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DIRECT DETERMINATION OF 4-HYDROXY-3-METHOXYPHENYLACETIC (*HOMOVANILLIC*) *ACID IN URINE BY HIGH-PERFORMANCE LIQUID* CHROMATOGRAPHY WITH AMPEROMETRIC DETECTION

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SUMMARY

The improvement of high-performance liquid chromatographic analysis with electrochemical detection for urinary homovanillic acid is described. The method permits the chromatographic resolution of authentic homovanillic acid from coeluting interfering compounds in human and nonhuman primate, and rat urine. The electrochemically derived results are compared with post-column derivatized fluorescence results, and quality-control checks necessary to maintain assay precision in automated analysis are described.

INTRODUCTION

Subtle alterations in the concentrations of the major final metabolites of the catecholamine neurotransmitters dopamine and norepinephrine — 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid, HVA) and dihydroxyphenylacetic acid (DOPAC) and 4-hydroxy-3-methoxyphenylglycol (MHPG), respectively — reflect changes in neuronal activity [1—3] and are sensitive indicators of altered mood and emotional state [4—6]. Silbergeld and Chisolm [7, 8] have shown that alterations in urinary concentrations of HVA may indicate exposure to the known neurotoxin lead. If sufficient analytical precision can be achieved, it is possible that HVA measurement may be of general applicability to studies of the effects of environmental pollution on neurological systems. In such studies, a specimen which is simple to collect is almost mandatory; in this case urine is the specimen of choice.

The most promising technique for accurate and precise urine analysis is high-performance liquid chromatography (HPLC) [9]. However, published HPLC methods for urinary HVA level determination were initially developed to

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screen patients for neural crest tumors where differences in HVA concentrations between patients and controls are very large, i.e. 5—10 times [9, 10]. Imprecision appears to be attributable to the complexity of sample preparation particularly with gas chromatographic (GC) methods; inaccuracy is due to small chromatographic interferences which go unnoticed when high levels in pathological specimens are measured.

We report an HPLC assay for urinary HVA based on the direct injection of urine which avoids sample preparation errors and gives specificity for HVA in human, monkey and rat urine. It may be employed over the long term of a chronic toxicological exposure so that definitive neurochemical evidence of subtle neuronal changes can be observed. For simplicity and to facilitate automated injection, an isocratic mobile phase is used which had been shown to separate the majority of catecholamines and their metabolites [11].

Accuracy is demonstrated by comparison with data obtained by HPLC with fluorescent detection [12].

EXPERIMENTAL

Materials

4-Hydroxy-3-methoxyphenylacetic acid (HVA) was purchased as the free acid from Sigma (St. Louis, MO, U.S.A.), potassium dihydrogen phosphate (anhydrous) from J.T. Baker (Phillipsburgh, NJ, U.S.A.), and methanol (HPLC grade) from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.).

Water was deionized, glass-distilled and stored in glass.

Mobile phase

The mobile phase was prepared by dissolving 13.61 g of potassium dihydrogen phosphate in 1 l of deionized, glass-distilled water to yield a 100 m*M* solution. The pH was adjusted accurately with 1 *M* hydrochloric acid before the addition of the organic modifier to avoid measurement in a non-aqueous solvent in which hydrogen ion activity is not precisely known. This solution (9 parts) was then mixed with methanol (1 part) and drawn under vacuum through a 0.45-μm Type HA filter (Millipore, Bedford, MA, U.S.A.) to filter and degas it.

Chromatography

Electrochemical assay. A Waters Assoc. (Milford, MA, U.S.A.) Model 6000A chromatographic pump was used to deliver the mobile phase at a rate of 1.0 ml/min. Samples were injected with an automated sample injector (WISP Model 710A, Waters Assoc.) onto two serially connected Waters μBondapak C_{18} columns (30 cm × 3.9 mm, 10 μm non-spherical particle size) heated to 40°C. No pre-column was used. An LC-5 glassy carbon electrode (BioAnalytical Systems, West Lafayette, IN, U.S.A.) with a silver/silver chloride reference electrode was used to oxidize the compounds of interest at 0.80 V potential versus the reference electrode. The resulting signal was amplified with a polarographic analyzer (PAR 174, EG&G; Princeton Applied Research, Princeton, NJ, U.S.A.) and recorded on a Hewlett-Packard (Palo Alto, CA, U.S.A.) 3385A automation system.

