Journal of Chromatography, 162 (1979) 394—400
Biomedical Applications

CHROMBIO. 276

Note

Analysis of urinary 4-hydroxy-3-methoxyphenylethylene glycol as vanillyl alcohol by high-performance liquid chromatography with amperometric detection

DOUGLAS N. BUCHANAN, FRANZ R. FUCEK and EDWARD F. DOMINO

Department of Pharmacology, Lafayette Clinic, 951 East Lafayette, Detroit, Mich. 48207 and the University of Michigan, Ann Arbor, Mich. 48109 (U.S.A.)

(Received June 12th, 1978)

4-Hydroxy-3-methoxyphenylethylene glycol (HMPG) is a major metabolite of norepinephrine [1] that is found in urine and cerebrospinal fluid (CSF). It has been determined that 25—50% of urinary HMPG is derived from brain norepinephrine [2—4].

Urinary HMPG exists as the non-conjugated, free molecule (HMPG-free), as the conjugate of sulfuric acid (HMPG-SO_4) and as the β-conjugate of glucuronic acid (HMPG-Glu). In human urine, HMPG exists mainly in the conjugated form [5—7]. The sulfate conjugate has been reported to be derived mainly from the metabolism of brain norepinephrine [2, 4, 8], while the β-glucuronide conjugate has been reported to be derived mainly from the metabolism of systemic norepinephrine [4, 7, 8]. The origin of HMPG-free remains uncertain although HMPG exists in CSF predominantly as the non-conjugated molecule [9].

Hydrolysis of conjugated HMPG, either enzymatically or under acidic or basic catalysis, affords the non-conjugated molecule. Acid catalyzed hydrolysis of conjugated HMPG is not recommended because HMPG has been reported to decompose under the conditions of the reaction [10]. Enzymatic hydrolysis of conjugated HMPG in urine can be selectively performed through the use of the appropriate enzymes [3, 5, 8]: Aryl sulfatase type VI (EC 3.1.6.1) liberates HMPG from HMPG-SO_4 [11]; β-gluconidase type V-A liberates HMPG from HMPG-Glu while β-glucuronidase type H-1 (contains aryl sulfatase activity) liberates HMPG from HMPG-SO_4 and HMPG-Glu. The sum of HMPG-free, HMPG-SO_4 and HMPG-Glu equals HMPG-total. It is HMPG-total that has been analyzed and reported in most clinical investigations.

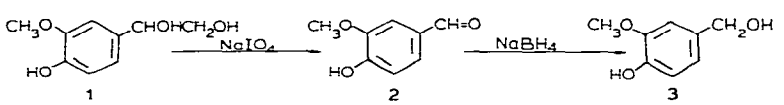
We were interested in ascertaining the differences in the excretion of HMPG-free, HMPG-SO_4, HMPG-Glu and HMPG-total by schizophrenic patients and mentally normal control subjects. It was hoped that one of these forms of

HMPG might provide a clue to brain norepinephrine metabolism in schizophrenia.

The quantitative analysis of HMPG in body fluids has been performed using gas—liquid chromatography (GLC) with flame ionization detection [12], electron capture detection [4, 8, 10, 13, 14] and gas chromatography—mass spectroscopy (GC—MS) [7, 15—17]. GC methods for the determination of HMPG in body fluids have the necessary sensitivity to detect HMPG-free (approximately 0.20 ng/μl in urine) and may have the necessary selectivity to detect HMPG-free. No matter what type of detector one employs in the GC analysis, HMPG must be derivatized to be volatile. The commonly used derivatives of HMPG are unstable and are not specific for HMPG. Other compounds present in the urine extract could become gas chromatographically active when subjected to the derivatization process and may interfere with the HMPG analysis. The derivatization process leaves a gas chromatographically active residue which may also interfere with the analysis.

