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SHORT COMMUNICATION

Determination of Vanillylmandelic, 5-Hydroxyindoleacetic and Homovanillic Acid in Urine by Isocratic Liquid Chromatography

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Summary: A new isocratic HPLC method, employing electrochemical detection, is described for the determination of urinary vanillylmandelic acid, 5-hydroxyindoleacetic acid and homovanillic acid. The main advantages of this technique are: simplicity, simultaneous determination of all analytes, the absence of an extraction procedure, isocratic elution and low cost. The diluted urine is injected onto a C18 reversed phase column. The mobile phase is potassium dihydrogenphosphate buffer containing 1-heptanesulphonic acid, methanol and acetonitrile. The calibration curves are linear from 0.1 to 50 mg/l; the precision data show CV less than 2.36% for within-day assay and less than 2.72% for day-to-day assays. The mean recoveries for supplemented samples are 98.2 to 102.0% for vanillylmandelic acid, 99.6 to 103.9% for 5-hydroxyindoleacetic acid and 98.7 to 102.0% for homovanillic acid. In comparisons of the present method with *Radjaipur*'s extraction method (Radjaipur M. et al., Eur J Clin Chem Clin Biochem 1994; 32:609–13) the slopes for the three analytes were nearly 1, and the confidence region of the intercepts was close to 0. In conclusion the technique seems to be suitable for routine determination of the three analytes, especially for mass screening purposes.

Introduction

Vanillylmandelic acid (4-hydroxy-3-methoxymandelic acid) and homovanillic acid (4-hydroxy-3-methoxy-phenylacetic acid), the major metabolites of catecholamines, and 5-hydroxyindoleacetic acid (5-hydroxy-3-indoleacetic acid), metabolite of serotonin, are often determined in urine for neurologic diagnosis and for monitoring the response to therapy in illnesses like phaeochromocytoma, neuroblastoma and carcinoid syndrome, respectively (1, 2).

High-performance liquid chromatographic (HPLC) methods for vanillylmandelic acid, homovanillic acid and 5-hydroxyindoleacetic acid have been reported, either without (3–13) or with (14–24) simultaneous determination of all three analytes. Combined analysis was proposed mainly for technical reasons, but the molecular characteristics of these metabolites also conveniently enable their simultaneous determination in a single HPLC run. However none of these techniques is totally free of technical problems, and the time of operation and high cost are still matters of concern. For instance many methods need an extraction step or sample pretreatment (14–22, 24): some determinations require gradient elution techniques and an excessive time for regeneration of the column after each run (14, 17, 18, 23, 24); in addition, some of these methods employ time-consuming procedures and/or need instrumentation not always available in a clinical laboratory (15, 18–20).

In this work we describe our new simultaneous HPLC technique, developed for an isocratic separation and quantitation of vanillylmandelic acid, 5-hydroxyindoleacetic acid and homovanillic acid in urine, using a reversed phase column and an electrochemical detector.

Materials and Methods

Patients

Urine samples were collected early in the morning from adult volunteers who did not consume walnuts, vegetable food, coffee, bananas and food containing vanilla after 1 p.m. of the previous day. In assessing the reliability of our method we expressed the concentrations of these metabolites in μmol/l, rather than μmol of compound per g creatinine which is used in routine analyses (19). Urine was stored at −20 °C after acidification with 6 mol/l hydrochloric acid to a pH of about 4. Under these conditions vanillylmandelic acid, 5-hydroxyindoleacetic acid and homovanillic acid are stable for at least four weeks (7).

Reagents

All reagents and chemicals were of analytical grade and were purchased from Carlo Erba (Milan, Italy) except for vanillylmandelic acid, 5-hydroindoleacetic acid, homovanillic acid and 1-heptanesulphonic acid which were from Sigma (St. Louis, MO, USA). Methanol and water, both of HPLC grade, were obtained from Merck (Darmstadt, Germany).

