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MASS FRAGMENTOGRAPHIC DETERMINATION OF 4-HYDROXY-3-METHOXYPHENYLGLYCOL (HMPG) IN URINE, CEREBROSPINAL FLUID, PLASMA AND TISSUES USING A DEUTERIUM-LABELLED INTERNAL STANDARD

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SUMMARY

4-Hydroxy-3-methoxyphenylglycol (HMPG) with the three hydrogen atoms in the side-chain replaced with deuterium (HMPG-D_3) was used as the internal standard in the mass fragmentographic determination of free and conjugated HMPG in human urine, cerebrospinal fluid and plasma and in rat urine, liver and brain. HMPG-D_3 was added to body fluids or homogenates followed by enzymatic hydrolysis of conjugates. HMPG was extracted with ethyl acetate, converted into the trifluoroacetyl derivative and analyzed by mass fragmentography. HMPG levels were 7.4 nmoles/ml $\pm$2.8% in human urine, 73 pmoles/ml $\pm$8.2% in human cerebrospinal fluid, 56 pmoles/ml $\pm$5.4% in human plasma, 24 nmoles/ml $\pm$3.6% in rat urine, 0.26 nmoles/g $\pm$6.2% in rat brain and 99 pmoles/g $\pm$13% in rat liver. The method is highly specific and sensitive, permitting analysis in small samples or in plasma and in tissues for which previously no methods for HMPG analysis were available.

INTRODUCTION

4-Hydroxy-3-methoxyphenylethane-1,2-diol (4-hydroxy-3-methoxyphenylglycol, HMPG) is the major metabolite of norepinephrine released from adrenergic neurons situated within the brain[1–4]. In rat urine, HMPG appears mainly conjugated with sulphate[5,6], whereas in humans HMPG is excreted as both sulphate and glucuronide[6,7]. Methods for the analysis of this important metabolite in urine are based on spectrophotometry[8,9] and gas–liquid chromatography[10] with electron capture detection[11–15] (GLC–ECD) or mass spectrometric detection[6]. In brain and cerebrospinal fluid (CSF), HMPG can be determined by GLC–ECD[2,12,16] or GLC–MS[22]. Similar techniques for the analysis of HMPG in plasma and in tissues such as liver are not yet available.

Mass fragmentography offers a mode of detection with unsurpassed specificity and sensitivity[17]. Using internal standards labelled with stable isotopes, the precision can be further increased. This paper describes the use of HMPG labelled with three

deuterium atoms as the internal standard in the mass fragmentographic determination of free and conjugated HMPG in rat and human urine, in rat liver and brain and in human plasma and CSF.

EXPERIMENTAL

Materials

2-(4-Hydroxy-3-methoxyphenyl)-2-hydroxyacetic acid (VMA) and the piperazine salt of HMPG were obtained from Calbiochem (Los Angeles, Calif., U.S.A.). HMPG sulphate, potassium salt, was a gift from Hoffman-La Roche (Basle, Switzerland). Sulphatase, type H-1 (23,000 units/g of phenolsulphatase and 300,000 units/g of β-glucuronidase) was supplied by Sigma (St. Louis, Mo., U.S.A.). This preparation is uncontaminated with material which, following incubation and extraction into ethyl acetate and treatment with trifluoroacetic anhydride (TFA), gives a mass fragmentographic signal corresponding to HMPG–TFA (*m/e* 472). The ethyl acetate and inorganic chemicals were of standard analytical grade. Drugs were obtained through the courtesy of the following pharmaceutical companies: pargyline hydrochloride from Abbot Labs. (North Chicago, Ill., U.S.A.); nialamide hydrochloride from Pfizer (New York, N.Y., U.S.A.); debrisoquine sulphate from Hoffmann-La Roche. Dr. B. Ho of the Texas Research Institute of Mental Sciences kindly supplied the 2,9-dimethyl-β-carbolinium iodide[18].

Urine was collected from healthy laboratory personnel. Plasma was obtained from healthy volunteers following fasting overnight. Rat urine was collected from 200–250-g male rats placed in metabolic cages and fed with a synthetic diet free from *l*-phenylalanine, *l*-tyrosine and *l*-dihydroxyphenylalanine. Rat or human urine was analyzed at once or stored frozen at −20° until required for analysis. Human CSF was obtained from diagnostic lumbar punctures on psychiatric and neurological patients. The CSF was pooled and stored frozen (−20°) until required for analysis.