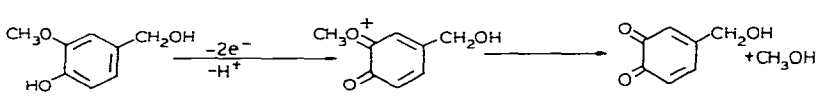
The quantitative analysis of HMPG in body fluids by reversed-phase high-performance liquid chromatography (HPLC) with amperometric detection was investigated because of the high selectivity afforded by both the μBondapak C_{18} HPLC column [18] and the oxidizing electrochemical detector [19—22] and the high sensitivity of the oxidizing electrochemical detector [19, 20] towards easily oxidizable organic functional groups such as phenols [22]. Reversed-phase HPLC columns and the amperometric detector permit the use of aqueous buffer as the mobile phase. This is ideal for the analysis of biological compounds isolated from body fluids. The pH of the buffer affects the mobility of the compounds on the reversed-phase column [23] thus affecting the selectivity of the system. The compounds being analyzed do not require derivatization but must be electrochemically oxidizable. Compounds that are electrochemically inactive cannot be detected. The oxidation potential of the detector can be varied so that functional groups with low oxidation potentials can be detected while functional groups with higher oxidation potentials are not detected. This increases both the selectivity and the sensitivity of the detector.

Utilizing the procedure of Felice and Kissinger [24], HMPG (1) was oxidized by periodate to vanillin (2) and the vanillin thus formed was reduced by sodium borohydride to vanillyl alcohol (3) via the procedure of Schwedt et al. [25].



1 2 3

At the carbon paste electrode of the detector, vanillyl alcohol (3) was converted into an *o*-quinone (4) via a two electron process [26].



3 4

MATERIALS AND METHODS

Materials

Glass distilled ethyl acetate, methanol and toluene were obtained from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.) and were used without further purification. Water was glass distilled from an alkaline permanganate solution. All chemicals were reagent grade. Aryl sulfatase type VI (EC 3.1.6.1), lot No. 127C-6820; β-glucuronidase type V-A (from *E. coli*), lot No. 107C-6810 and β-glucuronidase type H-1 (from *Helix pomatis*), lot No. 67C-0393 were obtained from Sigma (St. Louis, Mo., U.S.A.) and were used without further purification.

Solution

The internal standard, isovanillyl alcohol (Aldrich, Milwaukee, Wisc., U.S.A.), was used as a 500 ng/μl aqueous solution. A standard 58.6 ng/μl aqueous HMPG solution (free acid) was used.

Apparatus

The analyses were performed on an LC-50 liquid chromatograph (Bioanalytical Systems) with an oxidizing electrochemical detector, employing a carbon paste electrode and an Ag/AgCl reference electrode. A μBondapak C_{18} (Waters Assoc., Milford, Mass., U.S.A.) HPLC column (30 cm × 3.9 mm I.D.) containing octadecyl silane chemically bonded to 10 μm Porasil (10%, w/w) was used for the separation.

Analysis of HMPG-free, HMPG-SO_4, HMPG-Glu and HMPG-total from urine [24, 25]

Samples were prepared in duplicate as follows:

Sample 1: 3.00 ml of standard urine (24 h pool utilized for all analyses).

Sample 2: 3.00 ml of standard urine plus 25 μl of a 58.3 ng/μl HMPG solution.

Sample 3: 3.00 ml of standard urine plus 50 μl of a 58.3 ng/μl HMPG solution.

Sample 4: 3.00 ml of standard urine plus 100 μl of a 58.3 ng/μl HMPG solution.

Sample 5: 3.00 ml of unknown urine.

Sample 6: 3.00 ml of unknown urine plus 150 μl of aryl sulfatase type VI (EC 3.1.6.1).

Sample 7: 3.00 ml of unknown urine plus 30 mg of β-glucuronidase type V-A plus 15 μl $CHCl_3$.

Sample 8: 3.00 ml of unknown urine plus 30 mg of β-glucuronidase type H-1.

