Journal of Chromatography, 87 (1973) 147–153

CHROM. 6977

QUANTITATIVE DETERMINATION OF 4-HYDROXY-3-METHOXYPHENYL GLYCOL AND ITS CONJUGATES IN CEREBROSPINAL FLUID BY MASS FRAGMENTOGRAPHY

LEIF BERTILSSON

Departments of Clinical Pharmacology (at Huddinge Hospital), Pharmacology, Psychiatry (at Karolinska Hospital) and Toxicology (Swedish Medical Research Council), Karolinska Institutet, S-104 01 Stockholm 60 (Sweden)

(Received June 25th, 1973)

SUMMARY

A method for the quantitative determination of 4-hydroxy-3-methoxyphenyl glycol and its conjugates in human cerebrospinal fluid has been developed. Unconjugated 4-hydroxy-3-methoxyphenyl glycol and its conjugates after hydrolysis (81% efficiency) were reacted with trifluoroacetic anhydride. The derivative containing three trifluoroacetyl groups was analyzed by mass fragmentography. 4-Hydroxy-3-methoxyphenyl glycol labelled with two deuterium atoms was synthesized and used as internal standard. The molecular ions *m/e* 472 and 474 in the mass spectra were used for monitoring 4-hydroxy-3-methoxyphenyl glycol and labelled 4-hydroxy-3-methoxyphenyl glycol, respectively. This sensitive and specific method allows the quantitative determination of unconjugated and conjugated 4-hydroxy-3-methoxyphenyl glycol in 2 ml of the same cerebrospinal fluid specimen in concentrations down to 1 ng/ml. The standard deviation for the determination of unconjugated 4-hydroxy-3-methoxyphenyl glycol was ±7% in the concentration range 2–36 ng/ml. Synthetic 4-hydroxy-3-methoxyphenyl glycol sulphate added to cerebrospinal fluid (15 ng/ml) was recovered in a yield of 99.3±8.0%.

INTRODUCTION

Recent biochemical and pharmacological studies indicate that at least two subtypes of endogenous depression may exist[1], hypothetically called "noradrenaline" and "5-hydroxytryptamine" depressions. It has been suggested that the concentration of the major metabolite of 5-hydroxytryptamine, 5-hydroxyindole-3-acetic acid (5-HIAA) in the cerebrospinal fluid (CSF) of depressed patients has a bimodal distribution[2]. 5-HIAA and indole-3-acetic acid, the major metabolite of tryptamine, were measured by novel quantitative mass fragmentographic methods[3,4].

The major metabolites of noradrenaline in the central nervous system are unconjugated and conjugated 4-hydroxy-3-methoxyphenyl glycol[5,6] (HMPG; sometimes also abbreviated MHPG or MOPEG, although according to IUPAC rules phenyl substituents should be in alphabetical order). Gas chromatographic (GC) methods for determination of HMPG in CSF have been developed during the last

few years[7–9]. In these methods fluoroacyl esters of HMPG were prepared and after GC separation detected by electron capture. Shopsin *et al.*[10] have reported normal levels of HMPG, while Post *et al.*[11] have found decreased concentrations, in the CSF of depressed patients as compared to controls.

Mass fragmentography[12], which offers unique specificity and sensitivity, has recently been applied to quantitative determinations of biogenic amine metabolites in CSF[3,4,13]. In mass fragmentographic methods an ideal internal standard may be used, *i.e.* the compound being measured is labelled with stable isotopes[3].

This paper describes a method for an accurate quantitative determination of unconjugated and conjugated HMPG in CSF by mass fragmentography. HMPG labelled with two deuterium atoms was used as the internal standard.

