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Note

Determination of noncatecholic phenylethylamines and monomethylated derivatives of phenylethylamine

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There is growing evidence supporting the involvement of noncatecholic phenylethylamines (NCPEA), e.g., phenylethylamine (PEA), phenylethanolamine (OHPEA), the tyramines (TRMs) and octopamines (OCPs) in the pathophysiology of a number of disease states (refs. 1, 2 and references cited therein) including the major psychoses [3, 4], diabetes mellitus [5], Parkinsonism [6], hepatic encephalopathy [7], migraine [8], and phenylketonuria [9, 10]. Elucidation of the mechanisms by which these amines exert their physiological role and pharmacological effects has been, however, slowed by technical problems encountered in the development of relatively simple, sensitive, specific and reproducible methodologies for their determination in biological samples [11—15].

An analysis of the literature shows that despite the variety of techniques used in measuring these compounds such as spectrophotometry [16, 17], chromatography [10, 18—20], enzymology [11, 14, 21], radioimmunoassay [7] and mass spectrometry [13, 15, 22, 23], their actual levels in mammalian fluids and tissues is still a matter of great controversy (refs. 1, 2 and references cited therein). Similar discrepancies can be observed in the reported levels of these amines in invertebrates [11].

Results obtained using either gas—liquid chromatography (GLC), GLC—mass spectrometry or enzymatic isotopic (N-methylation) techniques are being actively reevaluated as several workers have questioned their specificity [11, 14, 15, 24]. These investigators have stressed the need for rigorous isolation of these substances from the original biological sample before their actual measurements [14, 15].

In this paper, we have addressed this problem particularly with regard to the parent NCPEA and to the biologically active monomethylated derivatives of PEA.

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MATERIALS AND METHODS

Reagents, solvents and gases used in these experiments were of the highest purity commercially available and were used without further purification. They were obtained from different sources; PEA (K & K, Labs., Plainview, NY, U.S.A.), the monomethylated PEAs, methylamine and nitroethane (Aldrich, Milwaukee, WI, U.S.A.), OHPEA, OCP, and *p*-TRM (Regis, Chicago, IL, U.S.A.), *m*-TRM (Vega-Fox Biochem., Tucson, AZ, U.S.A.), acetic acid, acetone, ammonia, benzene, chloroform, ethanol and sodium tetraborate (Sigma, St. Louis, MO, U.S.A.), ninhydrin (Fischer Scientific, Chicago, IL, U.S.A.); and 2,4-dinitrobenzenesulfonic acid (DNBS) and phenylacetic acid (PAAc) (Eastman Kodak, Rochester, NY, U.S.A.). Radioactive PEA, *p*-TRM, and *p*-OCP were purchased from New England Nuclear (Boston, MA, U.S.A.) and labeled PAAc was obtained from ICN (Irvine, CA, U.S.A.).

Thin-layer chromatography (TLC) plates were obtained either from Brinkman Inst. (Des Plaines, IL, U.S.A.; systems I and II) or Analtech (Newark, DE, U.S.A.; systems III, IV and V). Samples (5—10 μg standard compounds) were detected after ninhydrin spraying. N-Acetyl PEA was visualized under UV light (254 nm), whereas R_F values for PAAc were obtained by using autoradiography of plates spotted with ^{14}C-labeled acid.

For GLC analysis, whether individual or amine mixtures, these substances were reacted with DNBS and the resultant derivatives injected into a Beckman GC-65 equipped with flame ionization detector and coiled glass (U turn) 0.54 m × 4 mm I.D. column, packed with Diatoport S 80—100 mesh with a liquid OV-17 coating. Samples were run at the following temperatures: injection block, 250°C; detector line, 260°C; detector, 280°C; and oven, 220°C (all samples were run under isothermal conditions). Maintenance of the flame was obtained using hydrogen and air (42 ml/min and 300 ml/min, respectively); the carrier gas was nitrogen (80 ml/min). Amines were derivatized as previously described [18—20, 25, 26]. Briefly, an excess of freshly prepared DNBS reagent (0.25 *M* in saturated tetraborate, pH 7.86) was added to standard amine(s) (range 0.1—500 ng each, 0.001 *N* hydrochloric acid solution in glass test tube with screw top). The tubes were sealed, placed into a boiling water bath and reacted for 15 min. After cooling at room temperature, the corresponding dinitrophenyl (DNP) derivative(s) were extracted into benzene by twice adding 1 ml of benzene, shaking gently for 2 min, and centrifuging at 5000 *g* for 5 min. The organic layers were pooled, evaporated to dryness under a stream of dry nitrogen and the residue redissolved in benzene (10—100 μl) and analyzed by GLC (1—10 μl injections). Areas under peak-response were plotted as a function of amine concentration.

