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ESTIMATION OF β-3,4-DIMETHOXYPHENETHYLAMINE AND RELATED COMPOUNDS IN URINE EXTRACTS BY RADIOIMMUNOASSAY*

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Abstract—Antibodies to β-3,4-dimethoxyphenethylamine (DMPEA) were produced by immunizing rabbits with conjugates of *N*-succinyl-DMPEA coupled to poly L-lysine. *N*-(3′,4′-dimethoxyphenethyl)-4-hydroxyphenylacetamide was synthesized, iodinated to high specific activity, and used to develop a sensitive and specific radioimmunoassay (RIA) capable of measuring 100 pg DMPEA. While most of the binding energy of the antisera is directed toward the 3,4-dimethoxyphenyl nucleus, structural changes in the ethylamine side chain are also recognized. High pressure liquid chromatography was used in conjunction with RIA to identify the immunologically active material. In the chloroform extracts of six urine samples, all of this material cochromatographed with [^{3}H]DMPEA. In four other samples, the immunologically active component(s) did not correspond to DMPEA or to the metabolites that were tested. It is evident that normal individuals excrete different amounts of DMPEA.

Friedhoff and van Winkle [1] reported that β-3,4-dimethoxyphenethylamine (DMPEA) was present in the urine of fifteen out of nineteen schizophrenics but absent in the urine of fourteen normal controls. A plethora of papers appeared in the literature during the next 10 years, attempting to clarify the role of DMPEA in mental disease (for a review, see Wyatt *et al.* [2] and Boulton [3]). The "pink spot test" (staining with a ninhydrin-pyridine reagent followed by a modified Ehrlich's reagent [1]) was used to identify DMPEA, but in some studies, the area corresponding to authentic DMPEA in the chromatogram was eluted and identified by more rigorous means, such as mass spectrometry [4, 5], gas chromatography [6], or fluorescence [7–9]. Even among these investigations, there were conflicting results. In addition, von Studnitz and Nyman [10] and others [2, 11–18] suggested that DMPEA either was an artifact of food intake or originated from the intestinal flora.

In 1969, Van Vunakis *et al.* [19] reported the production of rabbit antibodies specific to DMPEA. With their complement fixation assay, 500 ng DMPEA could be detected. Since that time, a sensitive and specific radioimmunoassay—capable of measuring 100 pg of this compound—has been developed. The specificity of the antisera is such that many of the substances incorrectly identified as DMPEA (e.g. tyramine and acylcadaverines) do not interfere with this test. However, because other compounds having a 3,4-dimethoxyphenyl nucleus can cross-react with the antibodies, liquid chromatography was used in conjunction with the radioimmunoassay to identify the immuno-reactive material.

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MATERIALS AND METHODS

DMPEA and related compounds. DMPEA was purchased from Aldrich Chemical Co. [^{3}H]-DMPEA (sp. act., 19 mCi/m-mole) was purchased from New England Nuclear. 2,5-Dimethoxy-4-methylamphetamine (DOM) was the gift of Dr. S. H. Snyder of the Johns Hopkins University. *N*-methyl-mescaline, *N*,*N*-dimethyl-mescaline and 3,5-dimethoxyphenethylamine were the generous gifts of Dr. W. E. Scott of Hoffmann-LaRoche Pharmaceutical Co. Except for the compounds that were synthesized, all chemicals were obtained from the same sources listed in Van Vunakis *et al.* [20], were reagent grade, and used without further purification.

The following compounds were prepared according to published directions: *N*-acetyl-DMPEA [21], 3,4-dimethoxyphenylisopropylamine [22], β-dimethylamino-3,4-dimethoxypropiophenone [23], β-piperidyl-3,4-dimethoxypropiophenone [23], and *N*-acylcadaverine [17]. In each case, the melting point agreed with the reported value and the product was homogeneous by thin-layer chromatography in one or more solvent systems.

β-Pyrrolidyl-3,4-dimethoxypropiophenone-HCl. To a mixture of 8·1 g of 3,4-dimethoxyacetophenone (45 m-moles) and 1·83 g paraformaldehyde (61 m-moles) was

added 7·2 ml of 0·15 M hydrochloric acid in 95% ethanol and 5·0 ml pyrrolidine in 5·0 ml concentrated hydrochloric acid. The reaction mixture was refluxed for 24 hr. Acetone (40 ml) was added and the solution was cooled at 4° for 2 weeks. The precipitate was collected and dried at 25° (0·2 mm) to give 1·3 g (10%) β-pyrrolidyl-3,4-dimethoxypropiophenone-HCl, m.p. 190–192°.

