Journal of Chromatography, 181 (1980) 1—10
Biomedical Applications

CHROMBIO. 434

MODIFICATION OF ASSAYS FOR THE ROUTINE ANALYSIS OF 3-METHOXY-4-HYDROXYPHENYLGLYCOL IN URINE BY ELECTRON-CAPTURE GAS CHROMATOGRAPHY

LEON LOMBROZO, TIMOTHY J. ANDERSON, KATE KANASKE and LEO E. HOLLISTER

Psychopharmacology Research, Veterans Administration Hospital, Palo Alto, Calif. 94304 (U.S.A.) and Stanford University School of Medicine, Stanford, Calif. 94305 (U.S.A.)

(First received May 7th, 1979; revised manuscript received September 13th, 1979)

SUMMARY

Urinary 3-methoxy-4-hydroxyphenylglycol has been reliably assayed on a routine basis using an electron-capture detector method. Modifications of previous procedures include simplification of extraction and derivatization of urine, inclusion of an internal standard, prevention of losses during concentration, use of each urine as its own standard, and better chromatographic resolution by lengthening of columns and programming temperature. The assay shows a coefficient of variation of 3.1%.

INTRODUCTION

3-Methoxy-4-hydroxyphenylglycol (MHPG) was first measured with electron-capture gas chromatography by using trifluoroacetyl derivatives that enhanced the sensitivity and resolution of the analysis by Wilk et al. [1]. Their use of an AG 1-X4-chloride ion-exchange column removed freed phenolic acids, but introduced a 30% loss of MHPG. Dekirmenjian and Maas [2] simplified the procedure, and investigated many of the variables that contributed to the irreproducibility of the assay. Karoum et al. [3] utilized tryptophol as a ubiquitous internal standard with 3-methoxy-4-hydroxyphenethanol (MOPET) and MHPG added to one aliquot of a duplicate sample. Sharpless [4] has analyzed MHPG as the heptafluorobutyrate derivative and determined that there is no significant difference between MHPG in acidified and non-acidified urine that had been repeatedly thawed and refrozen during 10 months of storage.

The present assay is based on the method developed by Dekirmenjian and Maas [2]. Modifications were made in the initial sample size, transfer and derivatization procedure, use of an internal standard, and addition of isoamyl alcohol to prevent loss of MHPG during evaporative concentration. Each

urine sample is made to serve as its own standard curve, compensating for variations in extraction, hydrolytic and derivative losses, and instrument response.

EXPERIMENTAL

Materials

The di-MHPG piperazine salt, 3-methoxy-4-hydroxyphenethanol (MOPET) and β-glucuronidase—aryl sulfatase enzyme mixture (Cat. No. 34742) were obtained from Calbiochem (Los Angeles, Calif., U.S.A.). The (3-methoxy-4-sulfonyloxyphenyl)-glycol potassium salt (MHPG-SO_4) was obtained from Tridom Chemical (Hauppauge, N.Y., U.S.A.).

"Nanograde" ethyl acetate obtained from Mallinckrodt (St. Louis, Mo., U.S.A.) was distilled just prior to use and stored in the dark. Trifluoroacetic anhydride (TFAA) was obtained from Pierce (Rockford, Ill., U.S.A.). A solution of 1.0 *M*, pH 6.0 acetate buffer was mixed with a solution of 2% EDTA in a ratio of 2:1.

The gas chromatograph was a Model 5713A Hewlett-Packard equipped with an electron-capture detector. An on-column injection configuration 12 ft. × 2.0 mm I.D. glass column was used, packed with 1.5% OV-225 on 100—120 mesh Chromosorb G AW DMCS from Applied Science Labs. (State College, Pa., U.S.A.). A Hewlett-Packard automated sample injector Model 7671A was added to the chromatograph, allowing the overnight use of extended temperature programming from 130° to 190° at 1°/min. The injection port was maintained at 250°, and the detector at 350°. The carrier gas was 5% methane in argon, flow-rate 30 ml/min. The electron-capture detector contains 15 mCi of internally plated ^{63}Ni.

