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Phosphorimetric Assay for 3-Methoxy-4-hydroxyphenylethyleneglycol in Urine

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A sensitive phosphorimetric method is described for the assay of urinary free and total 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG), a major metabolite of norepinephrine. Free MHPG, after separation from urine by Amberlite CG-50 column chromatography, is oxidized with periodate to vanillin, which is then measured phosphorimetrically in a mixture of ether and ethanolic potassium hydroxide. Total MHPG is measured after β-glucuronidase and arylsulfatase mediated hydrolysis of conjugated MHPG. The assay requires less than 2 ml of urine to provide test, blank and standard samples, and offers lower limits of determination of 5 and 30 ng/ml for free and total MHPG, respectively.

Keywords——phosphorimetry; microassay; 3-methoxy-4-hydroxyphenylethyleneglycol; urine; vanillin; conjugated and free forms

In the previous phosphorimetric study of the catecholamines and related compounds, we found that vanillin phosphoresced intensely even at a pmol/ml concentration level in an alkaline medium.[2] This phenomenon is applicable to the determination of 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG), a major metabolite of catecholamines, which can be converted quantitatively to vanillin by periodate oxidation. This paper describes a sensitive phosphorimetric method for the assay of free MHPG and total MHPG [the total of free MHPG, its 4-O-sulfate and 4-O-glucuronide (conjugated forms)] in urine. The sensitivity of the method is much higher than that of spectrophotometric methods[3–5] based on the oxidation of MHPG to vanillin, higher than that of the fluorimetric method[6] based on oxidative conversion of MHPG to a fluorescent product, and comparable to that of gas chromatographic methods with electron capture detection[7–10] and with a mass fragmentographic technique[11,12].

Experimental

Chemicals and Apparatus——All chemicals were of reagent grade, unless otherwise specified. Double-distilled water and solvents were used. MHPG standard solutions (0.2 and 2.0 μg/ml) were prepared as aqueous solutions using the piperazine salt of MHPG (Sigma). Amberlite CG-50 (type I. H^+ form, 100—200 mesh; Rohm and Haas) was purified after the manner of Pisano[13] and equilibrated with 0.1 M phosphate buffer (pH 6.0—6.5). A glass column (10 mm i.d., 150 mm long) packed with 4.0 ml of the purified resin was washed with 10 ml of water before use.

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Glusulase (Sigma) used was a mixture of β-glucuronidase (92200 Sigma units/ml) and arylsulfatase (2990 Sigma units/ml).

The phosphorescence spectra and intensities were measured with the sample solution in the form of a clear solid frozen at liquid nitrogen temperature (77°K), using a Hitachi MPF-3 spectrofluorimeter equipped with a Hitachi phosphoroscope attachment using quartz sample tubes (4.0 mm i.d., 5.0 mm o.d., 200 mm long; sample volume required, 150 μl). The slit widths in the exciter and analyzer in terms of wavelength were both set at 10 nm. The phosphorescence lifetimes were measured on a Hitachi V-051 synchroscope. pH was measured with a Hitachi-Horiba M-7 pH meter.

Urine Collection——Twenty-four hr urine was collected in a bottle containing 10 ml of 10% EDTA-2Na solution from healthy volunteers in our laboratory. The urine could be stored in a refrigerator for at least 2 weeks without loss of free and total MHPG.

Procedure for Free MHPG Assay——The urine (2.0 ml) is adjusted to pH 6.2—6.5 with 0.1 M NaOH and poured into an Amberlite CG-50 column, and MHPG is eluted with 4.0 ml of H_2O. MHPG in the eluate is extracted with 16.0 ml of ethyl acetate after addition of 2 g of NaCl. The ethyl acetate extract (15.0 ml) is divided into three 5.0 ml portions (test, blank and standard).

For the test, 0.1 ml of H_2O, 3.0 ml of 4 M NH_4OH and 0.1 ml of 2.0% $NaIO_4$ solution are successively added to the extract (5.0 ml). The mixture is shaken for about 20 min and centrifuged briefly. The alkaline layer (2.5 ml) containing vanillin derived from MHPG is adjusted to pH 7.5—8.5 with concentrated H_3PO_4 (about 85%) under cooling in ice-water. Vanillin in the layer is extracted with 2.0 ml of ether, and 1.4 ml of the ether extract is diluted with 0.35 ml of ethanolic 0.5 M KOH.

The same procedure is carried out, except for replacement of the periodate solution with 0.1 ml of H_2O, for the blank. For the standard, 0.1 ml of 0.2 μg/0.1 ml MHPG standard solution is added in place of H_2O.

