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Letter to the Editor

Determination of free and sulphate-conjugated 3-methoxy-4-hydroxyphenylethylene glycol in human plasma by liquid chromatography with electrochemical detection

Sir,

In the mammalian central nervous system, 3-methoxy-4-hydroxyphenylethylene glycol (MHPG) is the major metabolite of norepinephrine (NE) [1]. Free MHPG is the predominant form of MHPG in the brain and cerebrospinal fluid; the sulphate conjugate is the major conjugated form. By contrast, the glucuronide conjugate represents only ca. 5% of the total MHPG concentration in the brain [2]. MHPG has been assayed in physiological samples by gas chromatography [3, 4] and by a gas chromatographic–mass fragmentometric procedure [5] that is both sensitive and selective but is also slow and very expensive for routine use. More recently, high-performance liquid chromatography (HPLC) with electrochemical detection (ED) has been proposed as the technique of choice [6–8]. We describe a rapid and accurate technique for the assay of free and sulphate-conjugated MHPG in plasma, in which HPLC is used after a simple clean-up procedure on a solid phase. Efficient column and electrochemical detection combined with the use of an internal standard (vanillyl alcohol or hydroxybenzyl alcohol) allow MHPG determinations in the picomole range; the solvent system and column are the same as those used for NE. Results obtained for controls are compared with literature reports. The position of the individual during blood sampling appears to be just as important for MHPG as for NE.

EXPERIMENTAL

Chromatography

The liquid chromatographic system consisted of a Model 114 M Beckman pump (Beckman, Gagny, France) a Model U6K injector (Waters, Saint-Quentin, France) and a 250 mm×4.6 mm I.D. column of 5-μm Ultrasphere ODS (Beckman). The Model 5100 A Coulochem Esa electrochemical detector (Cunow, Clichy, France) was equipped with a guard cell (Model 5020) and an analytical cell

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(Model 5011) with a positive double potential. The potential of the guard cell was set at +0.46 V to oxidize mobile phase impurities.

The potential of the first detector (T1) was set at +0.15 V; this improved the baseline and did not decrease the response of the second detector. The potential of the second detector (T2) was set at +0.46 V to obtain a high signal from MHPG oxidation. Measurement was based on estimation of peak heights using an integrator (Spectra-Physics, La Verpillière, France).

The solvent system consisted of a 95:5 (v/v) mixture of 0.1 *M* sodium acetate, 0.05 *M* citric acid containing 50 mg of EDTA and 255 mg of octanesulphonic acid sodium salt per litre (mobile phase pH 4.8) and methanol. This mixture was filtered through a 0.2-μm membrane filter and degassed.

Solvents and chemicals

HPLC-grade methanol was purchased from Carlo Erba (Milan, Italy). All other reagent-grade chemicals were obtained from Merck (Darmstadt, F.R.G.). The standard MHPG hemipiperazine salt and an internal standard (hydroxybenzyl alcohol) were purchased from Sigma (St. Louis, MO, U.S.A.). The internal standard, 4-hydroxy-3-methoxybenzyl alcohol (vanillyl alcohol), and octanesulphonic acid sodium salt were obtained from Fluka (Buchs, Switzerland) and sulphate-conjugated MHPG from Calbiochem (San Diego, CA, U.S.A.). Arylsulphatase from *Helix pomatia*, 5 U/mg (25°C, 4-nitrophenyl sulphate as substrate), was purchased from Boehringer Mannheim (Mannheim, F.R.G.). Bond-Elut containing 100 mg of C_{18} bonded silica (40 μm) was obtained from Analytical International (Prolabo, Paris, France).

In water, the 1 *M* MHPG solution is stable for one month at 4°C; the working solution is prepared every day. In methanol, the 10 *M* vanillyl alcohol is stable for one week at 4°C; the working solution is prepared daily.