To each sample was added 0.5 ml of a 2% EDTA solution. Each sample was further modified as follows:

Samples 1—5: Added 1.0 ml of 0.45 *M* acetate buffer, pH 6.8 and the pH of the sample was adjusted to 6.5.

Sample 6: Added 1.0 ml of 0.45 *M* acetate buffer, pH 7.0 and the pH of the sample was adjusted to 7.1 (at pH 7.0, sulfatase had no β-glucuronidase activity).

Sample 7: Added 1.0 ml of 0.45 *M* acetate buffer, pH 6.5 and the pH of the samples was adjusted to 6.8.

Sample 8: Added 1.0 ml of 0.45 *M* acetate buffer, pH 5.0 and the pH of the sample was adjusted to 5.2.

All samples were kept in a constant temperature bath for 18—22 h at 37°. After incubation, the pH of each sample was adjusted to 6.5. Each sample was extracted 3 times with 10 ml of ethyl acetate. The ethyl acetate pool from each sample was washed with 1.0 ml of 1.0 *M* $NaHCO_3$ to remove carboxylic acid contaminants. The ethyl acetate layer was taken to dryness at 37° under a stream of nitrogen.

The residue from each sample was redissolved in 1.5 ml of 1.0 *M* K_2CO_3. To each solution was added 0.1 ml of a 3% $NaIO_4$ solution. The samples were kept for 10 min at 39°. Excess $NaIO_4$ was decomposed by the addition of 0.1 ml of a 10% $Na_2S_2O_5$ solution to each sample. The pH of each sample was adjusted to 6.5 by the addition of 0.3 ml of 5.0 *M* acetic acid and 0.6 ml of 1.0 *M* acetate buffer, pH 6.3 [24]. Each sample was extracted 3 times with 5 ml of toluene [24]. To the pooled toluene from each sample was added 10 μl of a 500 ng/μl isovanillyl alcohol solution (internal standard). The pooled toluene was extracted 3 times with 1 ml of 1.0 *M* K_2CO_3. $NaBH_4$ (100 mg) was added to the pooled K_2CO_3 solution from each sample and allowed to react for 20 min at ambient temperature. Excess $NaBH_4$ was quenched and the pH readjusted to 8.6 by the addition of 0.7 ml of 5.0 *M* acetic acid to each sample. Each sample was extracted 3 times with 5 ml of ethyl acetate. The pooled organic layer from each sample was taken to dryness at 39° under a stream of nitrogen. The residue from each sample was redissolved in 1.0 ml of glass distilled water.

HPLC analysis. 20 μl of each solution was placed on to a μBondapak C_{18} column via a loop injector. The mobile phase, 0.5 *M* acetate buffer, pH 4.7 containing 15% methanol, was pumped through the column at a flow-rate of 1.5—1.7 ml/min. The electrochemical detector was set at + 0.85 V vs. an Ag/AgCl reference electrode. The sensitivity of the detector was set at 10 nA/V with the recorder set at 1.0 V full scale deflection and a chart speed of 0.5 cm/min. The average retention time for vanillyl alcohol was 5 min while the average retention time for isovanillyl alcohol was 7 min.

Calculations. A standard curve using the method of standard additions was prepared from the analytical results of samples 1—4 by plotting the vanillyl alcohol/isovanillyl alcohol peak height ratio versus the [HMPG] of the standard urine. Linear regression analysis was employed to afford the best linear graph. The equation for the standard curve was $y = 0.37x + 0.072$. The slope of the linear regression line was used to determine the HMPG in unknown urine samples. The average of each duplicate analysis was recorded.

Sample 5 afforded the HMPG-free of the unknown sample.

Sample 6 afforded the HMPG-free + HMPG-SO_4 of the unknown urine sample.

Sample 7 afforded the HMPG-free + HMPG-Glu of the unknown urine sample.

Sample 8 afforded the HMPG-total of the unknown urine sample.