Methods

Portions of the measured urine samples (20 ml) were stored frozen at —20° in glass screw-capped vials (scintillation vials) without any added preservative. For assay, four 1-ml aliquots of a urine sample are placed in 30-ml tubes with PTFE-lined screw caps. MHPG standard solution (2 mg/100 ml, as the free form) is added to give final concentrations of 0, 1, 2, and 4 μg/ml of urine. MOPET internal standard (200 μl of a 3 mg/100 ml solution) is added to each tube. Each tube also receives 500 μl of buffer-EDTA and 60 μl of enzyme. The final volumes are made constant by adding 200, 150, 100, and 0 μl of saline to the four tubes. A saline standard curve is prepared by substituting 1 ml of saline for the urine sample. The samples are mixed briefly and incubated at 37° for 24 h.

The incubated samples are extracted once with 12 ml of ethyl acetate by reciprocal shaking for 45 min, and briefly centrifuged. Ten milliliters of the ethyl acetate is transferred to a 50-ml round-bottomed flask (14/20 taper) and 1.2 ml of isoamyl alcohol is added. The sample is evaporated just to dryness on a Rinco rotary evaporator connected to a double-trapped vacuum pump. TFAA (0.4 ml) is added and the stoppered flask is kept at 37° for 1 h. The excess TFAA is evaporated with a gentle stream of nitrogen and the residue is distributed in 8 ml of toluene by vortex mixing. After a brief set-

tling period, a portion of the toluene is placed in red septa capped autosampler vials, and 3-μl aliquots are injected into the chromatograph at an attenuation of 512 or 1024. Fig. 1 shows a chromatogram of a urine sample containing additions of 6 μg of MOPET and 1 μg of MHPG per ml of urine.

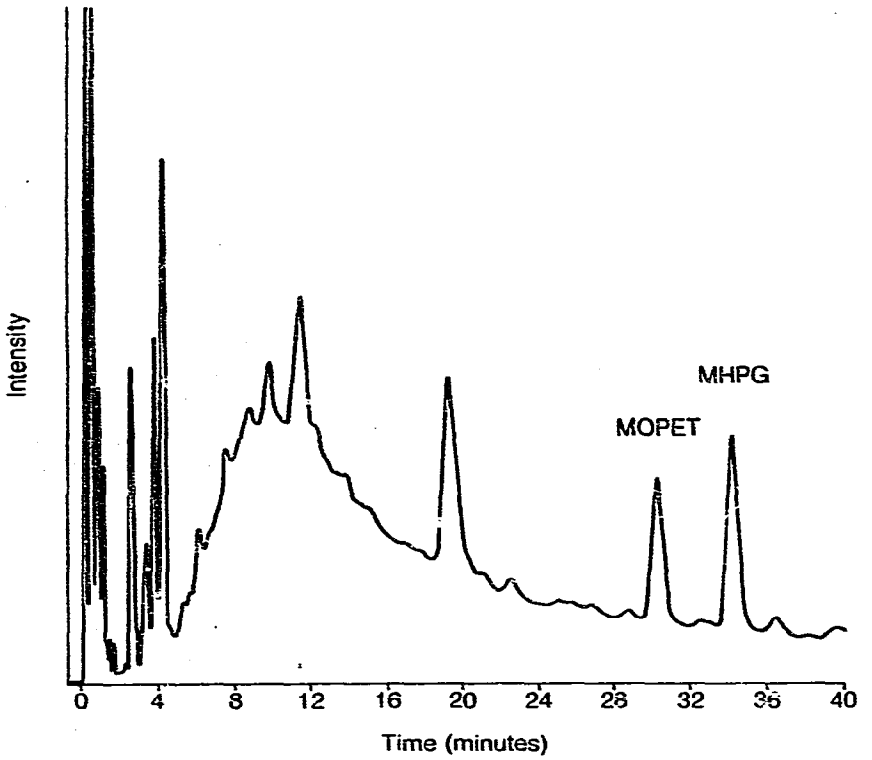

Fig. 1. An electron-capture—gas—liquid chromatogram of the TFAA derivatives of MOPET and MHPG obtained during analysis of urine with an initial 2.6 μg/ml MHPG content and additions of 6 μg/ml of MOPET and 1 μg/ml of MHPG.