Studies to ascertain the optimal conditions (pH and reaction time) for amine derivatization (100 ng pure compound plus trace amounts of the corresponding labeled amine, either PEA, *p*-TRM or *p*-OCP) were carried out by varying the pH of the amine solution (pH range, 0.76—13.08; pH range of the resultant reaction mixture 0.83—9.94, respectively) and the reaction time (range 5—50 min). The extent of the reaction was followed by TLC of the benzene phase (alumina-gel TLC plates; chloroform—ethanol (9:1); 2 h, 27°C). After identifying the radioactive area corresponding either to unreacted amine or to its

derivative [Packard radiochromatogram scanner; also ninhydrin spraying (amine) or UV light (derivative; 365 nm, yellowish color)] these compounds were scraped off the TLC plate and counted. The stability of the DNP derivatives of the above labeled amines (up to 14 h in benzene solution) was followed in a similar fashion.

RESULTS

As can be seen from Table I, the basic NCPEA, PEA, OHPEA, *p*-TRM and *p*-OCP can be separated from each other using either one of the TLC systems I—IV. All of these compounds reacted promptly with ninhydrin giving either a reddish-blue (PEA and *p*-TRM) or reddish-gray (OHPEA and *p*-OCP) coloured spot. Separation of the *m*- and *p*-TRM isomers is better accomplished by using system II. As could be expected from their chemical structures the monomethylated derivatives of PEA behave quite similarly in most TLC systems. System III provides, however, a clear separation for PEA and N-MePEA, whereas TLC system II shows significantly different R_F values for PEA, N-MePEA and *o*-MePEA. In this system *p*- and α-MePEA behave as PEA itself, whereas β-MePEA has an R_F value similar to that of *o*-MePEA. Table I shows a range for the R_F

TABLE I

TLC OF PHENYLETHYLAMINE AND RELATED COMPOUNDS

System I: cellulose-coated (0.1 mm thickness) glass plates. Solvent system: nitroethane—acetic acid—water (9:2.8:1.2), 2 h, 30°C.
System II: silica gel-coated glass plates (0.25 mm thickness, activated at 110°C for 40 min). Solvent system: acetone—1 *N* ammonia (10:3), 3—4 h, room temperature.
Systems III, IV and V: microcellulose-coated glass plates (0.25 mm thickness). Solvent systems were, respectively, nitroethane—acetic acid—water (45:14:16) 37°C; *tert.*-amyl alcohol—40% methylamine—water (8:1:1) and 96% ethanol—concentrated ammonia (20:1).
Amines were detected after spraying with ninhydrin; 0.2% in ethanol, at 100°C for 10 min.

Compound	R_F value				
	System I	System II	System III	System IV	System V
β-PEA	0.75—0.80	0.62—0.68	0.45—0.50	0.92—0.96	0.87—0.91
N-MePEA	0.79—0.83	0.20—0.24	0.75—0.80	—	—
o-MePEA	0.80—0.82	0.72—0.74	—	—	—
p-MePEA	0.81—0.82	0.62—0.64	—	—	—
α-MePEA	0.79—0.82	0.67—0.69	—	—	—
β-MePEA	0.79—0.81	0.71—0.73	—	—	—
OHPEA	0.67—0.69	0.82—0.84	0.35—0.40	0.80—0.84	0.86—0.88
p-Tyramine	0.51—0.61	0.54—0.61	0.23—0.28	0.66—0.70	0.82—0.86
m-Tyramine	0.60—0.62	0.63—0.65	—	—	—
p-Octopamine	0.43—0.45	0.78—0.80	0.18—0.20	0.50—0.53	—
N-Acetyl PEA*	0.89—0.91	0.87—0.89	—	—	—
PAAc**	0.95—0.97	0.77—0.78	0.01—0.02	0.01—0.02	0.29—0.35

*Visual detection under UV light (254 nm) on plates containing a fluorescent dye background.
**Detected by autoradiography utilizing [^{14}C]phenylacetic acid.

value of each amine in the various TLC systems, which possibly reflects the different sample composition. Similarly to the parent compound, PEA, these substances were easily detected with ninhydrin (reddish blue spot; β-MePEA gave a brown-charcoal colouration). R_F values for PAAc and N-acetyl PEA, the main PEA metabolites, are included for comparison.

Table II shows the relative and absolute retention times for DNP derivatives of PEA and related compounds. Non-derivatized amines are combusted along with the benzene solvent and therefore do not interfere with the DNP-amine peaks. The DNP derivatives of monomethylated PEA showed consistently a

TABLE II

GLC OF PHENYLETHYLAMINE AND RELATED COMPOUNDS*

Compound	Time** (min)	Relative retention time***
β-PEA	44	1.00
N-MePEA	20	0.45
o-MePEA	23	0.53
β-MePEA	29	0.66
α-MePEA	33	0.75
p-MePEA	35	0.79
OHPEA	97	2.23
m-Tyramine	148	3.36
p-Tyramine	160	3.64
p-Octopamine	260	5.91

*After reaction with 2,4-dinitrobenzenesulfonic acid (DNBS). Non-derivatized amines come off with the solvent peak.
**Retention times have a variation of no more than ± 2%.
***Relative to DNP-phenylethylamine (DNP-PEA).

