to Dowex 50W-X4 (Na^+). Acid elution (5 *M* HCl)	Organic extraction	
Käser and Thomke[2]	Non-hydrolysed urine	Dowex 50W-X8 (H^+) (pH 6.5). Acid discontinuous elution (1 *M* then 5 *M* HCl)	Fractionated elution (2 fractions). Separation (NE + E) and (DM + 3-MT); DM is destroyed by heating	
Geissbühler[1]	Urine (acid hydrolysis at 100° for 30 min)	Dowex 50W (NH_4^+) (pH 6). Acid elution (3 *M* HCl)	Not mentioned. Fate of DM uncertain	
Guldberg *et al.*[5]	Deproteinised brain	Dowex 50W-X8 (Na^+) (pH 6). Acid elution (2 *M* $HClO_4$)	Alumina (pH 7). Separation of 3-MT and DM	73
Kehr[13]	Brain	Dowex 50W-X4 (H^+) .Acid discontinuous elution (1 *M* aqueous HCl and 1 *M* ethanolic HCl, 50%)	Fractionated elution (3 fractions). Sepation of NE, DM, 3-MT. DM interference with extraction, 5%. Specific 3-MT fluorimetric assay	62.2 to 90
This paper	Urine (acid hydrolysis at 100° for 30 min)	Dowex 50W-X2 (H^+) (pH 5); sample (pH 6). Ammoniacal elution (5 *M* NH_4OH)	Amberlite CG-50 (NH_4^+) (pH 8.78). Fractionated elution (3 fractions). Separation of NM, NMN, 3-MT. DM is destroyed by 5 *M* NH_4OH	78 ± 4

* Continuous elution: a single eluent is used for separation of 3-MT.
** Discontinuous elution: two different eluents are used for separation of 3-MT.

olite of dopamine, results from the direct effect of catechol methyltransferase (EC 2.1.1.6) on the latter. It has been detected in human urine[1–3] and in several nervous tissues[4–8] also containing large amounts of dopamine. The specificity of 3-MT as a metabolite of dopamine gives rise to much interest in its determination in urine; quantitative studies on dopamine metabolism in various physiological conditions as well as in pathological situations resulting from increased synthesis of this amine (sympathetic tumours or the therapeutic use of L-DOPA) might benefit from this assay. Few analytical procedures are available for the simultaneous extraction and determination of the methoxyamines 3-MT, normetanephrine (NMN) and metanephrine (MN) in a single biological sample (see Table I).

The aim of this work has been rigorously to separate the three methoxyamines from a single urine sample and to determine them with minimal interference from pigments or related chemical compounds. To accomplish this, we have taken advantage of the properties of Dowex 50 and Amberlite CG-50 resins and have devised a highly efficient extraction procedure based on a method used by one of us for separating urinary MN and NMN[9].

The method consists in:

(1) purification of urine on Dowex 50W-X2 (H^+), with ammoniacal elution of the unseparated methoxyamines,

(2) rigorous separation, on Amberlite CG-50 (NH_4^+) with 0.5 *M* sodium borate (pH 8.78) as eluent of MN, NMN and 3-MT in the ammoniacal eluate, and

(3) specific fluorimetric assay of the three methoxyamines in the fractions separated on the Amberlite.

MATERIALS

The following materials and reagents have been applied: Pyrex glass columns for Dowex and Amberlite resins[9]; long-fibre glass-wool (Corning, Corning, N.Y., U.S.A.); acidic ascorbate solution [2 mg of ascorbic acid (E. Merck, Darmstadt, G.F.R.) in 1 ml of 5 *M* hydrochloric acid, prepared just before use]; 0.02 *M* iodine solution in absolute ethanol, stored at 4° in darkness and kept for no more than three weeks; alkaline sulphite solution (0.02 *M* anhydrous sodium sulphite in 4.5 *M* sodium hydroxide, freshly prepared each day); 0.5 *M* sodium tetraborate buffer, pH 8.78; 0.5 *M* sodium tetraborate buffer, pH 5.2, prepared from the pH 8.78 buffer by adding 0.5 *M* acetic acid; standard solution of 3-MT hydrochloride (mol. wt. 203.6; Sigma, St. Louis, Mo., U.S.A.); stock solution of 3-MT (100 μg/ml) (6.2 mg of 3-MT in 50 ml of 0.01 *M* hydrochloric acid, stored at 4° and kept for no more than three weeks); solutions of 3-MT (10 μg/ml and 1 μg/ml), prepared by diluting the stock solution with glass-distilled water (this solution must be prepared daily).

The reagents for the NMN and MN assay are those used previously[9].