Subtracting HMPG-free from the HMPG in samples 6 and 7 afforded HMPG-SO_4 and HMPG-Glu respectively.

Using this procedure, urinary HMPG concentrations of 50 pg/μl could be measured with a range of linearity from 30 pg/μl to 5.00 ng/μl.

RESULTS AND DISCUSSION

Attempts to analyze HMPG directly from the ethyl acetate extract of urine failed because of our inability to separate the HMPG peak from much larger interfering peaks. Conversion of the extracted HMPG to vanillin (2) using periodate oxidation according to the method of Felice and Kissinger [24] yielded a much cleaner chromatogram. However, we still could not completely resolve the vanillin peak from an interfering peak in the chromatogram. Felice and Kissinger [24] analyzed urinary vanilmandelic acid (VMA) as vanillin and reported no interfering peaks. VMA exists in urine in much higher concentrations than does HMPG-free. In their analysis, Felice and Kissinger utilized a detector sensitivity of 50 nA/V while we used a detector sensitivity of 10 nA/V. At our higher sensitivity, any small interfering peak unnoticed by Felice and Kissinger in their analysis would be very large in our HMPG analysis and would greatly interfere with vanillin derived from HMPG.

Oxidation of HMPG (1) to vanillin (2) by periodate followed by the reduction of the vanillin to vanillyl alcohol (3) by sodium borohydride was found to be specific for HMPG. No peaks other than those due to vanillyl alcohol and isovanillyl alcohol (internal standard) were present in the chromatogram (Fig. 1).

When a known amount of HMPG, 1.00 μg to 6.50 μg, was added to 3.00 ml aliquots of water and carried through the procedure, a straight line, $y=0.91x$

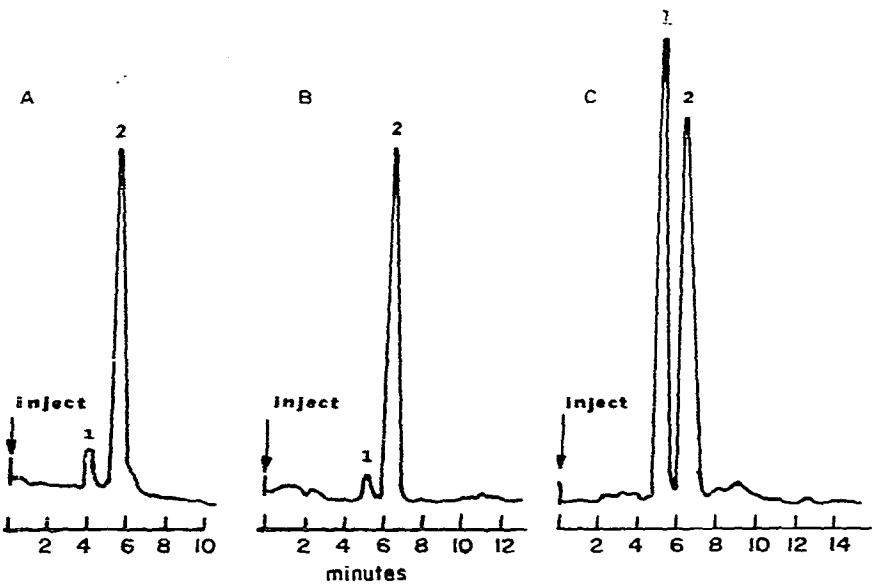


Fig. 1. Chromatogram of HMPG detected as vanillyl alcohol by HPLC with amperometric detection. Peaks: 1, vanillyl alcohol; 2, isovanillyl alcohol. (A) HMPG (0.33 ng/μl) from water; (B) HMPG-free (0.18 ng/μl) from urine; (C) HMPG-total (2.93 ng/μl) from urine. The mobile phase is 0.5 *M* sodium acetate, pH 4.7 containing 15% (v/v) methanol. The flow-rate is 1.5 ml/min. The detector was set at 10 nA/V at an oxidation potential of + 0.85 V vs. an Ag/AgCl reference electrode.

