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SEPARATION, DETECTION AND QUANTITATIVE ANALYSIS OF URINARY β-PHENYLETHYLAMINE

ALAN A. BOULTON AND L. MILWARD
Psychiatric Research Unit, University Hospital, Saskatoon, Sask. (Canada)

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SUMMARY

A method for the analysis of phenylethylamine based on the formation of a fluorophore with alloxan is described. The procedure is simple, relatively specific and fairly sensitive. A linear relationship exists between the concentration of the amine and fluorescence. Application to the analysis of urine obtained from a control population reveals that the excretion of free phenylethylamine is approximately 47 μg/24 h; conjugated phenylethylamine excretion is variable in the range 34–365 μg/24 h. Free urinary phenylethylamine seems not to be excreted by patients suffering with depression.

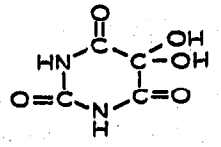
INTRODUCTION

Abnormalities in the urinary excretion of β-phenylethylamine have been reported in phenylketonuria[1] and depression[2]. Published procedures for the analysis of phenylethylamine have to date been based on the assessment of the ninhydrin chromaphore following separation, on paper, in certain solvent systems. Such a procedure is lacking in specificity and sensitivity and is, at best, only semiquantitative. A search of the literature revealed that although some described visualisation techniques could be applied to phenylethylamine they suffered from certain defects. For instance the fluorophore produced by formaldehyde–HCl–ethanol first described by PROCHÁZKA[3] and later extended by SEILER[4] is not specific with respect to its spectral characteristics, it is also not very sensitive. Furthermore, following the thin-layer chromatographic (TLC) separation of a crude urinary extract the number of fluorophores produced after spraying with Procházka's reagent is so numerous as to make an assessment of the plate impossible.

The fluorescent derivatives produced between primary amines and *ortho*-acetoacetylphenol or 2-acetoacetyl-4-methylphenol[5,6] seemed as though they would be appropriate but unfortunately these particular reagents are not commercially available and are difficult to synthesise.

Following an empirical survey of a variety of commercially available aldehydes and ketones we finally selected the fluorescent derivative produced between β-phenylethylamine as it exists after separation on a precoated layer of silica gel

(Eastman Kodak 6061) and the triketone reagent alloxan (2,4,5,6-tetraoxohexahydropyrimidine).



This fluorophore seems to possess the characteristics required for a simple, convenient, sensitive and relatively specific analysis.

(1) PREPARATION AND SEPARATION OF A URINE EXTRACT

Free phenylethylamine along with other non-conjugated basic substances were extracted from approximately 40 ml (1/25 of a 24-h urine sample) of urine at pH 12.0 with benzene (3 × 40 ml). The benzene extracts were aspirated, combined and after adding three drops of concentrated HCl rotary evaporated under reduced pressure at 40° to approximately 5 ml. This concentrated extract was then quantitatively transferred to a smaller flask (25 ml) and the evaporation continued to dryness. Quantitative transfer to a precoated thin layer (20 × 20 cm) of silica gel (Eastman Kodak 6061) was accomplished using 100 μl of 95% ethanol solution. The conjugated basic substances remaining in the extracted urine were hydrolysed at pH 1.5 on a steam-bath during 30 min and after adjustment to pH 12.0 extracted into benzene (3 × 45 ml). This benzene extract was then rotary evaporated, under reduced pressure, to dryness—loss of the volatile amines being prevented by conversion to HCl salts—dissolved in 95% ethanol solution (100 μl) and transferred to a different silica gel chromatogram. Aliquot parts of synthetic phenylethylamine—to serve as markers and for quantitative measurements—were applied to other thin-layer sheets and treated in parallel with the urine extracts.

Separation in the first dimension proceeded during a 6–8 h period in the solvent system butanol–acetic acid–water (4:1:1) and after air drying (1–2 h or overnight) in the second dimension with the solvent system pyridine–*tert.*-butanol–propylene glycol–methyl ethyl ketone (4:3:2:1). After air drying the chromatograms were treated as described in Section 5. Conditions for the optimum development of the alloxan fluorophore were obtained empirically as described in Sections 2, 3, and 4.