The cation-exchange resins are obtained from Fluka (Buchs, Switzerland), and are treated as follows. Amberlite CG-50 (NH_4^+; 200–400 mesh) is rigorously stabilized at pH 8.78 before use by successive washings with 0.5 *M* borate buffer (pH 8.78). Dowex 50W-X2 (H^-; 100–200 mesh) is rigorously adjusted to pH 5 before use by numerous washings with glass-distilled water. Both resins are purified before use as described elsewhere[9].

METHODS

Principle

The general procedure is based on the method used in our laboratory for extracting urinary metanephrines[9,10]. After initial purification of the urine hydrolysate on Dowex 50W-X2, the ammoniacal eluate is evaporated, and the residue is dissolved in borate buffer; the three methoxyamines are then separated on an Amberlite CG-50 (pH 8.78) column, with borate buffer of pH 8.78 as eluent, and collected in separate fractions for fluorimetric assay.

Urine collection and hydrolysis of conjugates

Urine (24-h specimen) is collected over hydrochloric acid (5 ml for 100 ml of urine). Methoxyamines are less sensitive to oxidation than are catecholamines; thus, acid urine (pH 1) may be stored at 4° for at least a month without addition of ascorbic acid or EDTA. Hydrolysis of conjugated methoxyamines is performed as described previously[9].

Methoxyamine extraction

Exchange on Dowex 50W-X2 (pH 5). The procedure is the same as that previously described for MN and NMN[9], but borate buffer of pH 8.78 is used instead of a buffer of pH 8.80.

Separation on Amberlite CG-50 (pH 8.78) column. The Dowex eluate is transferred to an Amberlite column, prepared as described previously[9], the only modification being that the borate buffer and the resin have a pH of 8.78 instead of 8.80. The effluent is collected as soon as the extract has been applied to the column; the first 15 ml of effluent (containing pigments and other coumpounds) is discarded. Elution takes place at a flow-rate of 15 ml/h and under atmospheric pressure and the compounds of interest are eluted as follows (see Fig. 1):

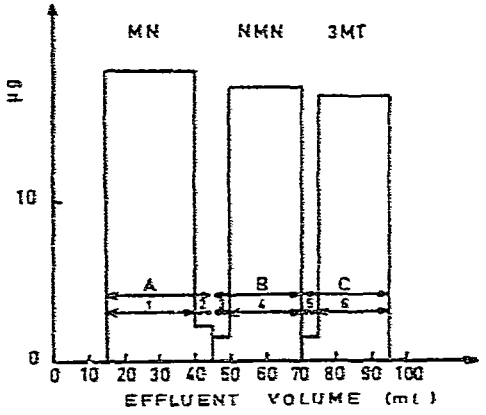


Fig. 1. Fractionation of methoxyamines on an Amberlite CG-50 column (11.5 cm × 1 cm) at pH 8.78. The solution applied is an ammoniacal eluate coming from filtration on a Dowex 50W-X2 column at pH 5 of urine (20 ml) diluted to 80 ml and containing added MN (20 μg), NMN (20 μg) and 3-MT (20 μg); the eluent is 0.5 *M* borate buffer of pH 8.78. The reaction steps are described in Table II and ref. 9.

MN in fraction A (15 to 40 ml)
NMN in fraction B (40 to 70 ml)
3-MT in fraction C (70 to 95 ml)

Each compound is collected in two fractions (see Fig. 1) (1 and 2 for MN; 3 and 4 for NMN; 5 and 6 for 3-MT) in order to highlight the overlap between the three amines and to indicate any displacement of the separation diagram[10]. The eluates from the Amberlite column can be stored at 4° for 24 h.

Fluorimetric assays

MN and NMN. Fluorimetric assay is performed for each eluate (A or B) adjusted to pH 4.5 or 5 and oxidized with potassium metaperiodate[9].

3-MT. The pH of Amberlite eluate C (fraction No. 5, 5 ml; fraction No. 6, 20 ml) is adjusted to 5.2 by means of glacial acetic acid (about 2 drops for each 5 ml), and the fluorescent derivative of 3-MT is obtained in three steps (see Table II) as follows: oxidation with 0.02 *M* iodine; cessation of oxidation by means of 0.02 *M* alkaline sulphite; stabilisation of the fluorescent derivative by acidification with 5 *M* hydrochloric acid.

For each eluate, two samples of different volumes (0.3 and 0.8 ml) are used, and an internal standard is prepared by adding 0.2 μg of 3-MT to 0.3 ml of eluate. The pattern of oxidation is as set out in Table II.

TABLE II

PROCEDURE FOR FLUORIMETRIC ASSAY OF URINARY 3-MT IN AMBERLITE CG-50 ELUATE

Spectrofluorimeter: Jobin-Yvon (Beam type); fluorescence wavelengths, 335–390 nm. If the final volume is inadequate for the cells, 1 ml of glass-distilled water may be added to each sample before fluorescence readings are made.