Extraction

Blood was collected in a heparinized tube and immediately centrifuged at 600 *g*. Plasma was stored at −20°C until assayed (within one month). A 500-μl sample of plasma was combined with 500 μl of 0.1 *M* sodium acetate buffer (pH 6) containing 0.1%. EDTA and 30 μl of 5 μM vanillyl alcohol. Sulphate-conjugated MHPG was hydrolysed with 50 μl of arylsulphatase (250 μg) by incubation for 18 h at 37°C. This hydrolysed plasma (1 ml), plus 30 μl of vanillyl alcohol, was applied to the Bond-Elut column previously conditioned with 1 ml of methanol and 1 ml of water [10]; for free MHPG determinations, untreated plasma samples were used. MHPG was eluted with 0.2 ml of a degassed mixture (6:4, v/v) of the mobile phase and methanol. A 20-μl sample was injected.

The appropriate standard curve constructed from spiked pooled plasma was used to evaluate the MHPG concentration in the test samples. Sulphate-conjugated MHPG was calculated by subtracting the MHPG peak height of the blank sample (without any enzyme) from the peak height of MHPG in the sample incubated with arylsulphatase.

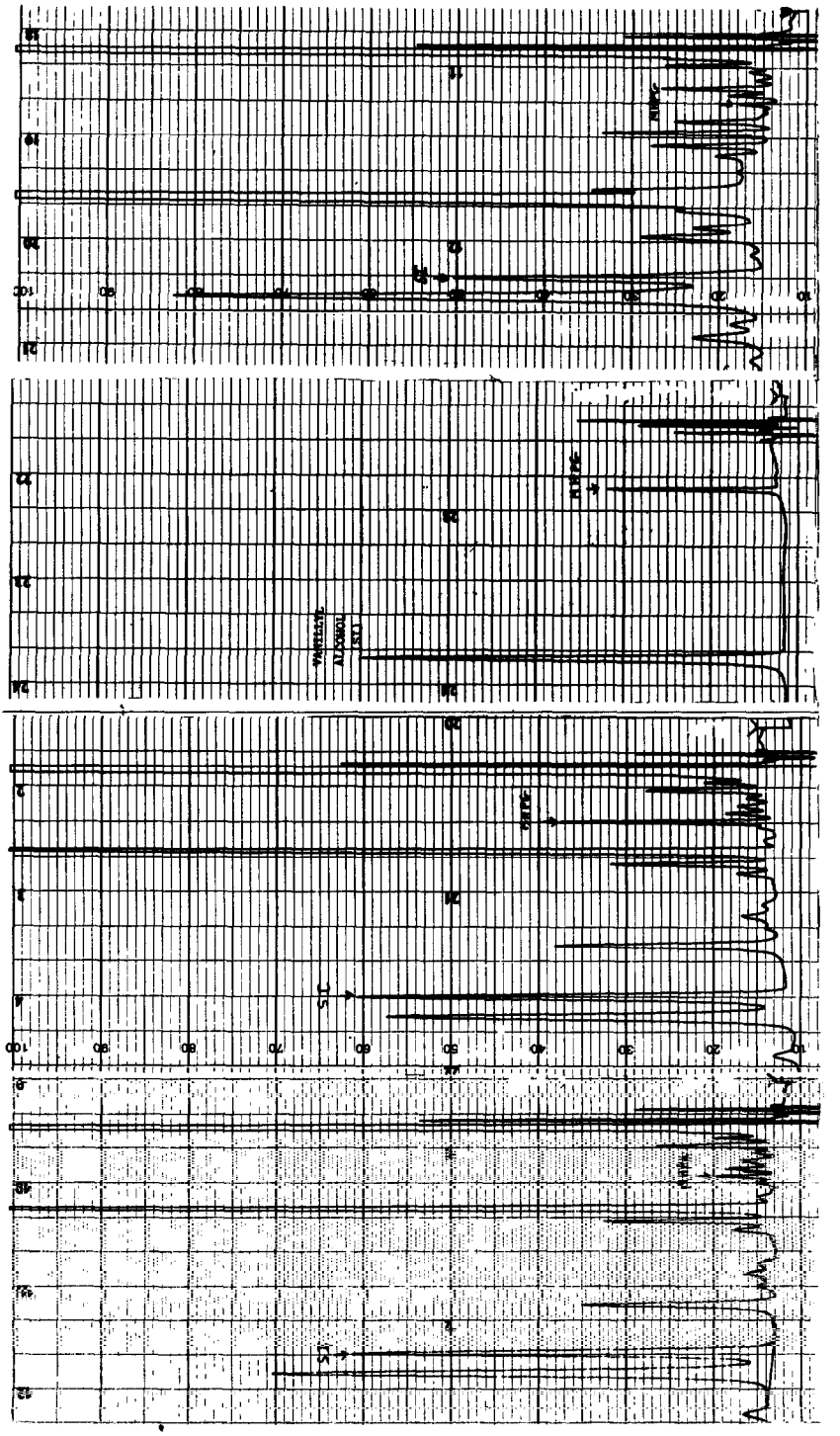

Fig. 1. Chromatograms of (A) a plasma sample, (B) a spiked plasma sample, (C) an aqueous standard solution of 50 nmol/l MHPG and 150 nmol/l vanillyl alcohol and (D) a hydrolysed plasma sample.

RESULTS AND DISCUSSION

The mobile phase and column used to assay MHPG were the same as for NE. Octanesulphonate did not affect chromatographic MHPG separation [9].

The main problems encountered during HPLC–ED determination of plasma MHPG concentrations concern selection of the appropriate extraction procedure and the internal standard; use of vanillyl alcohol and hydrobenzyl alcohol at the end of chromatography provided sensitive, reproducible MHPG analyses. The coefficient of variation ranged from 3.5% (same day) to 8.5% (between days).

Fig. 1. shows representative chromatograms of the standards and the human plasma extracts. Under the chromatographic conditions used, both MHPG and vanillyl alcohol gave sharp peaks. The retention time for the plasma peak was identical with that for reference MHPG; modification of the pH or methanol concentration caused an identical modification in the retention time.