(2) REACTION OF VARIOUS ALDEHYDES AND KETONES

Thin-layer silica gel chromatograms on which phenylethylamine at concentration levels in the range 0.05–15 μg had been unidimensionally separated were air dried, sprayed with the various aldehydes and ketones (0.1% solutions in acetone) listed in Table I and then heated for 10 min at 125°. After inspection in UV light (365 nm and 265 nm) the minimum amount of phenylethylamine detectable and the appearance of the fluorophore were noted. The results are tabulated in Table I.

Although several other aldehydes and ketones were tested they have not been recorded because they either did not react or else produced instead an absorptive zone or a substantial background fluorescence. From Table I it is apparent that alloxan and 5,7-dichloroisatin reacted to form the most sensitive and spectrally ac-

TABLE I

REACTION OF VARIOUS ALDEHYDES AND KETONES WITH β-PHENYLETHYLAMINE

Reagent	*Lower detection limit (μg)*	*Appearance of fluorophore*	
		265 mμ	*365 mμ*
Benzophenone	2.0	salmon	—
Acetophenone	3.0	buff	—
Acetaldehyde	0.8	yellow-white	yellow-gray
4-Aminoveratrole	12.0	gray	—
Alloxan	0.08	lemon-yellow	yellow
p-Anisaldehyde	2.0	buff	buff
Benzil	3.0	—[b]	yellow
p-Bromophenacil bromide	0.7	gray	white
p-[N,N-Bis(2-chloroethyl)amino]-benzaldehyde	0.6	yellow	—
tert.-Butylhydroquinone	0.7	white	gray
4-(*tert.*-Butyl)pyrocatechol	1.0	white	—
Cacothalein[a]	1.5	yellow	gray
d-10-Camphorsulphonic acid	2.0	yellow	—
Catechol	1.5	white	—
Carvacrol	2.0	yellow	—
Chloranilic acid	2.0	yellow[c]	—
1-Chloro-2:3-epoxypropane	1.5	yellow	—
p-Chlorobenzaldehyde	1.5	yellow	—
5-Bromoisatin	1.0	gray	gray
5-Nitroisatin	0.5	yellow	yellow
5,7-Dichloroisatin	0.08	gray	yellow-gray
N-Acetylisatin	0.7	yellow	yellow

[a] 0.1% suspension.
[b] = no observable fluorescent zone.
[c] This yellow fluorescence was present on a purple fluorescent background.

ceptable fluorophores. The 5,7-dichloroisatin derivative was rejected on the grounds that it produced a less pleasing yellow fluorescence than that of alloxan, in addition there existed a not insignificant contaminating background fluorescence.

Elution of the fluorescent zone from the alloxan-sprayed chromatograms revealed that the derivative was sufficiently stable to permit quantitative assessment in the Aminco-Bowman spectrophotofluorimeter. By trial and error methanol was selected as the best eluting solvent. In this solvent the fluorescent yield was highest and the background blank lowest.

(3) EFFECT OF THE REAGENT SOLVENT

In order to investigate the effect of the reagent solvent on the reaction, chromatograms on which 1 μg of phenylethylamine had been unidimensionally separated were sprayed with alloxan hydrate (0.1%) dissolved in a variety of organic solvents. As can be seen from Table II the fluorescent yield was highest in the case of *n*-propanol.