HMPG-D_3 was synthesized using VMA as the starting material. The benzylic hydrogen atom was exchanged for deuterium in $NaOD/D_2O$. This compound was then reduced with $LiAlD_4$ in tetrahydrofuran to yield HMPG-D_3. Details of the procedure have been published elsewhere[19]. The material was homogeneous with respect to gas–liquid and thin-layer chromatography. Mass spectrometry of the TFA derivative confirmed the quantitative substitution of deuterium for hydrogen in the side-chain (Fig. 1). The fragmentation pattern is similar to that described previously[20]. The molecular ion of the TFA derivative of the deuterium-labelled HMPG occurs at *m/e* 475 and in the corresponding derivative of HMPG at *m/e* 472. The ratio between the peak heights at *m/e* 472 and 475 for HMPG-D_3–TFA was 0.008. The HMPG-D_3 was dissolved in distilled water and stored frozen at −20°.

Analysis of urine

The same general procedure was used for both rat and human urine. If precipitation occurred on thawing, the sample was centrifuged prior to analysis.

A 100-μl aliquot of the urine was transferred into a centrifuge tube, to which were added 0.8 ml of 0.1 *M* citrate buffer of pH 6.4, 0.05 ml of a saturated solution of barium chloride and 10 nmoles of HMPG-D_3. The sample was mixed and centrifuged briefly at 1000 *g*.

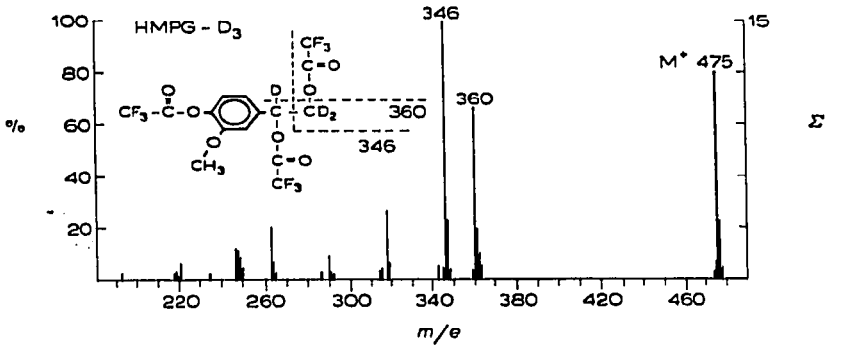

Fig. 1. Mass spectrum of the trifluoroacetyl derivative of HMPG-D_3.

To the supernatant were added 10 mg of sulphatase dissolved in 0.1 ml of distilled water. The test-tubes were capped and incubated at 37° for 12–16 h, usually overnight. After the enzymatic hydrolysis, the sample was extracted three times with 2 ml of ethyl acetate and the combined extracts were evaporated to dryness.

Analysis of human CSF

To 2 ml of CSF were added 2.5 nmole of HMPG-D_3 and 10 mg of sulphatase and the pH was adjusted to 6–7 prior to incubation at 37° overnight. The sample was extracted three times with 2 ml of ethyl acetate and the combined extracts were evaporated to dryness.

Analysis of human plasma

To 4 ml of plasma were added 5 nmoles of HMPG-D_3 and 10 mg of sulphatase and the mixture was incubated at 37° overnight. After shaking the sample with 4 ml of ethyl acetate, the mixture was centrifuged at 10,000 *g* for 45 min in order to separate the organic phase from the water. A second extraction was carried out with 3 ml of ethyl acetate and brief centrifugation at 1000 *g*, and the combined extracts were evaporated to dryness.

Analysis of rat liver and brain

The organs were removed immediately after death, cut into pieces, washed free from blood with saline and homogenized in 40% ethanol with an Ultra-Turrax Model TP 18/2 homogenizer. One nanomole of HMPG-D_3 was added to each sample and thoroughly mixed with the homogenate. Following centrifugation at 10,000 *g* for 45 min, the supernatant was decanted off, evaporated to dryness and the residue dissolved in water (pH 6–7). Sulphatase (10 mg) was added and the samples were incubated at 37° overnight. The tubes were centrifuged briefly at 1000 *g* before the supernatant (pH 6.5) was extracted three times with 2-ml portions of ethyl acetate . The combined extracts were evaporated to dryness and TFA derivatives prepared as described below.

