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Note

Method for total 3-methoxy-4-hydroxyphenylglycol extraction from urine, plasma and brain tissue using bonded-phase materials: comparison with the ethyl acetate extraction method

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Over the past two decades convincing evidence has accumulated indicating that 3-methoxy-4-hydroxyphenylglycol (MHPG) is the major metabolite of brain norepinephrine (NE) activity [1, 2]. Any biochemical investigation of central NE activity involves MHPG analysis. This analysis has been used in NE agonist and antagonist pharmacological studies [3], in classifying affective dis orders [4, 5] and in predicting an individual's treatment response to tricyclic antidepressant drugs [6]. Such studies require a rapid and accurate method for isolating and quantifying MHPG.

The methods currently available involve ethyl acetate extraction [7]. These methods include lengthy handling time, and concentration of the sample, which also leads to the concentration of non-desirable materials and to a decrease of accuracy.

The following method uses Bond-Elut® extraction columns which are specifically adsorbent surface-modified materials. This has the advantage of minimal handling time and very clean sample extraction with good recovery. Compared to the liquid—liquid extraction method, it is better suited for high-performance liquid chromatographic (HPLC) analysis and essentially addresses the problem of sample extraction for HPLC analysis.

Later on in the analysis, the classical fluorimetric method of detection is tedious because it requires derivatization. HPLC analysis combined with amperometric detection complements this improved preparative method and facilitates routine applications.

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EXPERIMENTAL

Instrumentation

The preparative step required: Bond-Elut columns, with 1 ml PH-bonded phase (Analytichem International, Harbor City, CA, U.S.A.), a micropartition system MPS-1® with YMB® membrane (Amicon, Danvers, MA, U.S.A.), a brain homogenizer Polytron® (Kinematica, Luzern, Switzerland), vacuum flasks, ultracentrifuge tubes, etc. The analytical step consisted of the HPLC system: a Model 6000A solvent-delivery system and Model U6K injector equipped with a μBondapak RP-18 column, all from Waters Assoc. (Milford, MA, U.S.A.) and a LC-4A amperometric detector (BAS, West Lafayette, IN, U.S.A.).

Quantification was achieved with a Data Module® integrator (Waters Assoc.). The method used was that of external standard where peak areas were computed using the width at half height.

Chromatographic conditions

The mobile phase consisted of 0.05 *M* citrate—phosphate buffer (pH 3.5)—methanol (10 : 1) filtered through a Millipore membrane (0.22 μm), degassed, then pumped through the column at a flow-rate of 1 ml/min. The electrode was a glassy carbon electrode TL-5 held at 0.85 V versus the Ag/AgCl reference electrode. The sensitivity was 20 nA/V.

Standards and reagents

HPLC-grade methanol and acetonitrile were obtained from Merck (Rahway, NJ, U.S.A.). Double-distilled deionized water was used to prepare aqueous solutions. MHPG hemi-piperazine salt, crude sulphatase (type H-2) and pure sulphatase (type H-1) were purchased from Sigma. MHPG sulphate was obtained from Fluka (Buchs, Switzerland).

Sample preparation

Brain tissue. The rat brain was removed and dissected into various structures. About 100—200 mg of brain tissue were homogenized with a Polytron in 0.2 *M* perchloric acid (1 ml per 100 mg of fresh tissue) containing 0.1% EDTA. After ultracentrifugation (15,000 *g*, 15 min), the supernatant was adjusted to pH 6 for enzymatic hydrolysis which was achieved with 50—100 μl of crude sulphatase at 41°C in 2 h.

Plasma. A 1-ml volume of plasma was isolated by centrifugation and adjusted to pH 6 with 1 ml of 0.1 *M* acetate buffer containing 0.1% EDTA. Enzymatic hydrolysis was achieved with 50 μl of crude sulphatase as for brain tissue. After hydrolysis, pools of plasma were spiked with known quantities of MHPG and then ultrafiltered on a YMB membrane using the MPS-1 micropartition system. This yielded 400—500 μl of ultrafiltered plasma (per ml of plasma) after 30 min of centrifugation at 3000 *g*.