(4) EFFECT OF TEMPERATURE AND TIME OF HEATING

Examination of precoated silica gel chromatograms on which 1 μg of phenyl-

TABLE II

EFFECT OF THE REAGENT SOLVENT ON THE INTENSITY OF FLUORESCENCE

Solvent	*Fluorescence*[a] *(arbitrary units)*
n-Butanol	0.34
Methanol[b]	0.51
Isoamyl alcohol	0.59
Water	0.86
Acetone[c]	1.09
Ethanol	1.84
Isopropanol	1.94
n-Propanol	2.92

[a] Average of duplicate samples.
[b] When this solvent was used the fluorophore was red and not the usual yellow.
[c] With this solvent the intensity of fluorescence varied quite erratically from chromatogram to chromatogram.

ethylamine had been unidimensionally separated, sprayed with the alloxan reagent and heated for 3 min, revealed on examination following their removal from the oven that a temperature of less than 115° was insufficient immediately to produce a fluorescent derivative. Although after prolonged standing (*i.e.* overnight) a fluorescent derivative forms it does not reach the value produced on heating. Temperatures in excess of 125° caused warping and disintegration of the Kodak sheets. A temperature of 125° was therefore selected as being convenient. The effect of time of exposure to this temperature on the amount of fluorescence produced is expressed graphically in Fig. 1. It can be seen that an 8-min exposure results in the maximum fluorescent yield.

(5) OPTIMUM REACTION CONDITIONS

The optimum reaction conditions finally selected for the analysis of urine extracts involved spraying the air-dried plates separated as described in Section 1 with the alloxan hydrate reagent (0.1% in *n*-propanol) followed by heating at 125° for 8 min. The lemon yellow phenylethylamine fluorophore (see Fig. 2) is dilineated in pencil and the zone scraped from the plate and eluted by shaking for 1 min with methanol (2 ml) in a 5 ml centrifuge tube. After low-speed centrifugation the supernatant is transferred to a 1-cm cuvette and measured in the spectrophotofluorimeter (exitation 435 nm, fluorescence 523 nm). A typical calibration curve, the useful working range and the errors involved in the analysis are shown in Fig. 3; the uncorrected activation and fluorescence spectra are illustrated in Fig. 4.

Although the overall recovery on different occasions is somewhat variable, being in the range 45–75%, it is constant during the extraction on any particular occasion. An internal standard (3 μg) of phenylethylamine is therefore always added to a duplicate urine sample in order to obtain the recovery value for that occasion. The minimum detectable amount of phenylethylamine using this technique is 0.05 μg. In a urine extract separated two dimensionally 0.1 μg is, in the most favourable condition, the smallest detectable amount.