EXPERIMENTAL

Reagents and reference compounds

Trifluoroacetic anhydride (TFAA, Pierce, Rockford, Ill., U.S.A.,) was obtained in 100-ml bottles. The ethyl acetate and diethyl ether were of nanograde and analytical quality, respectively (Mallinckrodt, St. Louis, Mo., U.S.A.,). Sulphatase (18,000 units per gram) containing β-glucuronidase (300,000 units per gram) (Type H-1, Sigma, St. Louis, Mo., U.S.A.) was dissolved in sodium citrate buffer (0.1 *M*; pH 5.2) in a concentration of 800 units of sulphatase per millilitre before use. In some experiments β-glucuronidase Type I (50,000 units per gram, Sigma Chemical Co.) dissolved in 0.2 *M* potassium phosphate buffer (pH 7.0) at a concentration of 2,500 units per millilitre was used. HMPG piperazine salt (Calbiochem., Los Angeles, Calif., U.S.A.,) and HMPG sulphate (HMPG SO_4), potassium salt, synthesized according to Hegedüs[14] (a gift from Hoffman-La Roche, Basle, Switzerland) were stored at 4°.

Amounts of unconjugated HMPG and its conjugates are expressed as non-ionized unconjugated HMPG.

1-(4-Hydroxy-3-methoxyphenyl)-2-[$^2H_2$]glycol (HMPG-D_2) was synthesized by reduction of 4-hydroxy-3-methoxymandelic acid (Koch-Light, Colnbrook, Great Britain) with lithium aluminium deuteride (isotopic purity ≥99% from E. Merck, Darmstadt, G.F.R.) in glycol dimethyl ether (redistilled over calcium hydride, from E. Merck) for 18 h at 20°. After addition of deuterium oxide (isotopic purity 99.7%, from Koch-Light) and then 2 *M* hydrochloric acid (to pH 5) the product was extracted into ethyl acetate. The organic phase was dried with anhydrous sodium sulphate and evaporated to dryness. The red oil thus obtained was dissolved in 10% aqueous acetone at a concentration of 1 μg of HMPG-D_2/ml (determined by mass fragmentography by comparison with known amounts of HMPG). This internal standard solution was stored frozen at −20°.

Gas chromatography–mass spectrometry

An LKB Model 9000 gas chromatograph–mass spectrometer (LKB-Produkter, Bromma, Sweden) was used. The separations were made on a 1.2 m × 3 mm I.D. silanized glass column packed with 3% XE-60 on Gas-Chrom P 100/120 M (Applied Science Lab., State College, Pa., U.S.A.), maintained at a temperature of 130°. The temperature of the flash heater was 200° and the ion source was kept at 270°. The flow-rate of the carrier gas (helium) was 25 ml/min. The ionizing potential and trap

current were 70 eV and 60 μA, respectively. This instrument was used to obtain conventional mass spectra of reference HMPG and HMPG-D_2 derivatives prepared from milligram amounts. For mass fragmentography a multiple ion detector (MID)[15] was added to permit quantification of the small amounts of HMPG that were present in the CSF samples.

The same instrumentation conditions as described above were used for mass fragmentography, but the temperature of the column was 145°. The mass spectrometer was adjusted to record the intensity of *m/e* 472 and 474 (molecular ions of HMPG and HMPG-D_2 derivatives, respectively) on two different channels. A description of the focussing procedure has recently been reported[3]. The intensity of the molecular ion of the HMPG derivative was measured at different ionizing potentials and was found to have a maximum at 50 eV. This ionization potential was thus used for mass fragmentography.

Procedure. CSF samples were obtained by lumbar puncture and stored in glass tubes at −20° until analyzed. To 2.0 ml of CSF in a 15-ml silanized glass-stoppered tube, 50 μl of the internal standard solution (see above) and 0.5 ml sodium citrate buffer (0.5 *M*; pH 5.2) were added. This was extracted twice with 6 ml of ethyl acetate with a centrifugation at 4000 × *g* for 5 min after each extraction. The aqueous phase was saved for the analysis of conjugated HMPG (see below). The combined organic phases were concentrated to about 1 ml in a silanized, pear-shaped 25-ml flask at 15 mm Hg in a 40° water-bath. The ethyl acetate solution was transferred to a 3-ml glass-stoppered tube and 0.25 ml of trifluoroacetic anhydride was added. After reaction for 1 h at 20°, the tube was stored at 4°. Just before the mass fragmentographic analysis, the reaction mixture was evaporated to dryness in a stream of nitrogen at 40°. The residue was dissolved in 40 μl of ethyl acetate and 1–3 μl injected into the gas chromatograph–mass spectrometer.