Urine. Urine samples (5 ml) were collected in 10-ml tubes containing an antioxidant (1 mg of sodium metabisulphite per ml of urine). Then 2 ml of a saturated barium chloride solution were added to remove phosphate and sulphate salts, and the sample was centrifuged at 3000 *g* for 10 min. The supernatant was adjusted at pH 6 with 3 ml of 0.1 *M* sodium acetate, and enzymatic

hydrolysis was achieved with 100 μl (per ml of urine) of crude sulphatase at 41°C in 3 h.

Hydrolysis efficiency

Due to the fact that MHPG exists in free and conjugated form it was not suitable to use an internal standard before hydrolysis. We estimated enzymatic hydrolysis by an external standard method, and the internal standard was added at the time of extraction. The efficiency of enzymatic hydrolysis was determined by using solutions of MHPG sulphate. The concentration of the standards and the number of enzyme units were similar to those of the biological samples. Pure and crude sulphatase were used and a comparative investigation of the two forms of enzyme was made.

Extraction procedure

A 1-ml PH Bond-Elut column for each sample was inserted into a 500-ml vacuum flask which was connected to a vacuum source. With the vacuum on, each column was washed with 1 ml of methanol, then with 1 ml of distilled water. The column was not kept dehydrated during the procedure. With the vacuum off, we loaded 400—500 μl of spiked or blank plasma ultrafiltrate (corresponding to 1 ml of total plasma) or 200 μl of homogenized urine or 1—2 ml of brain tissue extract (corresponding to 100—200 mg of brain tissue) onto the column. For each type of sample, parallel reference samples containing known quantities of added MHPG were made for extraction recovery calculation.

After applying the sample to the column, we washed the Bond-Elut with 200 μl of distilled water and eluted the sample with 100 μl of 2 m*M* potassium dihydrogen phosphate—acetonitrile (3 : 1). The pH of all samples was kept at about 5. At this point, the samples were ready for HPLC analysis.

We also assayed samples using the already known ethyl acetate extraction method for comparison. Ethyl acetate was previously washed and saturated with sodium chloride as described by Oishi et al. [8]: 3 vols. of ethyl acetate were used three times to extract the sample. The ethyl acetate was then evaporated at 30°C under reduced pressure. The sample was reconstituted with mobile phase to a volume smaller than the original volume.

RESULTS AND DISCUSSION

Recovery from enzymatic hydrolysis: comparison between pure and crude sulphatase activity

Crude sulphatase (50 μl of type H-2) and pure sulphatase (15 mg of type H-1) equivalent to 250 and 270 units, respectively, were assayed to 50 ng of MHPG sulphate. One unit of sulphatase is defined as the amount of enzyme which can hydrolyse 1 μmol of *p*-nitrocatechol sulphate per h at pH 5.0 and 37°C. MHPG sulphate (50 ng) was dissolved in sodium acetate buffer (0.1 *M*, 2 ml), pH 6.0, containing 0.1% EDTA. The 50 ng of MHPG sulphate were supposed to yield 50 ng of free MHPG. We also checked the presence of MHPG in each lot of enzyme freshly received in order to avoid any MHPG contamination from the enzyme.

TABLE I

HYDROLYSIS OF MHPG SULPHATE BY TWO TYPES OF SULPHATASE

MHPG sulphate (50 ng) was dissolved in 2 ml of 0.1 *M* sodium acetate buffer containing 0.1% EDTA, at pH 6, and was expected to yield 50 ng of free MHPG. Results are the means of four independent experiments and are expressed in ng.

Enzyme	Hydrolysed MHPG sulphate (ng)			
	Incubation period (h)			
	1	2	3	16
Crude sulphatase (H-2, 250 units)	50.6	49.7	50.1	49.1
Pure sulphatase (H-1, 270 units)	3.1	3.8	3.4	22.7