Sample	*3-MT standard (1 µg per ml solution)*	*0.5 M Borate buffer of pH 5.2 (ml)*	*0.02 M Iodine (ml)*	*2.5% Alkaline sulphite (ml)*	*5 M Hydrochloric acid (ml)*
External standard					
3-MT (0.2 µg)	0.2 ml	0.8			
Blank	—	1	0.2; allow 3 min for oxidation	0.2; allow 3 min for reaction with excess of iodine	0.4; allow 30 min for stabilization
Eluates at pH 5.2					
(a) 0.3 ml	—	0.7			
(b) 0.3 ml + 3-MT (0.2 µg)	0.2 ml	0.5			
(c) 0.8 ml	—	0.2			

Calculation. The amounts (Q_1 and Q_2) of 3-MT present in the volumes of eluate (V_1 and V_2; $V_1 = 5$ ml; $V_2 = 20$ ml) are derived from the fluorescence difference between the two samples (0.8 and 0.3 ml) and from the fluorescence intensity of the internal standard (E_t) by the following relationship (Q in μg):

$$Q = \frac{2(c - a) \cdot V}{5E_t}$$

where $E_t = (b - a)$, and a, b and c are the fluorescence intensities of the eluate samples a, b and c, respectively, referred to in Table II.

The 3-MT concentration in μg/l of urine (with a 20-ml sample) is given by

$$(Q_1 + Q_2) \times 50$$

RESULTS AND DISCUSSION

Choice of technique for extracting 3-MT

Some gas chromatographic procedures for 3-MT assay have been described[3,11], but none is suitable for studying normal urine; ion-exchange chromatography seems to be more suitable for isolation of 3-MT from biological samples: The most frequently used adsorbents (see Table I) are the cationic resins Dowex 50W-X2 (H^+)[12], -X4 (H^+)[13], -X4 (Na^+)[7,14,15], -X8 (H^+)[2,4], -X8 (Na^+)[5,16] or Amberlite CG-50 ($NH4^+$)[9,17], CG-120 (H^+)[18], CG-120 (Na^+)[19].

Dowex 50W retains 3-MT at pH values from 5 to 6.5, the compound being desorbed by acid[1,2,4–6,13] or alkaline elution[12,14]. However, the exclusive use of Dowex has two shortcomings.

(1) The acid elution is not specific for methoxylated amines; catecholamines

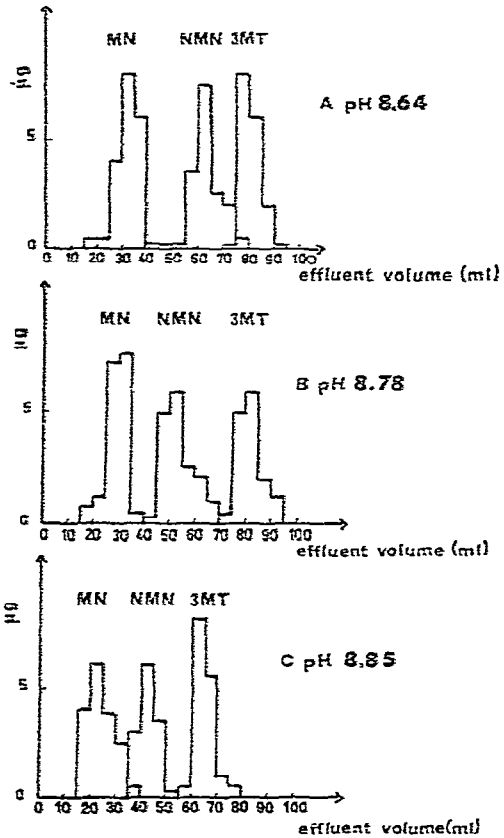


Fig. 2. Separation of methoxyamines on an Amberlite CG-50 column (11.5 cm × 1 cm) at the pH values shown. The solution applied is as in Fig. 1. The eluent is 0.5 *M* borate buffer of pH 8.64 (A), 8.78 (B), or 8.85 (C).

(including dopamine) are eluted as well, and, although dopamine can be partially removed by fractional acid elution[4], it is never completely separated from 3-MT[2].

(2) The basic elution has the advantage of destroying the catecholamines; however, the ammoniacal eluate cannot be used directly for fluorimetric assay, because it contains a large amount of urinary pigments.

The cationic Amberlite CG-50 or CG-120 retains the methoxylated amines at pH values to 8.8[9,10,18–20]. The use of an appropriate eluent (borate buffer, ammonium acetate buffer, or hydrochloric acid) leads to complete separation of MN, NMN and 3-MT, each of which can be determined without mutual interference. However, satisfactory separation on Amberlite is obtained only from a partially purified extract, and for this reason, an initial purification stage is usually performed either on Dowex 50[9,10] or on alumina[20].