For the analysis of conjugated HMPG, the aqueous phase from above was extracted with 6 ml of diethyl ether. After centrifugation at 4000 × *g* for 5 min the organic phase was discarded. To the aqueous phase 50 μl of the internal standard solution were added. The remaining traces of diethyl ether were removed by incubating the tube for 20 min at 37° in a water-bath. Hydrolysis of HMPG conjugates was performed after addition of 0.5 ml of the sulphatase (Type H-1) solution (400 units, see above) and incubation overnight (14–16 hours) at 37° in an agitated water-bath. The tubes were cooled to 4°, extracted twice with 6 ml of ethyl acetate and analyzed as described above for unconjugated HMPG.

In some experiments the hydrolysis of the HMPG conjugates was performed by using 0.4 ml of the β-glucuronidase (Type I) solution (1,000 units, see above) instead of the Type H-1 enzyme. Before the incubation the pH was adjusted to 7.0 and after incubation it was readjusted to 5.2.

RESULTS AND DISCUSSION

In the present method for the mass fragmentographic determination of HMPG in CSF, the extraction and derivatization procedures described by Wilk *et al.*[7] have been applied. Thus unconjugated HMPG is extracted at pH 5.2 from CSF by ethyl acetate and subsequently derivatized by trifluoroacetic anhydride. The mass spectrum and proposed fragmentation pattern of the derivative of HMPG prepared on a milli-

