QUENCHING EFFECTS OF VARIOUS COMPOUNDS ON FLUOROMETRIC ASSAY OF TRYPTOPHAN

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Received for publication August 25, 1979

Quenching effects of 26 kinds of compounds on the fluorometric assay of tryptophan were studied. Investigated compounds were monoamines, their precursors and metabolites, and drugs used in the study of monoamines. The fluorescence of 1 μ g of tryptophan was decreased in the presence of the same amount of L-DOPA, catecholamines, DOPAC, 5-HTP, 5-HT, MK 486 or Ro 4-4602 when these samples were prepared in 0.1 M phosphate buffer (pH 7.4) and estimated by the method of Denckla and Dewey. The degree of quenching was varied according to the diluent of the compounds and tryptophan. These results suggest that the direct application of the ultrafiltrate for the assay of free tryptophan might be quenched after administration of drugs. The decreased fluorescence of tryptophan by above compounds, except 5-HTP, was recovered after introducing separation procedure of tryptophan by the Dowex column.

INTRODUCTION

The synthesis of serotonin (5-hydroxytryptamine, 5-HT) in the central nervous system is regulated not only by the activity of the rate-limiting enzyme, tryptophan hydroxylase, but also by the concentration of tryptophan, since the enzyme is normally unsaturated with its substrate (Eccleston et al, 1965 ; Green and Sawyer, 1966 ; Moir and Eccleston, 1968 ; Jequier et al, 1969 ; Tagliamonte et al, 1971 ; Curzon et al, 1972). The brain concentration of tryptophan depends on the plasma level of the amino acid and on its transport rate into the brain (Eccleston et al, 1965 ; Oldendorf, 1971 ; Fernstrom and Wurtman, 1972).

In recent years, the significance of plasma levels of free and total (free + albumin bound) tryptophan on the cere-

bral metabolism of 5-HT has been investigated (Fernstrom and Wurtman, 1971 ; Knott and Curzon, 1972 ; Tagliamonte et al, 1973 ; Curzon and Knott, 1974 ; Brase and Loh, 1975 ; Wurtman and Fernstrom, 1976 ; Gál et al, 1978). Tryptophan in the plasma is most often determined by the fluorometric method of Denckla and Dewey (1967). The method is based on formation of the fluorophore, norharman, from tryptophan by condensation with formaldehyde and then oxidation with ferric chloride in the acid solution at 100°C for 1 hr. Usually, this reaction has been applied directly for an assay of tryptophan, i.e., free tryptophan in the ultrafiltrate of plasma and total tryptophan in the deproteinized plasma after the treatment with trichloroacetic acid or perchloric acid (PCA) (Fernstrom and Wurtman, 1971 ; Curzon et

al., 1972; Knott and Curzon, 1972; Eccleston, 1973; Tagliamonte et al, 1973; Mason et al, 1978). However, the direct application means that tryptophan is assayed in crude, only deproteinized extract from plasma, thus the assay, especially that of free tryptophan, might be quenched by such compounds in the plasma as produced after various treatments. Present study was undertaken to investigate how the fluorescence intensity of tryptophan was influenced by presence of various compounds, monoamines, their precursors and metabolites, and drugs generally used in the study of monoamines.

MATERIALS AND METHODS

Standard solution of tryptophan. L-tryptophan (WAKO PURE CHEMICAL INDUSTRIES, LTD.) was prepared at a concentration of 100 $\mu\text{g/ml}$ in 0.1 N NH_4OH and stored in a refrigerator up to a month.

Tested compounds. L-phenylalanine, L-tyrosine, histamine, tyramine hydrochloride (WAKO PURE CHEMICAL INDUSTRIES, LTD.), L- β -(3,4-dihydroxyphenyl)alanine(L-DOPA), L-adrenaline bitartrate, 4-hydroxy-3-methoxyphenylacetic acid(HVA), 3,4-dihydroxymandelic acid(DOMA), DL-4-hydroxy-3-methoxymandelic acid(VMA) (TOKYO CHEMICAL INDUSTRY CO. LTD.), dopamine hydrochloride, serotonin creatinine sulfate(5-HT), 5-hydroxyindole-3-acetic acid(5-HIAA), pargyline hydrochloride, DL-p-chlorophenylalanine (PCPA) (NAKARAI CHEMICALS LTD.), L-norepinephrine bitartrate (noradrenaline), 3,4-dihydroxyphenylacetic acid (DOPAC), DL-normetanephrine hydrochloride, DL-metanephrine hydrochloride, bis(4-hydroxy-3-methoxyphenyl-

glycol) piperazine salt (MHPG), kynuramine dihydrobromide, 6-hydroxydopamine hydrobromide(6-OHDA), DL- α -methyl-p-tyrosine(α -MT) (SIGMA), L-5-hydroxytryptophan (5-HTP) (KYOWA HAKKO CO LTD.), probenecid, carbidopa(MK 486) (NIPPON MERCK-BANYU), and benzerazide hydrochloride (Ro 4-4602) (NIPPON ROCHE K.K.). The samples containing these compounds were prepared daily.