Anal. Calcd. for $C_{15}H_{22}NO_3Cl$: C, 60·20; H, 7·36; N, 4·68. Found: C, 60·10; H, 7·17; N, 4·61.

Succinylated DMPEA–poly L-*lysine.* To a 10 mg solution of *N*-succinyl-DMPEA in 0·5 ml distilled water was added poly L-lysine-HBr (10 mg, mol. wt 100,000) in 0·5 ml distilled water. 1-Ethyl-3-(dimethylaminopropyl)-carbodiimide hydrochloride (10 mg, CDI) was added and the reaction mixture allowed to stand at room temperature overnight. It was then dialyzed exhaustively against 0·15 M sodium chloride–0·005 M phosphate buffer, pH 7·0.

Immunization. The protocol for the production of rabbit antibodies is similar to that already described [19].

Labeled hapten. N-(3′,4′-dimethoxyphenethyl)-4-hydroxyphenylacetamide was prepared by reacting DMPEA (90·5 mg, 0·5 m-mole) with an equimolar amount of *p*-acetoxyphenylacetic acid chloride [24] (106 mg) and triethylamine (21·5 mg) in 10 ml benzene. The solvent was evaporated; the residue was redissolved in ethanol, and an aliquot was chromatographed on precoated Silica gel G plates with fluorescent indicator using ethylacetate as solvent. An ultraviolet scan of the plate revealed one major spot (R_f = 0·44) which migrated differently from starting materials (R_f = 0·04 for DMPEA and R_f = 0·74 for *p*-acetoxyphenylacetic acid chloride) and which, after exposure to ammonium hydroxide vapors, gave a positive test with 1-nitroso-2-naphtholnitric acid [25]. A second aliquot was purified as described above. The spot (R_f = 0·44) was eluted with ethanol and concentrated *in vacuo*. After hydrolysis with ammonium hydroxide–ethanol (1:1) for 30 min, the solvent was evaporated to dryness *in vacuo* and the residue redissolved in distilled water. Its absorption spectrum showed a base shift at pH 12 due to the presence of the phenolic hydroxyl and a λ_{max} at 278 nm corresponding to the absorption maximum of DMPEA (Fig. 1). This derivative was iodinated with $Na^{125}I$ according to the procedure of Greenwood *et al.* [26] to a sp. act. of 10 Ci/mmole.

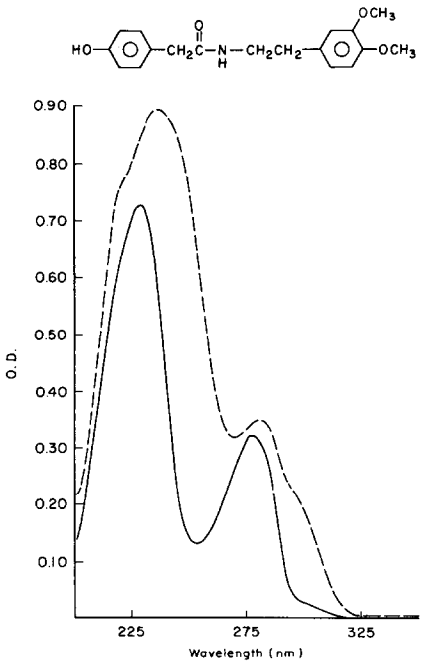


Fig. 1. Absorption spectrum of *N*-(3′,4′-dimethoxyphenethyl)-4-hydroxyphenylacetamide. Fifty-three μg/ml in ethanol (——) and in ethanolic NaOH, pH 12 (– – – –).

Radioimmunoassay procedure. The method used for radioimmunoassay of DMPEA is analogous to the double antibody technique for mescaline and 2,5-dimethoxy-4-methylamphetamine as described by Riceberg *et al.* [27].

Urine samples. Twenty-four-hr samples were obtained from 10 normal individuals working in the laboratory. To extract neutral and basic components, the pH of 275 ml urine was adjusted to 10·5 with 10 N sodium hydroxide. A known amount of [^{3}H]DMPEA was added and the sample extracted with chloroform using the continuous extraction apparatus described by Hershberg and Wolfe [28]. After 24 hr, at least 95 per cent of the radioactivity was found in the organic layer. The chloroform was evaporated to dryness and the residue was redissolved in 5·0 ml distilled water.

