Journal of Chromatography, 223 (1981) 305—314
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 824

QUANTITATIVE DETERMINATION OF 3-METHOXY-4-HYDROXYPHENYLETHYLENEGLYCOL AND ITS SULFATE CONJUGATE IN HUMAN LUMBAR CEREBROSPINAL FLUID USING LIQUID CHROMATOGRAPHY WITH AMPEROMETRIC DETECTION

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(Received September 25th, 1980)

SUMMARY

A sensitive and direct reversed-phase liquid chromatographic method with amperometric detection was developed for the determination of 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG). The concentrations of the free and sulfate conjugate of MHPG were measured in human lumbar cerebrospinal fluid. All samples were preconcentrated by extraction with ethyl acetate. Deconjugation of the sulfate form of MHPG was achieved by enzymatic hydrolysis with sulfatase.

Peaks were identified on the basis of chromatographic behavior, ratio of responses at several oxidation potentials and the stopped-flow UV spectra of the collected fractions.

The free MHPG content of 20 cerebrospinal fluid samples ranged between 0.720 and 19.51 ng/ml with the mean of 5.126 ± 4.652 (S.D.) ng/ml. The sulfate conjugate of MHPG in 12 samples of cerebrospinal fluid ranged between 0.08 and 0.850 ng/ml with the mean value of 0.2365 ± 0.2269 (S.D.) ng/ml. Although our results correlate well with the literature values, no attempt was made to interpret the quantitative data since samples were obtained from routine, diagnostic testing of patients admitted to the medical or neurologic services at the Mount Sinai Hospital.

INTRODUCTION

Since the biogenic amine hypothesis for affective disorders was proposed [1,2], considerable amount of research has been done in an effort to establish

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the functional concentrations of catecholamines (CA) in the central nervous system (CNS), both in normal and certain disease states. At the present time there is considerable evidence that central noradrenergic neurons play an important role in cardiovascular regulation [3], hypertension [4], and depressive illnesses [1,2]. In the absence of non-invasive analytical techniques, clinical studies of norepinephrine (NE) turnover and the degree of intra-neural communication usually rely on studies of the products of metabolic disposition of this important catecholamine.

3-Methoxy-4-hydroxyphenylethyleneglycol (MHPG) is a principal metabolite of NE in the brain of various mammals [5—7], and it is found in urine [8—10], plasma [11, 12] and cerebrospinal fluid (CSF) [13, 14]. Measurement of urinary MHPG provides little information on the central turnover since NE is extensively metabolized before leaving the CNS. Urinary MHPG exists as the non-conjugated molecule (free MHPG), as the conjugate of sulfuric acid (MHPG·SO_4) and β-conjugate of glucuronic acid (MHPG·Glu). The conjugated forms predominate in the urine and it has been suggested that the sulfate conjugate reflects the central NE metabolism [15] while the β-glucuronide is derived from the metabolism of systemic NE [16]. The MHPG content in CSF is composed mainly of free MHPG and a small fraction of MHPG·SO_4 [11]. The fact that the sulfate conjugate of urinary MHPG is derived mainly from the central NE metabolism has aroused considerable interest in the analysis of the CSF levels of MHPG, where findings are not likely to be clouded by any peripheral contributions.

Quantitative analysis of MHPG in body fluids has been performed using gas—liquid chromatography (GLC) with electron-capture detection [17] or flame ionization detection [18], alone or in combination with mass spectrometry (MS) [19—21] and, more recently, by high-performance liquid chromatography (HPLC) [9,22]. Although the GLC methods are highly sensitive and lend themselves readily to coupling with MS, a technique of unsurpassed identification potential, poor volatility and thermal instability of MHPG necessitate the use of derivatization procedures for enhancement of volatility. This not only introduces a new step in the analysis but also poses additional problems due to the lack of stable derivatives and specific derivatizing agents.

The ability of HPLC, particularly in its reversed-phase mode, to resolve complex mixtures in body fluids is well documented in the literature. However, until the advent of electrochemical detection, this technique could not be fully exploited due to the inadequate sensitivity of the most commonly used LC detectors. Since organic functional groups such as phenols can be easily oxidized, the use of the oxidative mode of amperometric detection is ideally suited for the analysis of physiological levels of CA metabolites [9]. Furthermore, since the reversed-phase separations are usually performed using mixtures of aqueous buffers and organic modifiers (methanol or acetonitrile), the main operational requirement for electrochemical detection is thus satisfield. Therefore, we have investigated the use of the reversed-phase mode of HPLC, coupled with amperometric detection in the direct analysis of endogenous levels of free MHPG and its sulfate conjugate in samples of human lumbar CSF.