As seen in Table I, crude sulphatase was more efficient than pure sulphatase. With the latter, more than 50% of MHPG sulphate was still not hydrolysed after 16 h of incubation, whereas with crude sulphatase 50 ng of free MHPG were produced after 1 h. This result is not surprising. The high efficiency of crude sulphatase has already been pointed out by Karoum et al. [9]. These authors recommended the use of crude sulphatase with biological materials and incubation for 1 h at 40°C. Such a result explains why many researchers are still incubating for 16 h or more for hydrolysis. However, we think that this depends on the type of enzyme used. Yet Karoum et al. [9] attributed this to the differences between enzyme subunits. From our experience, conditions for each lot of enzyme must be checked after its receipt in order to optimize hydrolysis. From such a result, no other recovery from enzymatic hydrolysis was necessary, since with 250 units of crude sulphatase all MHPG conjugates in 1 ml of plasma or 200 mg of brain were hydrolysed in 1 h. For absolute certainty we always incubated for 2 h. For urine, we slightly increased the time of hydrolysis since MHPG conjugates are more accumulated. We found 3 h to be sufficient.

Recovery and precision from Bond-Elut extraction

Ethyl acetate extraction was used parallel to Bond Elut extraction which allowed the two methods to be compared. Percentage recovery was determined

TABLE II

PERCENTAGE RECOVERIES OF MHPG FROM BIOLOGICAL MATERIALS USING BOND-ELUT AND ETHYL ACETATE EXTRACTION

Values are the means of four independent experiments.

Samples	Percentage recovery (± C.V.)	
	Bond-Elut	Ethyl acetate
MHPG standard	75 ± 0.9	98 ± 12
Urine	80 ± 2.4	93 ± 16
Plasma	60 ± 2.9	94 ± 9.7
Rat brain extract	80 ± 1.9	97 ± 9.9

for all samples and standard solutions using the standards addition method.

Table II shows the various percentage recoveries. It can be seen that the ethyl acetate extraction method yields the highest percentage recovery but that the Bond-Elut method shows better precision. The within-run precision of the Bond-Elut method was good as shown by a coefficient of variation never exceeding 3%, whereas with the ethyl acetate method the coefficient of variation reached 16%.

Linearity and sample clean-up

The linearity of this method is represented by an example which shows plasma MHPG extraction, with known levels of added MHPG ranging from 5 to 20 ng/ml (Fig. 1). This use of parallel references with added MHPG helps to validate the recovery over a large range of plasma MHPG levels. The discrepancy between levels of MHPG found in the same pool of plasma is due to the different recoveries obtained with the two methods. Although ethyl acetate shows the highest recovery, the best linearity was given by the Bond-Elut method.

Although not too high, the percentage recovery was sufficient to recommend this method.

The x-intercept represents the endogenous MHPG (Fig. 1).

The other argument in favour of the Bond-Elut method is given by the very clean chromatogram yielded by this method (Fig. 2C and D). The ethyl acetate extracted MHPG peak is not baseline-resolved and non-desirable peaks fused with the MHPG peak. When working with automatic integration, using computing areas, well resolved peaks are better than fused peaks as the reproducibility