The estimation of tryptophan. All compounds, except probenecid, were dissolved originally in 0.4 N PCA at a concentration of 1 mg/ml as a free base, and then diluted with 0.4 N PCA, 0.1 M $\text{Na}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$ buffer (pH 7.4) or 0.2 N CH_3COONa . Probenecid was dissolved in 0.1 N NaOH and treated in the same way as the others. Standard solution of tryptophan was diluted daily with 0.4 N PCA, 0.1 M phosphate buffer or 0.2 N CH_3COONa at a concentration of 1 $\mu\text{g}/4\text{ ml}$ of tryptophan alone or of tryptophan mixed with increasing amounts(1, 10, 50 μg) of various compounds dissolved in respective solutions. These samples were estimated by the method of Denckla and Dewey (1967) with a slight modification (Bloxam and Warren, 1974) as follows. The samples dissolved in 0.1 M phosphate buffer and 0.2 N CH_3COONa were acidified by 0.2 ml of 36 % HCl. To all samples in the three diluents, 0.15 ml of 18% HCHO and 0.15 ml of 0.01 M FeCl_3 dissolved in 0.1 N HCl were added, or only 0.15 ml of 0.01 M FeCl_3 to the samples for a blank, and the tubes containing the mixtures were firmly stoppered, heated in a boiling water bath for 1 hr, and brought to room temperature in running tap water. The fluorescence intensity was read at excitation and emission of 373 nm and 454 nm, respectively, using

a fluorescence spectrophotometer (HI-TACHI MPF-4). The values obtained by subtraction of each blank value were indicated as the percentage of the fluorescence of 1 μ g of tryptophan.

Separation of tryptophan through Dowex column. Tryptophan mixed with quenching substances was isolated through the Dowex column, and its fluorescence was measured by the same estimation method. For a preparation of the column, Dowex AG 50W $\times$ 4 (200/400 mesh, H⁺ form) (BIO•RAD Laboratories) suspended in distilled water was adjusted to pH 6.5 by 5 N NaOH, then bufferized with 0.1 M KH₂PO₄-K₂HPO₄ buffer (pH 6.5) and filled to a column (diamiter 0.6 cm, height 3 cm). The increasing amounts (1, 10, 50 μ g) of various compounds were mixed with 1 μ g of tryptophan in 4 ml of 0.4 N PCA, adjusted to pH 2.0-2.5 with 1 N NaOH, and passed through the column. After the column was washed with 4 ml of distilled water and 3 ml of 0.2 N CH₃-COONa, tryptophan was recovered into the following 8 ml of 0.2 N CH₃COONa. The fluorescence of each sample in 4 ml of the eluate was assayed as described above, and indicated as the percentage of the fluorescence of 1 μ g of tryptophan.

Assays of free and total tryptophan from the rat plasma. Male Wistar rats weighing 250-300 g were decapitated, and the blood collected from the body trunk into a heparinized tube was centrifuged at 3000 rpm for 20 min at 20°C. The plasma was stored at -45°C until assayed. The separation of free tryptophan in 1.25 ml of plasma was performed using the ultrafiltration cell with PM 10 membrane (AMICON, model 12) at room temperature. For the

direct estimation of tryptophan, 3.5 ml of 0.4 N PCA was added to 0.5 ml of the ultrafiltrate, and the free tryptophan was assayed by the same estimation method. Total tryptophan was assayed using 4 ml of the supernate obtained after addition of 5 ml of 0.4 N PCA to 0.2 ml of the plasma and centrifugation at 12000 rpm for 10 min in the cold. For the column separation of free and total plasma tryptophan, 0.5 ml of the ultrafiltrate or 4 ml of the PCA extract from the plasma was adjusted to pH 2.0-2.5 with 0.1 N HCl or with 1 N NaOH, respectively, and passed through the column as described above.

RESULTS

Quenching effects of 26 kinds of compounds diluted in 0.4 N PCA on the estimation of tryptophan are shown in Table 1. When 1 μ g of tryptophan was mixed with the same amount of the compounds in 0.4 N PCA, the fluorescence of tryptophan was slightly affected. However, the marked decrease of the fluorescence was found in the presence of the tenfold amounts of such compounds as catecholamines, 5-HT, their precursors (L-DOPA, 5-HTP) and metabolites (HVA, DOPAC, DOMA, MHPG, 5-HIAA), and related compounds (6-OHDA, Ro 4-4602). Phenylalanine, histamine, tyramine, pargyline, PCPA and α -MT did not decrease the fluorescence even in the presence of the compounds of which concentrations were 50 times amount of tryptophan.

