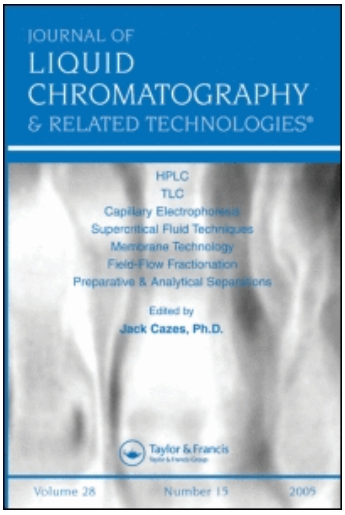

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Highly Sensitive and Simple Method for Determination of Free 3-Methoxy-4-Hydroxyphenylglycol in Plasma by High-Performance Liquid Chromatography Using a Sep-Pak Alumina B Cartridge

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HIGHLY SENSITIVE AND SIMPLE METHOD FOR DETERMINATION OF FREE 3-METHOXY--4-HYDROXYPHENYLGLYCOL IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING A SEP-PAK ALUMINA B CARTRIDGE

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ABSTRACT

A novel sample clean-up procedure for the determination of free 3-methoxy-4-hydroxyphenylglycol (MHPG) in plasma is described. MHPG was purified with Sep-Pak alumina B cartridge followed by ethyl acetate extraction from the cartridge. High-performance liquid chromatography with amperometric detection was used for separation and detection of MHPG and the internal standard 3-ethoxy-4-hydroxyphenylglycol (EHPG). This method provided good, clean chromatograms with base-line separation of the appropriate peaks. This technique is sensitive, reliable and less time-consuming than other methods. Thus, only 0.5 ml of plasma is needed and

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the within-analysis and between-analysis coefficients of variation were 5.2% and 13% respectively. The plasma MHPG values in normal control were in good agreement with those using more complex methods.

INTRODUCTION

3-Methoxy-4-hydroxyphenylglycol (MHPG) is the major metabolite of norepinephrine (1,2). Studies suggest that the level of MHPG in plasma can be used as an index of brain noradrenergic activity in human subjects (2) and, accordingly, MHPG has been extensively studied in various neuropsychiatric disorders (3).

MHPG levels in plasma have previously been determined by gas chromatography with either electron-capture detection (4-6) or mass spectrometry (7-12). Although the later technique has high sensitivity and specificity, it is not available in many clinical and research laboratories because of its high cost and technical complexity. Recently, high-performance liquid chromatography (HPLC) with electrochemical detection has been used to measure MHPG in plasma (13-23). These techniques require either deproteinization of plasma, complex column separation steps, or multiple solvent extractions, which often result in low sample recovery and are time-consuming procedures. The present paper describes an improved method for the determination of free MHPG in plasma by HPLC. The Sep-Pak alumina B cartridge were used to clean up 0.5 ml of plasma. The procedure is simple, rapid, accuracy and highly sensitive.

EXPERIMENTAL

Apparatus

A LC-154T liquid chromatograph (Bioanalytical Systems, West Lafayette, IN, USA) was used throughout this work. The system contained a guard column, 4.6 X 30 mm, 5 um particle size, RP-18 (Brownlee Labs. Santa Clara, CA, USA) and an analytical column, Ultrasphere ODS, 4.6 x 250 mm, 5 um particle size from Beckman (San Ramon, CA, USA). The column temperature was maintained at 26°C. The mobile phase was a mixture of 0.1 M soduium acetate with 0.09 M citric acid buffered to pH 3.75, 0.25 mM Na_2EDTA and containing 4% methanol. The solvent was filtered and degassed prior to use. The flow-rate was 1.5 ml/min. A TL-5 glassy carbon

electrode was used at a potential of 0.75 V vs Ag/AgCl reference electrode, with the sensitivity of the amperometric controller set at 0.5 nA. The chart speed of the recorder was set at 0.5 cm/min. Ethyl acetate extracts of plasma were dryed using Vortex - Evaporator (Haake-Buchler, Saddle Brook, NJ, USA).

Reagents

MHPG hemipiperazine salt was used as reference standard and was purchased from Sigman Chemical Co. (St. Louis, MO, USA). 3-Ethoxy-4-hydroxyphenylglycol (EHPG) was used as an internal standard and was kindly donated by Dr. Kenneth L. Kirk (Laboratory of Chemistry, NIADDKD, NIH, Bethesda, MD). Sep-Pak alumina B cartridges were obtained from Waters Associates, Inc. (Milford, MA, USA). Methanol, ethyl acetate, diethyl ether and water were HPLC grade from J.T. Baker Chemical Co. (Phillipsburg, NJ, USA). All other chemicals were reagent grade.

Procedure

Plasma samples were obtained from heparinized blood and stored at -70°C until analyzed.

The upper extension (syringe fitting) of the Sep-Pak alumina B cartridge was first shortened to about 5 mm to reduce the dead space, a 3 ml syringe was then inserted so that the syringe tip was flushed with the cartridge matrix chamber. The Sep-Pak alumina B cartridge was then prepared by washing through 3 ml of the following: water, methanol, ethyl acetate and diethyl ether via the syringe under vacuum. After allowing for the complete drying of diethyl ether the cartridge was slowly loaded with a 0.5 ml aliquot of plasma and 40 ul of EHPG (4 ng) which had been mixed thoroughly in the syringe. The front of the plasma mixture should not reach the botton of the alumina. The cartridge was then washed with 2 ml of diethyl ether. The MHPG and EHPG were eluted from the cartridge with 3 ml of ethyl acetate using a slow flow-rate and the eluate was collected into 12 x 75 mm glass test tubes. This extract was then evaporated to dryness in the Vortex-Evaporator at 35°C and the residue redissolved in 0.3 ml of water. Each reconstituted sample was filtered through 0.2 um pore size filter and then 50 ul of filtrate was analyzed by HPLC. The concentration

of MHPG in each sample was calculated from the peak height ratio for MHPG relative to EHPG by comparing this ratio to that obtained with pooled control plasma spiked with a known quantity of MHPG and the same amount of EHPG as the samples.