High pressure liquid chromatography. The urine extract was applied to an analytical diphenyl corasil column (2 ft × 0·125 in.) using methanol–0·1% ammonium carbonate (65: 35) as solvent at a flow rate of 1·0 ml/min (Waters Associates Liquid Chromatograph, model 202). One-half-ml fractions were collected, evaporated to dryness in a vacuum oven at 40° and redissolved in iso-Tris buffer (0·01 M Tris HCl–0·15 M sodium chloride, pH 7·5). Aliquots of each fraction were counted to determine the position of [^{3}H]-DMPEA and were analyzed for DMPEA by radioimmunoassay. Passage of the urine extracts through the same column using a second solvent system [methanol–water (1:9)] separated some of those compounds which were not resolved by chromatography in the first solvent system.

The chromatographic characteristics of several related compounds were determined by passing known samples through the column and relating V_e (the elution volume at peak height) to V_o (the void volume).

RESULTS

Sensitivity and specificity of the radioimmunoassay. The binding between the [^{125}I]-antigen and anti-DMPEA at different antibody dilutions is shown in Fig. 2A. The dilution of antibody precipitating

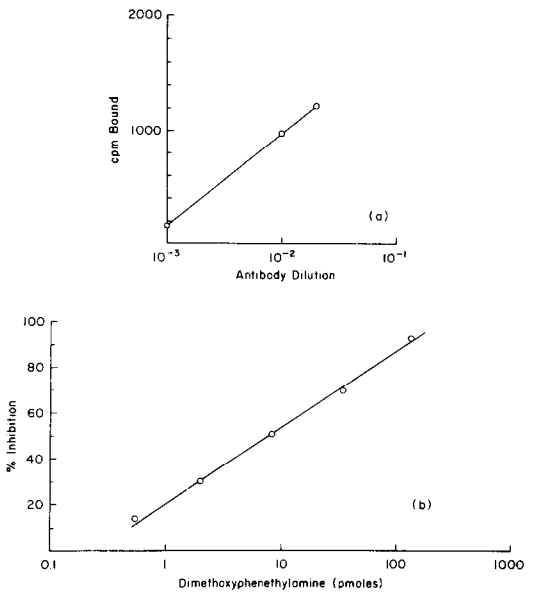


Fig. 2. Binding and inhibition characteristics of the [^{125}I]DMPEA* anti-DMPEA system. (a) The DMPEA antiserum (Ra 731 c-6) produced to the succinylated DMPEA–CDI–poly L-lysine conjugate was titred against the [^{125}I]-antigen (20,000 cpm/reaction mixture). (b) A dilution of antibody (1/50) precipitating 1500 cpm was chosen for assays. *[^{125}I]-*N*-(3′,4′-dimethoxyphenethyl)-4-hydroxyphenylacetamide.

1500 cpm (i.e. 1/50) was used to obtain the standard inhibition curve (Fig. 2B). Under these conditions, 9 pmoles (1·6 ng) DMPEA gives 50 per cent inhibition and as little as 600 femtomoles (100 pg) of this compound can be measured.

The ability of several other compounds to compete for the antibody is shown in Tables 1 and 2. While a large part of the binding energy is directed toward the 3,4-dimethoxyphenyl nucleus, there is some recognition of structural changes in the ethylamine side chain. Changing the length of the side chain (e.g. 3,4-dimethoxyaniline and 3,4-dimethoxybenzylamine) does not dramatically affect the amount of inhibition compared to DMPEA. Derivatives which are *N*-methylated, *N*-acetylated or alpha-methylated are two to four times more effective as inhibitors. The amino acid derivative of DMPEA inhibits to approximately the same extent as DMPEA, whereas the carboxy derivative (3,4-dimethoxyphenylacetic acid) is 100 times less effective. Mescaline, which has an additional methoxy group in the 5 position, gives 50 per cent inhibition at a level 100 times higher than DMPEA. Catecholamine metabolites (e.g. normetanephrine, 3-methoxytyramine and vanillylmandelic acid) and monoacetylated cadaverine are generally at least 5000 times less effective than DMPEA.

DMPEA and related compounds in urine. Since the concentration of DMPEA in some urine samples was too low to measure directly in the radioimmunoassay without encountering non-specific interference, continuous extraction with chloroform was used to isolate and concentrate this compound along with other immunologically reactive material.