Results obtained by coupling Dowex 50W-X2 and Amberlite CG-50

Dowex procedure. One essential condition for quantitative fixation of methoxyamines on Dowex 50 and good exchange of organic compounds on the resin is a low saline content of the sample; to achieve this, the urine should be diluted fourfold with glass-distilled water (until the creatinine content is less than 0.5 mg/ml).

Effect of Amberlite pH and buffer pH. The separation of the three methoxyamines is achieved by displacement chromatography with 0.5 *M* borate buffer on Amberlite CG-50. At constant buffer molarity, small variations in pH of Amberlite or buffer lead to modifications in the elution volumes and the separation pattern of the three amines (see Fig. 2). An increase in pH value seems to offer the advantage of reducing the elution volume, but detailed analysis of the diagrams obtained at pH 8.64 to 8.85 shows that separation of 3-MT from NMN was good over a wide range

TABLE III

MAIN PROCEDURES FOR FLUORIMETRIC ASSAY OF 3-MT

Reference	*Oxidation step*			
	Buffer	*pH*	*Reagent*	*Time (min)*
Carlsson and Waldeck[4]	1 *M* Citrate and 1 *M* phosphate	6.5	0.02 *M* Iodine (0.1 ml)	7
Laverty and Taylor[21]	0.05 *M* Borate	8.8	0.02 *M* Iodine (0.05 ml)	3
Geissbühler[1]	0.25 *M* Citrate and 0.25 *M* borate	6.5	0.02 *M* Iodine (0.05 ml)	7
Käser and Thomke[2]	0.5 *M* Citrate, 0.5 *M* borate and saturated sodium chloride	6.5	0.02 *M* Iodine (0.2 ml)	4
Guldberg *et al.*[5]	2 *M* Perchloric acid and potassium carbonate	7	0.02% Potassium ferricyanide (1 ml)	2
Kehr[13]	1 *M* Ethanolic (50%) hydrochloric acid and 10 *M* ammonium hydroxide. Heat mixture at 55° for 15 min		0.01% Potassium ferricyanide (0.05 ml)	3
This paper	0.5 *M* Borate	5.2	0.02 *M* Iodine (0.2 ml)	3

of pH (8.70 to 8.85), whereas MN and NMN were rigorously separated only at pH values between 8.75 and 8.80.

Indeed, the location of the elution peak of 3-MT is little modified over the pH range 8.64 to 8.78 and is clearly advanced at higher pH values (Fig. 2). The effect of pH is more important for metanephrines, which are retarded and better separated at pH values less than 8.75, but drawn forward and overlapped at pH values greater than 8.80 (Fig. 2). These facts led us to choose a pH of 8.78 (see Fig. 2b) for the Amberlite CG-50 and for the borate buffer used in the fractionation procedure.

Use of resins. The preparation, storage and use of the Dowex and Amberlite resins need particular care (see Materials). Dowex 50W-X2 (H^+), used for the first step, retains most of the urinary pigments; thus, it cannot be regenerated, and each sample must be purified on a newly prepared Dowex column. Amberlite CG-50 (NH_4^+) can be regenerated after each use by at least five successive washings with 0.5 *M* borate buffer of pH 8.78 and is stored in the same buffer. A single batch of Amberlite can be re-used 50 times, but, for each change of the resin, a new fractionation diagram has to be established; this is achieved by analysing a sample of urine to which 3-MT has been added.

A steady pH value of the resins is essential for reliable extraction of the methoxyamines. Moreover, under our conditions for preparing the Amberlite CG-50, and eluting the amines from the column, there is no interfering fluorescence between 335 and 390 nm; this contrasts with what happens when Amberlite is used with 1 *M* hydrochloric acid as eluent[18].

Purity of eluates. Urinary pigments in the ammoniacal eluate from the Dowex interfere seriously with fluorimetric assay; they are totally removed in the rejected fraction of eluate (0–15 ml) from the Amberlite column.

Reduction step		*Stabilization step*		*Temperature (°C)*	*Wavelength range (nm)*	*Sensitivity (ng per ml)*
Reagent	*Time (min)*	*Reagent*	*Time (min)*			
2.5% Alkaline sulphite (0.5 ml)	5	5 *M* Hydrochloric acid (0.7 ml)	15	20	335–385	Not mentioned
2.5% Alkaline sulphite (0.25 ml)	5	Glacial acetic acid (0.25 ml)	40	100	320–375	8
2.6% Alkaline sulphite (0.5 ml)	5	5 *M* Hydrochloric acid (1 ml)	30	45	340–380	Not mentioned
2.5% Alkaline sulphite (0.5 ml)	5	5 *M* Hydrochloric acid (1 ml)	30	80	330–385	Not mentioned
0.1% Cysteine (0.1 ml)	0				315–430	8
0.1% Cysteine (0.05 ml)	0				330–430	4.5
2.5% Alkaline sulphite (0.2 ml)	3	5 *M* Hydrochloric acid (0.4 ml)	30	20	335–390	5.5

