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DETERMINATION OF URINARY 3-METHOXY-4-HYDROXYPHENYLGLYCOL BY LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

The increasing popularity of urinary 3-methoxy-4-hydroxyphenylglycol (MHPG) determinations has placed a demand on clinical laboratories to provide these data. Previous methods for MHPG either exhibit unacceptable performance, are too slow, or utilize instrumentation not commonly employed in these laboratories. The liquid chromatography–electrochemical detection method described here has several positive features. MHPG is isolated by extraction, separated by liquid chromatography, and quantitated at an amperometric electrode. The precision, simplicity, and relatively low cost makes this new method an ideal choice for the clinical laboratory.

INTRODUCTION

A great deal of research has now established that there is a connection between brain biochemistry and affective disorders such as depression. Alterations in the metabolism of a number of biogenic amines have been discovered in these disorders. One of these biogenic amines in particular, norepinephrine (NE), is an integral mediator of brain reward systems and emotion centers. Deficiencies of norepinephrine at these receptor sites may produce certain depressions, while an excess may be the cause of mania and schizophrenia, although it is also recognized that these disorders are clinically and biochemically heterogeneous[1]. Thus, focusing attention on one biogenic amine may give misleading conclusions about exactly what the connection is between the brain biochemistry and the etiology of affective disorders.

In spite of this, many studies have shown the potential usefulness of monitoring norepinephrine metabolism in the diagnosis and treatment of these illnesses. For this purpose, a reliable and convenient marker of brain NE has been sought. The most common one is now the urinary concentration of 3-methoxy-4- hydroxyphenylglycol (MHPG), a major metabolite of brain NE. Around 50% of the urine MHPG in man seems to be derived from central nervous system noradrenergic activity[2]. The urinary excretion rate of MHPG has been shown to reflect total NE production[3]. Determination of urinary MHPG may allow patients to be separated into groups[4] or even

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subgroups[5]. Also, pretreatment concentrations of urinary MHPG have been used to predict outcomes of treatment with certain antidepressant drugs[6,7].

The increasing awareness by physicians of the utility of urinary MHPG measurements has placed demands on the clinical laboratories in the hospitals where these patients are treated. The method used in most of the early clinical studies was based on gas chromatography (GC) with electron-capture detection[8], but this is a cumbersome method requiring equipment not necessarily found in most laboratories. Recent modifications of this method still require over 3 h per analysis[9]. Liquid chromatographic methods do not require the preparation of volatile derivatives as in GC and also utilize instrumentation more common in today's clinical laboratory. For clinical use, a method for urinary MHPG must be precise, accurate, reliable, and simple enough to be used routinely. The method described here meets these criteria.

EXPERIMENTAL

Reagents and standards

Sodium acetate, sodium metabisulfite, boric acid, potassium bicarbonate, EDTA, ammonium phosphate, diethyl ether (anhydrous) and ethyl acetate were all of analytical reagent grade. Tetrabutylammonium hydroxide was a 40% aqueous solution from Aldrich, Milwaukee, WI, U.S.A. Acetonitrile was HPLC-grade from Fisher Scientific, Fair Lawn, NJ, U.S.A. The enzyme for hydrolysis was β-glucuronidase, type H-1, 420,000 U/gm (Sigma, St. Louis, MO, U.S.A.). Authentic MHPG standard (hemipiperazine salt, Sigma) was used to make 1 mg/ml stock solutions in deionized water. These were kept refrigerated in brown bottles and were stable for up to six months.

Quality controls

Several 24-h urines were collected from healthy individuals and were used to prepare urine pools. The urine was acidified to pH 4 with 30% acetic acid and further stabilized by adding 2 ml of 0.25 g/ml $NaHSO_3$. One urine pool was supplemented with a small amount of standard MHPG and became the calibrator, as described in the procedure section. Two other pools, one with added MHPG, were used to provide quality control materials. Aliquots of these urine pools were stored frozen in capped tubes. They are still stable after 8 months.

Apparatus

A Model 340 liquid chromatograph from Beckman Instruments (Fullerton, CA, U.S.A.) was equipped with an LC-4B electrochemical detector (Bioanalytical Systems, West Lafayette, IN, U.S.A.). A glassy carbon working electrode was used in all experiments. The analytical column was a Beckman 5 μm ODS 15 cm × 4.6 mm I.D. column. The detector output was monitored and plotted by a Hewlett-Packard 3390A integrator.

Chromatographic conditions

A mobile phase of 40 m*M* $(NH_4)_3PO_4$ buffered to pH 2.9 was supplemented with 4 m*M* butylammonium hydroxide, 0.1 m*M* EDTA, and 5% acetonitrile. The initial flow-rate of 0.6 ml/min was maintained for 10 min, when it was increased automatically to 1.5 ml/min for an additional 30 minutes. The electrode potential

was +0.85 V *vs.* Ag/AgCl. Ambient temperature was used throughout the chromatographic analysis.