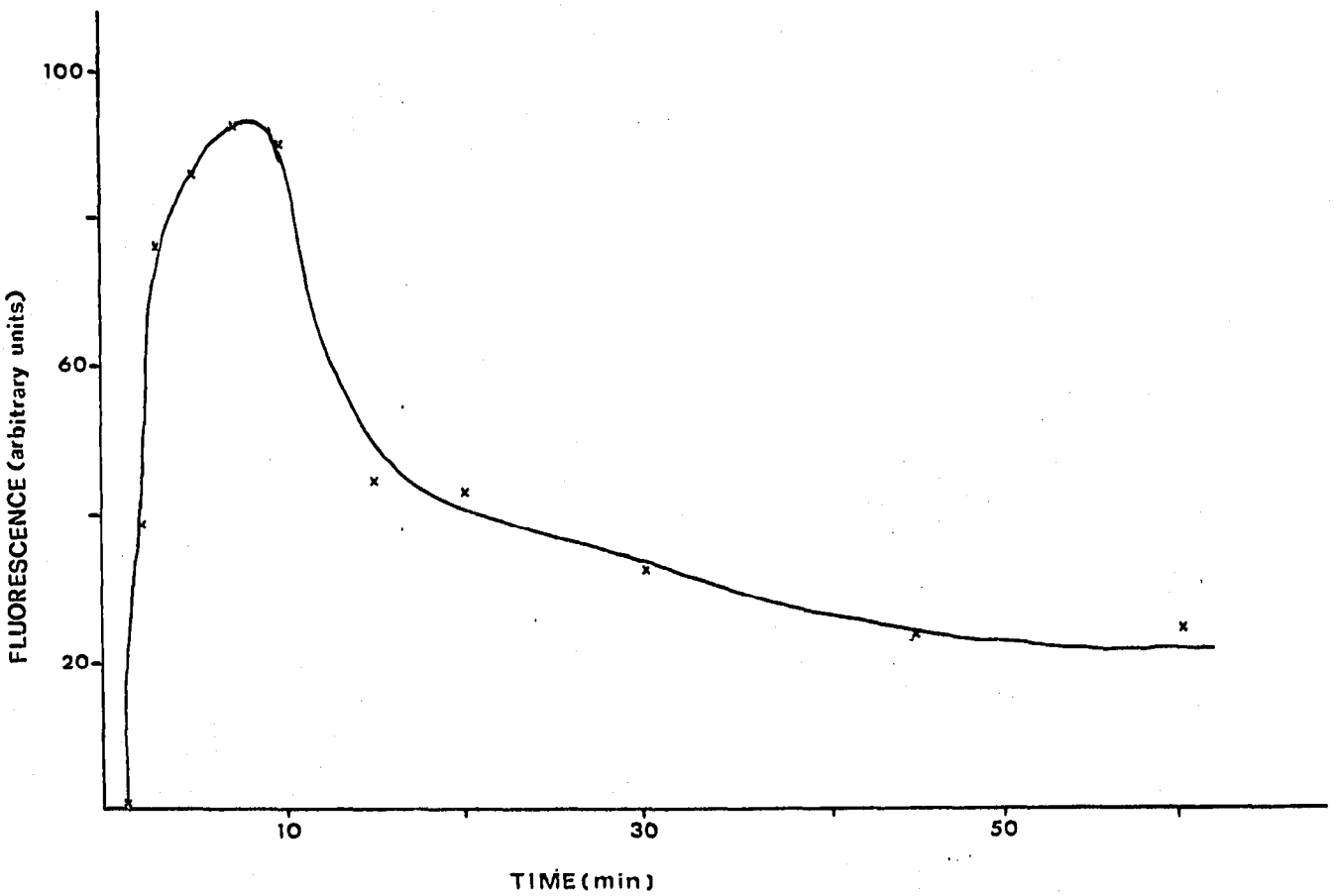


Fig. 1. Graphical representation of the effect of heating time on the intensity of fluorescence.

(6) SPECIFICITY

Numerous biogenic amines and synthetic amines have been tested. In the main the reaction seems to be specific for unsubstituted phenylalkylamines. Hydroxylation of the phenyl ring in susceptible cases causes a fluorescence shift towards the red end of the spectrum. *p*-Tyramine for example exhibits a weak golden coloured fluorescence, catecholamines (noradrenaline, dopamine) exhibit an orange fluorescence of even weaker intensity. Indoylalkylamines produce a grayish-white fluorescence. N-Methylated amines do not react. None of the reactive amines tested produced a fluorophore which after separation in the solvent systems quoted overlapped with the phenylethylamine fluorophore.

Further proof of the identity of phenylethylamine in the urine extracts in addition to the demonstration of isographic behaviour between the urinary phenylethylamine zone and synthetic phenylethylamine and the enhancement of the phenylethylamine zone when phenylethylamine was added to the urine sample before extraction was obtained in two different ways. First 1 nCi of [2-^{14}C]phenylethylamine (New England Nuclear, 11475 Côte de Liesse, Dorval, Quebec) was added to the urine before extraction. After processing as described in Sections 1 and 5 it was shown that all the radiocativity on the plate was associated with the phenylethylamine fluorophore zone. The recovery of the added label agreed with the recovery of added