EXPERIMENTAL

Equipment

A Model 6000A solvent delivery system, Model 660 solvent programmer and Model U6K universal injector, all from Waters Assoc. (Milford, MA, U.S.A.) were used throughout this study. Chromatographic peaks in the HPLC effluents were detected using a Metrohm/Brinkmann voltametric/amperometric detector, Model E611 with an EA 1096 detector cell (Brinkmann Instruments, Westbury, NY, U.S.A.). The amperometric detector operates on a wall-jet principle and employs glassy carbon working and auxiliary electrodes and a Ag/AgCl reference electrode.

In addition, a Model SF 770 Spectroflow Monitor (Kratos, Schoeffel Instrument Div., Westwood, NJ, U.S.A.) with a deuterium lamp and an 8-μl cell equipped with a 330A wavelength drive and MM 700 memory module, was used for obtaining stopped-flow UV spectra.

Areas of chromatographic peaks were electronically integrated using a Hewlett-Packard Model 3380A electronic integrator (Hewlett-Packard, Avondale, PA, U.S.A.). Stainless-steel columns (30 cm × 4.6 mm I.D.) were prepacked at the factory with 10-μm (average particle size) totally-porous support with a chemically-bonded octadecyl (C_{18}) moiety (Waters Assoc.).

Reagents

All reagents used were of highest purity (ACS Certified Grade). The MHPG reference compound and the enzyme sulfatase, Type V (β-glucuronidase activity: 2 units per mg of solid) were both purchased from Sigma (St. Louis, MO, U.S.A.); potassium dihydrogen phosphate from Mallinckrodt (St. Louis, MO, U.S.A.); methanol (distilled in glass) from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.), and ethyl acetate (pesticide grade) from Fisher Scientific (Springfield, NJ, U.S.A.). Reference solutions of MHPG were prepared in distilled-deionized water and kept refrigerated when not in use.

Chromatographic conditions

For the determination of the free and sulfate conjugate of MHPG, a gradient elution mode of the reversed-phase HPLC was used. The low-strength eluent was 0.1 *M* KH_2PO_4, pH 2.50, and the high-strength eluent was a mixture of anhydrous methanol and distilled-deionized water (3:2, v/v). The low-strength eluent was always filtered through a Millipore membrane filter (Millipore, Bedford, MA, U.S.A.), pore size 0.22 μm, and the high-strength eluent was regularly degassed under vacuum. A 45-min linear gradient from 0—60% of the high-strength eluent was used. The flow-rate was 1.2 ml/min and the temperature was ambient in all cases. Chromatographic peaks were detected amperometrically at oxidation potentials of +1.000 V and +0.700 V.

Sample preparation

Routine, unselected, diagnostic CSF specimens were obtained by lumbar puncture from 34 human subjects admitted to the medical and/or neurologic services at the Mount Sinai Hospital. For the analysis of free MHPG, 2—3-ml samples of CSF were acidified to a pH of 1.0 with 6 *M* HCl and extracted

three times with ethyl acetate (6 ml, 3 ml, 3 ml). The organic layers were pooled, evaporated to dryness under a stream of dry nitrogen, and reconstituted with water. The pH of the remaining aqueous layer was then adjusted to pH 5.2 and the samples were incubated for 16 h with 0.1 ml of sulfatase. Next, the pH was re-adjusted to 1 and the unconjugated compounds extracted three times with ethyl acetate, following the procedure analogous to the one used for the extraction of free MHPG. The protocol procedure is outlined in Fig. 1.

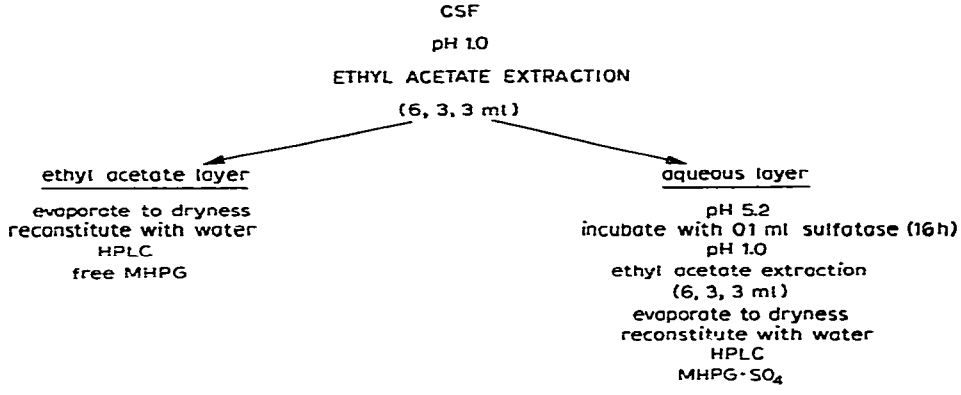

Fig. 1. Protocol procedure for the analysis of free MHPG and its sulfate conjugate.

