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SIMULTANEOUS DETERMINATION OF THE THREE MAJOR MONOAMINE METABOLITES IN CEREBROSPINAL FLUID BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A simple method is described for the simultaneous determination of the three monoamine metabolites, 4-hydroxy-3-methoxyphenylacetic acid, 4-hydroxy-3-methoxyphenylethyleneglycol and 5-hydroxyindole-3-acetic acid, in cerebrospinal fluid by high-performance liquid chromatography with electrochemical detection. Quantitation is accomplished by the standard addition technique. Chromatographic peak heights are corrected for volume effects by comparison with the signal obtained for an added auxiliary reference substance. Sample preparation is kept to a minimum, involving precipitation of proteins by means of perchloric acid and subsequent neutralization. The reproducibility was estimated to be 10%. For one of the metabolites, 4-hydroxy-3-methoxyphenylethyleneglycol, a correlation between the results obtained by this method and a mass fragmentographic method was made, and a satisfactory correlation (r = 0.904, slope = 0.914, intercept 3.26 ng/ml) found. The sensitivity of the method is in the picogram range. The methodology has been applied to measure biogenic amine metabolites in both rabbit and human cerebrospinal fluid. The levels found are in agreement with previously reported values.

INTRODUCTION

The diagnostic significance of the monoamine metabolites, 4-hydroxy-3-methoxyphenylacetic acid (HVA), 4-hydroxy-3-methoxyphenylethyleneglycol (MHPG) and 5-hydroxyindole-3-acetic acid (5-HIAA) is a subject of intensive research in the field of neuropharmacology. The outflow of HVA, MHPG and 5-HIAA in body fluids like cerebrospinal fluid (CSF), plasma and urine is an indication of the turnover of the corresponding biogenic amines, dopamine (DA), noradrenaline (NA) and 5-hydroxytryptamine (5-HT). As

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opposed to blood and urine, CSF is the source that probably best reflects the neuronal activity in the central nervous system.

Many assay methods for one or more of these metabolites in CSF have been described. The highest degree of sensitivity and specificity has been achieved using either gas chromatography with electron-capture or mass fragmentographic detection [1—15], or high-performance liquid chromatography (HPLC) using fluorimetric or electrochemical detection [16—21]. However, each of these techniques has shortcomings in at least one of the following criteria: cost of instrumentation, complex sample preparation involving multi-step derivatization, speed, sensitivity, specificity, flexibility, necessity of using internal standards to correct for and monitor the recovery during sample preparation. To our knowledge, only two studies, one employing gas chromatography with mass spectrometric detection, more specifically mass fragmentography, report the simultaneous determination of the three metabolites MHPG, HVA and 5-HIAA [11], and another, utilizing HPLC with electrochemical detection [21]. Although the technique of mass fragmentography is extremely well suited for the simultaneous determination of various compounds, it remains a very expensive technique which is only available in a few laboratories. Furthermore, the conditions are stringent: a derivatization and extraction procedure which works well for all compounds should be found, and deuterated analogues of the compounds should be available.

In this study, we describe a simple assay method which allows simultaneous determination of the title compounds, by HPLC with electrochemical detection. The method meets to a reasonable degree all the above mentioned criteria, so that it provides a valuable alternative to the more expensive and complex mass spectrometric methods. Compared to the published HPLC method [21], the present method has the advantage that the use of an auxiliary reference standard, i.e. 5-hydroxy-2-indolecarboxylic acid, allows correction for volume variations during sample work-up and injection, ensuring the precision of the determinations at the nanogram level.

MATERIALS AND METHODS

Chemicals

4-Hydroxy-3-methoxyphenylethyleneglycol piperazine salt (HMPG), DL-3-methoxy-4-hydroxymandelic acid (VMA) and DL-3,4-dihydroxymandelic acid (DOMA) were obtained from Calbiochem, Brussels, Belgium; 3,4-dihydroxyphenylacetic acid (DOPAC), DL-3,4-dihydroxyphenylglycol (DOPEG), 5-hydroxy-2-indolecarboxylic acid (5-HICA), homovanillic acid (HVA), 4-hydroxy-3-methoxybenzoic acid and 4-hydroxy-3-methoxybenzyl alcohol were purchased from Aldrich, Beerse, Belgium; β-(3,4-dihydroxyphenyl)-ethanol (DOPET) and 5-hydroxyindole-3-acetic acid (5-HIAA) were obtained from Serva, Heidelberg, G.F.R.; pentafluoropropionic acid anhydride (PFPA) was obtained from Pierce Chemical Co., Rockford, IL, U.S.A.; 1-(4-hydroxy-3-methoxy-phenyl)-1,2-ethane-1,2,2-[$^2H_3$]diol piperazine salt ([$^2H_3$]MHPG) was purchased from Merck, Sharp & Dohme, Pointe Claire-Dorval, Canada; and bovine serum albumin was from Sigma, St. Louis, MO, U.S.A. Inorganic salts and organic solvents were analytical grade from Merck, Darmstadt, G.F.R.; doubly distilled water was used throughout.