Apparatus

A model 116 Beckman (Palo Alto, CA, USA) pump, a Rheodyne (Berkeley, CA, USA) Model 7125 injector and a HP 3395 Integrator (Hewlett-Packard, Les Ulis, France) were used. We employed an ESA electrochemical detector Model 5110 Coulochem (Environmental Sciences Assoc. Bedford, MA, USA) with a Model 5011 analytical cell. Separation was carried out with a Supelcosil LC 18 DB 150 × 4.6 mm i.d. column (3-μm particle size) fitted with a 20 × 4.6 mm guard column (both from Supelco, Oakville, Canada).

Standards

A stock solution (500 mg/l each of vanillylmandelic acid, 5-hydroxyindoleacetic acid and homovanillic acid) was prepared by dissolving 50 mg of each compound in mobile phase and adjusting the volume to 100 ml. This was divided into several aliquots and frozen at −20 °C; under these conditions the stock solution is stable for at least 4 weeks and the working standards were prepared by diluting it with mobile phase.

Mobile phase

The mobile phase is a mixture of 990 ml of 35 mmol/l potassium dihydrogenphosphate buffer containing 100 mg/l of 1-heptanesul-

phonic acid as the ion-pair agent to reduce the interferences in the analysis (20) and 5 ml of methanol and 5 ml of acetonitrile to shorten the retention times; the pH of the solution was adjusted to 2.8 with phosphoric acid. Prior to use the phase was degassed and filtered through a 0.45 µm MF Millipore filter.

Samples

Intact urine (100 µl) was added to another tube containing 4.9 ml of mobile phase; 100 µl of this vortexed mixture was immediately injected into the chromatograph. When the urine sample contained insoluble impurities, the assay was performed using the supernatant after centrifugation (200 *g*, 5 min).

High performance liquid chromatography conditions

Diluted sample (100 µl) was injected into the HPLC. The flow rate for the isocratic elution was set at 1.0 ml/min and the column was maintained at room temperature. Since 5-hydroxyindoleacetic acid oxidizes much more easily than vanillylmandelic acid and homovanillic acid, the electroactive compounds were analysed electro-

