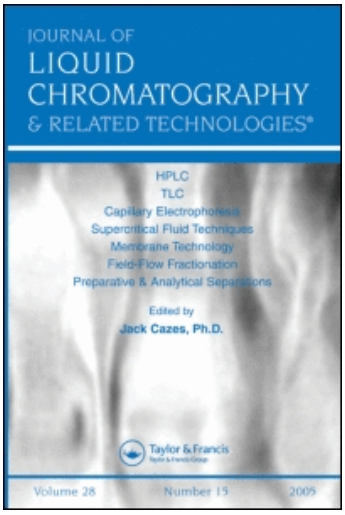

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E. Morier-teissier[a]; R. Rips[a]
[a] Unite de Pharmacologie Chimique, INSERM 17 rue du Fer à Moulin, Paris, France

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DETERMINATION OF 3-HYDROXY-4-METHOXY-PHENYLETHYLENEGLYCOL AND OTHER MONOAMINE METABOLITES IN BRAIN AREAS USING LC-EC AND A TWO-COLUMN SWITCHING TECHNIQUE

E. Morier-Teissier and R. Rips
Unite de Pharmacologie Chimique
INSERM
17 rue du Fer à Moulin
75005 Paris, France

ABSTRACT

A two-column switching technique has been developed for the separation of norepinephrine (NE) and its metabolite 3-hydroxy-4-methoxy-phenylethyleneglycol (MHPG) which are poorly resolved by a single-column technique, together with dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanilic acid (HVA), serotonin (5HT) and 5-hydroxy-3-indolacetic acid (5HIAA). The system uses two reversed phase columns (C8, C18) arranged in series and a valve so that the effluent from the primary column could be switched to the secondary column or directly to the detector. NE, MHPG, DA, DOPAC, HVA, 5HT and 5HIAA concentrations were determined in homogenates of a number of areas of the mouse brain. The detection limit was 0.25 pmole for MHPG and intra- and inter- assays variations were 1-3 % and 3-5%, respectively. Total analysis time was one hour. Mouse hypothalamus was found to contain a total of 110 ng of free MHPG per mg protein. Brain-stem contained 0.74 and hippocampus 0.19 ; the concentration of conjugated MHPG did not exceed 5 % of total MHPG.

INTRODUCTION

We have recently developed a method (1) for quantifying a variety of monoamines, catecholamines, indoleamines and their main metabolites in biological samples by high-performance liquid chromatography with electrochemical detection (LC-EC). This chromatographic system provides, for the first time, a mean of measuring these compounds simultaneously in crude biological samples, such as specific brain areas, cerebrospinal fluid (2), adrenal glands and heart. Because sample preparation is very fast labile compounds are protected from degradation, while the sensitivity of the method is high, as there is neither dilution nor loss of sample due to extraction. The simplicity of the method allows the automated measurement of a large number of samples. The risk of sample degradation is minimized by using a refrigerated automatic injector (3).

However, the method is not suitable for the measurement of products with relatively short retention times. Norepinephrine (NE) is eluted obscured by the frontal peak and products with short retention times such as 3-hydroxy 4-methoxy phenylethyleneglycol (MHPG), the major metabolite of NE, are masked by other endogenous compounds (1). Two strategies may be adopted for their measurement:

1. The sample may be prepurified before the injection.This can prolong the preparation, produce a low yield and increase the risk of degradation of labile compounds .
2. The selectivity of the system can be improved to allow direct injection of unfractionated homogenate supernatant.

The methods of quantifying the various forms of MHPG in plasma, cerebrospinal fluid, urine and body fluid which have been used to date include gas chromatographic methods, coupled

with electron capture detection (4-6) or mass spectrometry (7-9), the formation of a fluorophore of MHPG with ethylenediamine (10) and radioimmunological assay (11). More recent methods have been based on liquid chromatography combined with either fluorimetric detection (12) or, more generally, with electrochemical detection.

In the latter, injection is preceded by a prepurification step on ion exchange columns : Bio Rad AG 50W (13), DEAE-Sephadex A 25 (14), Amberlite and Dowex (15), on Sephadex G 10 columns (16) or on Bond-Elut adsorption columns (17), which sometimes includes solvent extraction.