Fluorescence assay. The fluorescence analysis was virtually identical to that of Rosano et al. [12]. The mobile phase consisted of 70 ml of acetonitrile and 2 ml of formic acid made up to 1 l in distilled water and delivered at a flow-rate of 1.0 ml/min. Urine was brought to pH 2.3 with 1 *M* hydrochloric acid, and injected on a single Waters μBondapak C_{18} column. The column effluent was reacted with an alkaline potassium ferricyanide reagent pumped into a 3-port T at 0.8 ml/min and reacted in a stainless-steel coil (2-ml injector loop from a Waters U6K injector) at room temperature. The fluorescent product was excited at 320 nm and the emission read at 420 nm on a fluorescence detector (Model 836, Dupont Instruments, Wilmington, DE, U.S.A.).

Standards and samples

Preparation of standard solutions. HVA was initially dissolved in glass-distilled, deionized water at a concentration of 100 μg/ml. Aliquots (1 ml) were then frozen at −80°C in polyethylene-capped microcentrifuge tubes (Markson Science, Del Mar, CA, U.S.A.). At the time of analysis a frozen aliquot was thawed and diluted to either 1 or 10 μg/ml with water.

Collection and preparation of urine samples. Urine was collected over a period of 24 h from humans into vessels containing 15 ml of 6 *M* hydrochloric acid and kept refrigerated; from monkeys (*Macaca nemestrina*) into icecooled containers; and from rats, into beakers under metabolism cages containing 1 ml of 3 *M* hydrochloric acid covered by 5 ml of paraffin oil (Fisher Scientific, Pittsburgh, PA, U.S.A.).

After collection, the pH of the urine was adjusted to < 2.5 with concentrated hydrochloric acid if necessary. It was then either filtered through Whatman No. 40 paper or centrifuged at 3200 *g* for 10 min. Aliquots (1 ml) were then frozen at −80°C until analysis. When thawed, each aliquot was diluted 10-fold with water and 20-μl injections were made with the WISP.

RESULTS

Successive rises in column temperature to 40°C showed an increase in theoretical plate efficiency for the mixture of standards and the apparent HVA peak in a normal human urine showed a shoulder, indicating the presence of an electroactive interferent (Fig. 1). Alterations in the pH of the mobile phase produced profound changes in the temporal relationship of HVA to potentially interfering compounds: the retention time of HVA was inversely related to the pH of the mobile phase within the ranges examined (Table I). Moreover, changes in the pH of the mobile phase caused the appearance and disappearance of interferents which were at times hidden under presumably pure HVA peaks. A pH of 4.00 and two columns in tandem gave a pure HVA peak (Fig. 2) on visual chromatographic evidence. The 6-ng peak shown in Fig. 2 yielded 22,000—25,000 integrator counts — clearly an HVA concentration one-tenth of that could be detected in a pure standard. However, in a complex chromatogram, sensitivity cannot be assessed so simply since occlusion of small peaks by large neighboring peaks must be considered. Because the concentration of endogenous HVA in urine of rats, human and non-human primates is in the range of 1—7 ng/μl, the detection limits in this assay pose no problem.

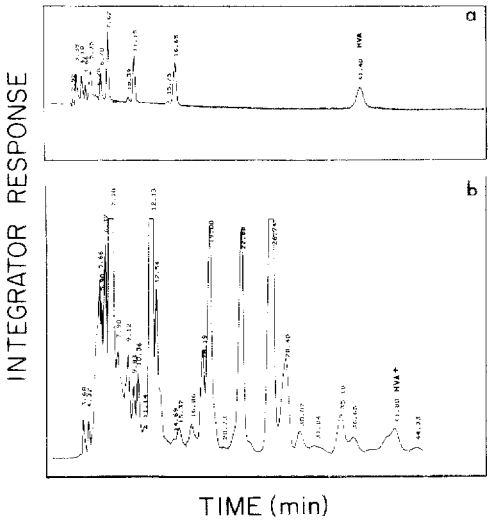

Fig. 1. (a) Chromatogram of a standard consisting of 6 ng of each of the following compounds: norepinephrine (5.35 min), dopamine (6.70 min), vanillylmandelic acid (7.62 min), MHPG (11.15 min), DOPAC (16.65 min), HVA (41.40 min). (b) Chromatogram of a 20-μl injection of filtered human urine diluted 1:10. Conditions: mobile phase, 100 m*M* KH_2PO_4 (pH 3.3) -10% methanol, at 40°C; electrolyzing voltage, +0.8 V vs. Ag/AgCl electrode. Note the appearance of a leading shoulder on the 41.80-min HVA peak, which indicates the presence of (an) interfering compound(s).