A standard curve is obtained for each urine sample, using the four supplemented points. The ratio of peak heights of MHPG to MOPET is plotted against the amount of MHPG (μg) added per milliliter of urine (Fig. 2). The line is extrapolated through the Y-axis to the X-axis, which gives the micrograms of MHPG originally present in the sample per milliliter of urine. This is equivalent to projecting a horizontal line from the Y-intercept to a theoretical standard curve originating at zero, and then down to the X-axis. Fig. 2 is an analysis of a urine sample calculated by linear regression to contain 1.53 μg of MHPG per milliliter of urine.

RESULTS AND DISCUSSION

Assay procedures

There is enough MHPG present in urine to permit the use of only a portion of the organic phase after a single extraction. However, the shaking time was

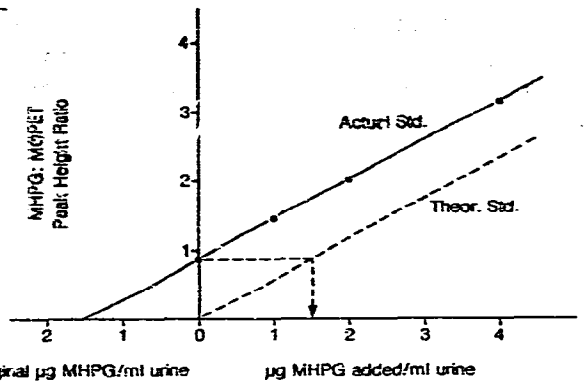

Fig. 2. Standard curve obtained for a urine sample initially containing 1.5 µg/ml of MHPG.

extended to ensure that the MHPG and MOPET had partitioned completely in the two phases. The urine samples often have different thicknesses of gel at the aqueous—organic interface, and transferring less than the total volume of ethyl acetate avoids contact with the gel. Removal of phosphates and sulfates with barium chloride [3, 4] accelerates the enzymatic hydrolysis of MHPG conjugates, but introduces additional steps that can be avoided by using enough enzyme and overnight incubation.

Dekirmenjian and Maas [2] propose a wash of the organic phase with a 1.0 *M* $KHCO_3$ solution to remove interfering acidic components. We found this step removed only chromatographic peaks that were remote from the MHPG and MOPET when using OV-225, and was deemed unnecessary.

Erratic losses in MHPG recovery were traced to the evaporative concentration step. Isoamyl alocohol in heptane was tried as an alternative solvent during the concentration step. The presence of isoamyl alcohol was found to decrease the MHPG losses during evaporation from heptane. The addition of 12% or more isoamyl alcohol to the transferred volume of ethyl acetate also decreased the MHPG losses during evaporation of the ethyl acetate.

Karoum et al. [3] preferred diethyl ether to ethyl acetate because of its better recovery of catecholamine alcoholic metabolites and easier evaporation. We avoided diethyl ether due to difficulty in its quantitative transfer and its potential danger in our hospital-based laboratory.

Direct derivatization of the vacuum dried residue eliminates re-solution and transfer steps. Evaporation of excess TFAA and resuspension in the same flask eliminates the effort of quantitative transfer and dilution to volume. Toluene used in this last dilution dissolves the MHPG—TFAA derivative, but leaves much of the more polar residue behind.

During preliminary studies, individual urine samples presented minor peaks that coincided with the MHPG and MOPET peaks. Slow peaks from previous runs also occasionally carried over into subsequent runs. The 12-ft. polar column and extended temperature programming increased the resolution and eluted most of the long retention-time peaks during each run. Karoum et al. [3] also found a long, polar column necessary (13 ft. 5% OV-210 + 1% SE-52) The temperature programming may be shortened (130°—170°, 1°/min) if overrunning peaks are not interfering.