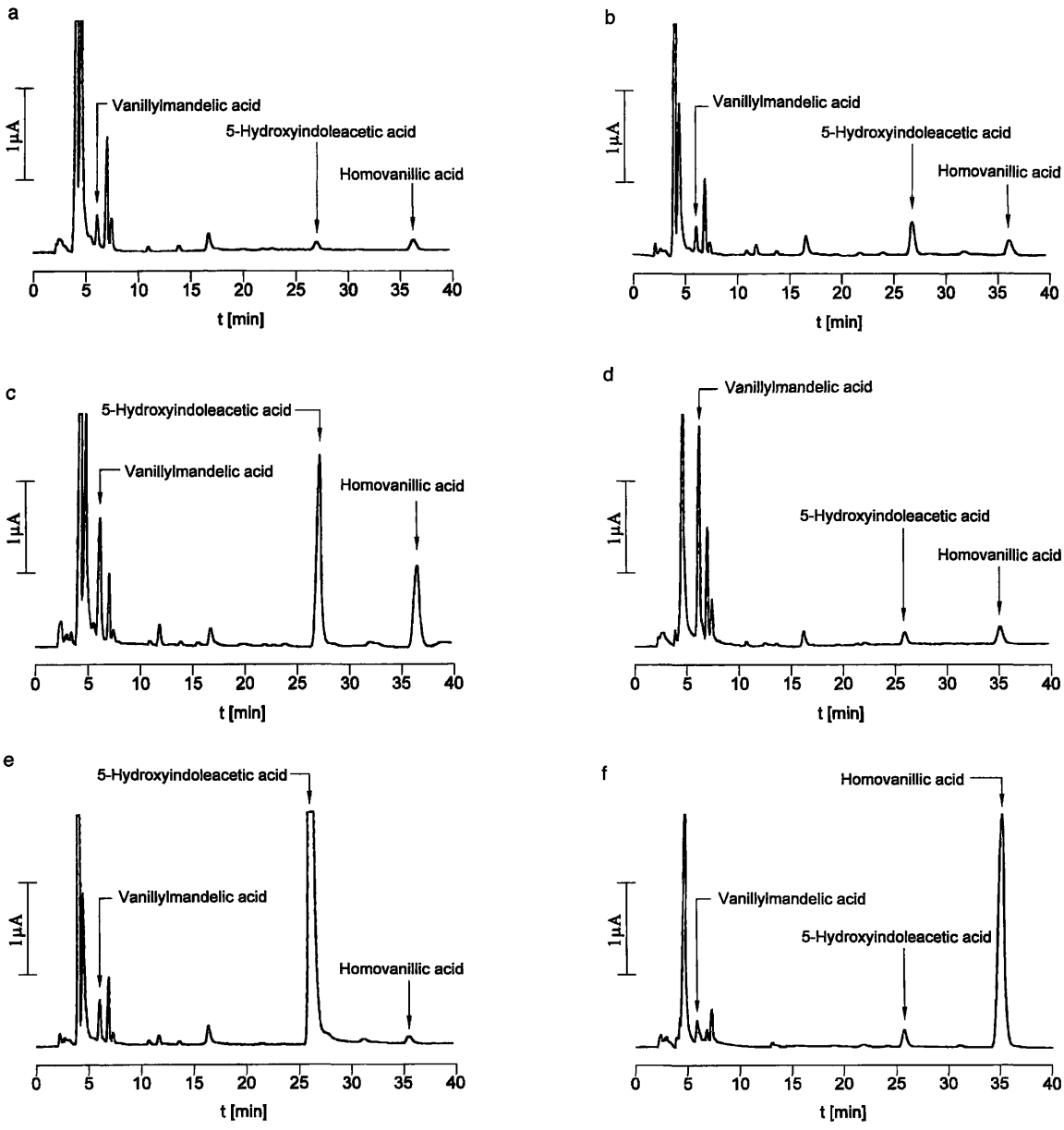

Fig. 1 Chromatograms of

(a) a normal urine sample containing 17.16 µmol/l vanillylmandelic acid, 10.09 µmol/l 5-hydroxyindoleacetic acid and 14.10 µmol/l homovanillic acid,

(b) a Quantitative Urine Normal Control (Lyphochek level I, Bio-Rad Laboratories, Anaheim, CA, USA) containing 14.11 µmol/l vanillylmandelic acid, 27.20 µmol/l 5-hydroxyindoleacetic acid and 15.03 µmol/l homovanillic acid,

(c) a Quantitative Urine Abnormal Control (Lyphochek level II) containing 62.92 µmol/l vanillylmandelic acid, 156.18 µmol/l 5-hydroxyindoleacetic acid and 89.86 µmol/l homovanillic acid,

(d) a pathological urine sample containing 111.42 µmol/l vanillylmandelic acid, 11.77 µmol/l 5-hydroxyindoleacetic acid and 18.72 µmol/l homovanillic acid,

(e) a pathological urine sample containing 22.45 µmol/l vanillylmandelic acid, 422.3 µmol/l 5-hydroxyindoleacetic acid and 10.92 µmol/l homovanillic acid,

(f) a pathological urine sample containing 13.42 µmol/l vanillylmandelic acid, 15.32 µmol/l 5-hydroxyindoleacetic acid and 253.59 µmol/l homovanillic acid.

See text for chromatographic conditions.

chemically with detector 1 operated at + 0.12 Volt, detector 2 at + 0.45 Volt and the guard cell at + 0.12 Volt.

Determination of vanillylmandelic acid, homovanillic acid and 5-hydroxyindoleacetic acid by an extraction method

For the method comparison we analysed 30 urine samples using our method and *Radjaipur*'s extraction method (20). Vanillylmandelic Acid, homovanillic acid and 5-hydroxyindoleacetic acid were extracted from urine samples using a cation exchange column (Pharmacia-LKB Freiburg, Germany) to remove neutral, basic, weakly acidic sample components and proteins. An aliquot of the eluate was injected into an isocratic HPLC system (pump: model 116 Beckman, Palo Alto, Ca, USA; injector: Model 7125, Rheodyne, Berkeley, CA, USA; integrator: HP 3395, Hewlett-Packard, Les Ulis, France). The samples were separated on a reversed phase column (Supelcosil LC 18 DB 150 × 4.6 mm i. d., Supelco, Oakville, Canada), detected electrochemically (Detector ESA Coulochem Model 5100A) and quantitatively evaluated with the help of the internal standards. The subsequent procedural steps and the HPLC analysis were performed according to *Radjaipur* (20).