Two assays have been recently proposed in which MHPG is measured without prior purification. Warnhoff (18) has described an improved chromatographic system in which each of the mobile phase parameters are optimized to obtain a good separation of a standard mixture of MHPG and catecholamines or their metabolites. However the technique was only applied to their measurement in the mediobasal hypothalamus, an area rich in NE and MHPG. The other approach (19) consists of a switching system with three columns and three pumps, which gives satisfactory results using a standard mixture. It has not yet been applied to the measurement of MHPG in biological samples.

We have improved the separation of NE and MHPG by using a two column switching technique. The initial step in the development of this method was the selection of the best pair of columns and optimization of the parameters of eluent composition and column switching protocols. The conditions, which were developed using standard mixtures, have been applied to the determination of free and sulfated MHPG in samples of mouse brain. The results obtained are compared with the published data obtained using other methods.

MATERIALS AND METHODS

Reagents

Norepinephrine hydrochloride (NE), 3-hydroxy-4-methoxy-phenylethyleneglycol (MHPG) hemipiperazine salt, dopamine hydrochloride (DA), 3,4-dihydroxy-phenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxy-3-indolacetic acid (5HIAA) and 5-hydroxy-tryptamine (5HT) were purchased from Aldrich (Strasbourg, France). MHPG sulfate, potassium salt, was from Sigma (St Louis, MO, USA). Heptane sulfonic acid (Pic B7) was purchased from Waters Assoc. (Maple, MA, USA), methanol (HPLC grade) from Prolabo (Paris, France). The resistivity of the demineralized water was 5 million ohms/cm. Helix pomatia juice sulfatase (HPS) was obtained from Industrie Biologique Française (Villeneuve la Garenne, France).

Apparatus

The HPLC system consisted of

a- A time-programable solvent delivery pump, allowing the control of valve switching (Model 590,Waters Assoc.)

b- A manual injector system (Model U 6 K, Waters Assoc.) or an automatic autosampler (Model ISS 100, Perkin Elmer, Norwalk, CT, USA). The sample tray was cooled to 0-5°C. Sample injection caused the pump timer to return to zero.

c- Rheodyne 6-port valve (Waters Assoc.) connected to a pressure source, a cylinder of nitrogen (working pressure, 2 bars).

d- Two radial-compressed columns (0.8 cm i.d x 10 cm) selected from among the following : Novapack, Radialpack CN, C8, C18, either 5 or 10 µm diameter packing (Waters Assoc.).

e- An electrochemical detector (LC 4B from BAS, West Lafayette, IN, USA) set up with a thin layer cell, reference electrode and

working vitreous carbon electrodes. The working electrode was maintained at a potential of + 0.75 V versus an Ag/AgCl reference electrode and the sensitivity was 1 to 10 nA.
f- The detector response was monitored on a Kipp and Zonen recorder (Paris, France) with a 1 V input.

Mobile Phase

The mobile phase was a mixture of 0.1 M KH_2PO_4, 0,1 mM EDTA, 2 mM heptane sulfonic acid and 7 % methanol (v/v), the pH was adjusted to an apparent value of 4.4 with 5 M NaOH. The mobile phase was filtered and degassed by vacuum filtration through a 0.45 µm membrane (Millipore, Bedford, MA, USA) before use. All separations were performed isocratically at room temperature at flow rates between 1 and 1.5 ml/min.

Standards and Calculations

The stock solutions of standards were prepared at a concentration of 10 mg/100 ml of active principle in 0.1 N HCl for catecholamines and in water for the others. These solutions were stored for up to one month at 4°C and diluted 1/1000 each day to give the working solutions containing 10 ng/100 µl.

The concentrations of monoamines and metabolites in the tissue were calculated by comparing their peak areas to those of the standard solutions measured in the same assay. The results are expressed in ng/mg of protein or in ng/g of wet tissue for comparison with the literature value. Statistical analyses were performed using the Mann-Whitney U-test.