The standard curve

We have found MOPET to be a readily available internal standard that closely follows the extraction and chromatographic resolution of MHPG. Since each urine sample contains a small but independent amount of MOPET each urine standard curve would have a slightly different MOPET divisor in its ratio calculation. This gives a different slope for each urine sample, and individual urine standard curves cannot be compared to a saline standard curve. However, supplementing the urine with MHPG makes each urine sample serve as its own standard curve and effectively determines the sample in quadruplicate. The curve is self-compensating for possible hydrolytic losses of MHPG during incubation, individual urine variations in partitioning during extraction, degradation of the derivatives, and daily variations in the response of the gas chromatograph.

These variables all change the slope but not the X-intercept. Fig. 3 demonstrates the theoretical effects on the observed slopes of a urine sample if it

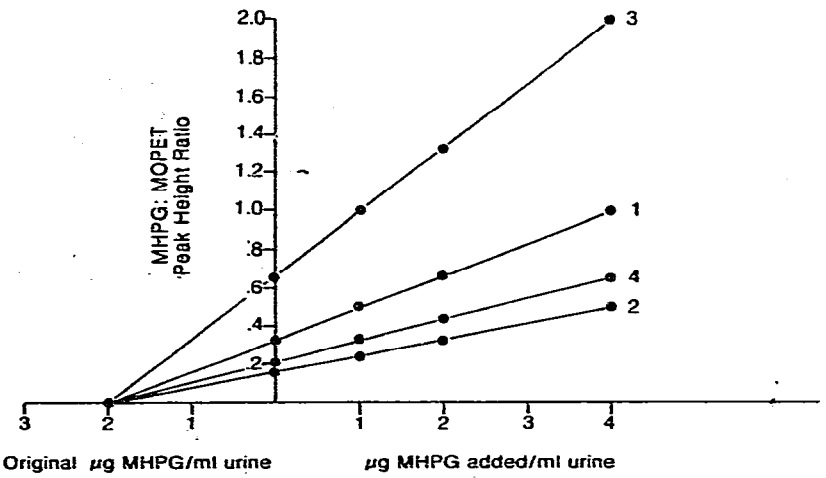

Fig. 3. Theoretical effect of consistent changes in original and added MHPG and MOPET. Chromatographic peak heights are assumed equal to the amounts of MHPG and MOPET originally present and added (MHPG, 2 μg/ml originally, 0—4 μg/ml added; MOPET, 0 or 3 μg/ml originally, 6 μg/ml added). (1) 100% MHPG:100% MOPET or 50% MHPG:50% MOPET; (2) 50% MHPG:100% MOPET; (3) 100% MHPG:50% MOPET; (4) 100% MHPG: 150% MOPET.

had systematic percentage losses in the total (endogenous and added) MHPG and/or MOPET during the analysis. The sample is assumed to contain 2 μg/ml of MHPG, 0 μg/ml of MOPET, and the peak heights are assumed proportional to the amounts added. The curves extrapolate to the same MHPG content for all the samples. The presence of 3 μg/ml of "endogenous" MOPET in a sample (100% MHPG, 150% MOPET) does not change the final MHPG value.

The effect of varying amounts of endogenous MOPET was simulated by adding small increments of MOPET (0, 0.3, 0.6, 1.2 μg/ml) to four aliquots of a urine sample. The four sets were assayed with the usual supplements of MOPET and MHPG. The increments of MOPET produced no differences (Fig. 4) in the calculated original MHPG content of the urine.