Statistical analysis

Data are expressed as means, SD and CV. *Passing & Bablok* (25) regression analysis was used for the comparison between methods.

Results

Figure 1 shows typical chromatograms obtained by direct-injection analysis of 100 µl of diluted sample. Good separation and baselines

Tab. 1 Precision data for within-day and day-to-day measurements of vanillylmandelic acid, 5-hydroxyindoleacetic acid and homovanillic acid.

Within-day (n = 10)			Day-to-day (n = 5)		
Compound	Concentration (mean ± SD) (µmol/l)	CV (%)	Compound	Concentration (mean ± SD) (µmol/l)	CV (%)
Vanillylmandelic acid	7.06 ± 0.10	1.28	Vanillylmandelic acid	7.01 ± 0.15	2.08
	26.44 ± 0.40	1.54		26.44 ± 0.45	1.64
	77.26 ± 1.21	1.57			
5-Hydroxyindoleacetic acid	8.89 ± 0.10	1.40	5-Hydroxyindoleacetic acid	8.84 ± 0.16	1.85
	23.48 ± 0.34	1.60		23.54 ± 0.42	1.84
	95.08 ± 1.93	2.04			
Homovanillic acid	9.28 ± 0.16	1.77	Homovanillic acid	9.39 ± 0.27	2.72
	33.75 ± 0.60	1.82		33.41 ± 0.60	1.81
	94.41 ± 2.19	2.36			

Tab. 2 Recovery of the method.

Analyte	Normal urine pool			Pathological urine pool		
	Initial concentration (µmol/l)	Within-day recovery* (%)	Day-to-day recovery* (%)	Initial concentration (µmol/l)	Within-day recovery* (%)	Day-to-day recovery* (%)
Vanillylmandelic acid	11.61	99.8 ± 2.5	98.2 ± 4.1	50.41	102.0 ± 2.6	101.1 ± 5.2
5-Hydroxyindoleacetic acid	17.78	102.0 ± 2.1	99.6 ± 3.7	108.92	103.8 ± 2.4	103.9 ± 3.4
Homovanillic acid	8.23	100.0 ± 1.7	102.0 ± 5.1	75.3	98.7 ± 3.6	100.8 ± 4.3

Quantity added: 5 mg/l in normal urine pool, 20 mg/l in pathological urine pool
* number of replicates: 5

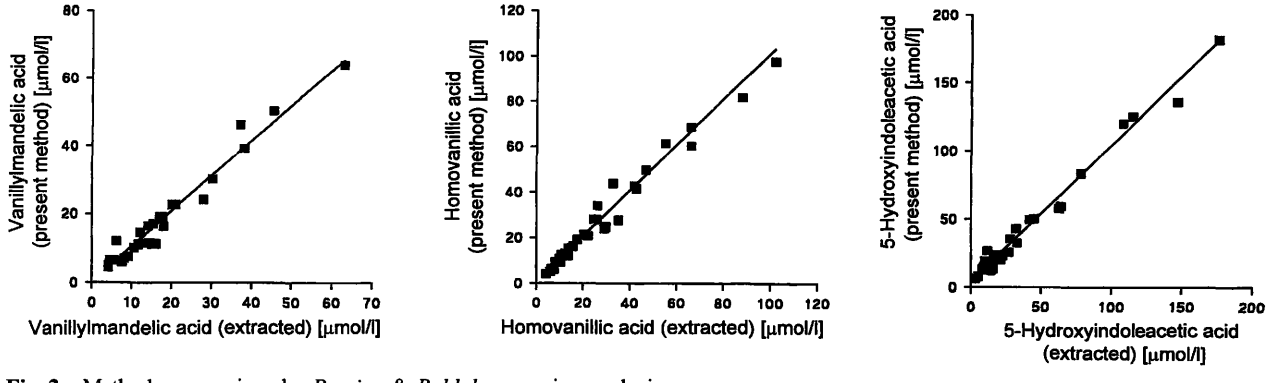

Fig. 2 Methods comparison by *Passing & Bablok* regression analysis.