- 0.03 (correlation coefficient 0.991) resulted. When a known amount of HMPG, 1.00 μg to 6.00 μg was added to 3.00-ml aliquots of standard urine (a 24-h urine pool) and carried through the procedure, a straight line, $y=0.37x + 0.072$ (correlation coefficient 0.983) resulted. It is more difficult to extract HMPG from urine than from water. Once the HMPG was removed from urine, all of the extractions were 91—97% efficient.

Because of the reactivity of *o*-methoxyphenols with periodate [27, 28] some loss of HMPG will occur during the oxidation of HMPG to vanillin. Allowing the reaction to proceed for a specific time span, 10 min at 37° gives the same percentage loss per sample as shown by the linearity of the results obtained from the addition of HMPG to water and urine. Isovanillyl alcohol, the internal standard, reacted completely with periodate during the 10 min oxidation reaction. Thus necessitating the addition of isovanillyl alcohol to each sample after the periodate oxidation. The procedure gave reproducible results.

The extent of enzymatic hydrolysis of conjugated HMPG varies with the urine sample. In some urine samples, endogenous inhibitors of the hydrolytic enzymes are present [7]. In other instances, the use of an excess of the enzyme will cause inhibition [7]. The sum of the experimentally determined HMPG-free + HMPG-SO_4 + HMPG-Glu should be 100 ± 20% of the experimentally determined HMPG-total. A lower limit of 80% was judged as being acceptable. This idealized condition was difficult to meet because of the variability of the

TABLE I

HMPG OBTAINED FROM THE HPLC-AMPEROMETRIC DETECTION ANALYSIS OF HUMAN URINE

Sample volumes (ml)	HMPG-free		HMPG-SO_4*		HMPG-Glu*		HMPG-total		Percent of HMPG-free + HMPG-conj as HMPG-total
	(ng/μl)	(mg)	(ng/μl)	(mg)	(ng/μl)	(mg)	(ng/μl)	(mg)	
24-h samples									
1258	0.13	0.16	0.48	0.60	1.21	1.52	2.14	2.69	85
1557	0.30	0.47	0.65	1.01	0.91	1.42	2.31	3.60	81
Individual samples									
264	0.16	0.04	1.34	0.35	1.72	0.45	3.83	1.01	84
113	0.23	0.03	1.51	0.17	1.59	0.18	3.84	0.43	87
130	0.15	0.02	1.16	0.15	1.42	0.18	3.05	0.40	90
280	0.14	0.02	0.44	0.12	0.76	0.21	1.68	0.47	80
340	0.12	0.04	0.31	0.11	0.31	0.11	0.75	0.26	99
240	0.13	0.03	0.54	0.13	1.39	0.33	2.36	0.57	86
388	0.13	0.05	0.76	0.30	0.72	0.28	1.98	0.77	82
82	0.29	0.02	1.02	0.08	1.45	0.12	2.96	0.24	93
124	0.31	0.04	0.72	0.09	1.35	0.17	2.02	0.25	118
100	0.47	0.05	1.92	0.19	2.32	0.23	4.09	0.41	115
96	0.31	0.03	0.57	0.06	0.94	0.90	2.31	0.22	82

*HMPG-free is not included in these values.

hydrolytic efficiency of the enzymes from urine sample to urine sample. In every determination, the same amount of enzyme was used per urine sample. This variability was not a result of a change in potency of the enzyme from lot to lot because only the same lot number was used for each analysis.

From the 24-h urine samples and the individual (non-24 h) urine samples that have been analyzed and which fall within the 100 ± 20% range, it can be seen that the HMPG-SO_4 is less than or equal to the HMPG-Glu (Table I). This is in contrast to the results of Bond and Howlett [8], who reported HMPG-Glu to be less than HMPG-SO_4 in human urine. Our results are comparable to those of Murray et al. [7] and Joseph et al. [4], who reported HMPG-SO_4 to be less than or equal to HMPG-Glu in 24-h human urine samples.