Procedure

Freshly voided urine or urine preserved as described above (0.5 ml) is pipetted into a 15-ml screw-cap culture tube. β-Glucuronidase solution (0.4 ml, 8000 U/ml 1 *M* sodium acetate, pH 6) is added, and after mixing and centrifuging this mixture, it is stoppered and incubated at 37°C. After 24 h, 250 μl of 0.1 *M* H_3BO_3 is added to the mixture, which is washed with three 2.0-ml portions of diethyl ether. After shaking and centrifuging, each ether layer is removed by suction. Following the third ether wash, 250 μl of saturated $KHCO_3$, containing EDTA and 0.5 g NaCl, is added to the aqueous layer. This mixture is extracted with three 2.0 ml portions of ethyl acetate. The ethyl acetate layers are transferred to a conical screw cap tube and evaporated just to dryness under nitrogen. The residue is dissolved in 0.5 ml mobile phase with vigorous agitation and a 20-μl sample is injected into the liquid chromatography–electrochemical detection (LC–ED) system. Each sample is quantitated by comparing the peak height with the calibrator sample. The concentration of the calibrator is determined by a standard addition method. Within each day's group of samples, one or both quality control (QC) pools should be determined. The data are judged acceptable if the QC samples are within 2 S.D. of their mean.

RESULTS

This method has been optimized for the determination of MHPG in urine. Fig. 1 shows a typical chromatogram obtained for one of several normal urine samples. The mean of these normal samples is currently 2.37 mg/day.

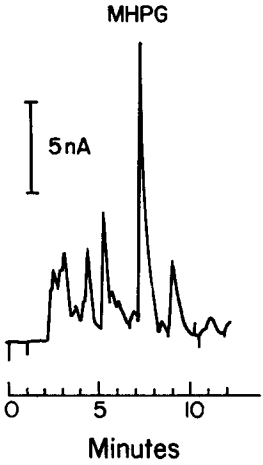

Fig. 1. A typical chromatogram obtained for the determination of urinary MHPG. Chromatographic conditions are described in the text. Concentration in this sample, 1.12 μg/ml.

The precision of the method has been tested by using the two QC pools. Table I lists these data, which indicate very acceptable precision throughout the range of concentrations likely to be encountered.

The relative recovery in 20 experiments averaged 99.1%. Many other catecholamines and metabolites tested for interference do not pass through the extraction

TABLE I

DAY-TO-DAY PRECISION (n = 20)

Level	*Mean (mg/l)*	*S.D.*	*C.V. (%)*
1	0.74	0.036	4.8
2	4.09	0.151	3.7

scheme. Other compounds that are extracted along with MHPG are either not eluted at the same time or do not interfere due to the selectivity of the detector. The purity of the urinary MHPG peak was established by comparing a multi-point hydrodynamic voltammogram of the urine sample with MHPG standard. The similarity of these curves indicated excellent selectivity.

An effort to determine the accuracy of the method was made by comparison with a method used at the Mayo Clinic (Mayo)[10]. This method, also based on liquid chromatography, but with fluorescence detection, was shown to agree closely with the GC procedures used in earlier studies. Fig. 2 is a comparison plot of μg/ml data obtained for 29 urines determined by the two LC procedures. Least squares statistics calculated from these data are University of Wisconsin method (UW) = 0.937 (Mayo) + 0.010. Correlation coefficient, R = 0.929.

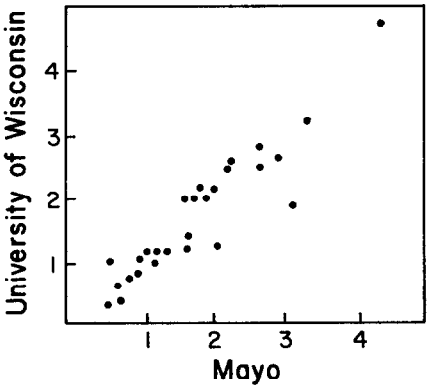

Fig. 2. Comparison plot of 29 urines, determined by our method and by the Mayo Clinic method. Statistics appear in the text.

DISCUSSION

The purpose of this study was to develop a method for urinary MHPG suitable for clinical laboratories. Important qualities in this setting are precision, accuracy, reliability, robustness, low cost, simplicity and availability of the required instrumentation. The method described here meets these needs.

Many peaks appear in the chromatogram; however, the selectivity seems assured by the combination of extraction procedure, chromatography, and potential of the electrode. The potential chosen, +0.85 V, gives a nearly optimal response to MHPG without causing other peaks to appear and increase the baseline noise.

Several deficiencies might be pointed out that cause this method to be less than ideal for clinical work, but each problem is either common to all MHPG methods or does not outweigh the advantages of the method. For instance, the 24-h incubation needed for enzymatic hydrolysis of conjugates delays results for at least one day. This step is required in any assay for urinary MHPG, however, as most of it is excreted in the form of conjugates, and these cannot be hydrolyzed by faster methods due to the instability of MHPG at extremes of pH. Also, inclusion of internal standard is usually desirable in methods of this type. The main purpose of this is to improve the precision. Our method shows more than adequate precision without an internal standard. Finally, the extra 30 min required to wash late-eluted peaks off the column after each injection lowers the throughput. Efforts to improve it continue, but evenso, a technician can assay 9 samples in an 8-h day. Except in the very busiest of laboratories, this should be more than adequate.

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