Hydrolysis of a Standard Solution of MHPG Sulfate and Stability of MHPG under Hydrolysis Conditions

A solution of the K salt of MHPG-SO_4 (16.4 mg/100 ml water) was diluted 1/1000 in 0.2 M sodium acetate pH 5.0 or in 0.1 M $HClO_4$. 1 ml of the acetate dilution or 1 ml of the $HClO_4$ dilution neutralized by 250 µl of a 5 M sodium acetate solution were mixed with 1 ml of a HPS solution (diluted 1/1000 in 0.2 M acetate pH 5.0) and the mixture was placed in a water bath at 37°C. A solution of MHPG-piperazine salt was treated in the same way. Aliquots were withdrawn at given intervals and injected directly onto the column (100 µl containing 5 ng MHPG and 1 ml HPS corresponding to 100 µg MHPG).

Standard curves were prepared by replicate additions of MHPG or MHPG-SO_4 to samples from a single pool of mouse brain homogenized either in 0.2 M sodium acetate or in 0.1 M $HClO_4$ brought to pH 5.5 by addition of 5 M sodium acetate.

The inter-assay reproducibility was calculated by assaying mouse brain samples to which had been added 1 ng of MHPG-SO_4. The measurement were repeated with fresh brain preparations each assay day.

Sample Preparation

Male Iffa Credo mice, 5 weeks old and weighing about 26 g, were killed by decapitation. Their brains were quickly removed, rinsed in ice cold saline, blotted dry ,and dissected on ice into 6 different areas (cerebellum, brain-stem, hypothalamus, hippocampus, striatum and the remainder) (20). The areas were frozen on dry ice and kept at -70°C for up to 2 months.

Each area was homogenized with an Ultra Turrax homogenizer (Labo Moderne, Paris, France) in 500 µl (1 ml/100 mg for the

areas weighing more than 50 mg) of 0.1 M $HClO_4$ containing 0.1 % EDTA and 0.1 % sodium metabisulfite. The homogenates were centrifuged at 5000 g for 10 min at 4 °C.

The quantities of sulfoconjugated metabolites were determined after incubation of 400 µl of sample supernatant, adjusted to pH 5.5, with 100 µl of HPS (diluted 1:1000 with 5 M acetate buffer) for 24 h at 4°C. For control samples the reaction mixture was prepared as above but not incubated. The samples were subsequently cooled in an ice bath and aliquots were directly injected into the HPLC system. Protein was analysed according to the method of Lowry (21) on the homogenate precipitates as it was not possible to weigh samples of less than 100 mg without partial thawing and risk of degradation.

RESULTS AND DISCUSSION

Caracteristics of the Columns Used

The columns tested were all radially compressed. The use of the radial compression system avoids the formation of voids, allowing a better separation of injected compounds. The C18-bonded silica columns provided good separations; most of the other columns tested (silica bonded to C8 or CN chains) were less lipophilic, providing less retention of injected material. Columns with lower percentage bonding (7 % C18 with end-capping) and smaller (5 µm diameter) particle size were also tested. Standard mixture retention times observed with these columns are compared in the table 1.

The retention times of the Radialpack columns increased from CN 10 µm through C8, 10 µm and C8, 10 µm to C18 10 µm .

TABLE 1

Retention (min) of Catecholamines, Indolamines and their Metabolites on 5 Types of Columns

Mobile phase 0.1 M KH_2PO_4, 5 mM heptane sulfonic acid, 15 % MeOH, pH 4.1 ; flow rate 1 ml/min ; detector potential 0.8 V; sensitivity 10 nA.

	RETENTION TIME (min)				
Column / Compounds	CN 10 µm	C8 10 µm	C18 10 µm	C18 5 µm	Nova 10 µm
NE	4.25	5.25	4.5	7.5	5.5
MHPG	5	5.5	4.0	7.0	4.75
DOPAC	5	7.0	7.25	12.5	9.0
DA	5	7.75	8.75	15.5	11,5
5HIAA	8	10.0	11.75	21.0	14.0
HVA	6.25	12.5	16.0	28.5	20.
5HT	8	14.0	20.75	38.5	22.5

The CN bonded column was not used in further studies as it did not provide sufficient separation on the first column with the mobile phase used. The separation on this column increased with decreasing polarity of the mobile phase, but the retention times on the second column became much too long.

