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# MEASUREMENT OF URINARY VANILMANDELIC ACID AND HOMOVANILLIC ACID BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION FOLLOWING EXTRACTION BY ION-EXCHANGE AND ION-MODERATED PARTITION

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## SUMMARY

An improved protocol has been developed to isolate homovanillic acid (HVA) and vanilmandelic acid (VMA) from urine with strong anion-exchange resin. The sample is diluted with acetate buffer and passed through a disposable column. HVA, uric acid, and many hydrophobic organic acids are removed with 1.0 *M* acetic acid—ethanol. Then VMA is eluted with 0.5 *M* phosphoric acid. Two isocratic mobile phases allow rapid high-performance liquid chromatographic measurement of VMA (5 min) and HVA (8 mins) on a 5-μm ODS column. Selective conditions were developed with dual-electrode coulometric detection to permit specific measurement of VMA, HVA, and internal standards, with less than 5% between-run variation.

## INTRODUCTION

Homovanillic acid (HVA) and vanilmandelic acid (VMA) are commonly measured in urine for the differential diagnosis of neuroblastoma, pheochromocytoma, and related tumors [1, 2]. Recently, VMA has been studied in evaluations of psychiatric patients [3], and HVA has been used to monitor chronic lead exposure [4] and response to medication during the treatment of Parkinson's disease [5, 6]. Although many techniques have been employed to separate and quantify these compounds, the use of high-performance liquid chromatography (HPLC) with reversed-phase columns has been increasingly common since the demonstration of this technique by Molnár and Horváth [7, 8].

The analysis of urinary phenolic acids by HPLC is complicated by the large number of compounds which are commonly observed in urine. Gradient

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elutions are often employed to improve resolution [9, 10]; isocratic separations have also been reported [11—13], but in both cases the analysis time is typically 30—60 min. For the analysis of individual compounds such as VMA and HVA, these procedures are quite time-consuming. Electrochemical detection [10, 14—16] and ion-pair chromatography [16] have been employed to shorten and simplify the HPLC analysis, and ethyl acetate [9] or diethyl ether [13] extraction has been used to reduce interferences. However, the chromatograms produced by these procedures show many peaks, indicating a lack of selectivity. Improvements in both the urine clean-up and the chromatographic system are needed to produce more selective methods.

The separation of VMA and HVA from other organic acids using an anion-exchange resin and salt gradients was first demonstrated by Weise et al. in 1961 [17]. The protocol was adapted to small disposable columns [18] and has been used in combination with the Pisano reaction [19] as a colorimetric test for VMA in urine. More recently, anion-exchange pre-treatment was combined with HPLC, in conjunction with electrochemical detection [16] or post-column derivatization [20].

The use of organic solvents to elute phenolic acids from resins was first demonstrated by Shelley and Umburger in 1959 [21]. Hydroxybenzoic acids were eluted from anion-exchange resin using 15% acetic acid in methanol [22]. The separation of HVA from other organic acids in brain tissue using acetic acid in ethanol was demonstrated by Shibuya et al. [23]. Since elution is not due to a change in ionic strength but rather the hydrophobic interaction between the eluting solvent and an anion which is electrostatically attached to the resin, this type of separation is termed "ion-moderated partition" (IMP) [24].

The purpose of this investigation was to develop an improved ion-exchange sample preparation to simplify HPLC analysis of urinary VMA and HVA. We used ion-moderated partition to first remove the hydrophobic compounds which are strongly retained by reversed-phase HPLC; this fraction contained HVA with a high degree of recovery. VMA was then eluted with dilute phosphoric acid. Finally, isocratic chromatographic separations were developed for analysis of each of these compounds using highly specific electrochemical conditions.

## EXPERIMENTAL

### *Materials*

1-, 3-, and 7-methyluric acids and 1,7-dimethyluric acid were purchased from Fluka Chemicals (New York, NY, U.S.A.). Iso-HVA was kindly supplied by the Research Labs. Hoffman-LaRoche (Nutley, NJ, U.S.A.). 5-Hydroxymethyl-2-furoic acid was synthesized from the corresponding aldehyde (furfural) in ethanolic sodium hydroxide (Cannizaro reaction). VMA, iso-VMA, HVA and all other organic acids were purchased from Sigma (St. Louis, MO, U.S.A.) or Aldrich (Milwaukee, WI, U.S.A.).