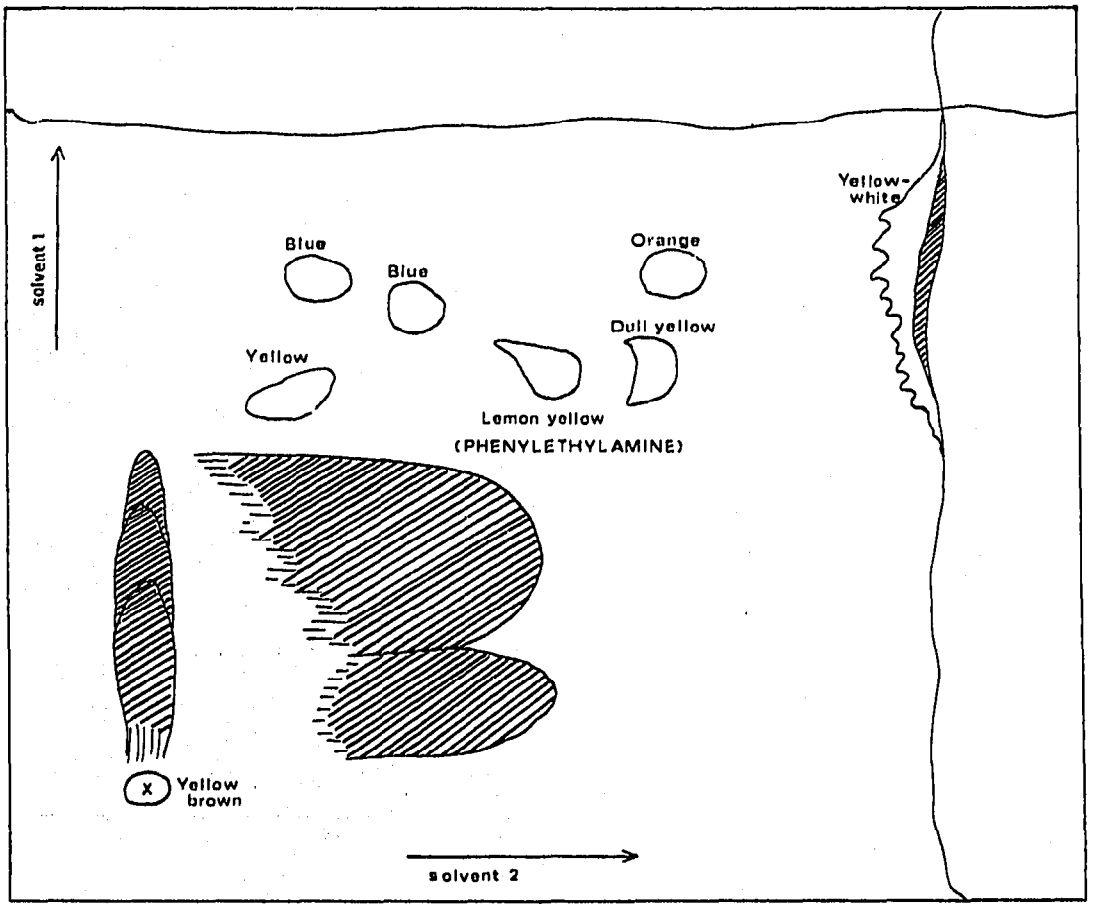


Fig. 2. Schematic representation of the appearance of a chromatogram on which a urine extract has been separated after treatment with the alloxan reagent. Hatched areas, absorptive zones; open areas, fluorescent zones.

phenylethylamine standard on this occasion. Secondly, instead of forming the fluorophore after the two-dimensional chromatographic separation the phenylethylamine zone was eluted in 90% methanol solution (2 ml) containing three drops of concentrated HCl. This eluate was then rotary evaporated to dryness under reduced pressure, dissolved in 0.1 *M* sodium bicarbonate solution and dansylated overnight as previously described[7,8]. The presence of DNS phenylethylamine in the eluate and the demonstration of identical behaviour with authentic DNS phenylethylamine was shown following separation, on precoated layers of silica gel, in numerous different solvent systems[9].

In a final comment about the alloxan reaction we must draw attention to the remarkable sensitivity of the reaction to ozone. Even the slightest exposure to ozone as for instance being in the vicinity of a high-pressure xenon arc after spraying with the alloxan reagent and before heating completely inhibits the reaction.