Extraction recoveries. At pH 8.78 and with the procedure described under Methods, recovery of 3-MT (mean ± standard error) is 80 ± 3% ($n = 5$) for pure solutions and 78 ± 4% ($n = 8$) for 3-MT added to urine samples. At the same pH, recoveries of MN or NMN are, respectively, 85 ± 3% ($n = 10$) and 97 ± 2% ($n = 10$).

Fluorimetric assay of 3-MT

3-MT can be converted into a fluorescent compound by oxidation with potassium ferricyanide[5,15,17] or iodine[1,2,4,21] (see Table III). The spectral features of the fluorescent compound differ according to the nature of the oxidant, suggesting that the fluorescent derivatives are not the same. For the assay of 3-MT, we have modified the procedure described by Fleming *et al.*[22] for dopamine assay. All the parameters of the reaction have been studied and are discussed below.

Oxidation time. The fluorescence intensity of the 3-MT fluorophore shows a rapid increase up to 1.5 min after iodine oxidation, after which it remains constant between 2.5 and 3 min, and then decreases (Fig. 3). As the oxidation time is critical, it is important not to oxidize more than 20 samples at once.

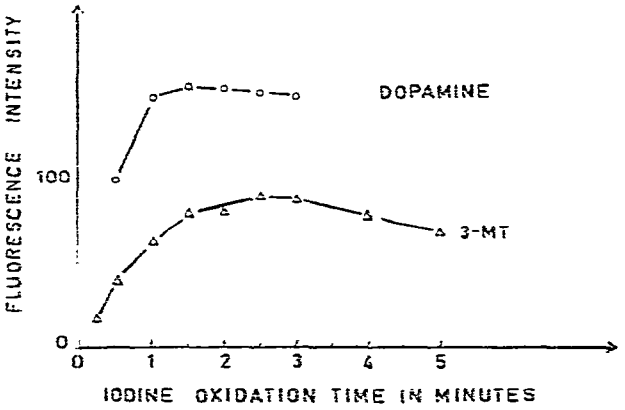


Fig. 3. Fluorescence intensity of 3-MT (1 μg) (△) and dopamine (1 μg) (○) as a function of iodine oxidation time. The sample is 1 ml of amines in 0.5 *M* borate buffer of pH 5.2. The reaction steps are described in Table II.

Kinetics of fluorescence development. At room temperature, the fluorescence intensity of 3-MT increases rapidly soon after the final acidification, but the maximum is reached only at 30 min (Fig. 4); the fluorescence intensity then remains constant for 3 h. The addition of ascorbic acid (2 mg in 1 ml of 5 *M* hydrochloric acid), as suggested by Fleming *et al.*[22] for dopamine assay, is not necessary.

Oxidation pH. In our experimental conditions, the fluorescence intensity of 3-MT (1 μg) is maximal when the initial pH before oxidation is 5.2 (Fig. 5). In fact, the optimum pH depends also on other parameters (the buffer molarity and the nature and concentration of the oxidising reagent). Thus, in the Laverty and Taylor procedure[21], the optimum pH for 3-MT assay is 8.8; better sensitivity can be obtained by adding 15 *M* ammonia[23] to the medium before oxidation. This was not confirmed in our procedure.

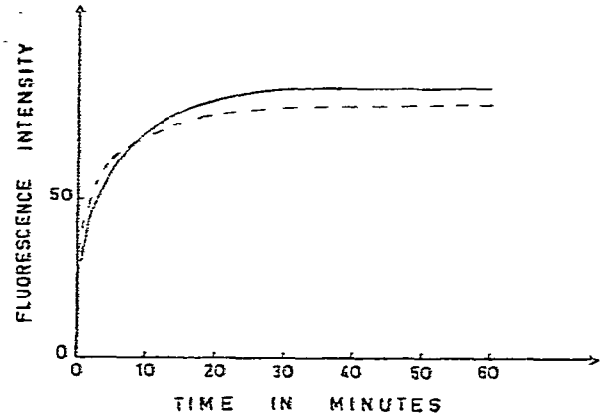


Fig. 4. Development of fluorescence intensity of dopamine (———) (1 μg) and 3-MT (— — —) (1 μg) oxidized as described in Table II. For dopamine, the stabilizing reagent is 0.4 ml of 5 *M* hydrochloric acid containing 2 mg of ascorbic acid per ml (see ref. 22); for 3-MT the fluorescence development is the same whether the stabilizing reagent is as above or only 5 *M* hydrochloric acid.