The C18 Novapack column gave results quite similar to those obtained with the ordinary C18 column with the same particle size in spite of the absence of free OH functional groups on the silica, indicating that the free OH groups do not play a major role in the separation of catecholamine and indolamine derivates. Retention times were a little shorter than with the Radialpack C18 columns but the loading rate was only 7 % instead of 13 %.

The C8 - 10 µm and C18 - 5 µm columns were adopted for all further studies. These two columns have very different capacity factors, which improves the switching technique (22).

Choice of Eluent Composition

The analysis was optimized to provide :

a- measurement of MHPG without prior purification.

b- an improvement in the measurement of NE.

c- simultaneous detection of the amines and their metabolites.

Counter-ion concentration, percentage of methanol and pH are the main determinants of solute separation. The influence of the variations of these factors with the two columns C8 and C18 was examined and the most appropriate values for each column were determined. The retention times of the amines, acids and neutral compounds can be varied in a complex manner by varying the concentration of the counter-ion. On a C18 column at pH 4.1, increasing the concentration of heptane sulfonic acid increased the retention time of amines such as NE. That of acids was only slightly increased whereas those of neutral compounds such as MHPG decreased. The best compromise for counter-ion concentration producing a good separation of NE and MHPG was found to be 2 mM (table 2).

The retention times of all substances decreased with increasing methanol concentration (1). The percentage chosen (7 %) was a function of two criteria : a good separation of the tested compounds and a minimum analysis time.

The retention times of acids are much more sensitive to pH changes in the pH 4 to 4.5 range than are those of the amines, which remained fully protonated (1); the glycol MHPG is unaffected. The separation between an acid and a neighbouring base could be improved by varying the pH. Thus at pH 4.1, the pairs DOPAC-DA and HVA-5HT were not well separated, but the resolution improved when the pH was increased to 4.4.

TABLE 2

Variation of Retention Times of NE and MHPG with Heptane Sulfonic Acid Concentration and Methanol Percentages

Column Radialpack C18 10 m ; mobile phase 0.1 M KH_2PO_4, pH 4.1 ; flow rate 1 ml/min; detector potential 0.8 V; sensitivity 10 nA.

Heptane sulfonic ac.		0 mM	1 mM	2 mM	5 mM
Methanol					
0 %	NE	5.5	6	12	15.5
	MHPG	33	19	18.5	-
5 %	NE	5	6	9	9.5
	MHPG	19	18.5	16	8.5
15 %	NE	-	-	3.5	4.5
	MHPG	-	-	4.5	4.0

As a result, the composition of the mobile phase was fixed at 2 mM heptane sulfonic acid, 7 % methanol and an apparent pH of 4.4.

Development of the Switching Technique for a Standard Mixture

The separation of a standard mixture on a C18 column is shown in figure 2 (left). This chromatogram was used to determine the times t_1 and t_2 at which the effluent flow was switched. In this case, the slow-moving compounds are NE, MHPG, DOPAC and DA and the fast-moving compounds 5HIAA, HVA and 5HT. The second column, C18, was connected and disconnected (as shown on the table 3 and the figure 1) between these two groups of compounds : t_1 must then be slightly longer than the retention time of DA and t_2 greater than that of 5HT.

TABLE 3

Program of Automatic Analysis by HPLC and two Columns Switching of a Mixture of Catecholamines, Indolamines and of their Metabolites

Event	time (min)	column used	sequence	
Starting			1	The two columns equilibrated together
Injection	0	C8 + C18		Injection sets the timer to 0.
			2	Fast eluting compounds are trapped on the 2nd column.
Switching	8	C8	3	Slow eluting compounds are directly eluted onto the detector.
Switching	20		4	Compounds trapped on the 2nd column are eluted onto the detector.
Injection	60/0	C8 + C18		Injection resets the timer to 0.