(7) ANALYSIS OF URINE

Using the technique described in Sections 1 and 5 the free and conjugated urinary phenylethylamine contents in a control population (laboratory and related

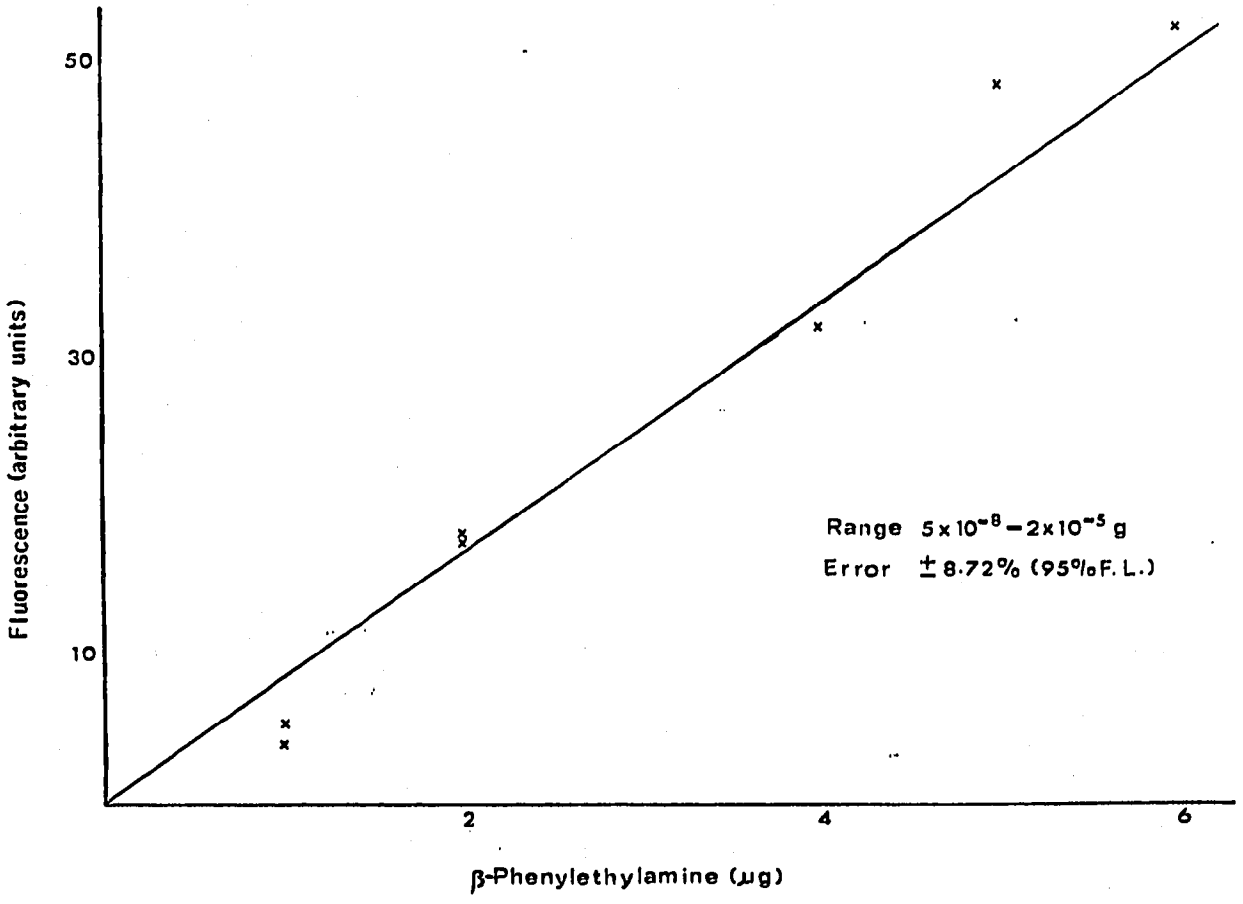


Fig. 3. Typical calibration curve for phenylethylamine.

personnel) and in patients suffering with depression have been determined. A schematic diagram of a typical urine chromatogram is shown in Fig. 2. The actual values obtained in μg/24 h for free and conjugated urinary phenylethylamine in a control population are listed in Table III. In the analysis of twenty patients suffering with various depressive illnesses (manic-depressive depressions and involutional melancholias) we have so far not been able to demonstrate any phenylethylamine at all in the zone corresponding to free urinary phenylethylamine. Whether this means that free phenylethylamine is not excreted in the urine obtained from these patients or whether it is excreted in such small amounts as to be below our detection level of approximately 5 μg/24 h is not at present known. It is clear that the excretion of urinary conjugated phenylethylamine exhibits quite marked person-to-person variation. In the case of the depressive population 43% excreted conjugated phenylethylamine in the range 23–149 μg/24 h. The remaining 57% did not excrete any measurable conjugated phenylethylamine.