TABLE I

CHROMATOGRAPHIC CHARACTERISTICS AND HVA CONCENTRATIONS OF TWO HUMAN URINES ASSAYED UNDER DIFFERENT MOBILE PHASE CONDITIONS

Mobile phase pH	Retention time (min)		Interfering peaks resolved	HVA (ng/μl)	
	HVA standard	HVA in urine		Urine with interfering peaks	Urine without interfering peaks
100 mM KH_2PO_4—10% methanol					
3.95	34.61	34.38	No	4.58	12.25
4.00	33.30	33.21	Yes	3.27	ND*
4.05	31.29	31.13	Yes	3.11	11.70
4.10	30.83	30.95	Yes	3.17	11.53
4.50	21.99	21.88	No	5.04	ND
60 mM KH_2PO_4—23% methanol					
3.00	10.10	9.96	No	4.74	12.43

*ND = Not determined.

Indeed, by selecting a higher gain on the amplifier the detection limits could be further lowered.

Numerical comparison of analyses of different urine samples supports the visual evidence that the HVA peak in Fig. 2 is pure. Table I shows the analysis

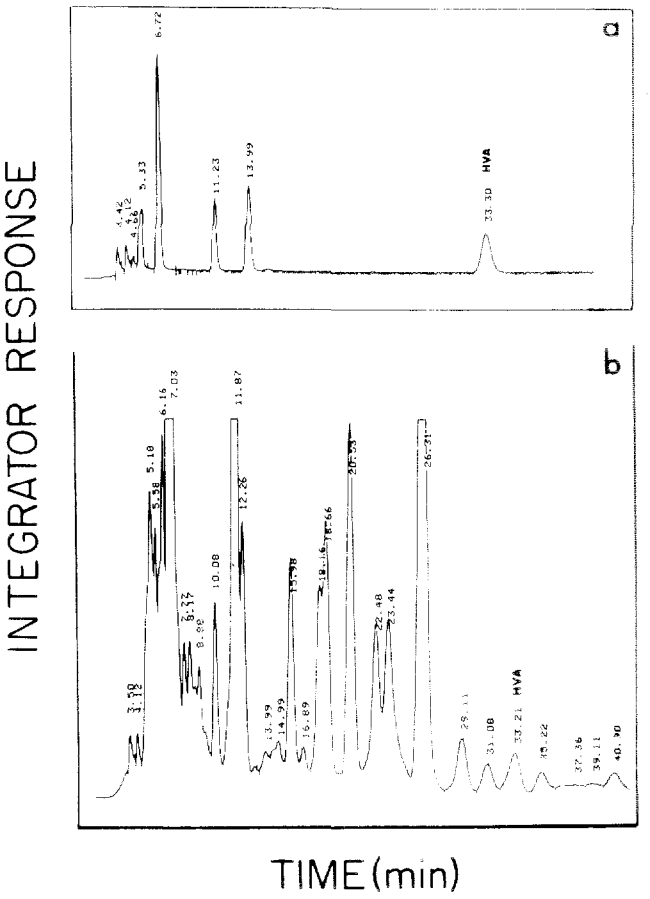

Fig. 2. (a) Chromatogram of the same standard as in Fig. 1a. Retention times are: norepinephrine (5.33 min), dopamine and vanillylmandelic acid (6.72 min), superimposed, MHPG (11.23 min), DOPAC (13.99 min), and HVA (33.30 min). (b) Chromatogram of a 20-μl injection of a human urine diluted 1:10. Conditions: mobile phase, 100 mM KH_2PO_4 (pH 4.0)—10% methanol, at 40°C; electrolyzing voltage, +0.8 V vs. Ag/AgCl electrode.

of two samples of urine, the first of a male laboratory scientist and the second of a patient who had a resected neuroblastoma. Notice the HVA analysis reaches a minimum at a pH near 4.05. Analysis of urine from five monkeys with a different mobile phase [13] yielded apparently higher HVA concentrations than those obtained under optimized conditions (Table II). Finally, chromatograms produced by the post-column derivatization method of Rosano et al. [12] determined the HVA level of the human urine used throughout this work to be 2.9 ng/ml and the neuroblastoma patient's urine to be 11.1 ng/ml, in good agreement with the result obtained at optimized pH (Table I).