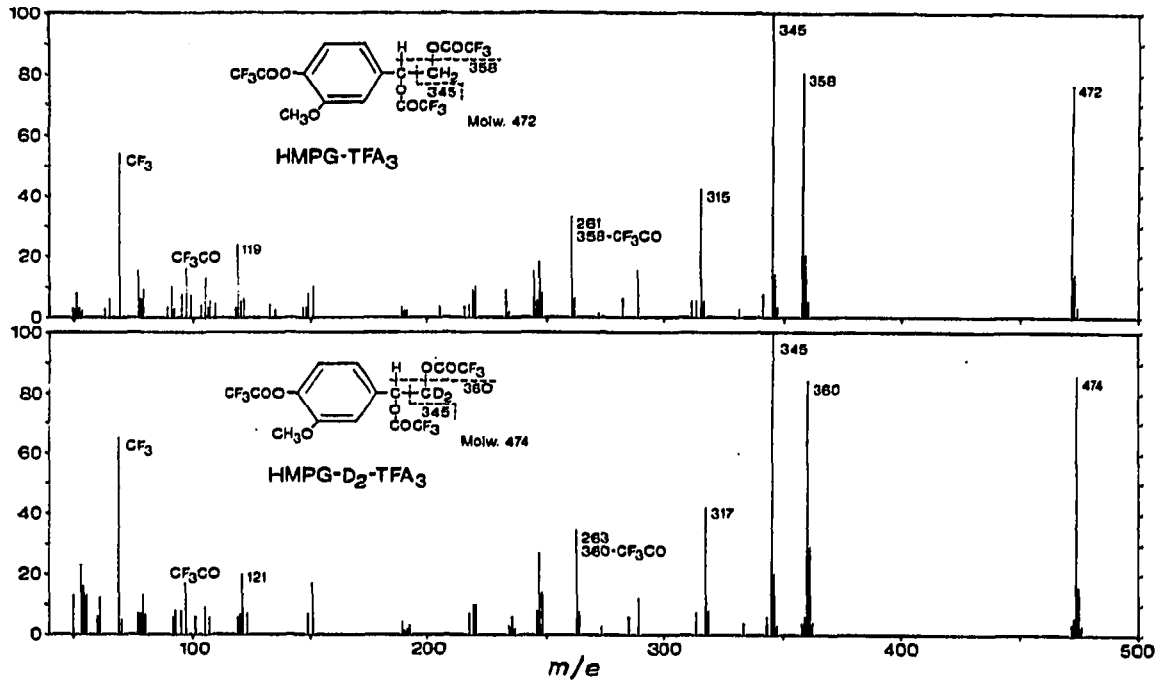

Fig. 1. Mass spectra and proposed fragmentation pattern of the trifluoroacetyl (TFA) derivatives prepared from reference HMPG (upper part) and HMPG-D_2 (lower part) used as an internal standard in the analysis of HMPG.

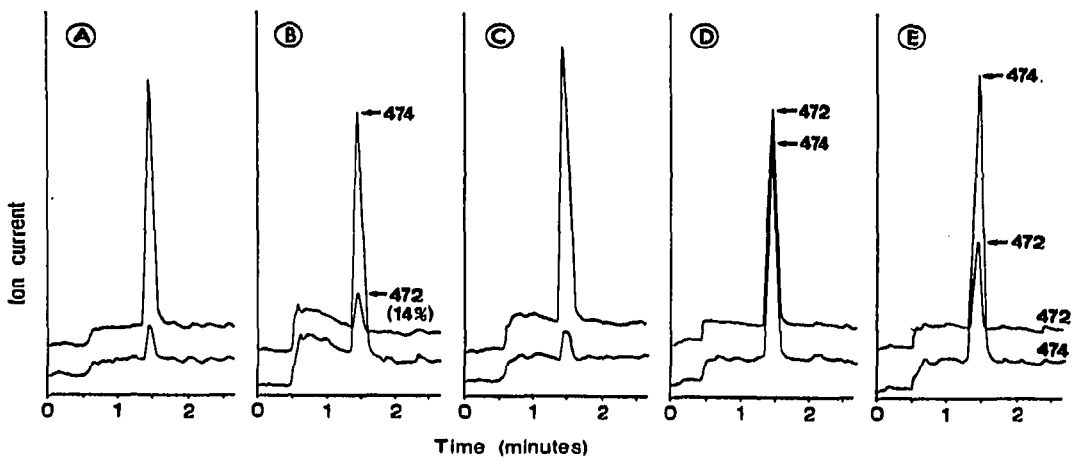

Fig. 2. Mass fragmentograms obtained from derivatives of: (A) reference HMPG; (B) reference HMPG-D_2; (C) material extracted from CSF (without hydrolysis); (D) same as (C) but the internal standard HMPG-D_2 was added to CSF; (E) material extracted from CSF after hydrolysis (HMPG-D_2 was added prior to hydrolysis). The CSF analyzed here (C–E) contained unconjugated and conjugated HMPG in concentrations of 24 and 6.7 ng/ml, respectively. The mass spectrometer was set to detect the molecular ions of HMPG (*m/e* 472, upper channel) and HMPG-D_2 (*m/e* 474, lower channel).

gram scale (Fig. 1, upper part) is analogous to that reported for the pentafluoropropionyl derivative of HMPG[16]. The most prominent peaks are *m/e* 472 (molecular ion), 358 (formed by the loss of trifluoroacetic acid) and 345 (base peak, formed by the loss of CH_2OCOCF_3).