DISCUSSION

To our knowledge alloxan has not hitherto been used as a spray reagent in the production of phenylalkylamine fluorophores. As such it seems to possess many

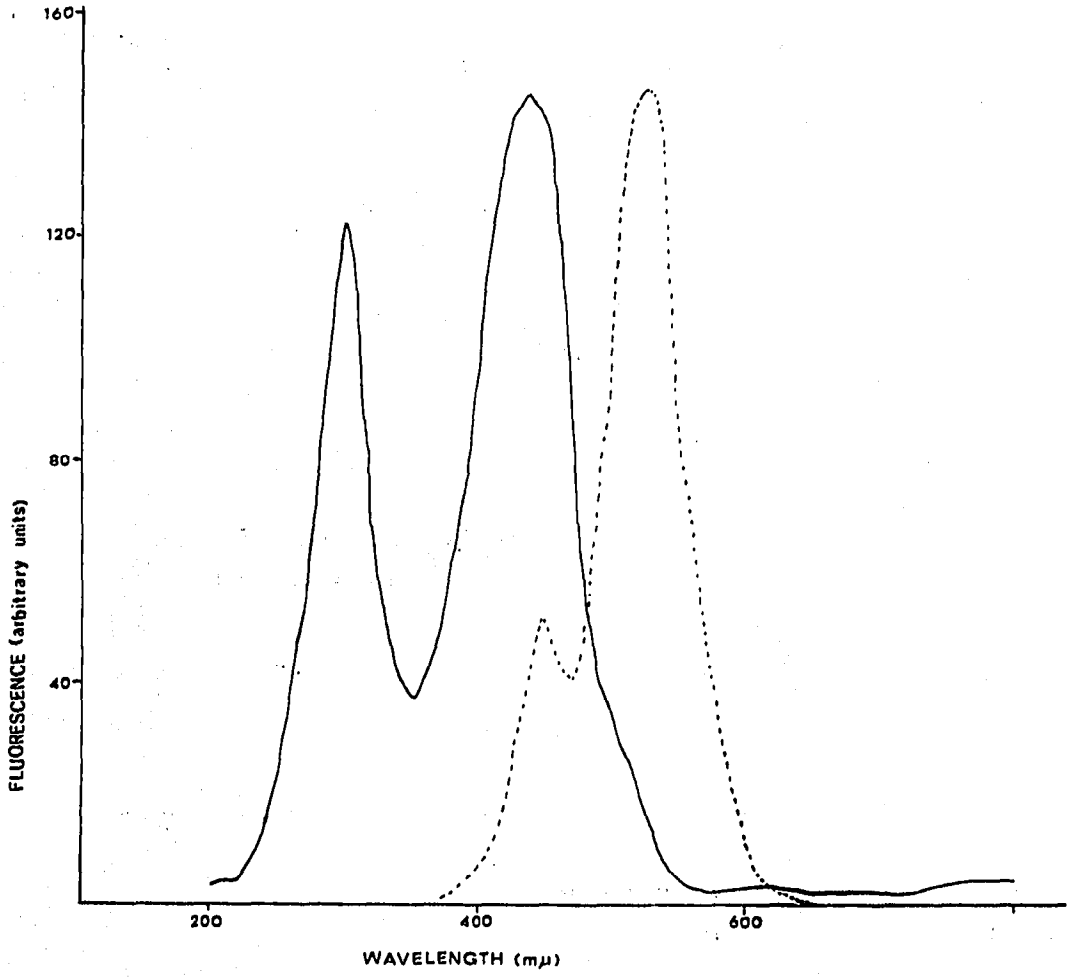


Fig. 4. Activation and emission spectra (uncorrected) of the alloxan–phenylethylamine fluorophore. (——) Activation; (— — —) emission.