HPLC reagent-grade alcohol (ethanol) and 2-propanol were purchased from Fisher Scientific (Fairlawn, NJ, U.S.A.). The other reagents and solutions used were reagent grade. Prepacked strong anion-exchange columns for VMA/HVA analysis were from Bio-Rad Labs. (Richmond, CA, U.S.A.) and pH paper, range 4.5—7.0, was from E. Merck (Rahway, NJ, U.S.A.).

Iso-VMA (450 mg/l) was prepared in 0.05 *M* hydrochloric acid and stored up to three months at 4°C. A stock solution of iso-vanillic acid (iso-VA) was prepared in 50% ethanol (10 mg/ml) and diluted to 500 mg/l with water for each run. Solutions of 1 mg/ml of VMA and HVA in 0.05 *M* hydrochloric acid were used to prepare a urine-based calibrator, which was subjected to the same clean-up as unknown urines.

*HPLC instrumentation*

The chromatographic system (Bio-Rad Labs.) was composed of a Model 1330 dual-piston pump, a Model 1305A variable-wavelength ultraviolet (UV) detector, a Model 450 column heater, a Model AS-48 automatic sampler with a 20-μl sample loop, and a Model 1322 dual-pen recorder. The column effluent was also monitored with a dual-electrode electrochemical ESA detector (Model 5100A Coulochem™ with a 5010 ESA analytical cell, Environmental Sciences Assoc., Bedford, MA, U.S.A.). A 150 × 4.6 mm, 5-μm ODS column with a 30 × 2.1 mm guard cartridge (Bio-Rad Labs.) was used for both analyses.

*Mobile phase preparation*

For VMA analysis, 0.05 *M* phosphate buffer, pH 3.00, was prepared from 2 *M* phosphoric acid and 1 *M* potassium dihydrogen phosphate, and 16 ml ethanol were added per 1000 ml buffer. The mixture, prepared daily, was filtered under vacuum through a 0.22-μm membrane (GVWP, Millipore, Bedford, MA, U.S.A.). For HVA analysis, 0.05 *M* phosphate buffer, pH 2.30, was prepared; 100 ml ethanol and 20 ml 2-propanol were added per 1000 ml buffer and the mixture was filtered as above.

*Urine collection and clean-up procedure*

Urines (24-h) were collected in plastic containers with 10 ml of 6 *M* hydrochloric acid as a preservative. Samples were stored at 4°C up to one week and at −20°C for longer periods. Urines were centrifuged at 2000 *g* to remove particulates.

The prepacked anion-exchange column was inverted to resuspend the resin, opened, and allowed to drain. Then 15 ml of 0.08 *M* sodium acetate buffer, pH 6.10, were used to prerinse the column. Urine (3.0 ml) was combined with 0.2 ml of iso-VMA, 0.2 ml iso-VA, 5 ml acetate buffer, and 0.1 ml of 0.5 *M* sodium hydroxide. The pH, which should be between 5.0 and 7.0, was verified with pH paper. Further pH adjustment with 0.5 *M* sodium hydroxide or 2 *M* acetic acid was performed if required. This mixture was poured onto the column and allowed to drain, then the column was rinsed again with 5 ml acetate buffer. HVA was eluted with 12 ml of 1.0 *M* acetic acid—ethanol (50:50, v/v). Then VMA was eluted with 18 ml of 0.5 *M* phosphoric acid. If only VMA is to be analyzed, the same procedure is followed, except the iso-VA is omitted and only 9 ml of acetic acid—ethanol are required.

*HPLC operating instructions*

For both analyses, the guard cartridge and analytical column were maintained at 35°C with a flow-rate of 1.5—2.0 ml/min. For VMA analysis, the first potential of the ESA cell was set at 0.18 V and the second potential

was at 0.30 V. For HVA, the first potential was set at 0.20 V and the second potential was set at 0.46 V. Equilibration from one mobile phase to the other required less than 10 min. The HPLC system was allowed to run overnight with the mobile phase flowing at 0.1 ml/min. Once a week, the system was rinsed with 30 ml distilled and filtered water, followed by 30 ml of 50% ethanol. The electrochemical cell was disconnected during the ethanol wash. No routine maintenance was required for the ESA detector, and one analytical column with two guard columns was sufficient for at least 600 analyses.

UV measurement of VMA was performed at 280 nm with 0.005 absorbance units full scale (a.u.f.s.).