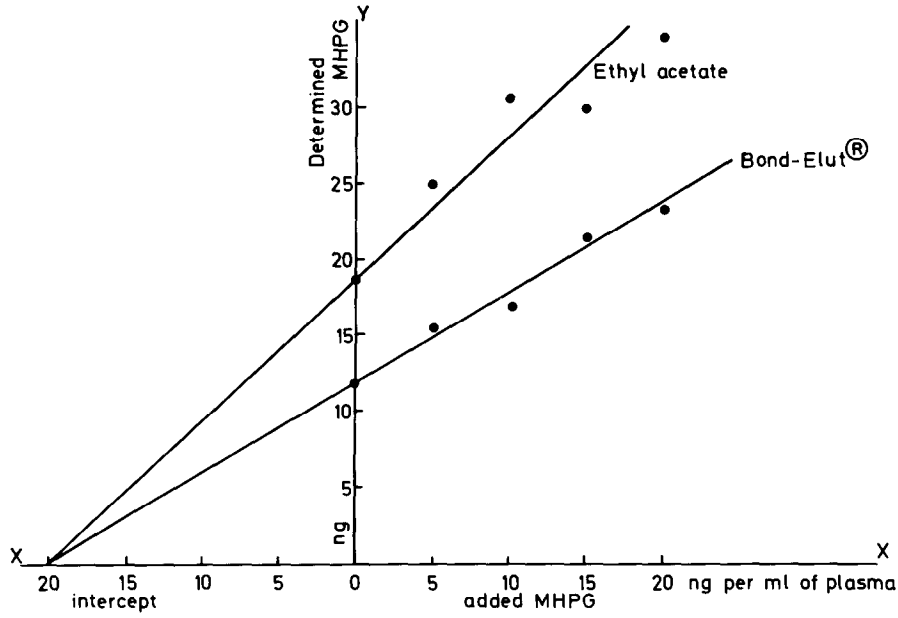

Fig. 1. Comparison between pooled plasma (1 ml) spiked with 5—20 ng of MHPG and extracted by the ethyl acetate or the Bond-Elut method to show the linearity of the two techniques. The x-intercept represents the total endogenous plasma MHPG.

is improved. Fig. 2A and B shows chromatograms from plasma and rat brain extracts, separated by the Bond-Elut method. The sample clean-up is clearly shown by these chromatograms. Finally, these preparative columns can be reused. Where plasma or brain tissue is concerned, we have seen that Bond-Elut columns can be reused one more time, after a good clean-up with 4—5 ml of distilled water, without affecting recovery. This recovery is sharply reduced when the column is used a second time for total urinary MHPG.

Recovery from the YMB membrane

Initially, we thought that the YMB membrane was responsible for the low percentage recovery from plasma. We determined the recovery of MHPG using the MPS-1 system with the YMB membrane. After hydrolysis, 2 ml of plasma were separated into two parts: 1 ml was ultrafiltered on MPS-1 with the YMB membrane and the ultrafiltrate was extracted in the same volume of ethyl acetate as the non-ultrafiltered plasma. The recovery of plasma MHPG in the YMB ultrafiltrate was 96.2% (±1.2). This result indicated that MHPG does not bind to the plasma proteins as Murray et al. [10] suggested occurs in dog plasma. Furthermore, MHPG passes through the YMB membrane very well. The relatively low recovery with Bond-Elut extraction could be due to the ionic environment of the plasma, and not to the low ultrafiltration of MHPG through the YMB membrane as previously thought.

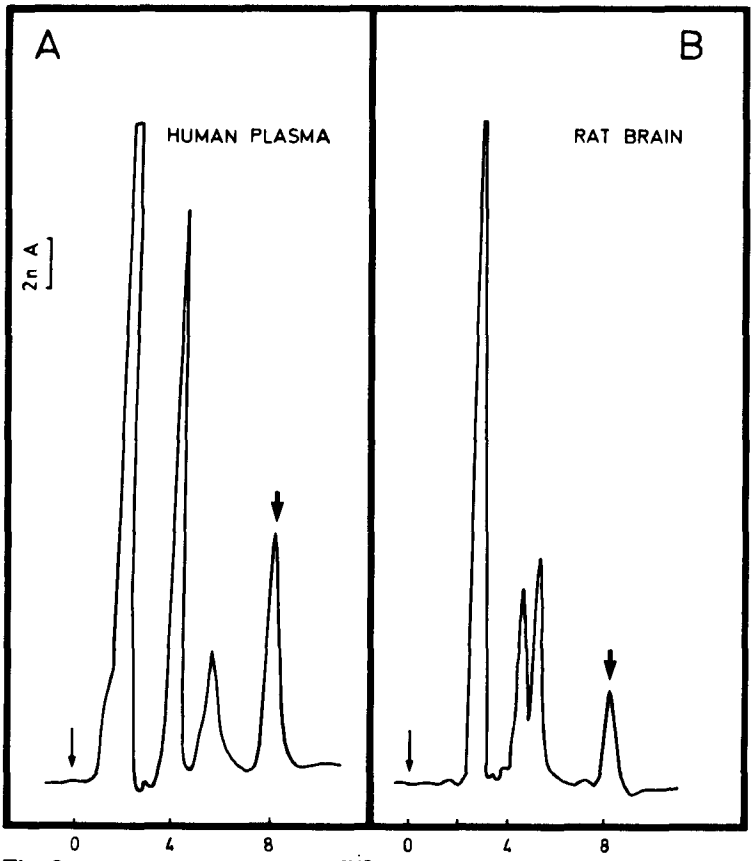

Fig. 2.