High-performance liquid chromatography

Instrumental conditions. The HPLC analyses were performed with a Model 6000 A solvent delivery system (Waters Assoc., Milford, MA, U.S.A.), equipped with a Model U6K universal sample injector (Waters Assoc.) and with an electrochemical detector (Model LC4, Bioanalytical Systems, West Lafayette, IN, U.S.A.) with a glassy carbon working electrode. The detector was operated at a potential setting of +0.8 V vs. the reference electrode. The chromatographic trace was registered by a recorder supplied by Kipp & Zonen. A reversed-phase column RSil C18 HL (Alltech, Eke, Belgium, particle size 10 μm, 250 × 4.6 mm I.D.) was used for all experiments. The eluent consisted of a 0.07 *M* NaH_2PO_4 buffer, with 0.1 m*M* disodium EDTA and methanol, in a ratio 9:1. The pH of the eluent was carefully adjusted to 5.4 with sodium hydroxide, since slight variations in eluent pH had a pronounced effect on the retention times, especially those of the acidic metabolites. The solvent flow-rate was 1.5 ml/min, with a corresponding pressure of 10.3 MPa. The eluent was filtered through a Millipore type RA 1.2 μm filter, and degassed before use in an ultrasonic bath (Bransonic 92).

Sample preparation. CSF samples were collected from the cisterna magna of urethane-anesthetized rabbits, by a method previously described [22]. The rabbits were of either sex, weighing between 2.5 and 3.0 kg. Human CSF samples were collected by lumbar puncture from patients suffering from various psychiatric disorders. Samples were immediately frozen until analysed. The further processing of samples before injection is very simple and does not include an extraction step, since this step gives rise to difficulties with the reproducibility of the quantitation (see below). The sample work-up only involves a deproteinization step with perchloric acid, followed by neutralization of the supernatant prior to injection. Of the CSF sample, two 100-μl aliquots are transferred to conical Eppendorf tubes. To one of the aliquots, a mixture of the three metabolites MHPG, HVA and 5-HIAA is added in a 100-μl volume, to the other, 100 μl of doubly distilled water are added. In this way, one part of the sample is spiked with an amount of the three metabolites, approximately equal to the expected amount present in the sample. Typically, additions of 2.5 ng of MHPG and 9.5 ng of HVA and 5-HIAA were used to spike a 100-μl rabbit CSF sample.

For both the spiked and the unspiked aliquot, the rest of the sample work-up is identical: 10 ng of the auxiliary standard 5-hydroxy-2-indolecarboxylic acid (5-HICA) are added in 50 μl, followed by 50 μl of a 2.4 *M* $HClO_4$ solution. Subsequently the samples were kept in the refrigerator or in an ice-bath for 15 min to allow protein precipitation, after which they were centrifuged for 5 min in an Eppendorf 5414 centrifuge. Of the supernatant, 250 μl were then transferred to another Eppendorf tube, which already contained 50 μl of a 5 *M* sodium acetate solution. Of the final neutralized and buffered sample, an aliquot of 100 μl was injected into the chromatographic system.

Quantitative analysis. The ratio of the detector response (peak height) for each compound versus that for the auxiliary standard (5-HICA) was determined, and will be referred to as *R*. In this way, a correction is carried out for volume variations during sample preparation is carried out for volume variations during sample preparation and injection. Calculation of the

metabolite concentration is based on comparison of the spiked and unspiked sample chromatograms, according to the equation

$$X = \frac{R_{\text{X unspiked}}}{R_{\text{X spiked}} - R_{\text{X unspiked}}} \times Y$$

where X = amount of metabolite X in the unspiked sample (ng), Y = amount of added metabolite X in the spiked sample (ng).