Measurable quantities of compounds reacting with the antibodies to DMPEA were found in all the extracts. The levels of DMPEA equivalents varied widely, between 2·6 μg and 1·2 mg/24-hr specimen. (Although much of the binding energy of the antibodies is directed toward the 3,4-dimethoxyphenyl nucleus, differences in the side chain do affect the inhibition within small limits. Therefore, in analyses of physiological fluids, where different reactive compounds may be encountered, the results are expressed as "DMPEA equivalents" rather than as DMPEA.)

For more rigorous identification of the active materials, high pressure liquid chromatography was used. The relative elution volumes (V_e/V_0) of DMPEA and other known cross-reacting derivatives are listed in Table 3. DMPEA is effectively separated from all controls except for overlap with 3,4-dimethoxyphenylisopropylamine. The immunological analyses of control samples of *N*-acetyl-DMPEA and DMPEA (extracted, concentrated and separated by liquid chromatography) indicated greater than 90 per cent recovery of the added material.

Figures 3 and 4 show the liquid chromatograms of two representative urine samples (A.L.-1 and L.R.-1 respectively). In urine A.L.-1, 85 per cent of the counts contributed by [^{3}H]-DMPEA (see Methods) was recovered after liquid chromatography in fractions 11–14. Radioimmunoassay of the individual fractions from the chromatogram shows activity only in this area corresponding to [^{3}H]-DMPEA, and essentially all of the immunologically reactive material applied to the column could be accounted for in the DMPEA

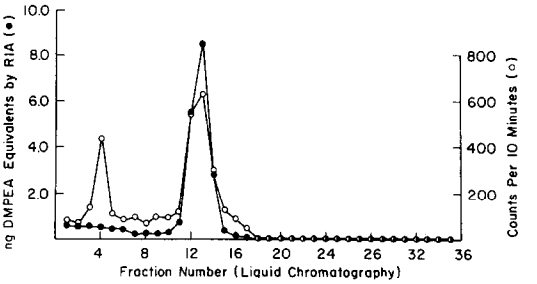
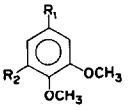


Fig. 3. Inhibition of the [^{125}I]DMPEA anti-DMPEA system by urine extract (A.L.-1). An aliquot of a continuously extracted urine sample was fractionated by liquid chromatography. The fractions were dried *in vacuo* and redissolved in buffer. An aliquot (0·1 ml) was analyzed for 3,4-dimethoxyphenyl compounds by radioimmunoassay (RIA) (●) and for [^{3}H]DMPEA (○) which had been added to the sample prior to extraction and which served as an internal control. (An impurity in the [^{3}H]DMPEA preparation migrated with the solvent front. Its concentration was the same before and after the extraction procedure.) The emergence of DMPEA in fractions 11–14 has been confirmed using unlabeled DMPEA.

Table 1. Inhibition values for β-3,4-dimethoxyphenethylamine and related compounds as measured by radioimmunoassay with an [^{125}I]-antigen



Compound	R_1	R_2	Nmoles required for 50% inhibition
3,4-Dimethoxyphenethylamine (homoveratrylamine)	$-CH_2CH_2NH_2$	$-H$	0·009
N,*N*-dimethyl-3,4-dimethoxyphenethylamine	$-CH_2CH_2N(CH_3)_2$	$-H$	0·002
N,*N*,*N*-trimethyl-3,4-dimethoxyphenethylammonium iodide (*O*,*O*-dimethylcoryneine iodide)	$-CH_2CH_2N^+(CH_3)_3I^-$	$-H$	0·016
3,4-Dimethoxybenzylamine (veratrylamine)	$-CH_2NH_2$	$-H$	0·025
3,4-Dimethoxyaniline	$-NH_2$	$-H$	0·010
N-acetyl-3,4-dimethoxyphenethylamine	$-CH_2CH_2NHCOCH_3$	$-H$	0·004
α-Methyl-3,4-dimethoxyphenethylamine	$-CH_2CHCH_3NH_2$	$-H$	0·004
3,4-Dimethoxyphenylalanine	$-CH_2CH(NH_2)COOH$	$-H$	0·015
3,4-Dimethoxyphenylacetic acid (homoveratric acid)	$-CH_2COOH$	$-H$	1·1
3,4-Dimethoxybenzoic acid (veratric acid)	$-COOH$	$-H$	1·0
β-Dimethylamino-3,4-dimethoxypropiophenone	$-COCH_2CH_2N(CH_3)_2$	$-H$	0·028
β-(1-Piperidyl)-3,4-dimethoxypropiophenone	$-COCH_2CH_2N$⬡	$-H$	0·025
β-(1-Pyrrolidyl)-3,4-dimethoxypropiophenone	$-COCH_2CH_2N$⬡	$-H$	0·025
3,4,5-Trimethoxyphenethylamine (mescaline)	$-CH_2CH_2NH_2$	$-OCH_3$	1·0
N-methyl-3,4,5-trimethoxyphenethylamine	$-CH_2CH_2NHCH_3$	$-OCH_3$	2·0
N,*N*-dimethyl-3,4,5-trimethoxyphenethylamine	$-CH_2CH_2N(CH_3)_2$	$-OCH_3$	2·0