*Recovery studies*

Urine samples were spiked with 10 μg/ml VMA and 20 μg/ml HVA to determine recovery. Absolute recovery was calculated against aqueous standards diluted with mobile phase. Analytical recovery was determined by comparison of VMA/iso-VMA and HVA/iso-VA ratios in the spiked urines to the aqueous standards.

Elution profiles for VMA and iso-VMA were prepared after a 9-ml alcohol wash by collecting successive 2-ml fractions of 0.5 *M* phosphoric acid.

Elution profiles for VMA, HVA, iso-VMA, iso-HVA, uric acid, 1-methyluric acid, 1,3-dimethyluric acid, *p*-hydroxyphenylacetic acid (*p*-OH-PAC), 5-hydroxyindole-3-acetic acid (5-HIAA), indole-3-acetic acid (IAA), and caffeic acid were prepared by spiking normal urines with 40 μg/ml of each compound. Ten columns were prepared and compounds were eluted with acetic acid—ethanol in amounts from 2—20 ml. This approach was required, as the collection of successive 2-ml applications of reagent to the same column gave substantially different results.

For comparison of different eluents, a normal urine was applied to the columns by the usual protocol but with no internal standards, and eluted directly with 0.5 *M* phosphoric acid or with 3 *M* sodium chloride in 0.08 *M* acetate buffer, pH 6.10.

## RESULTS

The use of a two-step elution for urine yields a considerable improvement in the specificity of VMA analysis (Fig. 1a). The potentials chosen on the ESA detector enhance the analysis by removing easily oxidized compounds at the first electrode and reducing the background signal of the mobile phase. Detection at the second electrode is performed at a potential where most monohydroxyphenolic acids are not electrochemically active. Under these conditions, isocratic analysis is possible in 5 min and a very stable baseline is commonly observed.

The analysis of HVA is completed within 8 min (Fig. 1b). VA is commonly observed in small amounts between HVA and iso-VA (internal standard). By developing conditions where HVA, iso-HVA, VA and iso-VA were resolved, we were able to use iso-VA as an internal standard without interference from iso-HVA (Fig. 2). Small peaks from IAA are commonly observed at 15—16 min; injections may be made at one-half the elution time of IAA to avoid interference from this compound.