Gas chromatography—mass spectrometry

Instrumental conditions. Gas chromatography—mass spectrometry (GC—MS) analysis was carried out on a Finnigan 4000 instrument, connected to an Incos 2000 data system, using the jet separator as GC—MS interface and with the mass spectrometer operated in the electron-impact ionization mode. GC was performed on a 2 m × 2 mm I.D. glass column, packed with 3% SP-2100 coated on Gas-Chrom Q 100—120 mesh (Supelco, Bellefonte, PA, U.S.A.), which was operated isothermally at 155°C. Helium was used as carrier gas at a flow-rate of 25 ml/min. Injector and GC—MS interface temperatures were 250°C and 230°C, respectively. The MS conditions were: electron energy, 70 eV; emission current, 0.3 mA; ion source temperature, 230°C. Using the Incos datasystem software, the mass spectrometer was programmed to monitor ions at *m/z* 311 and 622 for the detection of MHPG and at *m/z* 313 and 625 for the detection of [$^2H_3$]MHPG.

Sample preparation. For the mass fragmentographic analysis of MHPG, the procedure described by Bertilsson [7] was applied with minor modifications. The sample size used was 200 μl for analysis of rabbit CSF and 500 μl for human CSF. After addition of 0.1 volume of a 5% aqueous ascorbic acid solution and of [$^2H_3$]MHPG (16 and 8 ng for rabbit and human CSF, respectively) into 2 volumes of sodium acetate buffer (2.5 *M*, pH 6.2; saturated with NaCl), the samples were allowed to equilibrate for 1 h at room temperature. Subsequently, the samples were extracted twice with 3 ml of ethyl acetate. The combined extracts were evaporated under a nitrogen stream and the residues reacted with 100 μl of a mixture of PFPA and ethyl acetate (1:1, v/v) for 30 min at 60°C. Immediately before GC—MS analysis, the derivatizing reagent was evaporated and the residues redissolved into 15 μl of ethyl acetate. Aliquots of 1.5 μl were used for GC—MS analysis.

Quantitative analysis. Standard curves were obtained with five aqueous standard solutions, each containing known amounts of MHPG covering the concentration range of interest and bovine serum albumin (0.3 mg/ml), and by carrying these standards through the extraction and derivatization steps. Quantitative calculations were based on peak area ratios of the molecular ion record (at *m/z* 622) versus those of its $^2H_3$-labeled analogue (at *m/z* 625). Ratios for the standard mixtures were plotted against the concentration in ng/ml, and an unweighted least-squares linear regression analysis was performed. Using the regression parameters of the calibration curve, the unknown MHPG concentrations and their associated standard errors were estimated. The precision of the assay was found to be 3% (C.V.; n = 5) at the 30 ng/ml level.

RESULTS AND DISCUSSION

Fig. 1 shows the chromatographic separation of a series of metabolites (DOMA, VMA, DOPEG, DOPAC, MHPG, 5-HIAA, HVA, DOPET) and the auxiliary standard (5-HICA), under the experimental conditions mentioned in the previous section. The position of peaks in the chromatogram can easily be modified by changing the methanol content or the pH of the eluent. Adding methanol will decrease the retention time of all compounds, whereas an increase in pH will decrease selectively the retention times of the acidic compounds (HVA, 5-HIAA, DOMA, VMA, DOPAC, 5-HICA) and will only slightly influence the position of neutral metabolites in the chromatogram. The pH effect is rather drastic (see also ref. 23). Therefore it is absolutely necessary to adjust the pH of the eluent very carefully in order to obtain identical chromatograms with satisfactory resolution between peaks, when proceeding with fresh eluent. We believe that it is possible to obtain a good separation of all these compounds with any commercially available reversed-phase column, simply by optimizing the pH and the methanol content of the eluent.

In order to determine quantitatively the metabolites in CSF, various procedures can be used. Precise and accurate results will only be obtained by