RESULTS AND DISCUSSION

Representative chromatograms of reference standards, human and rat plasma extracts and human plasma with internal standard (EHPG) are illustrated in Fig. 1. Under these conditions, MHPG and EHPG which have retention time of 9 min and 22 min respectively gave sharp peaks, well separated from other electroactive constituents of plasma. No interfering peaks with a retention time corresponding to that of EHPG were seen in human and rat plasma samples (Fig. 1 B and D). Elution of plasma extract was complete within 30 min and there were no slow eluting peaks which would interfere with the next chromatogram (14,21). Indeed, no late eluting peaks were observed even after many samples had been continuously injected into the column. When reversed-phase columns from different manufacturers were used, the plasma extract peak for MHPG was identifiable by retention time with that for reference MHPG, and it changed in an identical manner when the pH, and/or methanol concentration of the mobile phase was changed. Hydrodynamic voltammograms for a plasma extract and authentic MHPG were identical.

It was found that the resolution of HPLC columns from different manufacturers varied for this assay. The results shown in Fig. 1 were obtained by using an Ultrasphere ODS column from Beckman, and similar results were given by the Sota C-18E column, but both of these columns varied widely in performance from column to column. The uBondapak C-18 columns from Water Associates and a Biophase ODS column from BAS were suitable for the separation of MHPG, as had been reported by others (17,21) but not for EHPG because of peak broadening in proximity to interfering peaks.

The sample clean-up procedure using the alumina B cartridge was chosen because it provided clean chromatograms, rapid sample preparation and easy handling when compared with the liquid-liquid extraction method

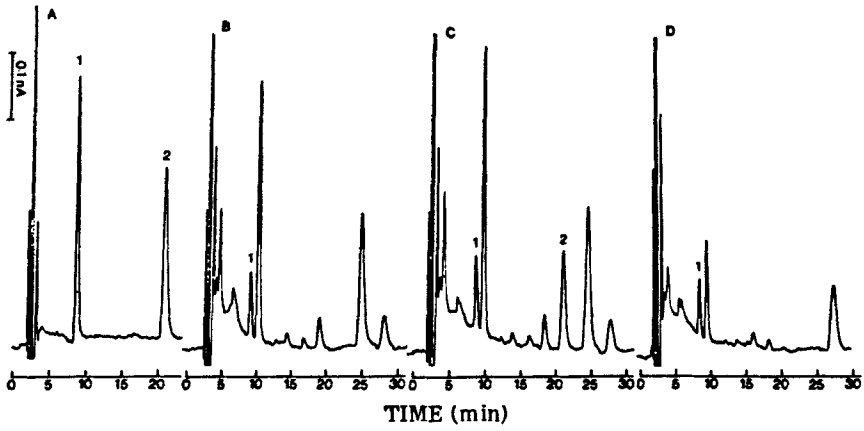

FIGURE 1. Chromatograms of (A) MHPG and EHPG standards (1 ng each), (B) extract of human plasma, (C) extract of human plasma with EHPG added, (D) extract of rat plasma.
Peaks: 1 = MHPG and 2 = EHPG. For conditions, see text.

(13-15, 18-19, 21, 22) and the liquid-solid extraction method (16,17,20). The plasma samples can be directly loaded onto the cartridge without deproteinization. The relatively clean chromatograms obtained by this method of sample preparation may be accounted for by two factors: firstly, adsorption of undesired substances to the alumina matrix of the cartridge, and secondly, elution of contaminating species by the diethyl ether wash. The effectiveness of this clean-up contributed to prolongation of column life, and more than 1500 injections of sample extraction have been injected with no change in column performance. Furthermore, the same working electrode has been used during this extended period without the necessity of resurfacing. Because MHPG is not adsorbed directly by the alumina (24) the sample volume should be regulated so as not to exceed the volume capacity of the cartridge. We found 0.56 ml to be the maximum volume usable within this constraint.

The recovery of MHPG was 72.3 $\pm$ 14.8% at the concentration of 0.5-10 ng/0.5 ml of plasma (mean $\pm$ SD, n = 40) while that for EHPG was 65.0 $\pm$

16.8 at the concentration of 4 - 8 ng/0.5 ml of plasma (n = 358). The primary source of variation in recovery was due to unavoidable heterogeneity in the alumina cartridges, but MHPG and EHPG were found to vary in concert and thus provide a constant peak ratio. The alumina B cartridge can be reused several times after washing with the same solvent as for initial preparation. Recovery of MHPG and EHPG from reused cartridges was found to be very constant.

The overall within-assay variance (C.V.) calculated for 10 samples was 5.2%, while the variation between-assays was 13.0% after 16 assays. The average value for blood obtained from normal untreated control subjects was 3.56 $\pm$ 0.50 ng/ml (n = 5), which compares with observed values (8,10,14-16,18-20,23).

During preparation of this manuscript Gupta et al (23) described an "Extrelut" column extraction method in which an ethyl acetate eluate was mixed with pentane and back-extracted into water. Whereas their method was simple and the chromatograms appeared clean, a cuolometric detector was necessary and furthermore, no suitable internal standard was available for this particular solvent-extraction procedure.

In conclusion, we have developed a simple, sensitive HPLC assay for free MHPG in plasma using the Sep-Pak alumina B cartridge for samples clean-up. The method is suitable for routine clinical analysis and neurochemical research.

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