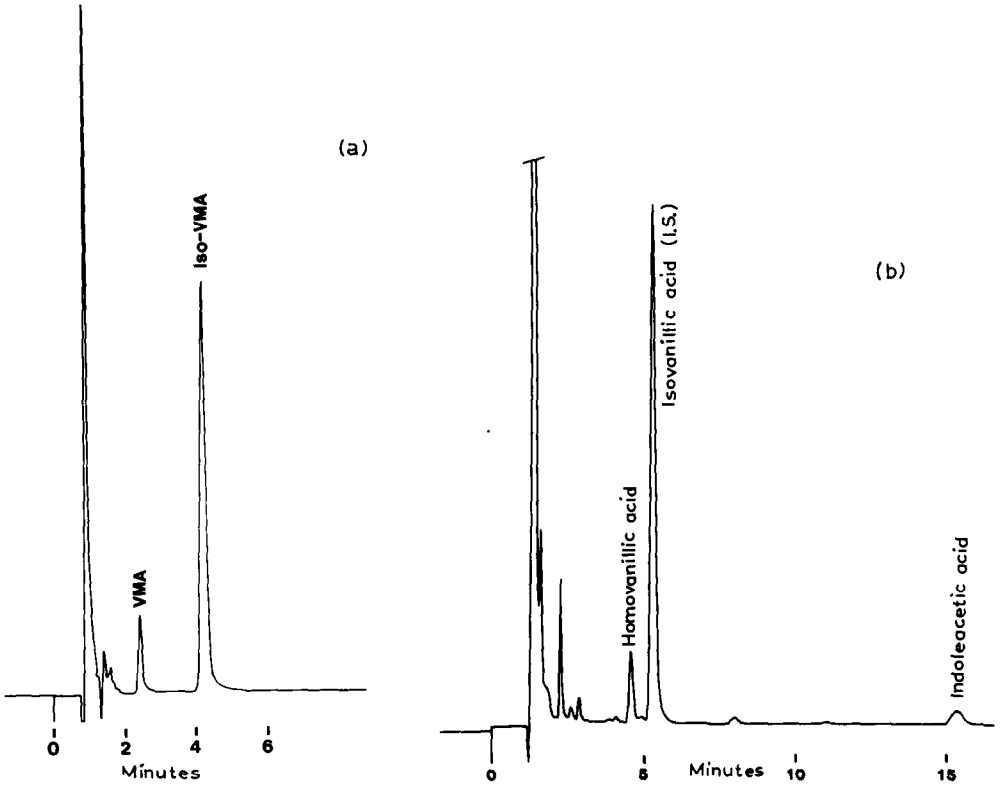


Fig. 1. (a) Chromatogram of a normal urine following pretreatment described in the text. Mobile phase: 0.05 *M* phosphate buffer, pH 3.00—1.6% ethanol. Flow-rate is 2.0 ml/min at 35°C. ESA detector gain is 10 × 1 at 0.30 V. (b) Chromatogram of a normal urine following pretreatment described in the text. Mobile phase: 0.05 *M* phosphate buffer, pH 2.30—ethanol—2-propanol (100:10:2). Flow-rate is 1.5 ml/min at 35°C. ESA detector gain is 12 × 1 at 0.46 V.

Electrochemical response is linear for HVA up to at least 40 mg/l. For VMA, we dilute samples reading higher than 20 mg/l.

The phosphoric acid eluates were also used for UV analysis of VMA (see Fig. 3). Analysis time was 15 min and hippuric acid was the last peak observed in normal urines. Results by the UV method ($X$) showed excellent agreement with the electrochemical method ($Y$); $Y = 1.002X + 0.02$, $n = 73$, $r = 0.990$, with values up to 25 mg/l. This confirms the selectivity of the HPLC conditions for VMA and iso-VMA. However, the chromatograms were more complex, and pH adjustment of the mobile phase was sometimes required to achieve a satisfactory separation of VMA from *m*-hydroxyhippuric acid and 2-furoyl chloride. Some urines from hospitalized patients contained unusual UV-absorbing peaks which eluted after hippuric acid. Also, some urines contained 5-hydroxymethyl-2-furoic acid in amounts up to 1 g/l. This compound is derived from dietary sources containing fructose, including intravenous solutions [25, 26]. Although it elutes 1 min after VMA, the very broad peak produced by high concentrations made quantitation of VMA difficult in a few cases. Satisfactory resolution could be obtained by increasing the ionic strength of the mobile phase and using a lower flow-rate for these urines.

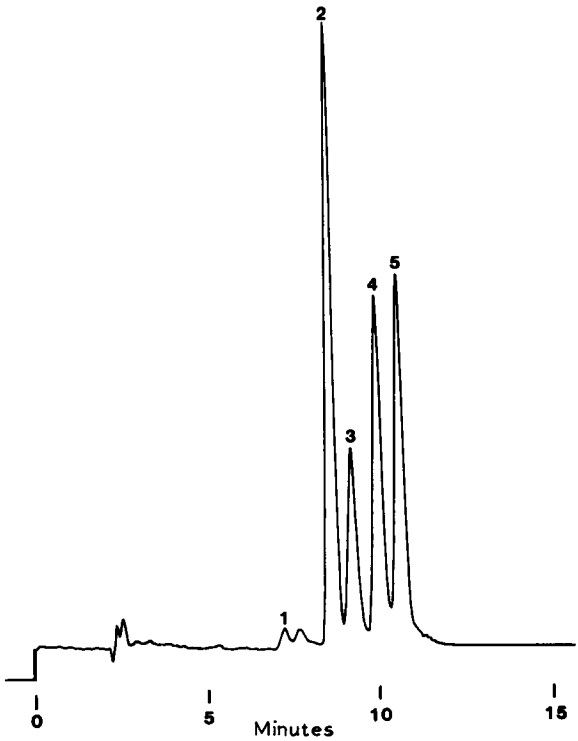


Fig. 2. Separation of HVA from related compounds. Mobile phase: 0.05 *M* phosphate buffer, pH 2.10—ethanol—2-propanol (100:10:2). Flow-rate is 0.8 ml/min at 35°C. ESA detector gain is 40 × 10 at 0.46 V. Test mix contains 1 ng each of the following compounds: *p*-hydroxyphenylacetic acid (1), homovanillic acid (2), vanillic acid (3), iso-vanillic acid (4), and iso-homovanillic acid (5).