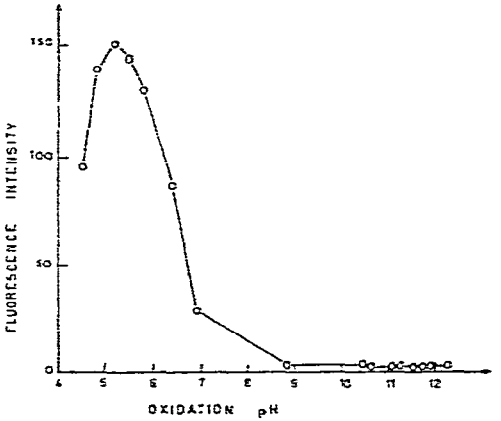


Fig. 5. Effect of pH of 0.5 *M* borate buffer on fluorescence intensity of 3-MT (0.5 μg). Alkaline pH values (above 10) are obtained by adding glacial ammonia to the medium before oxidation. The sample is 1 ml of 3-MT in 0.5 *M* borate of varying pH. The reaction steps are described in Table II.

Molarity of medium. The molarity before oxidation differs in the methods used by different authors (see Table III), varying from 0.05 to 1. Kaser and Thomke[2] used a sodium chloride-saturated medium before oxidation. A study of the effect of borate buffer molarity (Fig. 6) shows that the fluorescence intensity due to 3-MT is constant at molarities from 0.3 to 1, but decreases greatly when the molarity exceeds 1. Thus, in order to ensure good fluorimetric sensitivity, the eluates must not be diluted by a factor greater than 5.

Stabilizing pH. By varying the molarity of the hydrochloric acid added at the final step to the oxidized sample, a final pH ranging from 0.4 to 11.5 can be obtained. A high fluorescence intensity is reached only when the final pH is acid; the optimal value (1.6) is obtained by adding 0.4 ml of 2.5 *M* hydrochloric acid to the oxidized

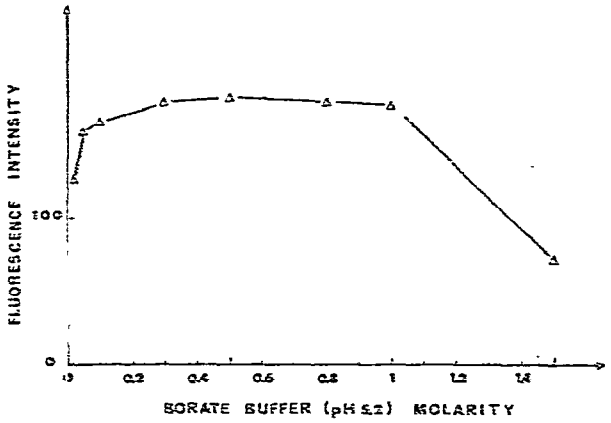


Fig. 6. Effect of molarity of borate buffer of pH 5.2 on fluorescence intensity of 3-MT (0.5 μg). The sample is 1 ml of 3-MT in borate buffer of varying molarity. The reaction steps are described in Table II.

mixture (Fig. 7). However, this pH is not easily reproducible because of the unsatisfactory stability of the pH of 2.5 *M* hydrochloric acid and of acetate ions present in eluates with high buffer strength; for these reasons, in spite of a 10% loss in assay sensitivity, 5 *M* hydrochloric acid has to be used to ensure good reproducibility. In fact, as has been shown for the oxidation pH, the final optimum pH depends on the operating conditions: thus, for the method of Laverty and Taylor[21], the optimal pH is 4.5.

Specificity. Possible interference in the fluorimetric assay of 3-MT by equimolar amounts of related compounds is indicated in Table IV. Only dopamine (150%),

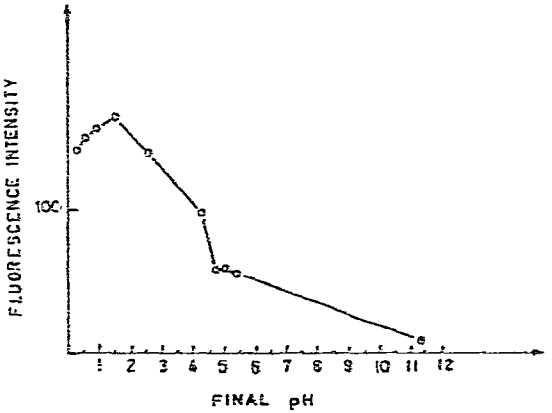


Fig. 7. Fluorescence intensity of 3-MT (0.5 μg) as a function of pH of the final mixture. The sample is 1 ml of 3-MT in 0.5 *M* borate buffer of pH 5.2. The fluorescence reaction is as described in Table II. The final stabilisation is achieved by adding hydrochloric acid (0.4 ml) of the following molarity: 5 for pH 0.4; 4 for pH 0.6; 3 for pH 1; 2.5 for pH 1.6; 2.4 for pH 2.7; 2.3 for pH 4.4; 2.2 for pH 4.9; 2.1 for pH 5.2; 2 for pH 5.5; and 1 for pH 11.5. Each point represents the mean of five determinations.