shorter retention time than PEA itself, whereas the derivatized products of OHPEA, *m*- and *p*-TRM and *p*-OCP have a retention time substantially longer than DNP-PEA. When analyzed in mixture these compounds were easily distinguishable from each other. Conditions for derivatization were found to be optimal when the reaction mixture was boiled for 15 min at pH 7.74 (range of 92—98%, 73—87% and 70—78% completion respectively, for PEA, *p*-TRM and *p*-OCP). These derivatives were stable in benzene for at least 14 h at room temperature. This GLC technique could detect as little as 0.5 ng of the amines studied and the detector response was linear (area under the peak) for the range of concentrations studied (0.1—500 ng).

DISCUSSION

A growing body of clinical and animal observations suggest a role for the NCPEA in body function and dysfunction (refs. 1, 2 and references cited therein). Several workers have proposed the use of the levels of these amines in biological samples (blood, urine, cerebrospinal fluid) as markers for a number of diseases, specially neuropsychiatric disorders [3, 6, 16, 27, 28]. Initial work in this field, mostly involving the basic NCPEA, PEA, *p*-TRM, *p*-OCP and OHPEA, has now been extended to include other related amines showing

biological activity. Similar to their parent compounds, some of these derivatives are endogenous substances, e.g., the *o*- and *m*-isomers of TRM [13] and OCP [12], and N-MePEA [29] whereas others, e.g., α-, *o*-, *m*- and *p*-MePEA, do not appear to be normally present in the species so far studied. However, the possibility of their formation in pathological states should not be entirely discarded. In fact, we have recently described rather striking behavioral and analgesic properties for these monomethylated PEAs [30, 31]. Although work on these substances, which easily cross the blood—brain barrier and are rapidly metabolized by MAO type B [31, 32], is only of a preliminary nature, these studies may be crucial for the understanding of the mechanisms involved in some of the actions of α-methyl PEA (amphetamine) and of PEA itself. So far, only N-MePEA has been shown to be present in the brain of pargyline administered rabbits, and to be synthesized in vivo by rabbit liver and brain preparations [29]. However, one should keep in mind that perhaps other monomethyl derivatives of PEA could also be present in mammalian tissues.

Progress in the elucidation of the possible physiological role and pharmacological actions of these compounds has been slowed by several factors, such as the behavioral and toxic effects reported after their administration to laboratory animals, which appears to be also the case for humans [1, 2, 30, 31], their short half-life [32, 33] and technological problems involved in their measurement in biological samples (refs. 1, 2 and references cited therein). Earlier techniques, e.g., visual estimation from spots on thin-layer or paper chromatograms [34], UV absorption or fluorescence spectrophotometry [17, 35], have been substituted by newer methodologies claimed to be highly sensitive and specific. However, and despite the elaborate amine separation and quantitation procedures involved in some of these assays, e.g., integrated ion-current mass spectroscopy [23], GLC—mass fragmentography [22], enzymatic isotopic assay [21], derivatization followed by GLC [19], and radioimmuncassay [36], the actual levels of noncatecholic PEAs in biological samples remain a conflictive issue [1, 2].

There is agreement among most workers in the field that the main remaining problem with these techniques is specificity [11, 13—15, 21, 22, 24, 36]. For example, accurate estimation of either *p*-TRM or of *p*-OCT in the presence of their *o*- and *m*-isomers and/or their N- and N,N-methylated derivatives has proven to be a formidable task [14, 15]. The same can be said for the methylated derivatives of PEA and OHPEA [29].

Using a combination of TLC plates and solvent systems, we have been able to clearly separate from each other the four endogenous, biologically active basic NCPEA as well as *m*-TRM and N-MePEA (Table I). These systems also allowed for the separation of the different monomethylated PEAs suggesting their possible use to isolate the methylated derivatives of the other basic NCPEA.

Isolation of these compounds from biological samples by selective solvent extraction procedures [17, 26, 29, 37], followed by further separation by TLC, derivatization and GLC analysis provides a relatively simple, specific, sensitive and reproducible technique to determine their concentration in biological samples. GLC analysis of DNP derivatives of a number of related amines and amino acids, e.g., dopamine, epinephrine, norepinephrine, serotonin, *p*-chloro PEA,

phenylalanine, tyrosine and others, show that these substances do not interfere with the accurate estimation of the compounds used in this study [26] (Table II). When using the correct derivatization pH, reaction time and temperature, the corresponding derivatives are produced with consistent, relatively high yields (see Results). These conditions are critical as small variations in the pH of the reaction mixture or increased reaction times (> 20 min) result in sharply lower yields of DNP-amine derivatives [23]. With the use of appropriate radioactive internal standards to correct for amine recoveries from biological samples and completeness of DNP-derivatization, the present technique could prove a useful tool in the elucidation of the biological role of NCPEA.

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