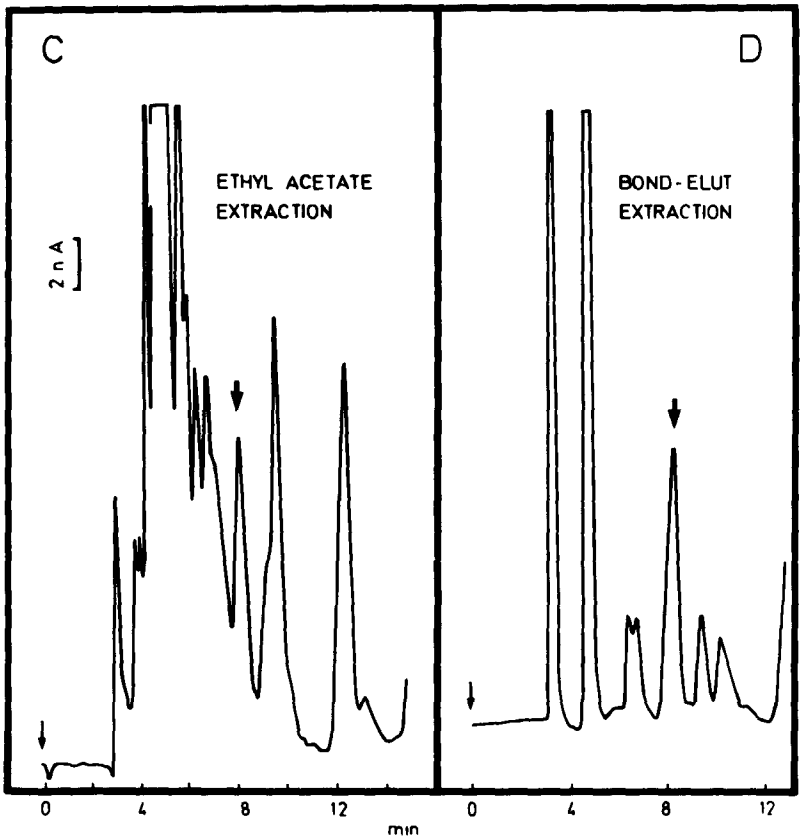

Fig. 2. (A and B): Typical chromatograms using the reversed-phase HPLC system of actual plasma (A) and rat brain (B) extract carried through the Bond-Elut extraction procedure. For chromatographic conditions see Experimental. (C and D): Typical chromatograms using the reversed-phase HPLC system of actual urine carried through the ethyl acetate (C) and Bond-Elut (D) extraction procedures. For chromatographic conditions see Experimental.

Comparison with other techniques

Table III presents results from our technique compared to data reported in the literature. Although this kind of comparison is not suitable for several reasons (differences in condition techniques, type of rat strain or monoamine

TABLE III

RESULTS OF MHPG EXTRACTED WITH BOND-ELUT COLUMNS COMPARED TO THOSE OF THE DIFFERENT TECHNIQUES FOUND IN THE LITERATURE

	MHPG extracted			
	Plasma (ng/ml)		Urine (μg per 24 h)	Rat brain (ng/g)
	Men	Women		
Bond-Elut	18.8 ± 4.5	15.6 ± 3.1	1600—2200	92 ± 10
Literature	22 ± 0.6 [11]	15.7 ± 2.0 [12]	1873 [11]	108 [13]

diet of subjects), nevertheless it does indicate that our results are similar to those reported in the literature. Furthermore, the difference between MHPG levels in men and women shown in Table III argues for the sensitivity of the method.

CONCLUSIONS

The aim of this study was to find a rapid, low-cost and precise method of determining MHPG, as this might be helpful in the diagnosis of certain forms of psychopathology. Although the ethyl acetate extraction method gives a relatively high recovery, the method is not precise enough, is time-consuming, and requires repeated assays in order to show mean data.

Bonded-phase materials for chromatography have existed for a number of years and seem to improve the isolation. It was Analytichem who first suggested isolating urine MHPG with PH-Bond-Elut columns [14]. In this study I was able to improve the conditions for isolation of MHPG from plasma and rat brain and to adapt the Analytichem method for total MHPG extraction. We believe that this method is well suited for HPLC analysis because of its precision and the clean sample chromatograms, and because it is simple enough to be reliable in routine use.

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