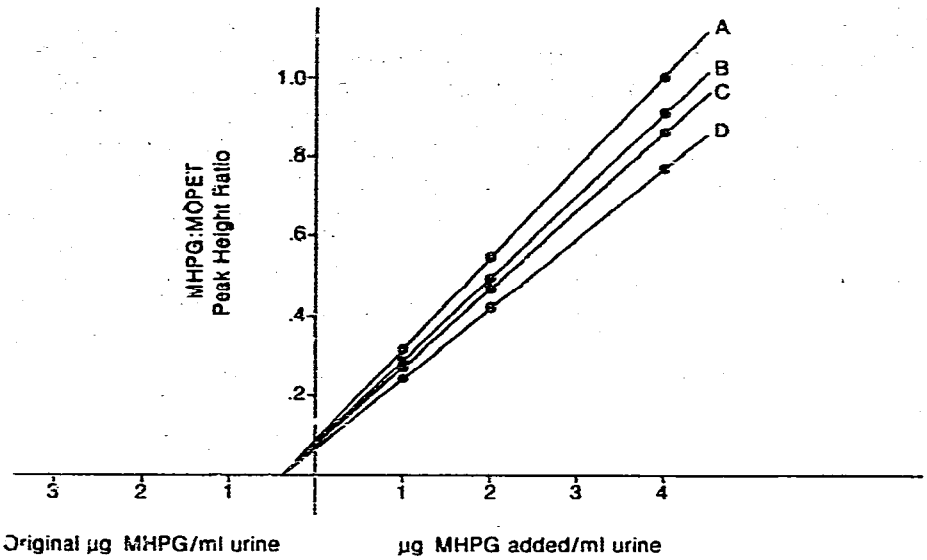

Fig. 4. Effect of increments of MOPET on determination of MHPG content of a urine sample. All the members of a urine set received either: A, 6.0; B, 6.3; C, 6.6; or D, 7.2 µg/ml of MOPET. Each set was then supplemented with 0, 1, 2, or 4 µg/ml of MHPG before assay.

The amount of MOPET present in a sample may be determined by supplementing the set with increasing amounts of MOPET, using MHPG as a constant internal standard.

The saline standard curve

The saline standard curve is not necessary for data calculation, but should be included in the assay periodically as a precautionary measure. The curve will intercept the X-axis to the right of zero if a fixed amount of MHPG is lost from each sample by evaporative losses or column degradation. The curve will intercept the X-axis to the left of zero if contaminating MHPG is brought into the assay by the reagents. The Calbiochem enzyme preparation has never introduced a visible MHPG peak at the gas chromatographic attenuations used in this assay. MHPG losses during the gas chromatographic analysis are avoided by the 4—6 injections of an MHPG-containing sample at 1-min intervals at the beginning of a run.

Precision of the assay

A urine sample was analyzed by ten sequential assays. The MHPG content and slopes were determined for each set of four points by the linear regression method. Data from these analyses revealed a coefficient of variation (C.V.) of the slope of 1.1% and of the value for MHPG of 3.1%.

Wilk et al. [1] report carrying two sets of eight identical samples through the procedure and obtaining standard deviations from the mean (C.V.) of 6.9% and 5.7%. Recalculation of the data of Table I of Karoum et al. [3] indicates that duplicate determinations of three 24-h urine samples gave a mean C.V.

of 5.5%. Sharpless [4] found the mean ± S.D. recovery of free MHPG added to urine was 78.5 ± 12.7% (n = 25), in effect a C.V. of 16.2%.

Stability of MOPET and MHPG

Dekirmenjian and Maas [2] showed that MHPG loses stability below pH 6.0. Enzymatic hydrolysis is most effective at slightly acidic pH, and introduces a possible loss of MHPG during incubation.

MHPG and MOPET stabilities under assay conditions were studied by an extended four-day incubation of nine sets of a urine sample. Each set was supplemented with 0, 1, 2, 3 or 4 μg/ml of MHPG, 6 μg/ml of MOPET and 60 μl of enzyme. The sets were then incubated for 0, 3, 6, 12, 18, 24, 48, 72, or 96 h, and each set was assayed for MHPG and MOPET. The MHPG content of the urine sample was calculated for each set by the extrapolation method, and the change in original MHPG content with time of incubation is shown in Fig. 5. The incubation period was extended to deliberately produce losses in the original and supplemented MHPG and MOPET content (see below). The extrapolation method compensated for the losses and presented a constant final value for MHPG after enzymatic hydrolysis (Fig. 5).