The response curve was linear in the concentration range 5–150 nmol/l (after subtraction of free plasma MHPG). Results were compared with those obtained by MHPG extraction with 0.1 *M* phosphate buffer (pH 7.4) and with the values obtained without extraction to determine recovery. MHPG recovery from plasma (10–150 nmol/l) using Bond-Elut was $67 \pm 3\%$; the recovery with phosphate buffer was $72 \pm 2.5\%$. Although not very high, plasma recovery is sufficient for recommendation of the method. Classical ethyl acetate extraction gave the highest recovery percentage, but was time-consuming and not accurate enough [11].

Free MHPG concentrations measured in five pooled plasma samples were 29.3 ± 5.7 nmol/l; values in six healthy volunteers who were supine for 20 min prior to sampling through an indwelling catheter, were 15.8 ± 4.1 nmol/l. These figures agree with recent values obtained using HPLC–ED [10–13], gas chromatography [3, 4, 14] and gas chromatography–mass spectrometry [5, 15]. The MHPG concentration was significantly higher in a patient with pheochromocytoma (1025 nmol/l).

Every fresh batch of enzyme was examined for the presence of MHPG to avoid MHPG contamination by the enzyme. The enzyme activity in a 50-μl aliquot completely hydrolysed the sulphated MHPG in the samples. Only slight loss of MHPG (5%) was observed after 18 h at 37°C (pH 6). The efficiency of enzymatic hydrolysis was determined using an MHPG sulphate solution. The sulphate-conjugated MHPG concentrations in plasma from two healthy volunteers were, respectively, 41 and 49 nmol/l, which concurs with published data [2].

In conclusion, HPLC coupled with sensitive differential ED, combined with a simple clean-up procedure, is a fast and inexpensive method for measurement of free plasma MHPG and its sulphate conjugate. It is an attractive alternative for monitoring the plasma MHPG levels in patients with psychiatric and metabolic disorders.

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