Tab. 3 Comparison of the present method with the extraction method for vanillylmandelic acid, 5-hydroxyindoleacetic acid and homovanillic acid determinations in urine. *Passing & Bablok* regression analysis in the form y = a + bx. The confidence interval (n = 30) is in parenthesis.

	Intercept (a)	Slope (b)
Vanillylmandelic acid	0.1063 (−1.6425−1.2537)	1.0392 (0.9582−1.1397)
5-Hydroxyindoleacetic acid	3.71 (1.943−5.0668)	1.010 (0.9167−1.0753)
Homovanillic acid	0.3807 (−0.945−1.6769)	1.0105 (0.9326−1.0877)

with low background are observed in normal and pathological samples. As shown in the figure, about 35 min are required for the elution of vanillylmandelic acid, 5-hydroxyindoleacetic acid and homovanillic acid. The calibration curves for all compounds, prepared with chemical standards, were linear in the range 0.1 to 50 mg/l ($r > 0.999$) with a negligible bias. The results for within-day ($n = 10$) and day-to-day precision ($n = 5$) are reported in table 1. Coefficients of variation less than 2.36% for within-day and less than 2.72% for day-to-day were obtained. Linearity was studied by adding known amounts of the three acids (5, 10 and 20 mg/l) to two human urine pool samples (normal and pathological levels) on three different occasions. Results: vanillylmandelic acid $r > 0.998$, 5-hydroxyindoleacetic acid $r > 0.997$, homovanillic acid $r > 0.997$. Detection limits were below 0.1 mg/l for all three metabolites at a signal-to-noise ratio of 3. Recovery was assessed from replicate analysis ($n = 5$) for two days by adding 5 mg/l and 20 mg/l of vanillylmandelic acid, 5-hydroxyindoleacetic acid and homovanillic acid to normal and pathological urine pool samples respectively (tab. 2). Figure 2 reports the comparison between vanillylmandelic acid, homovanillic acid and 5-hydroxyindoleacetic acid concentrations calculated by the present method and by *Radjaipur*'s extraction method; the data of the regression analysis are reported in table 3. The slopes of the three analytes were close to 1, and the confidence region of the intercepts were close to 0. Then we evaluated potential interference from common urine constituents and drugs, which may be eluted close to the peaks of interest (16), by adding known amounts of these to the urine samples. None of the tested compounds (epinephrine, serotonin, vanillic acid, uric acid, ascorbic acid, acetaminophen, *L*-DOPA, carbi-DOPA and acetylsalicylic acid) showed substantial interference with the determination of vanillylmandelic acid, homovanillic acid and 5-hydroxyindoleacetic acid, because they gave recoveries < 0.1% or no peak at all.

Discussion

Clinical diagnosis of some neurological disorders needs precise, rapid and simple determination of catecholamine metabolites and 5-hydroxyindoleacetic acid in urine. Low-cost methods are necessary to satisfy the rising demand for mass screening in childhood (26−30). In view of its analytical characteristics, our technique could represent a new solution to these requests. The key advantage of this method is the ability of ion-pair chromatography (heptanesulphonic acid is added in the mobile phase) to reduce interference by a large number of compounds commonly observed in urine (20). The use of ion-pair chromatography and the above mentioned HPLC conditions resulted in clean peaks of these three substances; the extraction step was avoided without losing precision, accuracy and linearity. Although no interferences have been shown in our study, we think that a further investigation of the influence of dietary components is needed to confirm its specificity. However the combination of all these characteristics ensures good reliability. In conclusion our HPLC method is very fast, easy and inexpensive and requires an apparatus easily accessible to all clinical laboratories equipped with HPLC instrumentation, and it is certainly useful for routine determination and for mass screening for neurological impairment.

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