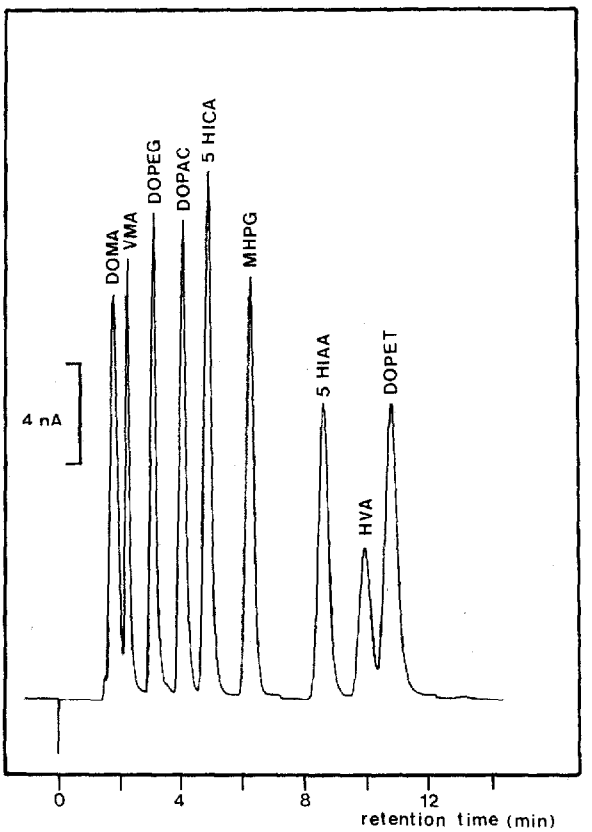

Fig. 1. Chromatogram of a standard mixture containing 25 ng each of DOMA, VMA, DOPEG, DOPAC, 5-HICA, MHPG, 5-HIAA, HVA and DOPET. Experimental conditions as mentioned in Materials and Methods.

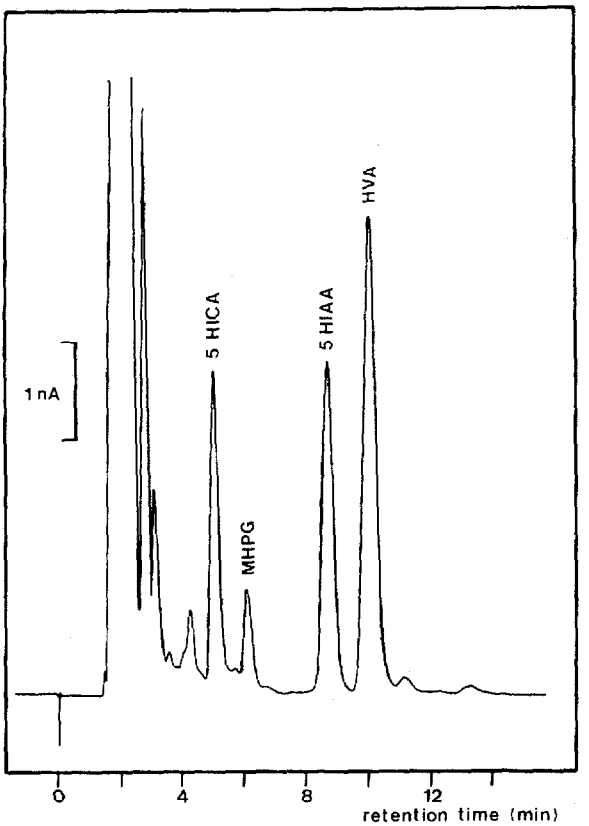

Fig. 2. Chromatogram obtained from an unspiked rabbit cisternal CSF sample. Experimental conditions and sample work-up as mentioned in Materials and Methods.

using an internal standard method. As internal standard one should preferably choose structurally related products, e.g. homologues, of the compounds to be determined, to correct for losses during the extraction procedure or other manipulations in the sample work-up. In preliminary experiments we tried to quantitate the monoamine metabolites with 5-HICA, 4-hydroxy-3-methoxybenzoic acid and 4-hydroxy-3-methoxybenzyl alcohol as internal standards for, respectively, 5-HIAA, HVA and MHPG, and with an ethyl acetate extraction as prepurification step. This procedure gave irreproducible results, even after the internal standard correction, probably because the compounds to be determined and their respective internal standards do not behave similarly during the extraction with ethyl acetate. Therefore we decided to use the standard addition technique, i.e. to use the products themselves as internal standards, and to calculate the metabolite content by comparison of the chromatograms of the spiked and non-spiked samples. Again this time, use of the extraction step gave rise to irreproducible results. Consequently, we decided to eliminate the extraction step by injecting the deproteinized and neutralized CSF supernatant directly into the HPLC system, as described in the Methods section. By proceeding in this manner, we were able to obtain chromatograms for CSF samples that were free from interference, as illustrated in Fig. 2. Satisfactory

TABLE I

METABOLITE LEVELS IN HUMAN AND RABBIT CSF, DETERMINED BY THE PRESENT METHOD

Results are expressed as mean ± standard deviation. The number of samples is indicated in parentheses.