The quenching effects of 14 kinds of compounds dissolved in 0.1 M phosphate buffer (pH 7.4) and 0.2 N CH₃-COONa were compared with those in 0.4 N PCA. Among the three diluents, the tested compounds tended to de-

crease the fluorescence of tryptophan most potently when they were dissolved in 0.1 M phosphate buffer, and more

TABLE 1

Effects of various compounds on the fluorescence intensity of tryptophan

compounds	fluorescence intensities (%)		
	amounts of compounds mixed with 1 μ g of tryptophan		
	1 μ g	10 μ g	50 μ g
phenylalanine	102	99	100
tyrosine	101	89	70
L-DOPA	92	38	0
dopamine	96	38	0
noradrenaline	94	44	1
adrenaline	96	44	1
HVA	97	71	18
DOPAC	99	47	4
DOMA	100	62	5
normetanephine	96	83	44
metanephine	96	83	50
VMA	106	94	76
MHPG	96	53	9
5-HTP	102	79	0
5-HT	105	73	4
5-HIAA	106	74	8
kynuramine	109	103	83
histamine	103	98	98
pargyline	103	94	94
probenecid	110	105	81
tyramine	103	106	105
PCPA	101	104	95
6-OHDA	94	46	0
α -MT	103	102	95
MK 486	104	94	70
Ro 4-4602	94	75	13

Tryptophan and above compounds were dissolved in 0.4 N PCA, and the estimation procedure for tryptophan was carried out. Fluorescence intensities are indicated as the percentage of fluorescence of 1 μ g of tryptophan after being corrected by each blank value. Values are indicated as the mean of duplicate samples.

potently in 0.2 N CH_3COONa than in 0.4 N PCA (See Tables 1 and 2). Being diluted in 0.1 M phosphate buffer, even 1 μ g of L-DOPA, catecholamines, DOPAC and Ro 4-4602 reduced the fluorescence of 1 μ g of tryptophan to less than 80 %.

In successive experiment, we examined whether the quenching of the compounds, the values of which were less than 90 % in the presence of 10 μ g of these compounds in the Table 1, can be abolished by introducing the separation procedure with the Dowex column.

TABLE 2

Effects of various compounds in the different diluents on fluorescence intensity of tryptophan

compounds	diluents			
	0.1 M phosphate buffer (pH 7.4)		0.2 N CH ₃ COONa	
	amounts of compounds mixed with 1 μg of tryptophan			
	1 μg	10 μg	1 μg	10 μg
L-DOPA	78	17	84	16
dopamine	74	15	83	14
noradrenaline	71	17	82	19
adrenaline	78	15	81	21
DOPAC	79	19	83	19
HVA	111	61	95	49
5-HTP	86	29	88	31
5-HT	89	27	84	30
5-HIAA	92	29	90	30
α-MT	102	102	105	104
MK 486	80	44	101	58
Ro 4-4602	71	39	95	47
probenecid	98	103	100	100
pargyline	99	103	101	99

Tryptophan and above compounds were diluted with 0.1 M phosphate buffer (pH 7.4) or 0.2 N CH_3COONa , and the estimation procedure for tryptophan was carried out. Further explanations are in the legend of Table 1.

TABLE 3

Effects of various compounds on the assay of tryptophan through the column separation

compounds	fluorescence intensities (%)		
	amounts of compounds mixed with 1 μ g of tryptophan		
	1 μ g	10 μ g	50 μ g
tyrosine	100	105	106
L-DOPA	102	102	99
dopamine	103	102	92
noradrenaline	105	109	111
adrenaline	106	111	116
HVA	97	111	116
DOPAC	100	107	100
DOMA	100	107	116
normetanephrine	104	100	104
metanephrine	100	100	104
MHPG	101	103	107
5-HTP	106	89	57
5-HT	103	111	109
5-HIAA	104	109	113
6-OHDA	106	107	101
MK 486	110	113	116
Ro 4-4602	103	103	106

Tryptophan mixed with above compounds was isolated through the Dowex column as described in the text, and estimated for tryptophan. Further explanations are in the legend of Table 1.

As shown in Table 3, decreasing effects on the fluorescence of tryptophan were diminished after the column separation in most compounds, however, the large amount of 5-HTP still showed the effect.