Analysis of free and conjugated HMPG separately

After addition of the internal standard HMPG-D_3, the sample was extracted twice with 3-ml portions of ethyl acetate. The organic phase was evaporated to dryness

and the residue analyzed for free HMPG. The remaining aqueous phase was extracted once with diethyl ether and the organic phase was discarded. The remaining traces of diethyl ether were removed by placing the sample for 20 min in a water-bath at 37°. HMPG-D_3 and sulphatase were added before incubation at 37° overnight. The released HMPG was extracted and analyzed as described above.

Animal experiments

Male rats, 200–250 g in weight, were given intraperitoneal injections of saline, pargyline hydrochloride (150 mg/kg), nialamide hydrochloride (30 mg/kg), debrisoquine sulphate (100 mg/kg) or 2,9-dimethyl-β-carbolinium iodide (10 mg/kg). Four hours after injection, the animals were decapitated with a guillotine, the liver and brain were rapidly removed and treated as described above.

Preparation of derivatives

The samples were re-dissolved in 50 μl of ethyl acetate and 50 μl of TFA (Pierce, Rockford, Ill., U.S.A.) were added. After mixing, the contents were allowed to stand for at least 15 min, then evaporated to dryness, and the residue was re-dissolved in 50 μl of ethyl acetate and injected into the gas chromatograph–mass spectrometer (GC–MS). If the samples had to be stored for more than a few hours, TFA was added to the ethyl acetate to a concentration of 1 %.

Instrument conditions

An LKB 9000 gas chromatograph – Mass spectrometer, equipped with a multiple ion detector (MID), was used. The focusing of the MID was guided automatically by a device initiated when the intensity of a fragment exceeded 2.5% of the maximal turn of the scale. The standard conditions used were a 1% SE-30 column operated at 150° or a 3% OV-17 column at 160°. The energy of the electrons at the ion source was 22.5 eV. The signal at *m/e* 472 (HMPG–TFA) and *m/e* 475 (HMPG-D_3–TFA) was registered by three galvanometers (amplifying 1, 10 and 100 times) on UV-sensitive paper.

Calculation of results

The peak heights at *m/e* 472 and 475 were measured manually. A standard curve was constructed from samples with known ratios of HMPG/HMPG-D_3 or HMPG-SO_4/HMPG-D_3 in water. These samples were incubated and extracted and derivatives were prepared as described for the biological samples. The unknown amount of endogenous HMPG and HMPG-SO_4 was then calculated via the standard curve and the known amount of internal standard (HMPG-D_3) in the sample.

RESULTS

In order to check the amount of enzyme necessary to achieve maximal hydrolysis, samples from the same pool of urine were subjected to hydrolysis with different amounts of sulphatase added. The results are shown in Fig. 2. It appears that for rat urine, 10 mg of sulphatase are sufficient to hydrolyze the conjugated HMPG. Routinely, 10 mg of sulphatase were used for the hydrolysis of the HMPG conjugates present in 0.1 ml of urine. A curve of the time course for the release of free HMPG (Fig. 3)

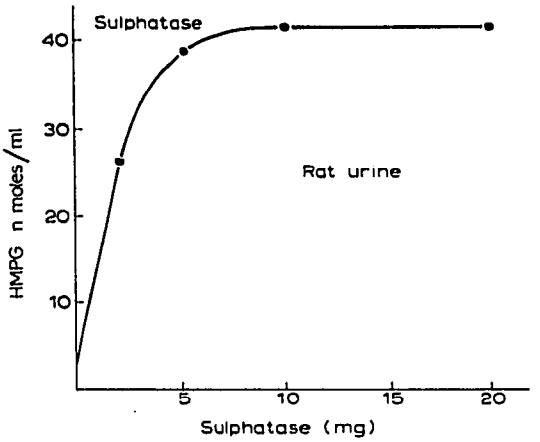

Fig. 2. Hydrolysis of conjugated HMPG in rat urine using sulphatase. Incubation time, 16 h.

indicated that the hydrolysis was maximal after 10 h with 10 mg of sulphatase in human urine also. The same amount of sulphatase was sufficient to hydrolyze the conjugated HMPG in plasma, liver and brain.