Peak identification

Initial identification of MHPG in chromatograms of CSF was performed on the basis of retention behavior and co-chromatography with the MHPG reference solution. Since the oxidation potential of the amperometric detector can be varied, high sensitivity and selectivity can thus be achieved by careful selection of the detection potential. Furthermore, ratios of responses at several oxidation potentials were computed for the MHPG reference compound and compared with those for the peaks in CSF samples.

In addition, UV spectra of the MHPG reference compounds and the peaks with the same retention time in a pooled CSF sample were obtained. Due to the low levels of MHPG in the CSF samples, UV spectroscopy is not sufficiently sensitive for its detection. In order to circumvent this problem and gain further insight into the identity of the peak with the retention time of MHPG, several CSF samples were pooled, the sample re-chromatographed and the fraction collected. The corrected stopped-flow UV spectra were obtained on-line with the HPLC system and the details of this procedure are described in the literature [23]. Although these spectra characteristically lack in fine structure, they are nevertheless an important fingerprint of the absorber. The identity of the peaks was deduced on the basis of evidence accumulated from all identification steps.

Extraction efficiency

Prior to the analysis of CSF extracts, the efficiency of the extraction procedure was determined by adding known amounts (ng) of reference MHPG

to the CSF matrix, buffered to a pH of 1, 5 and 7 and extracting it with ethyl acetate. The highest recovery (96.5%) was obtained at the pH of 1.

Reproducibility of retention times and peak areas

CSF samples were kept frozen and their stability was tested periodically over a period of one month to ensure that no sample decomposition was taking place upon storage. The reproducibilities of peak areas and retention times were determined on a day-to-day and within-a-day basis from ten repeated injections. The percent standard deviations for the peak areas and retention times were 1.1% and 0.60%, respectively.

Detection limits and linearity of response

In order to optimize the sensitivity and selectivity of amperometric detection, samples were monitored at oxidation potentials between +0.400 V and +1.000 V. By monitoring the HPLC effluents at +0.700 V, adequate sensitivity is achieved with no loss of selectivity. In addition, the use of low oxidation potentials prevents the deterioration of the glassy carbon electrodes and extends their life-time. Although a 25% increase in the MHPG response can be obtained at an oxidation potential of +1.000 V, this is accompanied by a concomitant decrease in detection selectivity. However, MHPG is completely resolved from the remaining CSF constituents and can thus be detected free from interference at either potential. The detection limit from MHPG detected at +1.000 V was found to be approximately 50 pg.

Linearity of detector response was determined by adding ng amounts of the MHPG reference substance to the CSF matrix. The plot of peak area versus the amount injected was found to be linear over the concentration range of 1 ng to 1 μg. When the sample MHPG content was subtracted from the points on the calibration curve, the line intercepted the axes at the origin.

Interferences

Under the chromatographic conditions used, no interferences with other naturally-occurring CSF constituents were observed at oxidation potentials of +1.000 V and +0.700 V. The hydrolysis of conjugated MHPG can be carried out using several procedures: acid- or base-catalyzed cleavage and enzymatic hydrolysis using aryl sulfatase or glusulase (β-glucuronidase with aryl sulfatase). However, hydrolysis under acidic or basic conditions is not recommended, due to instability of MHPG [24]. Incubation with glusulase, which affords deconjugation of both the MHPG·SO_4 and MHPG·Glu, was not used due to insufficient purity of the enzyme preparation. Aryl sulfatase solutions were found to be of sufficient purity and did not give rise to any background interferences.

RESULTS AND DISCUSSION

Preliminary experiments were conducted in order to establish the best analytical conditions for the separation of MHPG from other naturally-occurring CSF constituents and to achieve sensitive and selective detection. The reversed-phase gradient elution mode of HPLC was adopted since it affords rapid analyses of MHPG and enables simultaneous detection of other CSF constituents.