We studied the elution profile of VMA and iso-VMA in 0.5 *M* phosphoric acid to demonstrate that both compounds eluted at the same rate and to determine the optimum elution volume. Peaks were slightly higher after 12-ml elution, but this represented only 65% of the total VMA. By using 18 ml eluent, the recovery was near 80% and further elution removed little additional VMA. Iso-VMA and VMA were present in the same ratio at all eluent volumes from 4 to 20 ml. Lower concentrations of phosphoric acid slowed the elution rate. We also found that the pH of urine at application did not affect the VMA/iso-VMA ratio. Recovery was highest when application pH was between 5 and 7.

The total capacity of the clean-up column is 1.1 mequiv., which is adequate to retain all the organic acids present in 3 ml urine. However, all urines contain chloride, and concentrations of chloride above 0.5 *M* will substantially reduce recovery and affect quantitation. Since the main source of chloride is typically the hydrochloric acid added as a preservative, over-acidification during collection must be avoided, and urines which are received with a pH less than 1.5 should be diluted before assay.

The elution profiles observed with the acetic acid—ethanol eluent varied a great deal for the compounds studied (see Fig. 4). Uric acid and methylurates were washed quickly off the column; 12 ml of eluent removed HVA, iso-HVA,

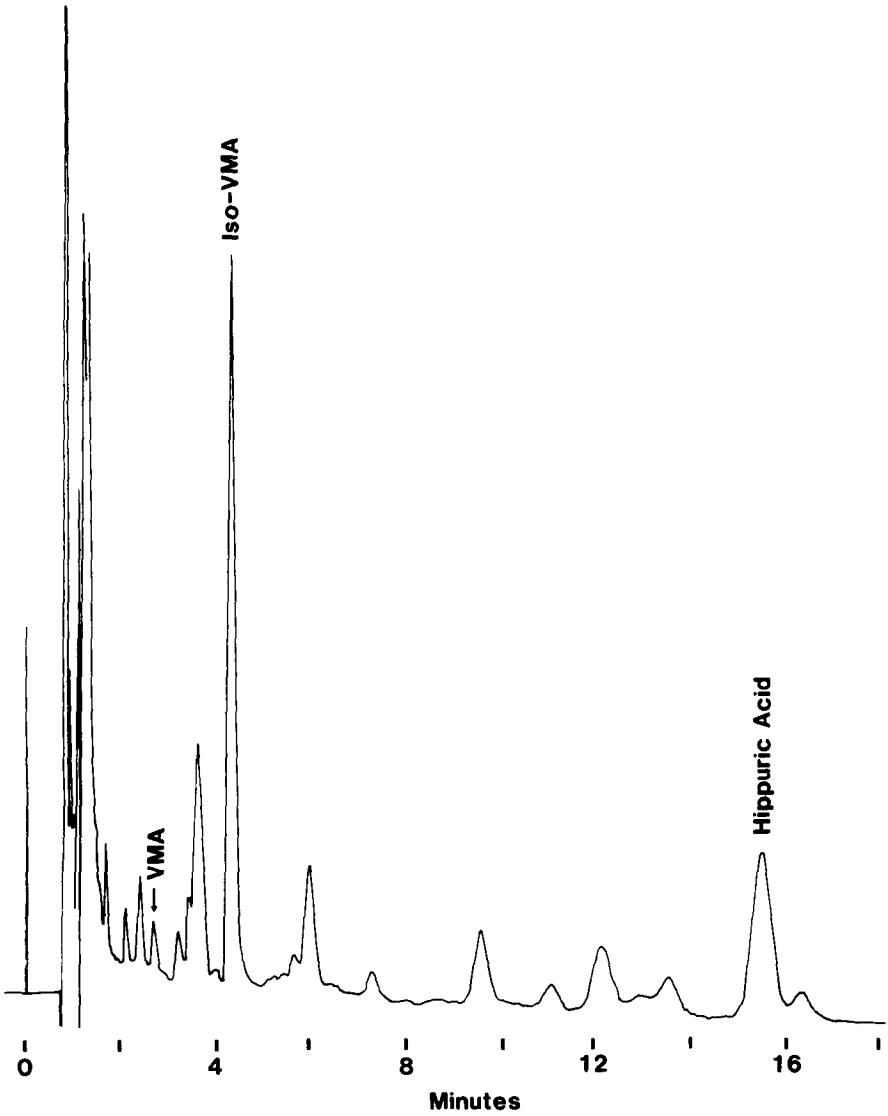


Fig. 3. Chromatogram of a normal urine measured by UV detection following pretreatment described in the text. Mobile phase: 0.01 *M* phosphate buffer, pH 3.00—1.6% ethanol. Flow-rate is 2.0 ml/min at 35°C. UV detection at 280 nm and 0.005 a.u.f.s.