Automated injection yielded the results shown in Fig. 3A, taken from a long series of 20 μl filtered, 1:10 diluted urine injections. A standard HVA solution was run after every fourth urine, and a control urine was run every eighth sample.

To avoid interference from non-polar electroactive compounds from preceding injections a hold period of 20 min is allowed after the emergence of

TABLE II

HVA CONCENTRATIONS (ng/μl) IN 24-h URINE SAMPLES FROM *MACACA NEMESTRINA* MEASURED UNDER TWO MOBILE PHASE CONDITIONS

Monkey name	Mobile phase		Overestimation (%)
	60 m*M* KH_2PO_4—23% methanol (pH 3.0)	100 m*M* KH_2PO_4—10% methanol (pH 4.1)	
Madison	7.98	3.94	102
	9.70	5.14	89
Mars	2.96	2.50	18
Adams	7.79	7.01	11
Earth	4.77	3.86	24
Venus	6.90	4.50	53

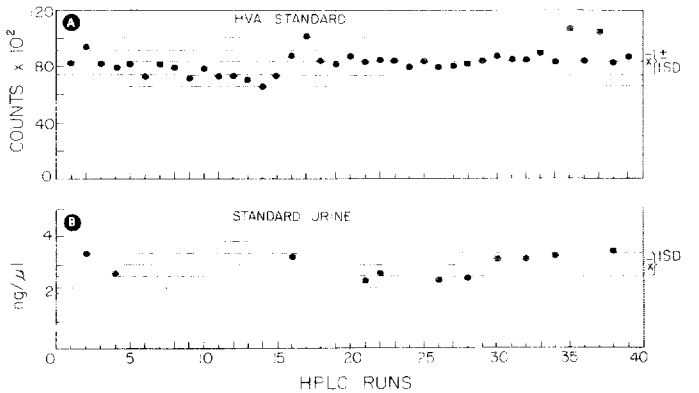

Fig. 3. (A) Integrator responses (counts × 10^3) for 36 μl of an HVA standard (1/6 ng/μl) injected after every fourth urine sample over a series of approximately 150 injections. The mean of all standard runs 1 × S.D. (— — —), and 2 × S.D. (——) are shown. (B) HVA concentration expressed as ng/μl for 20 μl of a control urine diluted 1:10 and injected every eighth sample.

HVA, before the next injection. Any irreversible build-up of non-polar material on the column slowly causes a reduction of retention time and a degradation in resolution. The column was flushed periodically with acetonitrile—water (3:2). This is illustrated in Fig. 4 after the automated runs of 3-9-82 and 3-19-82.

DISCUSSION

The mobile phase chosen for this work, 0.100 *M* aqueous potassium dihydrogen phosphate had been shown to separate a wide variety of catecholamine metabolites [11] and it would be expected to be nearly ideal as a support electrolyte for anodic polarography, being a small singly charged unoxidizable anion. Methanol was chosen as an organic modifier since we have found that it can be obtained electrochemically pure, and at concentrations

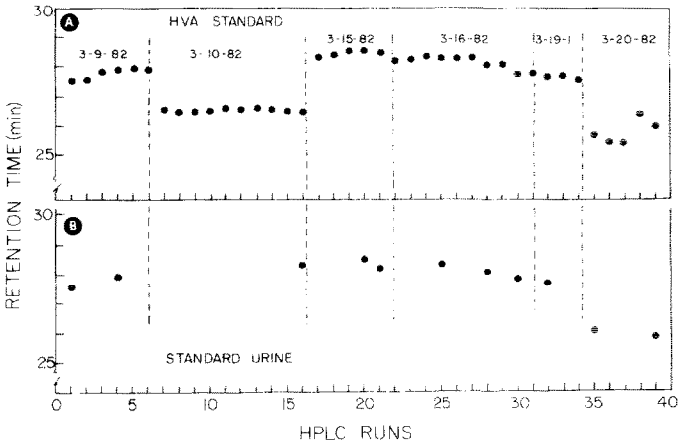

Fig. 4. (A) Retention time (min) for an HVA standard injected every fourth run over a series of approximately 150 runs. (B) Retention time (min) for the standard urine. All injection volumes were as described in Fig. 3.