The standard curves were straight lines that virtually coincided for HMPG/HMPG-D_3 and HMPG-SO_4/HMPG-D_3. Simply mixing HMPG and HMPG-D_3 in the same ratios and making derivatives gave the same result.

Analysis of 0.5–25 nmoles of synthetic *d,l*-HMPG-SO_4 added to 2 ml of distilled water containing 10 mg of sulphatase and 25 nmoles of HMPG-D_3 gave a recovery of 101 $\pm$ 7.2 % ($n = 7$). Addition of 0.5–25 nmoles of synthetic *d,l*-HMPG to 2 ml of distilled water containing 25 nmoles of HMPH-D_3 gave a recovery of 100 $\pm$ 5.1 % ($n = 7$).

The mass fragmentographic analysis of HMPG–TFA was performed with the instrument focused at *m/e* 472, representing endogenous HMPG–TFA, and at *m/e* 475, representing HMPG-D_3–TFA. Virtually the only peaks that appeared were those

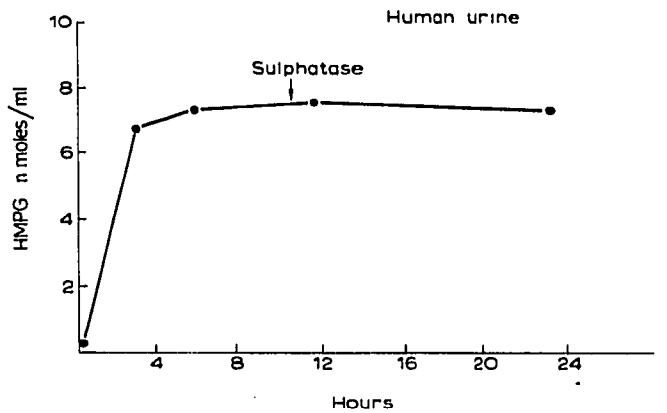

Fig. 3. Time curve showing the hydrolysis of conjugated HMPG in human urine using 10 mg of sulphatase.

with a retention time identical with that of authentic HMPG–TFA. This is shown in Fig. 4 for rat urine and brain and human plasma, representing analyses in which different sensitivities are required. In order to check the specificity, both the fragment ratios *m/e* 358/360 (see Fig. 1) and *m/e* 472/475 were focused for a human urine pool ($n = 10$). The mean value and coefficient of variation were found to be 8.21 nmoles ±5.6% and 7.15 nmoles ±4.5%, respectively. In human urine, about 6% of the total amount of HMPG found appeared as free HMPG. The corresponding values for human serum and CSF were 10% and 75%, respectively.

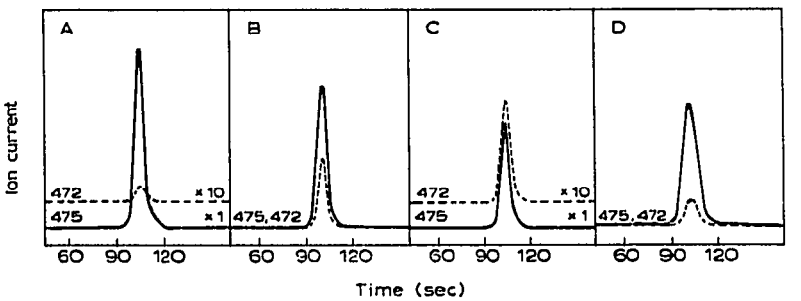

Fig. 4. Mass fragmentographic traces from analysis of HMPG. (A) Internal standard, HMPG-D_3; (B) rat urine; (C) rat brain; (D) human plasma. Solid line, signal from HMPG-D_3–TFA at *m/e* 475; broken line, signal from HMPG–TFA at *m/e* 472.