Sample origin	Concentration (ng/ml)		
	MHPG	5-HIAA	HVA
Human lumbar CSF	5.3 ± 2.4 (7)	19.8 ± 5.1 (11)	40.7 ± 20 (11)
Rabbit cisternal CSF	28 ± 9 (11)	111 ± 17 (12)	136 ± 31 (12)
Rabbit CSF pool*	45 ± 4 (9)	170 ± 15 (9)	298 ± 38 (9)

*CSF pool originating from non-treated and drug-treated rabbits.

In Table I are summarized the results of the present assay method, as applied to both human and rabbit CSF samples. The data for human lumbar CSF samples are in agreement with previously reported values [11]. It should be mentioned that the value obtained for human CSF only represents a mean of values obtained from patients with various psychiatric disorders, different age, sex, etc. The figures for rabbit cisternal CSF are consistently higher than for human CSF and are also in agreement with literature values [24–26]. The standard deviation is smaller here, probably because all animals were healthy, of the same weight and were treated in the same manner (anesthetized with urethane). To test the reproducibility of the present method, a CSF pool, originating from non-treated and drug-treated animals, was divided into 100-μl aliquots and analysed. As shown in Table I, the reproducibility (expressed as standard deviation) is about 9% for MHPG and 5-HIAA, and 13% for HVA. In order to obtain reproducible results it is essential to use precise volumetric material (i.e. glass micropipettes) for the transfer of CSF samples and for the addition of standards. The volumetric errors associated with the further processing of the sample are corrected for by comparison with the auxiliary reference substance (5-HICA).

For a number of CSF samples (both human and rabbit) we determined MHPG both by the present HPLC method and by the mass fragmentographic method described by Bertilsson [7] (see also Methods section). Since mass fragmentography is by far the most sensitive and specific method for quantitative analysis, a comparison of the results of the two methods will give an idea of the validity of the alternative HPLC method. A correlation of $r = 0.904$ was found, which is acceptable. The regression line equation is $Y = 0.914X + 3.26$ (ng/ml) (Fig. 4).

From the results of this correlation, and the reproducibility data given in Table I, we can conclude that for the determination of MHPG, and probably also for the other metabolites 5-HIAA and HVA which occur at higher levels in CSF, the present method offers an alternative which can easily be applied to routine analysis, and which does not require costly instrumentation and extensive samples preparation. For most of the samples we analysed, the

resolution is obtained for the three major monoamine metabolites MHPG, 5-HIAA and HVA, and the auxiliary standard 5-HICA. Injections can be repeated every 12 min. Eventually a short precolumn, with backflush possibility, can be incorporated into the chromatographic system, in order to avoid possible contamination of the analytical column. It is our experience, however, that several hundreds of injections directly on the analytical column can be performed without loss of column performance. Peak identification was based on retention behavior and co-chromatography with the standard reference compounds under various chromatographic conditions. The linearity of the method was evaluated by spiking CSF pool samples with increasing amounts of the three metabolites. Plotting the R values (metabolite peak height versus 5-HICA peak height) versus the amount of added metabolite gave linear relationships with satisfactory correlation coefficients (r = 0.9987, 0.9955 and 0.9878 for MHPG, 5-HIAA and HVA, respectively), as is illustrated in Fig. 3. The intercept of the regression lines in Fig. 3 (calculated by linear least-squares analysis), divided by their slopes, gives the amount of metabolite (ng) present in the initial pool sample. For routine measurements, only one spiked sample with one unspiked sample is analysed.