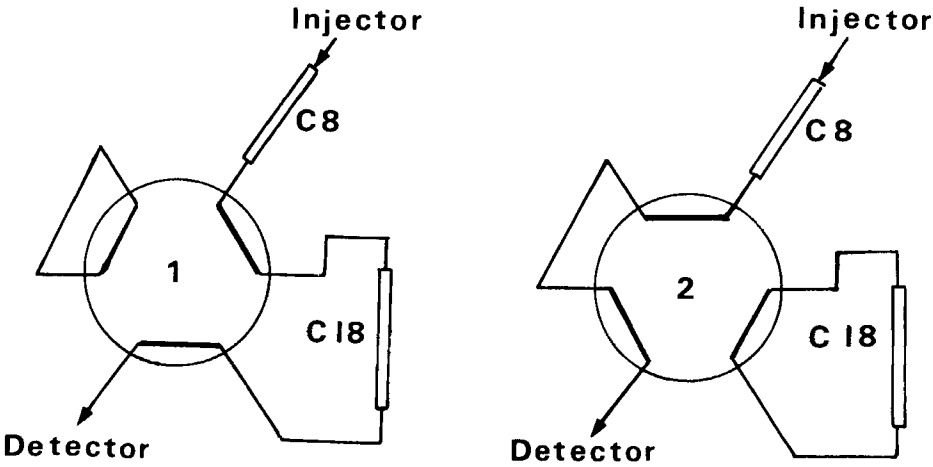

FIGURE 1. Connections and positions of the valve used for column-switching LC. Status (1), trapping or elution of fast moving compounds. Status (2) direct elution of slow-moving compounds.

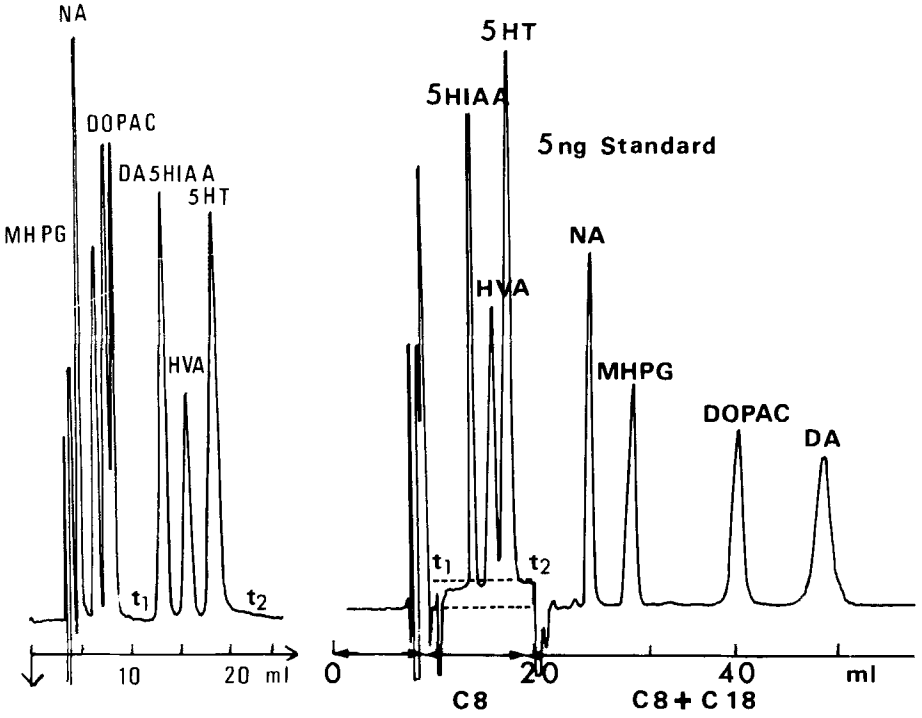

FIGURE 2. Chromatogram of a standard mixture ; mobile phase 0.1 M KH_2PO_4, 2 mM heptane sulfonic acid, 0.1 mM EDTA, 10 % methanol (v/v), pH 4.4; detector potential 0.75 V, sensitivity 5 nA, injection volume 50 µl containing 5 ng of each compound. (left) column Radialpack C8 10 µm . (right) columns Radialpack C8 10 µm and C18 5 µm with the switching programme described in table 6.

This combination of mobile phase and the two (C8 and C18) columns produced a very good separation of a standard mixture containing NE-MHPG while at the same time improving the separation of other derivatives of DA and 5HT (Figure 2(right)). The peaks DOPAC-DA were very well separated in the two-column system whereas they had a tendency to coalesce as the column aged in the one-column method. Finally, the sensitivity is increased for 5HIAA, HVA and 5HT as, since they are eluted

faster, their width is smaller and their peaks easier to quantify. The more slowly migrating group of products appears first on the chromatogram.