less than 20% and voltages less than +1 V relative to the Ag/AgCl electrode, the standing current observed is tolerable in that it does not produce noticeable pump pulses at the amplification required for the analysis and can be offset with the standard polarograph we employ. A temperature greater than 40°C was not employed to avoid problems of compound stability in spite of the fact that chromatographic efficiency still continued to improve up to this temperature. Temperature has recently been shown to be an important separative factor [14, 15].

Slight drift in the retention time of HVA during long series of runs can be corrected by the automated recalibration system of the Hewlett-Packard reporting integrator. Larger changes in retention time between days can be corrected by slight adjustments of the pH of the mobile phase or slight changes in the buffer—methanol ratio, but large changes in the retention time of the HVA standard (as shown in runs 6–16 of Fig. 4A) are associated with poor resolution of HVA in the control urine and often indicate column deterioration.

The electrolyzing voltage was changed in 0.1-V steps from 0 to 1.2 V and approximate dynamic half-wave potentials for the catechol derivatives were found to be +0.35 V vs. the Ag/AgCl electrode, whereas the monomethoxy derivatives HVA, MHPG and vanillylmandelic acid and dynamic halfwave potentials of +0.6 V vs. the Ag/AgCl electrode in agreement with previous work in other support electrolytes [10]. Several of the waves showed polarographic maxima [16] unlike the voltammograms previously reported which were observed in the presence of an ion-pairing detergent which would have suppressed maxima. Hence, an oxidizing voltage of +0.8 V was chosen for analysis to ensure diffusion-controlled electrolysis. The electrolyzing potential is known to drift slowly if the salt bridge of the reference electrode is modified by the flowing mobile phase. However, our choice of a relatively high electrolyzing voltage has resulted in no noticeable drift in response as is illustrated by Fig. 3. Changes in the response of the oxidizing electrode caused by coating of the

surface with coeluting urine components are controlled by injecting an HVA standard at frequent intervals in an automated run (every fourth sample), and if the integrator responses for the HVA standard are diminished, the glassy carbon electrode is polished according to techniques described by Bio-Analytical Systems. Experience with these diluted urine samples has shown that repolishing is rarely required.

Care must be taken in the interpretation of results of HPLC electrochemical assays for urinary HVA. Symmetrical Gaussian-shaped peaks with retention times similar or identical to those of the standard are not sufficient proof that chromatographic separation has been achieved. Careful analytical development must include validation by additional techniques (e.g. HPLC with fluorescent detection or gas chromatography). Indeed, the particular post-column reaction and fluorescent detection of urinary HVA used here only responded to HVA and resulted in a selective chromatogram whose numerical values for HVA compared favorably with those obtained by electrochemical detection with a mobile phase at the optimum pH. Electrochemical detection was chosen for this work in spite of the selectivity of the fluorescence method because it is our intention to extend the method, with direct urine injections, to other dopamine metabolites in due course. A supply of control urine should be maintained frozen in convenient-sized aliquots, to provide a check on HVA retention time and chromatographic resolution from potential interferents.

Several organic solvent extraction techniques [9] were tried to improve chromatographic prints. Methods based on ethyl acetate, diethyl ether and toluene extractions all carried electroactive interferents to the HPLC analysis and greatly increased sample preparation time. The present method allows fifteen analyses to be carried out in a 24-h period; this includes all the quality control samples mentioned. One technician can prepare the samples in less than 1 h.

Accurate HVA measurements may be useful in monitoring nervous system function after human exposure to toxic agents. Changes in HVA concentrations, for example, provide an objective, clinically applicable means of assessing nervous system damage caused by chronic lead exposure. When this approach is used in conjunction with a peripheral monoamine oxidase inhibitor, such as debrisoquin sulfate [17, 18], an accurate estimate of central dopaminergic function can be obtained. This technique also provides an experimental means of obtaining multiple, nonstressful estimates of dopaminergic function.

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