useful advantages; it is cheap and commercially available, simple to use, relatively specific to phenylalkylamines, relatively sensitive and capable of producing linear calibration curves in the 0.1–7.5 μg range following two dimensional separation and elution from thin-layer silica gel sheets. That only phenylethylamine is being measured when the technique described in this paper is utilised would seem to be established by virtue of the inherent specificity of a two-dimensional chromatographic separation when this is followed by a relatively selective visualisation procedure. That phenylethylamine is definitely present in the measured fluorescent zone was established by showing that this was the only zone to contain radioactivity on the final chromatogram when ^{14}C-labelled phenylethylamine was added to the urine before extraction. In addition dansylation of the phenylethylamine zone eluate after two-dimensional separation of the urine extract but before spraying with the alloxan reagent followed by chromatographic separation of the formed dansyl derivatives showed that DNS phenylethylamine was present.

Phenylethylamine has been shown to be present in both the free and conjugated forms in the urine of a control population. The level of excretion of the free form—

TABLE III

PHENYLETHYLAMINE EXCRETION IN THE URINE OF A CONTROL POPULATION

Subject	*Phenylethylamine*[a] *(μg/24 h)*	
	Free	*Conjugated*
E.Z.	56	—[b]
A.Z.	18	—
L.Q.	45	—
A.N.	32	—
L.M.	9	—
T.M.	5	—
H.P.	24	—
N.A.	17	—
L.M.	13	119
D.W.	25	365
S.M.	53	128
T.P.	85	45
A.N.	16	34
R.B.	140	[c]
H.H.	35	[c]
J.J.	167	[c]
S.B.	50	82
W.R.	8	[c]
E.S.	93	35

[a] Corrected for recovery.
[b] — = these samples were not analysed.
[c] Contamination in the phenylethylamine fluorophore zone prevented analysis in these cases.

47 ± 44 μg/24 h (n = 19)—is somewhat below the amounts of other free urinary primary aromatic amines[10]—*p*-tyramine, 312 ± 162 μg/g creatinine (n = 31) and tryptamine, 83 ± 36 μg/g creatinine (n = 16). The level of urinary free phenylethylamine estimated by this technique is in quite good agreement with a previous assessment[2].

Levels of conjugated urinary phenylethylamine for the control group are somewhat more difficult to obtain because of contamination on the chromatogram around the phenylethylamine zone. In those urines that were quantitated (n = 7) the amount of phenylethylamine varied widely between subjects, being in the range 34–365 μg/24 h. Other workers seem not to have analysed the conjugated form of this amine. Such a wide variation in excretion values and the difficulty of analysis of conjugated urinary extracts is again similar to the situation obtaining for the other urinary aromatic primary amines[10,11]. The excretion of urinary conjugated *p*-tyramine is in the range 0–3640 μg/g creatinine while tryptamine excretion is in the range 4–98 μg/g creatinine.

The finding of little, if any, urinary free phenylethylamine in patients suffering with depression confirms the observation first made by FISCHER[2]. Whether the excretion is actually nil or merely below our present lower detection level of approximately 5 μg/24 h must await the development of more sensitive and refined techniques. If the excretion were actually zero this would raise the interesting possibility of some enzyme deficiency in this condition. Whether the excretion of urinary free

phenylethylamine appears, increases or returns to normal values following an improvement in the condition as a consequence of remission or therapy is currently under study. The observation that 43% of our depressed patients excreted conjugated phenylethylamine in the range 23–149 μg/24 h is interesting. The remaining 57% either excreted none or else the amount was below our detection level of 5 μg/24 h. Why conjugated phenylethylamine might be present when free phenylethylamine is absent is at present not understood. Efforts aimed towards understanding more about the metabolism of phenylethylamine both in the whole body and in the brain are presently under investigation in our laboratory. It is of interest to note that phenylethylamine has recently been identified as a cerebral constituent of both the rat[8] and man[12].

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