The successful system had to satisfy the following criteria:

a- The compounds of the first group have to be well resolved from the others at the output of the second column, whereas the second group has to be well resolved at the output of the first C8 column.

b- During the period, $0-t_1$, where the first group is held on the second column, the first product of this group (in this case NE) must not be eluted before the last one (in this case DA) has entered it. This is achieved because of retention differences between the two columns.

c- The time between the elution of the two groups must be long enough to ensure that the baseline can become stable again after the switching. The major change encountered when switching from a single column to two columns in series is an increase in back pressure (from 13.7-17.2 bar for the C8 column alone to 31.0-34.4 bar with the C8 and C18 in series). Since the amperometric detector is sensitive to mobile phase pressure changes, an increase in the baseline (depending on the sensitivity) is observed. Thus, this method is less suitable for analyses which require a very high sensitivity. This is one of the limitations of the method, but compensation is possible using an integrator or an on-line micro-computer.

The characteristics of the procedure adopted are as follows:

a- The relationship between the injected amount of standards and the detector response is linear for each of the seven compounds measured over the range 0.2 to 5 ng.

TABLE 4

Resolution and Analysis Times Using C8 and C18 Columns, Alone, in Series or in the Switching Mode

	C8	C18	C8 + C18 (series)	C8 + C18 (switching)
NE-MHPG	2.25	2.55	2.71	2.71
DOPAC-DA	1.45	3.00	2.27	2.22
HVA-5HT	2.10	3.71	3.25	2.00
Analysis time min	28	88	116	60

b- The limit of sensitivity is 50 pg or 0.25 pmole for MHPG and HVA and 0.10 pmole for the other compounds. The sensitivity limit was taken as twice the detector noise level at 1 nA.

c- The serial system C8 + C18 provides increased resolution but also analysis time over those obtained using either C8 or C18 columns alone (table 4). On the contrary, if the columns C8 + C18 are in the switching mode, the resolution is the same as in the serial system but the analysis time is divided by two.

Application to the Measurement of Free MHPG and Other Catecholamines and Indolamines in a Biological Sample

The system described for a standard mixture produces a good separation of NE and its metabolite MHPG in the presence of compounds such as DA and its metabolites DOPAC and HVA, 5HT and its metabolite 5HIAA in crude brain samples as : brain-stem, hippocampus, striatum and the remaining portion of mouse brain (Figure 3 a,b,c,d).

The intra and inter-assays variations were 1-3 % and 3-5 % respectively, the latter value includes variations between