Thus, the developed chromatographic method is general and yet selective for this important catabolite. Prior to the establishment of the protocol procedure, a study was conducted in order to determine the optimal oxidation potential which would afford high sensitivity without a concomitant loss in selectivity. Fig. 2 illustrates the change in response with detection potential. Although any

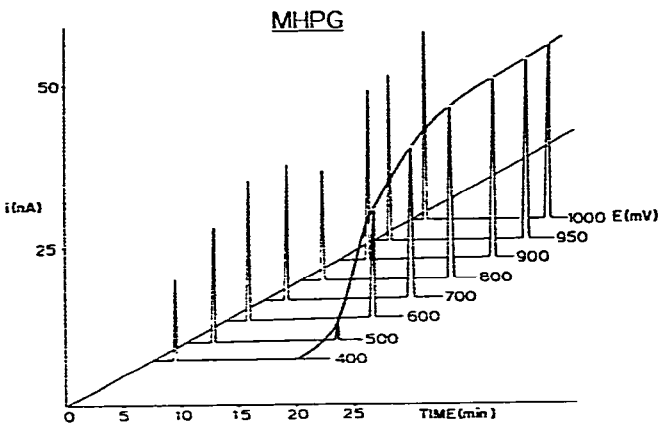

Fig. 2. Variation in the MHPG response with change in oxidation potential; amount injected: 3.0 μg; chromatographic conditions: column, μBondapak C_{18}; eluents: (low strength) 0.1 *M* KH_2PO_4, pH 2.50; (high strength) methanol—water (3:2, v/v); gradient: linear, from 0—60% of the high-concentration eluent in 45 min; flow-rate: 1.2 ml/min; temperature: ambient; detection: amperometric; sensitivity: 2 μA f.s.; recorder attenuation: x 1024.

potential between +0.700 V and +1.000 V offers adequate sensitivity, the use of the former potential is advantageous since the detection selectivity is enhanced and the electrode life-time extended. The use of both potentials is illustrated with the analysis of free MHPG in CSF samples, shown in Figs. 3 and 4, respectively. Although no interferences with other naturally-occurring CSF constituents were observed at either potential in the course of this work, injection of large volumes of CSF may cause problems at high oxidation potentials (> +0.700 V), due to the relatively low levels of MHPG compared to other CSF constituents. In addition, extended use of high oxidation potentials might have a deleterious effect on the stability of glassy carbon electrodes.

The selectivity of the assay is further increased by means of a simple extraction procedure with ethyl acetate, since the phenolic acids are thus separated from the neutral and basic compounds. In order to avoid changes in the sample concentration due to the high volatility of ethyl acetate, the extracts were evaporated to dryness under a stream of dry nitrogen and reconstituted with water. Chromatographic peaks were identified on the basis of retention behavior, co-chromatography with the reference compound and ratios of responses at several oxidation potentials. Additional proof of the identity of the peak was obtained from the comparison of the UV spectra of the collected peak with the retention time of MHPG and the reference compound (Fig. 5).

For the analysis of conjugated MHPG, two enzymatic deconjugation pro-

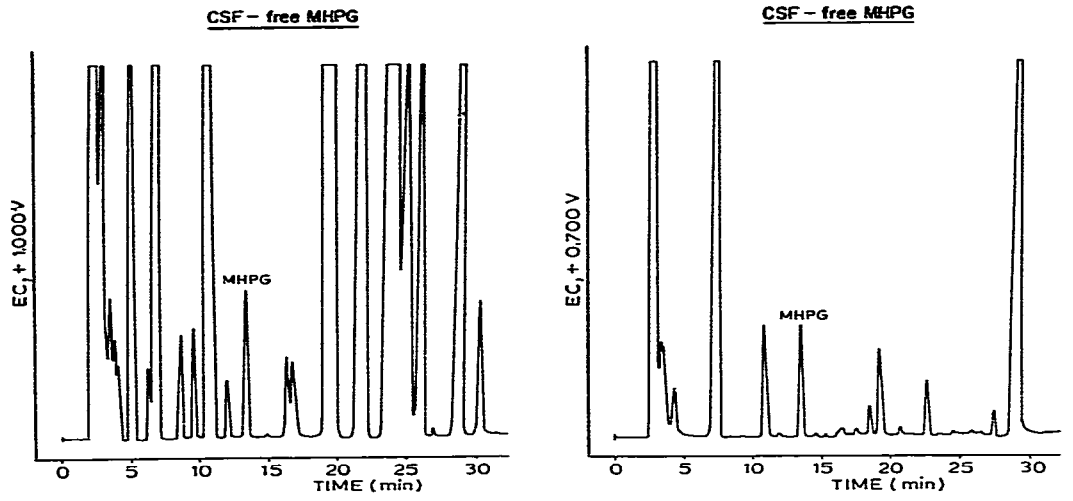

Fig. 3. Chromatogram of the ethyl acetate extract of CSF. Volume injected: 25 μl (corresponding to 0.52 ml of CSF); detection: amperometric, +1.000 V; recorder attenuation: X 256. Chromatographic conditions same as in Fig. 2.