TABLE IV

FLUORESCENCE OF EQUIMOLAR AMOUNTS OF 3-MT AND RELATED COMPOUNDS

Different compounds (in 1 ml of 0.5 *M* borate buffer of pH 5.2) are oxidized as described for 3-MT in Table II.

Compound	*Relative fluorescence*
3-MT	100
Dopamine	150
DOPA	30
α-Methyl-DOPA	14
3-O-Methyl-DOPA	7
3,4-Dihydroxyphenylacetic acid	5
Epinine	4
Aleudrine	3
NMN	3
MN	3
Norepinephrine	2
Parasympathol	2
3-Methoxy-4-hydroxyphenylethanediol	2
Serotonin	2
Epinephrine	1
Homovanillic acid	1
Vannilylmandelic acid	1
3,4-Dihydroxymandelic acid	1
Octopamine	0
Tyramine	0
Dihydroxyphenylserine	0

DOPA (30%) and 3-O-methyl-DOPA (7%) exhibit interfering fluorescence. α-Methyl-DOPA gives a fluorescence of 14%, but this compound is not present in normal urine. Dopamine is totally destroyed by the ammoniacal elution from Dowex 50; most of the DOPA is destroyed by 5 *M* ammonia and the remainder is eluted from the Amberlite CG-50 in the first fraction of effluent. 3-O-Methyl-DOPA resists alkaline treatment, but is eluted from Amberlite CG-50 (pH 8.78) in the fraction from 4.5 to 17 ml of eluate; under the same conditions, 3-MT is eluted in the fraction 70 to 95 ml. In contrast to other techniques (see Table I), the 3-MT extraction procedure described here eliminates dopamine, interference from which would make fluorimetric estimation of 3-MT impossible.

Sensitivity and linearity of reaction. The sensitivity of the fluorescence reaction (calculated for a fluorescence value twice that of the blank) is 30 ng of 3-MT in the reaction mixture. However, 10 ng of 3-MT, *i.e.*, 5.5 ng/ml of sample can be easily detected (Fig. 8A). The fluorescence intensity is linear for up to 4 μg of 3-MT in the reaction mixture (Fig. 8B).

"*Eluate blank*". The determination of the eluate blank is important. Blanks, determined on urinary eluates by replacing the oxidant by 10% EDTA solution (0.2 ml) or by inverting the order of adding reagents (hydrochloric acid, sulphite, iodine) often give high fluorescence values. It is therefore more convenient to take increasing volumes of eluate (0.3 and 0.8 ml) and to calculate the amounts of fluorophore formed by the difference in fluorescence between them ($F_{0.8\text{ ml}} - F_{0.3\text{ ml}}$). This procedure is suitable only if the eluate blank fluorescence is slight with respect to the sample value and can therefore be regarded as constant.

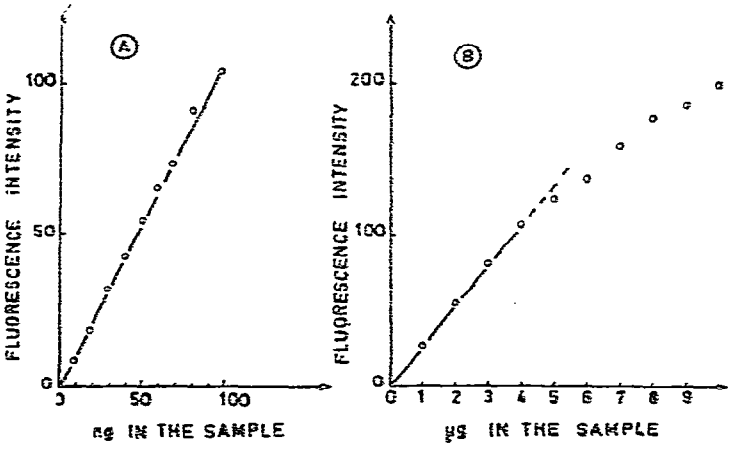


Fig. 8. Sensitivity (A) and linearity (B) of the fluorimetric estimation of 3-MT. The sample is 1 ml of 3-MT in 0.5 *M* borate buffer of pH 5.2. For the fluorescence procedure see Table II.

