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Note

Simultaneous determination of urinary vanillylmandelic acid, 5-hydroxyindoleacetic acid and homovanillic acid by liquid chromatography

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Liquid chromatographic (LC) methods for quantitative analysis of urinary vanillylmandelic acid (VMA), 5-hydroxyindoleacetic acid (5HIAA) and homovanillic acid (HVA) are replacing older non-specific colorimetric methods. LC methods which detect these compounds by absorbance at 280 nm have been reported but the chromatograms are complicated by the large number of compounds detected [1]. More specific techniques, which involve the use of ion-exchange chromatography prior to LC, have improved resolution of these analytes [2–4]. Recently, Parker et al. [5] reported on an LC method which simultaneously quantifies VMA and 5HIAA, as well as catecholamines and metanephrines. They combine ion-pair chromatography and electrochemical detection. However, they used a relatively laborious isolation procedure consisting of liquid–liquid extraction followed by solid-phase column extraction. Their report did not examine potentially interfering endogenous urinary constituents or possible drug interference.

We describe a method which simultaneously quantifies VMA, 5HIAA and HVA using a one-step solid-phase extraction technique. Additionally, we report on potentially interfering urinary constituents and drugs.

EXPERIMENTAL

Apparatus

The LC system consisted of a Beckman Model 100A solvent metering system (Beckman Instruments, Palo Alto, CA, U.S.A.) and an RCM-100 radial compres-

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sion module with a 4-μm C_{18} Nova Pak 100 mm×8 mm I.D. column (Waters Chromatography Division, Millipore, Milford, MA, U.S.A.). The detector was a Model LC-17 flow-cell (Bioanalytical Systems, West Lafayette, IN, U.S.A.). Data were reported through a Model 3390A reporting integrator (Hewlett-Packard, Santa Clara, CA, U.S.A.).

Extraction columns

Extraction of analytes from urine is accomplished using a solid-phase strong anion-exchange column (Bond Elut SAX column, Analytichem International, Harbor City, CA, U.S.A.).

Mobile phase preparation

To a 2-l flask half-full of water were added 12.0 g monochloroacetic acid, 3.5 g sodium hydroxide, 1.2 g disodium EDTA and 21 g citric acid monohydrate. The contents were mixed until dissolved and then 0.3 g sodium octyl sulfate was added and dissolved, followed by 0.9 ml diethylamine and 165 ml acetonitrile. Water was added to volume and the pH was adjusted to 2.61 ± 0.02 with solid monochloroacetic acid. The mobile phase is stable for three months at room temperature.

Chromatographic conditions

The analytes were detected at a potential of +0.75 V vs. an Ag/AgCl reference electrode. The mobile phase flow-rate was 1.0 ml/min at ambient temperature.

Procedure

The analytes and internal standard (50 mg/l isovanillylmandelic acid in 0.50 mmol/l aqueous hydrochloric acid) were extracted as follows. To 100 μl of urine, 100 μl internal standard and 1.0 ml of 160 mmol/l sodium acetate buffer (pH 7.0) were added and mixed. The Bond Elut SAX column was prepared by washing the column with 1.0 ml of 160 mmol/l sodium acetate buffer (pH 7.0). After the column had drained the prepared urine sample was passed through the column and allowed to drain. The bound analytes and internal standard were eluted with 1.0 ml of 2 mol/l phosphoric acid and the eluate was collected. After mixing, an aliquot of the eluate was injected onto the analytical column (see Fig. 1). Results were calculated by ratioing the unknown analytes to that of the internal standard.

RESULTS

Linearity, recovery and accuracy were determined by adding known amounts of VMA, 5HIAA and HVA to a urine previously determined to have a low but known concentration of each analyte of interest. An aliquot of urine at each concentration was processed as described in *Procedure*.

The calibration plots for each analyte were linear to a concentration of 50 mg/l. The relative analytical recovery was tested at concentrations of 5, 10, 20, 30, 40 and 50 mg/l for each analyte. The mean recovery of VMA was 103% (range 95–108%), for 5HIAA 102% (97–109%) and for HVA 105% (99–110%). The recovery at each concentration was tested on two separate days.

The within-day precision was tested by processing twenty aliquots of the same urine sample which was prepared by mixing aliquots of urine containing abnormal amounts of VMA, 5HIAA and HVA. Between-day precision was tested by processing, on consecutive work days, an aliquot of frozen urine. The precision of the assay is described in Table I.