Fig. 4. Chromatogram of the ethyl acetate extract of CSF shown in Fig. 3. Detection: amperometric at +0.7000 V; recorder attenuation: X 256. Chromatographic conditions same as in Fig. 2.

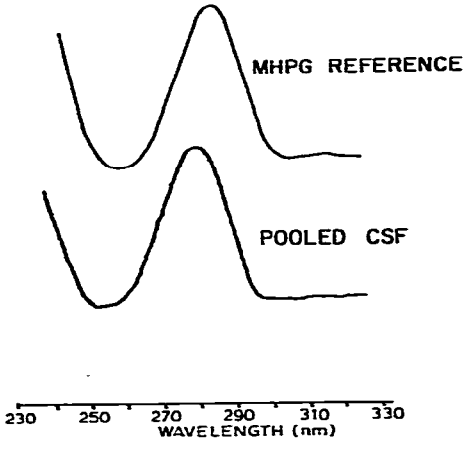

Fig. 5. Stopped-flow UV spectra of the MHPG reference compound and the peak with the same retention time in the pooled sample of CSF. Scanning rate: 100 nm/min; absorbance: 0.1 a.u.f.s. (reference MHPG), 0.02 a.u.f.s. (sample).

cedures were tried. The use of glusulase for the deconjugation of both the MHPG-SO_4 and MHPG-Glu gave rise to interferences due to insufficient purity of the enzyme preparation. In order to determine the enzyme blank, the pH of a sample of distilled-deionized water was adjusted to 5.2 and incubated with 0.1 ml of glusulase. The mixture was then extracted three times with ethyl acetate, the organic layers pooled, evaporated to dryness and reconstituted with water. A chromatogram of the enzyme blank is shown in Fig. 6. In addition, 9 ml of ethyl acetate were evaporated to dryness, reconstituted

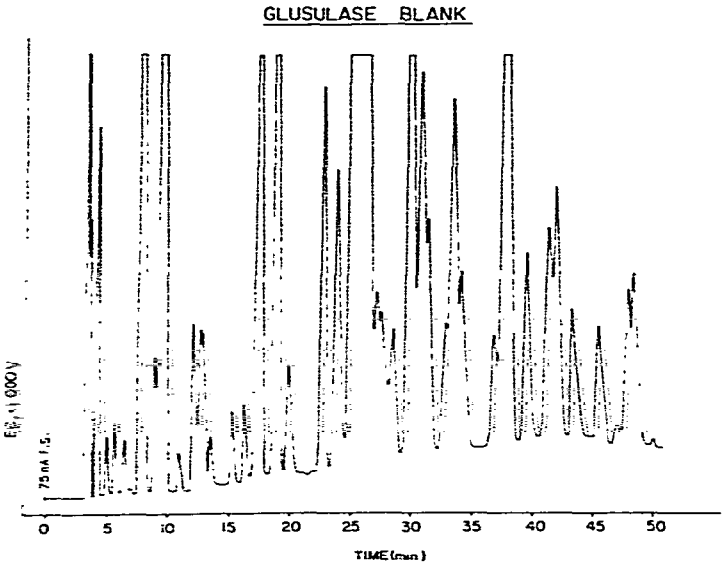

Fig. 6. Chromatogram of the ethyl acetate extract of a water sample incubated with glusulase under conditions indicated in Fig. 1. Volume injected: 100 μl; recorder attenuation: X 256. Chromatographic conditions same as in Fig. 2.

with water and subsequent chromatographing of the sample under identical conditions indicated that no peaks were present. Because of the presence of impurities in the glusulase preparation, this enzyme was not used for deconjugation. Since the literature reports indicate that the conjugated MHPG is present in CSF mostly as the sulfate form [19], the use of sulfatase was investigated. The enzyme preparation was found to be pure and no peaks were detected in the enzyme blank. A chromatogram of the ethyl acetate extract of a CSF sample incubated with sulfatase is shown in Fig. 7. Since the levels of MHPG·SO_4 are low, it is advantageous to use the oxidation potential of +1.000 V.