The HMPG derivative in the previously reported methods[7-9] was detected by electron capture after GC separation. In the present paper the specificity of the detection is increased by using the mass spectrometer as a detector for the GC effluent. Mass fragmentography has independently been used for the determination of HMPG in human and rat urine[17]. For the analysis of HMPG in CSF the mass spectrometer was set to detect the molecular ion at *m/e* 472. Fig. 2A shows a reference mass fragmentogram of HMPG, which has a retention time of 1.5 min. The mass fragmentogram of HMPG from human CSF (Fig. 2C) lacked other peaks and the derivative had the same properties as the synthetic reference. To confirm the identity of HMPG in CSF, the mass spectrometer was also focussed on *m/e* 345 and 358. This gave identical results.

In previous techniques for the quantitative determination of HMPG no internal standard[8,9], or internal standards with little similarity to HMPG, *i.e.* tyramine[7] and tryptophol[17], were used. HMPG-D_2 has been synthesized and used as an internal standard in the present paper. 4-Hydroxy-3-methoxymandelic acid was reduced to HMPG-D_2 by lithium aluminium deuteride. The mass spectrum of the trifluoroacetyl derivative of HMPG-D_2 (Fig. 1, lower part) showed that the isotopic purity of the compound was satisfactory for the present purpose. For the recording of HMPG-D_2 by mass fragmentography the molecular ion at *m/e* 474 was used.

The standard curves for the quantitative determination of unconjugated and conjugated HMPG in CSF (Fig. 3) were prepared using HMPG-D_2 as the internal standard. After the unconjugated HMPG had been extracted from the CSF, the conjugates were enzymatically hydrolyzed by arylsulphatase containing β-glucuronidase.

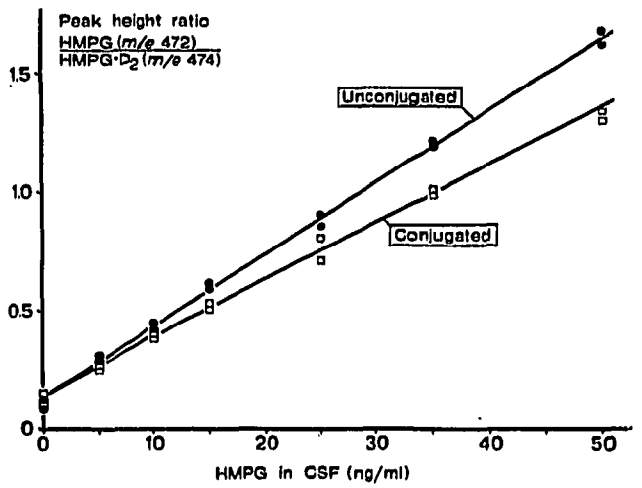

Fig 3. Standard curves for the quantitative determination of unconjugated and conjugated HMPG in CSF. The curves were prepared by analyzing standard solutions of HMPG and HMPG SO_4 in artificial CSF by the entire procedure described in the Experimental section. Concentrations of conjugated HMPG are given as nanograms of unionized unconjugated HMPG per millilitre of CSF.

The efficiency of the hydrolysis was almost constant (81%, as the same peak height ratio was obtained from 50 ng of HMPG SO_4/ml and 40.5 ng of HMPG/ml) in all standard CSF samples, as the peak height ratio of the duplicates were similar and the standard curve was a straight line. The intercept of the ordinate at 0.14 is the same as the peak height ratio between *m/e* 472 and 474 in HMPG-D_2 (Fig. 2B). The standard deviation for the determination of unconjugated HMPG was ±7.0% of the mean (12.6 ng/ml) when eighteen human CSF samples (concentration range 2–36 ng/ml) were analyzed in duplicates. When 30.0 ng of synthetic HMPG SO_4 was added to one of 2.0-ml duplicates of human CSF ($n=12$), the difference in concentration between the two samples was 29.8±2.4 ng (S.D.) (99.3±8.0% recovery). This shows that the hydrolysis of HMPG SO_4 was the same in the human CSF as in the samples of artificial CSF used for the preparation of the standard curve.