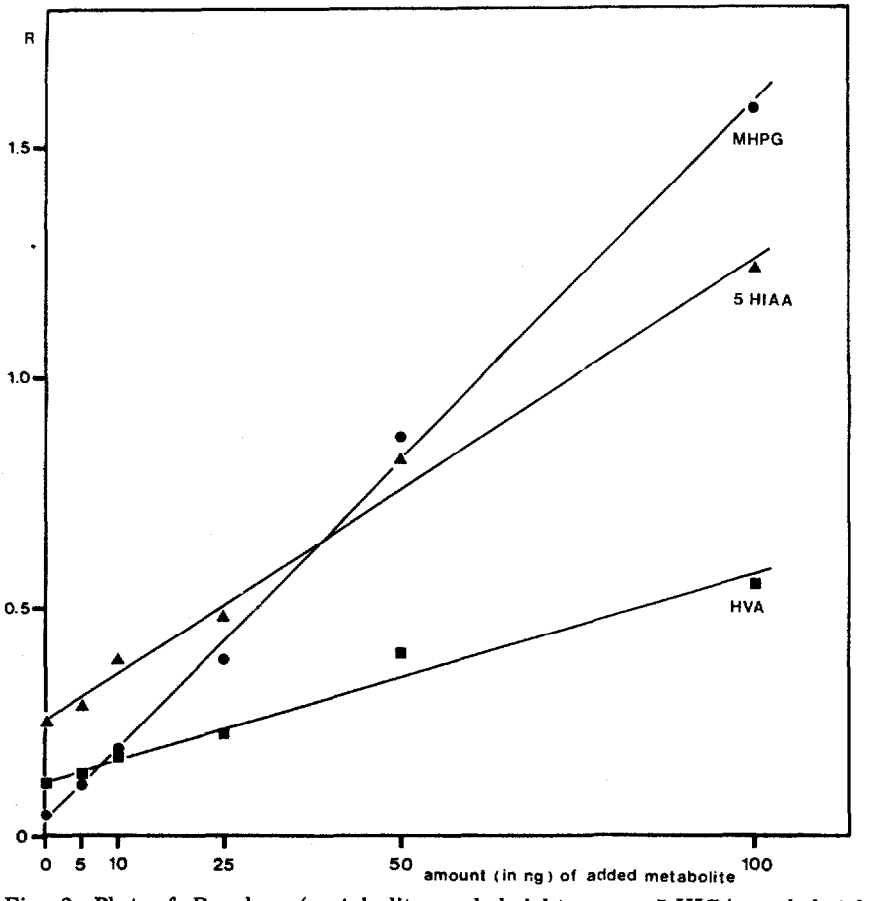

Fig. 3. Plot of R values (metabolite peak height versus 5-HICA peak height) versus the amount of metabolite added (in ng) to a rabbit cisternal CSF sample. Experimental conditions and sample work-up as described in Materials and Methods.

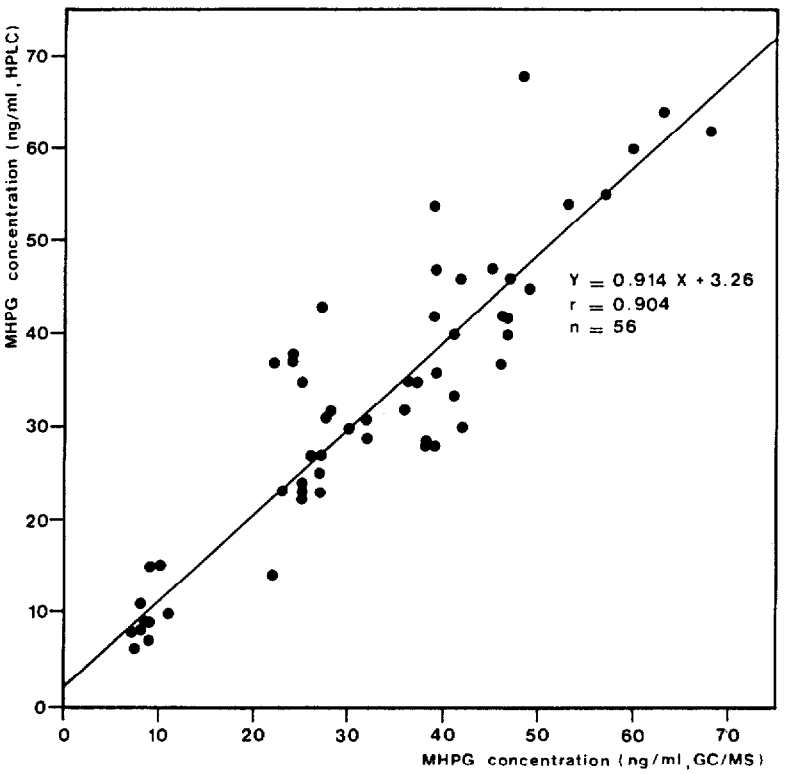

Fig. 4. Correlation between data sets obtained by mass fragmentography and the HPLC method, for MHPG levels in rabbit cisternal and human lumbar CSF. Experimental conditions and sample work-up for both methods are described in Materials and Methods.

metabolite levels were high enough to allow accurate detection, starting from a 100-μl CSF sample. The detector response remains appropriate for most metabolite levels encountered, which correspond typically with injections on column between 500 pg (for MHPG) and 15 ng (for HVA). Only when high sensitivity is required, e.g. for the determination of MHPG in human lumbar CSF, where the reproducibility of the HPLC method remains rather poor, does mass fragmentography remain the technique of choice.

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