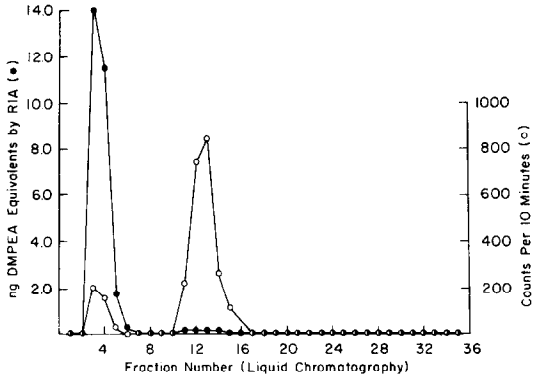
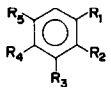


Fig. 4. Inhibition of the [^{125}I]DMPEA anti-DMPEA system by urine extract (L.R.-1). Procedures and symbols are the same as those described in Fig. 3.

peak. Urine samples from five other donors gave qualitatively similar results.

In urine L.R.-1 (and three other samples), however, radioimmunoassay shows that fractions 3–5 contain essentially all of the DMPEA immunoreactive material. The internal control ([^{3}H]-DMPEA) again assayed in fractions 11–14. Among the known derivatives which were tested, 3,4-dimethoxyphenylacetic acid, 3,4-dimethoxybenzoic acid, 3,4-dimethoxyphenylalanine and *N*-acetyl-3,4-dimethoxyphenethylamine would emerge at the solvent front in methanol–0·1% ammonium carbonate (65:35). Therefore, a second chromatogram with a different solvent system was run to further identify the immunologically reactive material. Radioimmunoassay of the urine L.R.-1 fractionated using methanol–water (1:9) did not correspond to any one of the compounds listed in Table 3.

Table 2. Inhibition by substituted phenyl compounds as measured by radioimmunoassay with an [^{125}I]-antigen



Compound	R_1	R_2	R_3	R_4	R_5	nmoles Giving less than 10% inhibition
p-Methoxyphenethylamine	$-CH_2CH_2NH_2$	−H	−H	$-OCH_3$	−H	30*
m-Methoxyphenethylamine	$-CH_2CH_2NH_2$	−H	$-OCH_3$	−H	−H	14*
4-Hydroxy-3-methoxyphenethylamine	$-CH_2CH_2NH_2$	−H	$-OCH_3$	−OH	−H	17*
dl-m-O-methylepinephrine (*dl*-metanephrine)	$-CHOHCH_2NHCH_3$	−H	$-OCH_3$	−OH	−H	50
dl-m-O-methylnorepinephrine (*dl*-normetanephrine)	$-CHOHCH_2NH_2$	−H	$-OCH_3$	−OH	−H	46
4-Hydroxy-3-methoxybenzoic acid (vanillic acid)	−COOH	−H	$-OCH_3$	−OH	−H	60
dl-4-Hydroxy-3-methoxymandelic acid (*dl*-vanillylmandelic acid)	−CHOHCOOH	−H	$-OCH_3$	−OH	−H	51
3,5-Dimethoxyphenethylamine	$-CH_2CH_2NH_2$	−H	$-OCH_3$	−H	$-OCH_3$	45
3,4-Methylenedioxyphenylisopropylamine	$-CH_2CH(CH_3)NH_2$	−H	$-O-CH_2-O-$		−H	47
p-Hydroxyphenethylamine (tyramine)	$-CH_2CH_2NH_2$	−H	−H	−OH	−H	50
N,N,N-trimethyl-*p*-hydroxyphenethylammonium iodide	$-CH_2CH_2N^+(CH_3)_3I^-$	−H	−H	−OH	−H	50
4-Methyl-2,5-dimethoxyphenylisopropylamine (DOM)	$-CH_2CH(CH_3)NH_2$	$-OCH_3$	−H	$-CH_3$	$-OCH_3$	6·6*
dl-α-Methylphenethylamine	$-CH_2CH(CH_3)NH_2$	−H	−H	−H	−H	69

* Fifty per cent inhibition at this level.