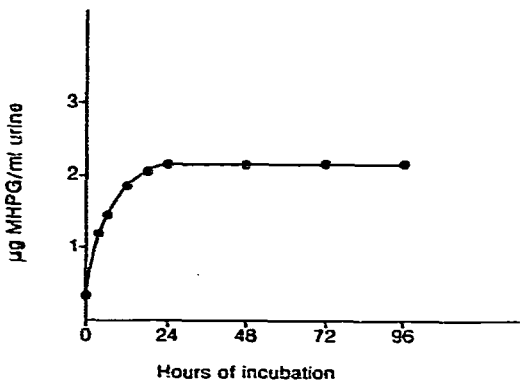

Fig. 5. The original MHPG content of a urine sample during extended incubation as determined by the extrapolation method.

The detected units of MOPET internal standards were well grouped for each time period (Table I), but showed a rapid 13% loss during the first six h of incubation. Little further change occurred during the normal incubation period. These changes do not prevent the use of MOPET as an internal standard.

Stability of MHPG was determined by replotting the curves without the internal standard. The peak heights of the 4 μg MHPG per ml urine supplemented points were taken from the curves for each supplemented set and replotted against time (Fig. 6). An analysis of the 12—96-h values by linear regression indicates that MHPG is lost from the incubation mixture at the rate of 1.0% per 24 h.

Stability of the derivatives

The appropriate amounts of MHPG and MOPET dissolved in ethyl acetate

TABLE I

STABILITY OF MOPET DURING EXTENDED INCUBATION

Incubation time (h)	MOPET units (S.D.)*	C.V.	Percentage loss
0	5.36 (0.11)	2.05	—
3	4.82 (0.10)	2.07	10.1
6	4.65 (0.20)	4.30	13.3
12	4.83 (0.11)	2.56	9.9
18	4.52 (0.04)	0.88	15.7
24	4.53 (0.17)	3.75	15.5
48	4.50 (0.13)	2.89	16.0
72	4.48 (0.07)	1.56	16.4
96	4.29 (0.09)	2.10	20.0

*cm of peak height at 1024 attenuation, 5 points averaged per time period.

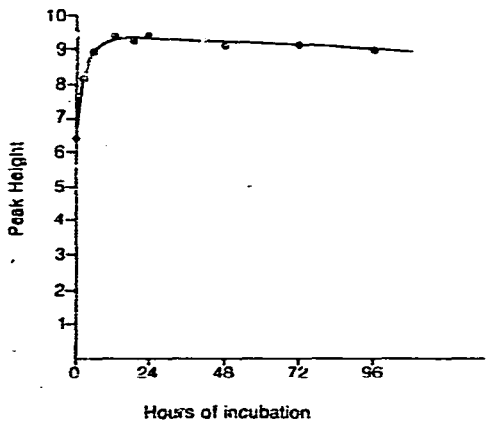

Fig. 6. The peak heights of the 4 μg MHPG per ml supplemented points during extended incubation. Peak heights are in cm at 1024 attenuation, taken from curves developed for each incubation time.

were added to round-bottom flasks to which were also transferred the ethyl acetate extracts of duplicate saline standard curves containing no standards. After normal preparation, the samples were repeatedly analyzed on the gas chromatograph over a 69-h period to determine the stability of the derivatized standards at room temperature. The slopes calculated by the MHPG:MOPET ratio remained linear, but decreased with time. The peak height of MHPG decreased continually with time, showing a 46-h half-life (calculated by the linear regression method) for the first 24 h, and a 198-h half-life thereafter. The MOPET peak height fell for the first 24 h ($t_{½}$ = 89 h) and remained steady thereafter. The ratios calculated with these varying values showed a half-life of 102 h. These losses would be theoretically compensated for during an analysis using the extrapolation method.