In the urine of rat HMPG is mainly found as the sulphate conjugate, but man excretes about equal amounts of HMPG as the sulphate and glucuronide[17]. In the present method for the determination of HMPG in CSF an enzyme containing high activities of both arylsulphatase and β-glucuronidase (Type H-1, Sigma) was used for the hydrolysis of conjugated HMPG. In some experiments incubations with β-glucuronidase (Type I, Sigma) were performed. This gave less than 50% of the yield obtained by the other enzyme in both standard (HMPG SO_4 in artificial CSF) and human CSF specimens. It was not possible to increase the recovery by adding the Type I enzyme to incubations with the Type H-1 enzyme. From these experiments it is concluded that the hydrolysis of conjugated HMPG is performed under optimal conditions using the Type H-1 enzyme.

In a group of fourteen depressed patients the total HMPG concentration in CSF was 13.4±3.5 ng/ml (S.D.) before antidepressive treatment, which is within the range previously reported[10,11]. Of this 10.9±9.3% (S.D.) consisted of the conjugated metabolite. The results of this study will be reported elsewhere.

ACKNOWLEDGEMENTS

I thank Å. Hallström and L. Roswall for expert technical assistance, J. R. Althaus for help with the synthesis of HMPG-D_2 and Hoffmann-La Roche for the kind supply of HMPG SO_4. This project was supported by grants from the Bank of Sweden Tercentenary Fund, the Swedish Medical Research Council (B72-40Y-2375-05), the National Institute of Mental Health, Chevy Chase, Md., U.S.A., (Grant No. 12007), the Wallenberg Foundation and by funds from the Karolinska Institute.

REFERENCES

1 F. Sjöqvist, *Int. Pharmacopsychiat.*, 6 (1971) 147.
2 M. Åsberg, L. Bertilsson, D. Tuck, B. Cronholm and F. Sjöqvist, *Clin. Pharmacol. Ther.*, 14 (1973) 277.
3 L. Bertilsson, A. J. Atkinson, Jr., J. R. Althaus, Å. Härfast, J. E. Lindgren and B. Holmstedt, *Anal. Chem.*, 44 (1972) 1434.
4 L. Bertilsson and L. Palmér, *Science*, 177 (1972) 74.
5 S. M. Schanberg, J. J. Schildkraut, G. R. Breese and I. J. Kopin, *Biochem. Pharmacol.*, 17 (1968) 247.
6 S. M. Schanberg, G. R. Breese, J. J. Schildkraut, E. K. Gordon and I. J. Kopin, *Biochem. Pharmacol.*, 17 (1968) 2006.

7 S. Wilk, K. L. Davis and S. B. Thacker, *Anal. Biochem.*, 39 (1971) 498.
8 E. K. Gordon and J. Oliver, *Clin. Chim. Acta*, 35 (1971) 145.
9 F. Karoum, C. R. J. Ruthven and M. Sandler, *Biochem. Med.*, 5 (1971) 505.
10 B. Shopsin, S. Wilk, S. Gershon, K. Davis and M. Suhl, *Arch. Gen. Psychiat.*, 28 (1973) 230.
11 R. M. Post, E. K. Gordon, F. K. Goodwin and W. E. Bunney, Jr., *Science*, 179 (1973) 1002.
12 C. G. Hammar, B. Holmstedt and R. Ryhage, *Anal. Biochem.*, 25 (1968) 532.
13 B. Sjöqvist and E. Änggård, *Anal. Chem.*, 44 (1972) 2297.
14 B. Hegedüs, *Helv. Chim. Acta*, 46 (1963) 2604.
15 C. G. Hammar and R. Hessling, *Anal. Chem.*, 43 (1971) 298.
16 E. Änggård and G. Sedvall, *Anal. Chem.*, 41 (1969) 1250.
17 F. Karoum, H. LeFèvre, L. B. Bigelow and E. Costa, *Clin. Chim. Acta*, 43 (1973) 127.