Table 3. Relative elution volumes (V_e/V_o) of β-3,4-dimethoxyphenethylamine and related compounds as determined by liquid chromatography

Compounds	Methanol–0·1% ammonium carbonate (65:35) (V_e/V_o)	Methanol–water (1:9) (V_e/V_o)
3,4-Dimethoxyphenethylamine	11·0	>25·0
N-acetyl-3,4-dimethoxyphenethylamine	1·0	2·5
3,4-Dimethoxyphenylacetic acid	1·0	1·25
3,4-Dimethoxybenzoic acid	1·0	1·25
3,4-Dimethoxybenzaldehyde	1·25	3·0
3,4-Dimethoxyphenethanol	1·0	2·0
3,4-Dimethoxyphenylalanine	1·0	1·25
3,4-Dimethoxyphenylisopropylamine	10·5	>25·0
β-Dimethylamino-3,4-dimethoxypropiophenone	12·5	
β-(1-Piperidyl)-3,4-dimethoxypropiophenone	13·5	
β-(1-Pyrrolidyl)-3,4-dimethoxypropiophenone	20·0	
Tyramine	9·5	
3-Methoxytyramine	10·0	
Metanephrine	8·75	
Normetanephrine	6·0	
Epinephrine	6·5	

DISCUSSION

The radioimmunoassay is capable of measuring 100 pg DMPEA which is at least 1/5000 the amount previously required for identification and quantitation by gas chromatography [6] or fluorescence [7–9]. Structurally unrelated compounds such as variously substituted cadaverines and tyramine which were not distinguished from DMPEA on the basis of paper chromatography and the "pink spot test" do not interfere with this assay. Substances closely related to DMPEA (i.e. those that possess the 3,4-dimethoxy nucleus) react with the antibody, but when necessary, DMPEA can be effectively separated from such derivatives by liquid chromatography prior to quantitation by radioimmunoassay.

Invariably we have found material in urine which reacts with the antibody to DMPEA. The levels of DMPEA equivalents ranged from 2·6 μg to 1·2 mg/24-hr specimen. In six of ten urine extracts which had been fractionated by high pressure liquid chromatography, all of the immunologically reactive material was present in a peak which had the same relative elution volume as authentic DMPEA. In four other samples, however, all of the immunologically reactive material eluted with the solvent front. *N*-acetyl DMPEA and 3,4-dimethoxyphenylacetic acid, which are normal metabolites of DMPEA [29], also emerge in the void volume when methanol–0·1% ammonium carbonate (65:35) is used as solvent. A further fractionation of the DMPEA-like material with a second solvent system (methanol–water, 1:9) indicated that the material was different from the compounds listed in Table 3. β-Piperidyl-3,4-dimethoxypropiophenone, β-dimethylamino-3,4-dimethoxypropiophenone and β-pyrrolidyl-3,4-dimethoxypropiophenone, implicated in the metabolism of methyl eugenol [30–32], have significantly different retention times and are, therefore, not present in these extracts. The immunologically active compound(s) in urine L.R.-1 has still to be identified.

From our results, it is apparent that different individuals excrete different amounts of DMPEA. The origin of DMPEA in body fluids has been disputed. Some sources suggest that it is derived from plant matter. Stabeneau *et al.* [18] found that normal subjects who drank a liter of tea in 24 hr were DMPEA positive by the "pink spot test," while the same individuals fed glucose, citric acid and water had no DMPEA in their urine. Friedhoff *et al.* [33], however, have refuted these results. In our laboratory, DMPEA itself was not found in an aqueous Soxhlet extract of tea, although compounds which reacted with the [^{125}I]-DMPEA* anti-DMPEA system were present. The specific and sensitive radioimmunoassay can be used to quantitate picogram amounts of 3,4-dimethoxyphenyl compounds and, in combination with proper extraction and liquid chromatographic techniques, can be used to estimate and identify DMPEA and related compounds in urine and in foodstuffs.

* [^{125}I]-*N*-(3′,4′-dimethoxyphenethyl)-4-hydroxyphenylacetamide.

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