The phosphorescence intensities of the test (I_t), the blank (I_b) and the standard (I_s) are measured at 475 nm with excitation at 340 nm. The concentration of free MHPG in 24 hr urine is calculated from the equation:

$$\text{Free MHPG } (\mu g) = V/2 \times 0.2 \times (I_s - I_b)/(I_t - I_s) \times 100/84 \times 3$$
$$= 0.357V \times (I_s - I_b)/(I_t - I_s)$$

where V is the volume of 24 hr urine and 84 is the recovery ratio of free MHPG.[14]

Procedure for Total MHPG Assay——The urine (2.0 ml) is adjusted to pH 11.0—11.5 with 1.0 M NaOH. To remove the inorganic phosphate and sulfate which act as inhibitors of β-glucuronidase and sulfatase, 0.1 ml of saturated $BaCl_2$ solution is added. The resulting mixture is centrifuged briefly. The whole supernatant is adjusted to pH 5.0 with 1.0 M HCl, 0.2 ml of glusulase is added for the hydrolysis of conjugated MHPG, and the whole is incubated at 37° for approximately 12 hr. After adjusting the hydrolysate to pH 6.2—6.5 with 0.1 M NaOH, the same procedure as for free MHPG assay is followed, except that the concentration of MHPG standard is 2.0 μg/0.1 ml and the volume of ethyl acetate used for the extraction is 23.0 ml. The ethyl acetate extract (15.0 ml) is divided into three 5.0 ml portions (test, blank and standard), as for free MHPG assay. The concentration of total MHPG in 24 hr urine is calculated from the equation:

$$\text{Total MHPG } (\mu g) = V/2 \times 2.0 \times (I_s - I_b)/(I_t - I_s) \times 100/53 \times 3$$
$$= 0.566V \times (I_s - I_b)/(I_t - I_s)$$

where 53 is the recovery ratio of total MHPG.[15]

Results and Discussion

The phosphorescence excitation (maximum, 340 nm) and emission (maximum, 475 nm) spectra and the lifetimes (0.2 s) of the final solutions in free and total MHPG assays were identical with those of vanillin dissolved in the same alkaline medium (Fig. 1).

The glusulase-catalyzed hydrolysis of conjugated MHPG was accomplished by the usual methods.[3,4,6] MHPG in urine was successfully separated from other substances such as metanephrine and normetanephrine by the ion exchange chromatography on Amberlite CG-50 column.[3,4] MHPG was completely eluted with 4 ml of water. Ethyl acetate has been used for the extraction of MHPG from the eluate.[3,4] In the present study, a maximum and con-

14) 84 = (recovery of MHPG on extraction from the eluate into AcOEt) × (recovery of MHPG on column chromatography) × 15/16 × 100 = 0.95 × 0.94 × 15/16 × 100.

15) 53 = (recovery of MHPG on hydrolysis of the conjugated MHPG) × (recovery of MHPG on extraction from the eluate into AcOEt) × (recovery of MHPG on column chromatography) × 15/23 × 100 = 0.92 × 0.93 × 0.95 × 15/23 × 100.

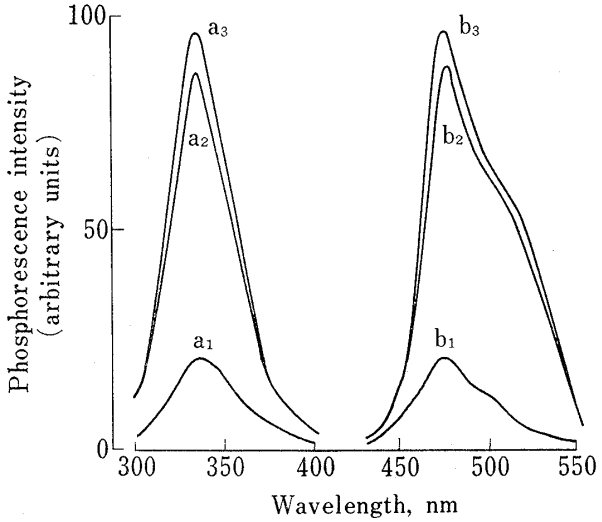

Fig. 1. Excitation and Emission Spectra of the Phosphorescence of the Final Solutions in Free and Total MHPG Assay and of Vanillin

a: excitation spectra; b: emission spectra.
a_1 and b_1: Portions (2.0 ml) of urine containing 0.22 μg of free MHPG were treated according to the standard procedure.
a_2 and b_2: Portions (2.0 ml) of urine containing 2.40 μg of total MHPG were treated according to the standard procedure.
a_3 and b_3: Vanillin (0.58 μg/ml) was dissolved in ether–ethanolic 0.5 M potassium hydroxide (4: 1, v/v).
In a_1, b_1, a_2 and b_2, the corresponding blanks were subtracted.

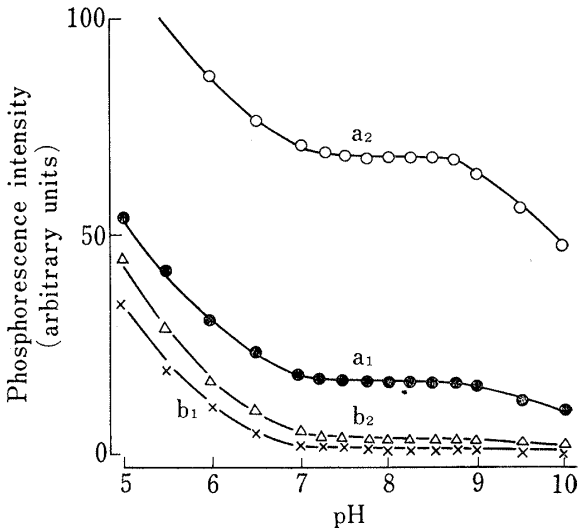

Fig. 2. Effect of pH on the Extraction of Vanillin from Ammonium Hydroxide Extracts with Ether

a_1 and a_2: Portions (2.0 ml) of urine containing 0.20 μg of free MHPG and 1.20 μg of total MHPG were treated according to the standard procedure, but the ammonium hydroxide extracts were adjusted to various pHs with phosphoric acid.
b_1 and b_2: The blanks corresponding to a_1 and a_2, respectively.