Nature of fluorescent product. The technical steps of fluorimetric assay for 3-MT are the same as for dopamine[24]. The dopamine estimation is based on oxidation of the molecule to quinone and cyclisation to give aminochrome; this undergoes intramolecular rearrangement to form 5,6-dihydroxyindole, the fluorescent derivative. The similarities in activation and fluorescence spectra of the dopamine and 3-MT derivatives (Fig. 9) suggest that they are the same. This observation can be compared with the established fact[10] that oxidation of MN and NMN by potassium periodate leads

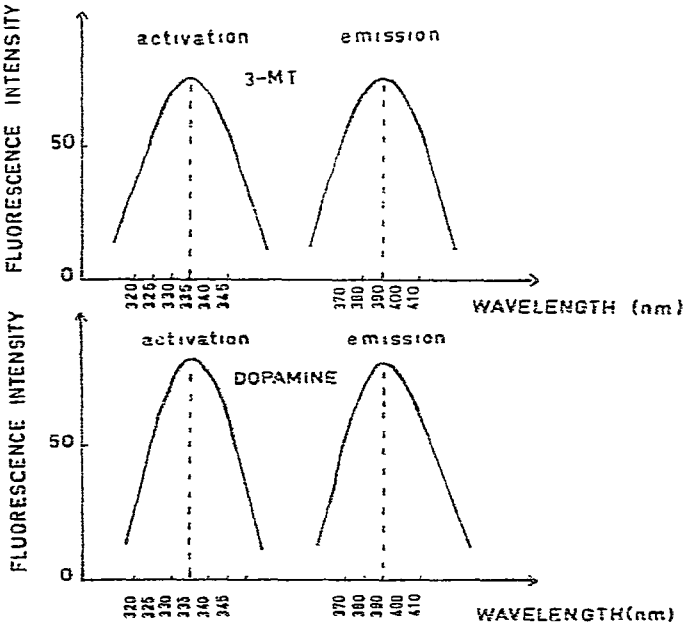
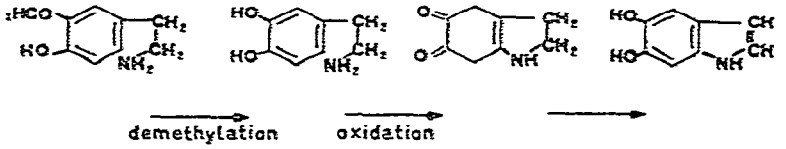


Fig. 9. Activation and fluorescence spectra of 3-MT (1 µg) and dopamine (1 µg).

to the formation of a fluorophore exhibiting the same activation and fluorescence spectra as epinephrine and norepinephrine, respectively. Adler and Magnusson[25] explain the spectral similarities between these amines and corresponding methoxyamines (MN and NMN) by demethylation of the latter during oxidation.

In the same way, we suggest that 3-MT might be demethylated during oxidation and that the subsequent chemical steps are the same as for the iodine oxidation of dopamine (Fig. 10). This hypothesis is supported by the fact that the iodine oxidation of dopamine is quicker than that of 3-MT. The greatest fluorescence intensity is reached in 1.5 min for dopamine and in 2.5 to 3 min for 3-MT (Fig. 3). In addition, the kinetics of fluorescence stabilization are the same for 3-MT and dopamine (Fig. 4).



3-O-METHYLDOPAMINE DOPAMINE AMINOCHROME 5,6-DIHYDROXYINDOLE

Fig. 10. Suggested scheme for formation of the fluorescent derivative of 3-MT.

BIOLOGICAL APPLICATIONS

The method described here has been successfully applied to the determination of urinary 3-MT in physiological and pathological situations, with reproducible results. Under physiological conditions, the mean excretion of total (free and conjugated) 3-MT for 43 adults (aged from 18 to 55 years) was 98.4 ± 8.3 μg per 24 h* or 8.8 ± 0.8 μg per 100 mg of creatinine*. Few results for urinary 3-MT in man have been published, and our values are lower that those previously reported[1,2,26]. This may be because most of the other procedures are insufficiently specific for 3-MT assay and do not completely eliminate interference from dopamine.

Our procedure is highly specific for 3-MT even in urine from patients with such diseases as phaeochromocytoma, where high concentrations of MN and NMN and normal levels of 3-MT are found: NMN and MN excretion are respectively 49.5 ± 10.5 and 42 ± 7.2 times the control values, whilst 3-MT is increased by a factor of only 1.4 ± 0.3. Also, urinary 3-MT estimation is of value for adrenergic tumour prognosis in children and adults and can assist in determining the degree of malignancy of tumours[27]. The assay is not affected by high concentrations of dopamine; for instance, in parkinsonian patients receiving L-DOPA urinary dopamine levels are increased 110 times, whereas excretion of 3-MT is only 44 times the control value[28].

* Mean ± standard error.

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