In addition to the recovery experiments, the accuracy of VMA values was tested by comparing this LC method with a modification of the LC method of Binder and Sivorinosky [2] which has been our routine method for two years. The regression analysis of the two methods was: $r=0.980$, $m=0.910$, y-intercept $=0.24$, the range of concentration tested was 0.5–31.6 mg/l VMA and $n=37$. The accuracy of the 5HIAA levels was primarily tested by the recovery experiments as reported. However, we compared the results obtained by this LC method

TABLE I

PRECISION OF ASSAY FOR VMA, 5HIAA AND HVA IN URINE

Analyte	Within-day			Between-day		
	Range (mg/l)	C.V. (%)	n	Range (mg/l)	C.V. (%)	n
VMA	17.4–19.4	3.3	15	4.5– 6.1	5.9	31
				27.7–34.1	6.5	15
5HIAA	7.3– 8.4	3.9	15	15.7–20.1	7.7	15
HVA	23.4–25.7	2.5	15	5.1– 6.2	7.0	15
				32.4–42.6	8.6	15

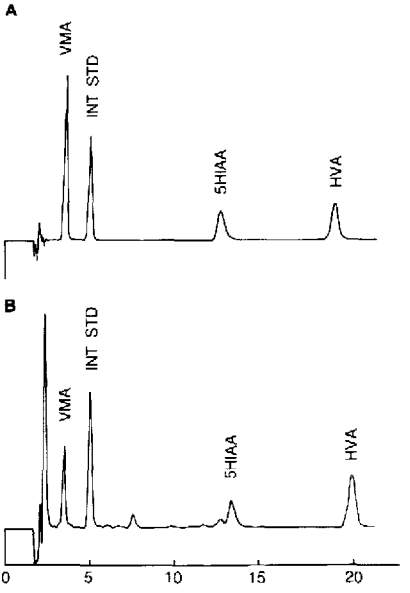

Fig. 1. Representative chromatograms of (A) reference standards and (B) abnormal urine containing 19.1 mg/l VMA, 7.1 mg/l 5HIAA and 20.7 mg/l HVA. The full scale recorder sensitivity was 100 mA.

with those obtained by other LC methods reported by participants in the College of American Pathologists (CAP) Urine Chemistry Survey Set N-A, 1987. The result of our LC method for specimen N-I was 3.6 mg/l 5HIAA, the CAP participants ranged from 2.4 to 5.8 mg/l 5HIAA; for CAP specimen N-2 the result of our LC method was 167 mg/l 5HIAA and the CAP participants ranged from 138 to 182 mg/l 5HIAA. The accuracy of the HVA procedure was tested by comparison of this LC method with the method of Binder and Sivorinosky [2]. The regression analysis of the two methods was: $r = 0.963$, $m = 1.07$, y-intercept $= 0.13$, the range of concentration tested was 1.3–16.6 mg/l HVA and $n = 14$.

Interferences

The potential interferences caused by common urine constituents and drugs were studied by chromatographing selected compounds. Any compound which eluted sufficiently close to an analyte or the internal standard was further studied by adding known amounts of that compound to urine and evaluating the quantitative effect. The following compounds did not interfere; epinephrine, dopamine, metanephrine, normetanephrine, serotonin, isovanillic and vanillic acid, methoxytyramine, tyramine, 3,4-dihydroxymandelic acid, 3-methoxytyrosine, methyl-DOPA, 3,4-dihydroxyphenylacetic acid, deoxyepinephrine, 3,4-dihydroxymandelic acid, 7-methyluric acid, 1-methyluric acid, uric acid, ascorbic acid, hippuric acid, caffeine, nicotine, urea, creatinine, tryptophan, tyrosine, theophylline, amobarbital, phenobarbital, butabarbital, pentobarbital, secobarbital, diphenylhydantoin and acetaminophen. Three compounds, epinephrine (EPI) 3,4-dihydroxyphenylalanine (DOPA) and 4-hydroxy-3-methoxyphenylethyleneglycol (HMPG) potentially interfered. HMPG eluted as a tailing peak on the VMA peak, however, when urine was spiked at 10 mg/l HMPG only 12% of the HMPG was recovered from the SAX column. HMPG exists in human urine at a concentration of 1.4–4.6 mg per 24 h and is excreted partially as a glucuronide [6]. When 10 mg/l free HMPG was added to urine containing 12.7 mg/l VMA, and processed as described, the resulting VMA concentration was found to be 13.5 mg/l VMA. The compounds EPI and DOPA potentially interfered with the internal standard. However, less than 25% of a 10 mg/l concentration of each in urine were recovered from the SAX column. Additionally, DOPA exists in human urine at a concentration up to 46 μg per 24 h [7] and EPI at a concentration up to 25 μg per 24 h [6]. Because both DOPA and EPI are found in urine at such low concentration and they are poorly reovered from urine, they would not be expected to significantly interfere with the results.

CONCLUSION

We describe a simple, accurate, precise and rapid LC method to simultaneously estimate VMA, 5HIAA and HVA in urine. Sample preparation requires minimal time and labor and chromatography is complete in 20 min.

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