In order to illustrate the usefulness of the method in a biological application requiring high sensitivity, the following experiment was performed. Rats received intraperitoneal injections of saline or the monoamine oxidase inhibitors (MAOI) pargyline hydrochloride, nialamide hydrochloride, debrisoquine sulphate or 2,9-dimethyl-β-carbolinium iodide. The last two compounds do not pass the blood–brain barrier. After 4 h, the animals were killed and the brain and liver were analyzed for HMPG as

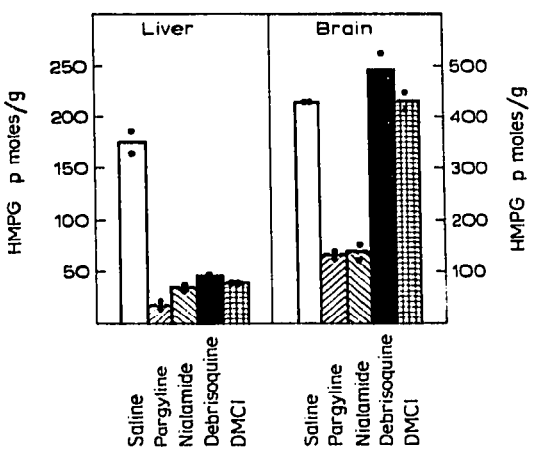

Fig. 5. Concentration of HMPG in rat liver and brain 4 h after injection of saline, pargyline hydrochloride, nialamide hydrochloride, debrisoquine sulphate and 2,9-dimethyl-β-carbolinium iodide (DMCI).

described above. The results are shown in Fig. 5. The good agreement between the duplicates in tissues from animals treated with MAOI indicates a high precision at levels of only 30–40 pmoles/g. These levels of HMPG are almost 1000 times lower than those present in the urine of untreated rats (Table I).

TABLE I

LEVELS OF HMPG IN TISSUES AND BODY FLUIDS FROM HUMANS AND RATS

Fluid or tissue	*HMPG level (nmoles/ml or nmoles/g)*	*Number of analyses, n*	*Coefficient of variation (%)*
Human plasma	0.056 ± 0.003	18	5.4
Human urine	7.4 ± 0.21	20	2.8
Human CSF	0.073 ± 0.006	16	8.2
Rat urine	24 ± 0.86	6	3.6
Rat brain	0.26 ± 0.016	10	6.2
Rat liver	0.099 ± 0.013	6	13

The precision of the method was examined by repetitive analyses of pooled urine, plasma and CSF from healthy adult humans and of pooled rat urines and homogenates of brain and liver. The results are shown in Table I. With urine, the precision was high, as shown by a coefficient of variation of less than 4%. With human plasma, CSF and rat tissues, in which the levels are 10–100 times lower, the coefficient of variation was 5–13%.

DISCUSSION

The methods developed for the analysis of the important catecholamine metabolite HMPG over the last 10 years illustrate well the general advances in the analytical techniques that have been made. Although spectrometric methods[8,9] are relatively simple and cheap, the sensitivity and specificity are not sufficient. Gas chromatographic methods[11–16], especially those using the electron capture detector, have improved the analysis in these respects, but often suffer from difficulties with the background and the precision and have relatively low capacity.

The mass fragmentographic technique represents the latest advance in the analysis of catecholamines and their metabolites, having unparalleled specificity and sensitivity. The high specificity makes the isolation procedure less critical and labour consuming and therefore permits many samples to be analyzed within a short time.

Many of these advantages are exemplified in the mass fragmentographic techniques for urinary- and brain-HMPG analysis described by Karoum *et al.*[6] and Braestrup[16], respectively. They used tryptophol or VMA as the internal standard. However, as pointed out by Gréen *et al.*[21] and others, it should be more advantageous to use the deuterated species as the internal standard, as it would be expected to behave similarly throughout the isolation procedure, but be differentiated in the final mass spectrometric analysis.

In this study, we have prepared HMPG containing three deuterium atoms as

the internal standard for the mass fragmentographic determination of HMPG in a number of tissues and body fluids. Bertilsson previously described[22] the use of HMPG-D_2 for the mass fragmentographic analysis of HMPG in human CSF. The incorporation of deuterium in this standard was not quantitative. The peak height ratio between the HMPG–TFA and HMPG-D_2–TFA was 0.14. The corresponding ratio for our standard was 0.008. A low background contribution from the internal standard is of value particularly for analysis requiring high sensitivity.

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