stant ratio of extraction of MHPG was achieved with more than 16.0 ml of ethyl acetate for free MHPG [recovery of MHPG, 95±3% (mean±SD), n=10] and 23.0 ml for total MHPG [recovery of MHPG, 93±2% (mean±SD), n=10] in the presence of sodium chloride. MHPG in the ethyl acetate extract, after oxidation with sodium metaperiodate to vanillin, was extracted with ammonium hydroxide solution [recovery of vanillin, 98±3% (mean±SD), n=20]. A maximum and reproducible phosphorescence intensitiy was obtained at concentrations of ammonium hydroxide and periodate higher than 3 M and 2.0%, respectively.

The vanillin was effectively extracted from the ammonium hydroxide extract with ether when the pH of the extract was adjusted to 7.5—8.5 with phosphoric acid (Fig. 2). When the pH of the resulting solution was lower than 7.5, the test and blank solutions showed intense phosphorescence overlapping with complex spectra due to unidentified substances (Fig. 2). No phosphorescence was detected when hydrochloric acid was used instead of phosphoric acid.

To obtain reproducible phosphorescence intensity, the ether extract should be frozen at 77°K to provide a clear solid. When ethanol was added to the ether extract at a volume ratio to the extract of 2—5, a clear solid was obtained. Thus, a ratio of ethanol to the extract of 4 was used in the procedure. Potassium hydroxide, which intensified the phosphorescence of vanillin,[2] was dissolved in ethanol; 0.5 M potassium hydroxide solution provided a maximum and reproducible phosphorescence.

Linear relationships were obtained between the phosphorescence intensity and the amount of MHPG up to at least 30 μg in 2.0 ml of urine for free and total MHPG. This indicates that the present standard addition method permits the assay of free and total MHPG in urine over wide ranges.

Metanephrine, normetanephrine and vanilmandelic acid (VMA) are converted to vanillin, and octopamine is converted to *p*-hydroxybenzaldehyde when they are oxidized with periodate under suitable conditions.[2] However, they did not interfere with the assay of MHPG even when present in unusually large amounts (2—5 μg/2.0 ml urine), because these amines were strongly retained by Amberlite CG-50. Further, VMA was not extracted with ethyl acetate

from the aqueous solution at pH 6.2—6.5 and the conditions of oxidation of MHPG in the procedure were unsuitable for VMA.

MHPG-O-sulfate added to urine at a concentration of 5 μg/2.0 ml did not affect the recovery of free MHPG.

The lower limits of determination for free and total MHPG in urine were 5 and 30 ng (27 and 160 pmol)/ml, respectively, which gave a phosphorescence intensity of twice the blank.

The within-day precision was examined using urines with mean values of free MHPG of 0.18 and 0.23 μg/2.0 ml (n=20 each), and mean values of total MHPG of 2.16 and 2.46 μg/2.0 ml (n=20 each). The coefficients of variation (CV) were 3.7 and 5.1% for free MHPG, and 3.2 and 5.4% for total MHPG, respectively. The day-to-day precision was obtained by repeating the assay for 10 days on urines stored at −5° with a mean value of free MHPG of 0.18 μg/2.0 ml (n=20), and a mean value of total MHPG of 2.46 μg/2.0 ml (n=20). The CVs were 3.8 and 5.4%, respectively.

The amounts of free and total MHPG in 24 hr urines of 17 healthy persons assayed by the present method are shown in Table I. The mean values of free and total MHPG are not very different from those obtained by other methods.[3-12]

Table I. Urinary Excretion of Free and Total MHPG in Normal Persons in 24 hr

Age	Sex[a]	Free MHPG (mg)	Total MHPG (mg)
45	M	0.161	2.10
33	M	0.185	2.33
30	M	0.098	2.23
29	M	0.162	2.21
28	M	0.210	3.42
28	M	0.205	2.54
27	M	0.192	1.80
26	M	0.158	2.29
25	M	0.172	1.56
24	M	0.135	2.47
23	M	0.118	1.61
54	F	0.148	2.10
35	F	0.122	1.97
30	F	0.203	1.70
27	F	0.193	2.08
24	F	0.140	2.32
22	F	0.112	1.84
Mean±SD		0.160±0.04	2.15±0.43

a) M, male; F, female.

The phosphorimetric method permits the assay of MHPG in a small amount of urine and gives reliable results, although the sample must be measured at 77°K with a phosphoroscope. We believe the present work represents the first phosphorimetric method ever reported for the determination of biological materials.