Prior to the quantitative analysis, a calibration plot was obtained by chromatographing a sample of CSF to which increasing amounts of MHPG were added. The relationship between the peak area and the amount of MHPG injected was found to be linear over the concentration range of interest (Fig. 8). The quantitative data for free MHPG and MHPG·SO_4 in human lumbar CSF samples, obtained by external calibration method, are shown in Table I. The mean value for free MHPG in 20 samples was 5.126 ± 4.652 (S.D.) ng per ml of CSF, and the range was 0.72—19.51 ng per ml of CSF. The mean values and the range for MHPG·SO_4 in 12 samples of CSF were 0.2365 ± 0.2269 (S.D.) and 0.08—0.850 ng per ml of CSF, respectively. Since unselected, diagnostic CSF specimens were used, it is difficult to interpret the quantitative data and correlate it with the medical and/or neurologic diagnosis. In addition, no effort was made to restrict medications. However, the quantitative results of this preliminary study are in agreement with the literature values [19]. The primary objective of this report was to demonstrate the usefulness of a direct HPLC method coupled with amperometric detection, in the analysis of en-

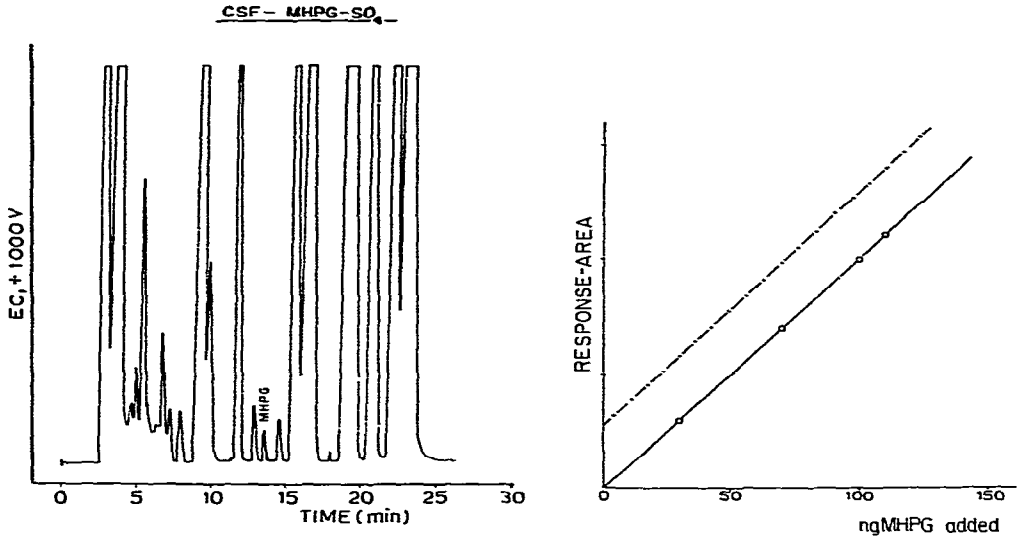

Fig. 7. Chromatogram of the ethyl acetate extract of a CSF sample incubated with sulfatase. Volume injected: 100 μl (corresponding to 2.1 ml of CSF); recorder attenuation: X 256. Chromatographic conditions same as in Fig. 2.
Fig. 8. The standard addition curve.

TABLE I

QUANTITATIVE ANALYSES OF FREE MHPG AND MHPG - SO_4 IN CEREBROSPINAL FLUID

Amounts expressed in ng per ml cerebrospinal fluid.

	N	Range	Mean
Free MHPG	20	0.720—19.510	5.126 ± 4.652 (S.D.)
MHPG - SO_4	12	0.081—0.850	0.2365 ± 0.2269 (S.D.)

dogenous levels of MHPG. The assay is simple, selective and quantitative and does not require derivatization of MHPG. Sample preparation is minimal and only ethyl acetate extraction is needed. Since this method circumvents many problems associated with other currently available analyses for MHPG, we believe it will give the clinicians a tool for routine assessment of the levels of this important catabolite.

ACKNOWLEDGEMENTS

The authors wish to thank Waters Assoc., Kratos, and Brinkmann Instruments for their technical assistance, Dr. L. Sarkozi of the Chemistry Department of Mount Sinai School of Medicine for the CSF samples, and Ms. Sandra Cerqueira for sample preparation. This study was supported in part by a grant from Ayerst Laboratories.

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