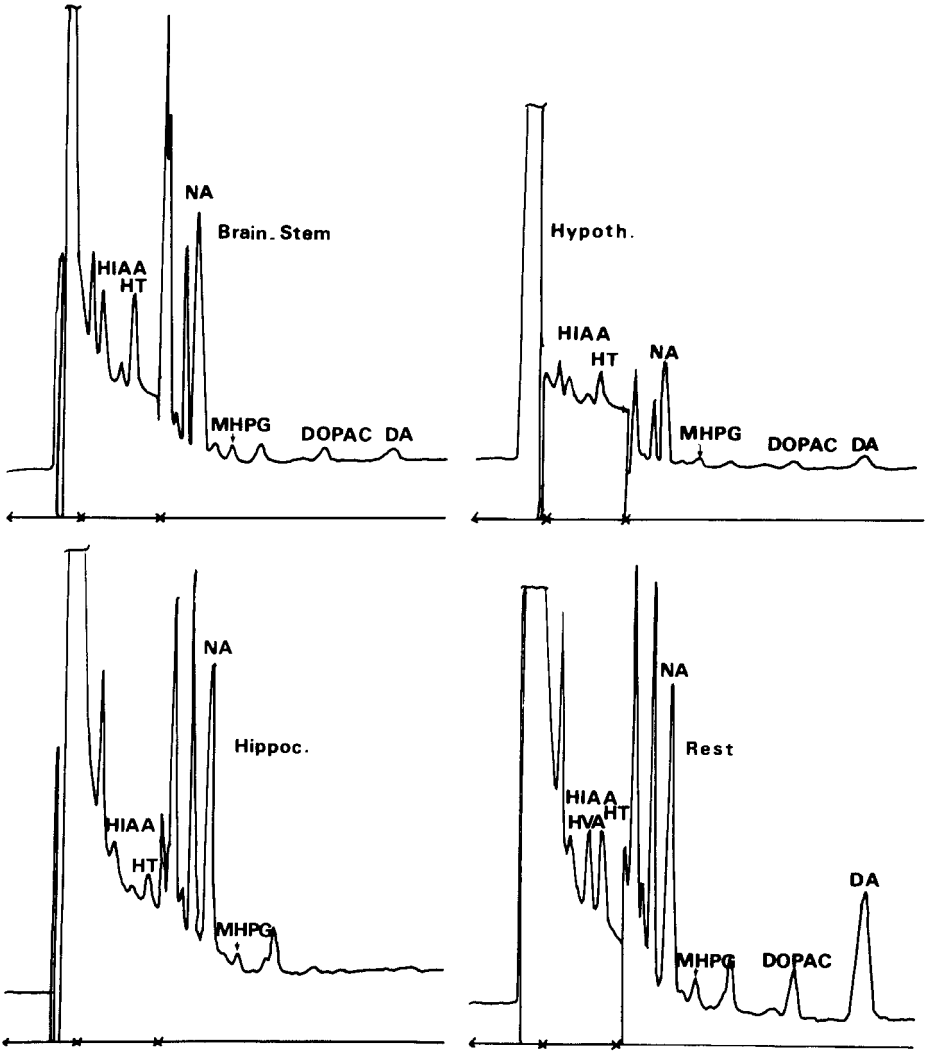

FIGURE 3. Typical chromatograms of mouse brain regions processed as described in materials and methods. Conditions are the same as in Fig. 2 (right).

animals. These values (except for MHPG) may be compared to those obtained using a single column (table 6). The samples analysed were identical, prepared in the same way from the same animals and used the same standard. There was no significant difference between the results obtained with either of the methods, but the switching method gives a more symmetrical NE peak which is therefore better quantified. The regional distribution of free MHPG in the mouse brain, obtained with the switching method, is shown in table 5.

The highest concentration of free MHPG was found in the hypothalamus followed by the brain-stem and hippocampus, all other areas tested showed low MHPG concentration.

In mice, and even more so in rats, MHPG exists in either its free or conjugated form (mainly as sulfate). MHPG-SO_4 is hydrolysed to free MHPG by enzymatic hydrolysis at 37°C in the presence of a crude sulfatase preparation from Helix Pomatia (HPS). The activity of the sulfatase is slower in the presence of $HClO_4$ brought to pH 5.5 by AcONa, than in the presence of AcONa only. We found that 1 ml of HPS hydrolyses 100 µg of MHPG-SO_4 at pH 5.5 in 24 hours. MHPG is not destroyed in these conditions (Figure 4). In the presence of a brain homogenate spiked with MHPG-SO_4 the kinetics are the same. This dilution of HPS could be used without prior purification as it did not interfere with the chromatogram of the samples. To measure free and bound MHPG, each sample was divided into three parts :

- The first was measured directly to obtain the quantity of free MHPG.
- The second was spiked with MHPG sulfate and incubated with a sulfatase preparation from Helix Pomatia (HPS) to verify the yield of the enzymatic hydrolysis, and

TABLE 5
Level (in ng/mg of Protein) of Catechol and Indolamine Derivatives in Mouse Brain Regions : Comparison of Results Obtained with a C18 Column and with C8 + C18 Columns in Switching Mode (N = 10)

	Brain stem		Hypothalamus		Hippocampus		Striatum	
	C18	Switching	C18	Switching	C18	Switching	C18	Switching
NE	6.46±0.48	6.24±0.24	15.77±1.38	16.25±1.25	3.80±0.61	4.01±0.53	-	-
DOPAC	-	-	1.02±0.07	1.32±0.12	-	-	12.76±0.59	17.93±0.62
DA	-	-	2.54±0.13	2.94±0.15	-	-	85.43±1.85	83.15±1.31
5HIAA	4.09±0.31	3.67±0.37	3.59±0.25	4.41±0.30	2.88±0.70	3.37±0.65	2.68±0.61	2.53±0.69
HVA	-	-	-	-	0.52±0.17	9.41±0.58	10.66±0.48	
5HT	4.66±0.35	3.97±0.16	6.67±0.71	6.51±0.82	3.98±0.62	3.51±0.56	3.03±0.41	3.49±0.45
Free MHPG	-	0.74±0.04	-	1.10±0.03	-	0.19±0.03	-	-