TABLE II

URINARY MHPG EXCRETION OF NORMAL SUBJECTS ANALYZED BY GAS CHROMATOGRAPHY

Source	Group	No. of subjects	μg MHPG/24 h (mean ± S.D.)	Range	μg MHPG/mg creatinine (mean ± S.D.)	Range
Hollister et al. [5] 1978	Men	11	2082 ± 842*	1158–4225**	1.80 ± 0.30*	0.68–1.92**
	Women	6	1634 ± 200*	1003–2018**	1.50 ± 0.31*	0.90–3.40**
	All	17	1924 ± 711*	1003–4225**	1.29 ± 0.35*	0.68–3.40**
Taube et al. [6] 1978	Women	10	1029***			
Sharpless [4] 1977	Men	6	2105	1158–2808	1.43	
	Women	5	1618	859–2016	1.32	
	All	11	1884	859–2808		
Maas [8] 1975	Men	19	1674			
	Women	21	1348			
Bond and Howlett [7] 1974	Men	7	2080		1.41	
	Women	6	1920		1.51	
Karoum et al. [3] 1973	All	9	1863	1130–2220	1.27	0.94–1.82
Dekirmenjian and Maas [2] 1970	Men	5	1600		0.95	
	Women	6	1320		1.09	
Maas et al. [9] 1968	Men	5	1600		1.15	
	Women	6	1397		1.16	
	All	11			1.16	
Wilk et al. [1] 1967	All	35			0.86	0.25–1.49

*Three consecutive 24-h urine collections averaged per subject.
**Individual sample range.
***2–4 separate 24-h MHPG values averaged per subject.

Completeness of enzymatic hydrolysis

The extent of enzymatic hydrolysis during incubation was determined by comparing the amount of MHPG detected after supplementing sets of a urine with MHPG or the equivalent amount of MHPG-SO_4. MOPET (6 μg/ml) was used as the internal standard. As the added MHPG-SO_4 is hydrolyzed, the slope of the curve increases and the X-intercept approaches that of the MHPG-supplemented sample. At complete hydrolysis the two curves are identical.

The use of 30 μl of enzyme gave incomplete (81%) hydrolysis in 24 h. Complete hydrolysis occurred when the incubation time was doubled or additional enzyme was added at 24 h and the incubation continued to 48 h.

Under the conditions of this assay, 60 μl of enzyme and 24 h incubation, the MHPG-SO_4 is completely hydrolyzed. The slopes remain superimposed after adding additional enzyme or extending the incubation time.

Application of the method

This method was used in a study of urinary excretion of MHPG in normals [5]. In Table II the results of this investigation are compared with others which measured excretion of MHPG in normal subjects. Some investigators obtain somewhat lower amounts of MHPG in their normal subjects than do others. The reasons for this difference are not entirely clear and could be many. Recent reports of MHPG excretion, both in normals as well as in depressed subjects, tend to report higher values than those originally reported. It is likely that modifications in the technique of measurement, such as those described in this paper, may have increased the extraction of MHPG and prevented its loss, resulting in the trend toward higher values.

ACKNOWLEDGEMENTS

Supported by grant MH 03030 and the Research Service, Veterans Administration Medical Center, Palo Alto, Calif., U.S.A.

REFERENCES

1 S. Wilk, S.E. Gitlow, D.D. Clark and D.H. Paley, Clin. Chim. Acta, 16 (1967) 403.
2 H. Dekirmenjian and J. Maas, Anal. Biochem., 35 (1970) 113.
3 F. Karoum, H. Lefevre, L.B. Bigelow and E. Costa, Clin. Chim. Acta, 43 (1973) 127.
4 N.S. Sharpless, Res. Commun. Chem. Pathol. Pharmacol., 18 (1977) 257.
5 L.E. Hollister, K.L. Davis, J.E. Overall and T. Anderson, Arch. Gen. Psychiat., 35 (1978) 1410.
6 S.L. Taube, L.S. Kirsten, D.R. Sweeney, G.R. Heninger and J.W. Maas, Amer. J. Psychiat., 135 (1978) 78.
7 P.A. Bond and D.R. Howlett, Biochem. Med., 10 (1974) 219.
8 J.W. Maas, Arch. Gen. Psychiat., 32 (1975) 1357.
9 J.W. Maas, J. Fawcett and H. Dekirmenjian, Arch. Gen. Psychiat., 19 (1968) 129.