The values of free and total tryptophan in the rat plasma both with the direct estimation and with the estimation after the column separation are indicated in Table 4. The values obtained from normal plasma were much alike between the two methods, how-

TABLE 4

Concentrations of free and total tryptophan in the rat plasma

	tryptophan free (μ g/ml)	concentra- tions total (μ g/ml)
<i>direct estimation</i>		
normal rat	2.07 ± 0.11	22.50 ± 0.97
L-DOPA-treated rat ¹	0.82 ± 0.09	15.75 ± 0.77
<i>estimation after column separation²</i>		
normal rat	1.95 ± 0.19	21.81 ± 1.03
L-DOPA-treated rat ¹	3.44 ± 0.26	17.40 ± 0.65

Each value represents the mean $\pm$ SEM of 6-7 determinations. 1) ; the rats were decapitated at 60 min after an i. p. injection of L-DOPA, 200 mg/kg. 2) ; the ultrafiltrates or the 0.4 N PCA extracts of the plasma were shaken with activated alumina at pH 7.5-8.0, and the supernatants were adjusted to pH 2.0-2.5 with 1 N HCl and applied to the column, and treated as described in the text.

ever, those obtained from the L-DOPA-treated rat showed large discrepancy depending on the methods.

DISCUSSION

Hess and Udenfriend (1969) devised originally the formation of norharman, which was highly specific to only tryptophan or tryptamine, and Denckla and Dewey (1967) adapted the reaction to determine tryptophan in the plasma or in the other tissues. However, the assay was reported to be quenched by certain metal ions (Denckla and Dewey, 1967), L-DOPA (Curzon and Knott, 1974) or sucrose and other keto sugars (Woodcock and Khairallah, 1976) and also by a light (Lehman, 1971). In the present study, quenching effects of 26 kinds of compounds on the assay

was studied. The standard amount of tryptophan, 1 μ g, is equivalent to the amount of free tryptophan in 0.5 ml of the ultrafiltrate of the rat plasma. As shown in Tables 1 and 2, the fluorescence of tryptophan was decreased markedly by many compounds according to their increasing amounts, and potentiated slightly by a few compounds. Although the chemical explanations of these phenomenon were not defined, further addition of HCHO or FeCl₃ could not recover the quenching effects and moreover decreased the fluorescence of tryptophan itself.

The degree of the quenching was changed according to the kind of diluents of the compounds. L-DOPA, catecholamines, DOPAC, 5-HTP, 5-HT, 5-HIAA and Ro 4-4602 showed more marked reduction of the fluorescence of tryptophan when dissolved in somewhat alkaline solution than in 0.4 N PCA as shown in Tables 1 and 2. These findings suggest the possibility that not only the compounds themselves, but also their degraded compounds decreased the fluorescence of tryptophan.

When tryptophan was separated by the column, the mean recovery of the aminoacid was 93%, and none of the compounds tested, except 5-HTP, decreased the fluorescence of tryptophan. On the contrary, some of the catecholamines, HVA, DOPAC, DOMA, 5-HT, 5-HIAA and MK 486 increased slightly the fluorescence of tryptophan. When tryptophan mixed with L-DOPA, catecholamines, DOPAC or MK 486 was shaken with activated alumina under pH 7.5-8.0, then adjusted to pH 2.0-2.5 with 1 N HCl and passed through the column, the quenching effect of the compounds could be removed completely.

Even after the column separation, it should be needed to examine the

existence of quenching effects. We observed that not only tryptophan but also these quenching compounds, L-DOPA, dopamine, 5-HTP, 5-HT and MK 486, were eluted in more concentrated way than with 0.2 N CH₃COONa when the aminoacid retained by the resin was eluted with 0.1-0.5 N NH₄OH proposed by Korobath et al. (1971).

Although the quenching effects of various compounds were studied *in vitro*, the results suggest that the effects are likely to occur in the assay of tryptophan in tissue samples if the estimation method is applied directly to their deproteinized extracts. Actually, the changes of plasma tryptophan in the L-DOPA-treated rat were different according to the assay methods, and the difference was more remarkable in the results of free tryptophan than in those of total tryptophan (See in Table 4).

The assay of plasma tryptophan by the direct method might not be interfered with the endogenous substances under physiological condition in which their concentrations were not so high (See in Table 4).

The method of Denckla and Dewey is simple and sensitive, and has been generally adopted in determination of plasma tryptophan by manually operated and autoanalyzed methods. However, our results serve a notice to the direct application after administration of drugs, especially to the application for the assay of free tryptophan, which controls brain tryptophan level and 5-HT synthesis (Knott and Curzon, 1972; Tagliamonte et al, 1973). For the accurate assay of tryptophan in the tissue samples, the estimation method should be applied after introducing appropriate separation procedure for tryptophan to remove quenching compounds.

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