The normal 24-h urinary HMPG values obtained by this method are: HMPG-free (0.16—0.47 mg); HMPG-SO_4 (0.60—1.01 mg); HMPG-Glu (1.42—1.52 mg); HMPG-total (2.69—3.60 mg).

REFERENCES

1 J. Axelrod, I.J. Kopin and J.P. Mann, Biochim. Biophys. Acta, 36 (1959) 576.
2 P.A. Bond, M. Dimitrakoudi, D.R. Howlett and F.A. Jenner, Psychol. Med., 5 (1975) 279.
3 K. Grunspan, J.J. Schildkraut, E.K. Gordon, L. Baer, M.S. Aronoff and J. Durell, J. Psychiat. Res., 7 (1970) 1971.
4 M.H. Joseph, H.F. Baker, E.C. Johnstone and T.J. Crow, Psychopharmacology, 51 (1976) 47.
5 B.R. Martin, M.C. Timmons and J. Prange, Jr., Clin. Chim. Acta, 38 (1972) 271.
6 F. Karoum, H. Lefevre, L.B. Bigelow and E. Costa, Clin. Chim. Acta, 43 (1973) 127.
7 S. Murray, T.A. Baillie and D.S. Davis, J. Chromatogr., 143 (1977) 541.
8 P.A. Bond and D.R. Howlett, Biochem. Med., 10 (1974) 219.
9 S. Murray, D. Huw Jones, O.S. Davies, C.T. Dollery and J.L. Reid, Clin. Chim. Acta, 79 (1977) 63.
10 H. Dekirmenjian and J.W. Maas, Anal. Biochem., 35 (1970) 113.
11 Shows no β-Glucuronidase activity at pH 7.0, Sigma,St. Louis, Mo., product literature.
12 P.A. Biondi, M. Cagnasso and C. Secchi, J. Chromatogr., 143 (1977) 513.
13 S. Wilk, S.E. Gitlow, D.D. Clarke and D.H. Paley, Clin. Chim. Acta, 16 (1967) 403.
14 A.E. Halaris, E.M. Demet and M.E. Halari, Clin. Chim. Acta, 78 (1977) 285.
15 F. Karoum, J.C. Gillin, R.J. Wyatt and E. Costa, Biomed. Mass Spectrom., 2 (1975) 183.
16 L. Bertilsson, J. Chromatogr., 87 (1973) 147.
17 S. Takahashi, D.D. Godse, J.J. Warsh and H.C. Stancer, Clin. Chim. Acta, 81 (1977) 183.
18 I. Molnár and C. Horváth, Clin. Chem., 22 (1976) 1497.
19 P.T. Kissinger, C. Refshange, R. Drieling and R.N. Adams, Anal. Lett., 6 (1973) 465.
20 P.T. Kissinger, Anal. Chem., 49 (1977) 448A.
21 P.T. Kissinger, L.J. Felice, R.M. Riggin, L.A. Pachla and D.C. Wenke, Clin. Chem., 20 (1974) 992.
22 B. Fleet and C.J. Little, J. Chromatogr. Sci., 12 (1974) 747.
23 C. Horváth, W. Melander and I. Molnár, J. Chromatogr., 125 (1976) 129.
24 L.J. Felice and P.T. Kissinger, Clin. Chim. Acta, 76 (1977) 317.
25 G. Schwedt, A. Blödorn and H. Bussemas, Clin. Chim. Acta, 65 (1975) 309.
26 R.E. Shoup and P.T. Kissinger, Clin. Chem., 23 (1977) 1268.
27 J. March, Advanced Organic Reactions, Mechanisms and Structure, McGraw-Hill, New York, 1968, p. 866.
28 E. Adler, I. Falkehag and B. Smith, Acta Chem. Scand., 16 (1962) 529.