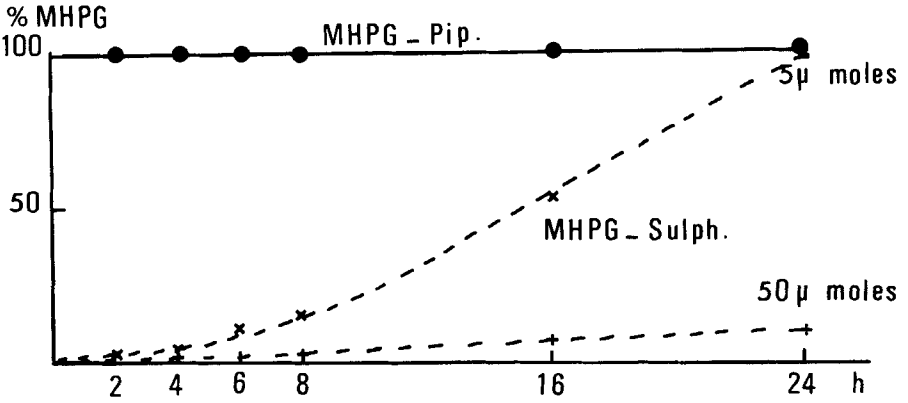

FIGURE 4. Rate of hydrolysis and stability of MHPG under hydrolysis conditions. MHPG-SO_4 and MHPG (100 and 1000 µg or 5 and 50 moles MHPG/1ml HPS) in 400 µl 0.1 M $HClO_4$ + 100 µl 5 M sodium acetate were allowed to react with 400 µl of crude Helix Pomatia sulfatase (1/1000 in 0.2 M sodium acetate). Aliquots were analysed by LC-EC at given intervals.

- the third was incubated in the same condition to find the total quantity of MHPG.

As the concentration of conjugated MHPG was found to be very low, not exceeding 5 %, it was not measured in each brain areas.

Whereas there are many values in the literature for the rat brain, data on the regional distribution of MHPG in mouse brain are scarce because of the low sensitivity of existing assay methods. Rat brain MHPG is mainly present as a conjugate, in contrast to the mouse brain in which it is more than 90% free. Warsh and al. (16) found by gas chromatography and mass fragmentation that mouse brain contained 43.1 ± 1.03 ng/g free MHPG and 43.8 ± 0.4 ng/g of total MHPG i.e. 98 % of the MHPG was in the free form. MHPG is most frequently measured in the hypothalamus. Sharman (5) pooled the hypothalami from six mice for measurement of the free MHPG without hydrolysis using gas

chromatography and electron capture and obtained 49 ng/g of tissue. Caesar et al. (8), also using gas chromatography and electron capture, gave the distribution of free MHPG in seven mouse brain areas, but found no MHPG sulfate in the whole mouse brain using a fluorometric method. Recently, Oka et al.(17) developed a method requiring a prepurification on Amberlite and Dowex, followed by three HPLC runs with three different mobile phases to measure the constituents of mouse brains without hydrolysis. The values found were somewhat higher than those of other workers (eg. hypothalamus : 84 ng/g).

The values obtained in this study i.e. 1.10 ng/mg protein (equivalent to about 110 ng/g of tissue) in the hypothalamus are higher than those obtained using extraction and either gas chromatography (5,16) or HPLC (17). This difference is probably a reflection of differences in the methods of sample preparation, brain area dissection technique and variations due to the breed, age and sex of the mice used. MHPG quantities in other mouse brain areas was not described.

This procedure, by which all important metabolites involved can be analysed simultaneously in the same sample, has a detection limit on the order of 1 ng/sample, sufficient for the determination of these substances in mouse brain regions without sample pooling. In the automatic mode up to 24 samples can be analyzed each day.

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