VA, and iso-VA. Other compounds such as VMA, iso-VMA, and caffeic acid elute very slowly, and volumes in excess of 30 ml would be required to remove them completely. IAA and *p*-OH-PAC could be collected in 15—18 ml, but 5-HIAA eluted very slowly. Elution volumes did not correlate with $pK_a$ values or HPLC elution order, and probably reflect a mixture of several retention mechanisms. To remove 1-methyluric acid and 7-methyluric acid, metabolites of caffeine which are found in most urines, 9 ml of acid—ethanol eluent is required. The removal of these compounds prior to VMA measurement is especially important as theophylline, a common anti-asthmatic drug, produces

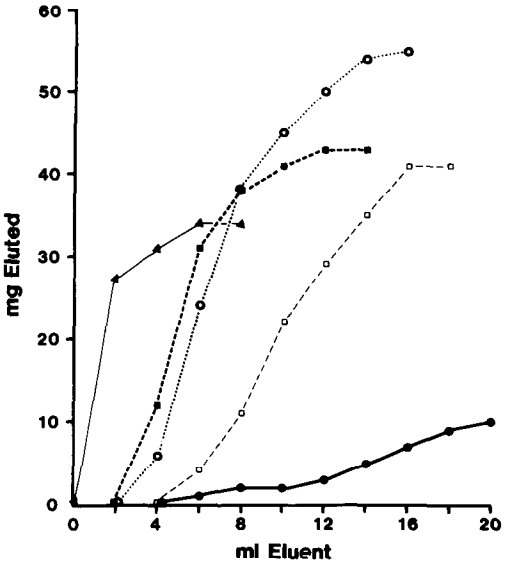


Fig. 4. Elution of different compounds from the anion-exchange column by 1.0 *M* acetic acid—ethanol (50:50). Compounds shown are homovanillic acid (▪), vanilmandelic acid (•), 1,3-dimethyluric acid (▲), indole-3-acetic acid (▫) and *p*-hydroxyphenylacetic acid (○). Protocol is described in Experimental.

TABLE I

RETENTION TIMES AND DETECTION CHARACTERISTICS FOR POSSIBLE INTERFERENCES IN VMA ANALYSIS

For VMA mobile phase and HPLC conditions, see Experimental. Flow-rate, 2.0 ml/min.

| Compound | Chromatographic retention time (min) | Potential required for oxidation (V) |
|---|---|---|
| Uric acid | 1.3 | >0.10 |
| *p*-Hydroxymandelic acid | 1.8 | >0.25 |
| 3-Methyluric acid | 2.0 | >0.05 |
| 4-Hydroxy-3-methoxymandelic acid (VMA) | 2.5 | >0.20 |
| *m*-Hydroxymandelic acid | 2.7 | >0.45 |
| *m*-Hydroxyhippuric acid | 2.8 | >0.60 |
| 7-Methyluric acid | 2.8 | >0.10 |
| 1-Methyluric acid | 3.5 | >0.05 |
| 2,5-Dihydroxyphenylacetic acid | 3.6 | 0.01 |
| 3-Hydroxy-4-methoxymandelic acid (internal standard, I.S.) | 4.0 | >0.20 |
| 3-Indoxyl sulfate | 4.8 | >0.15 |
| 3-Hydroxyanthranilic acid | 6.4 | >0.05 |
| 1,3-Dimethyluric acid | 6.9 | >0.10 |
| 3,4-Dihydroxyphenylacetic acid | 8.3 | 0.01 |
| *p*-Hydroxybenzoic acid | 9.3 | >0.40 |
| *p*-Hydroxyphenyllactic acid | 10.0 | >0.30 |
| 1,7-Dimethyluric acid | 11.5 | >0.20 |
| Hydrocaffeic acid | 15.5 | 0.01 |
| *p*-Hydroxyphenylacetic acid | 17.0 | >0.35 |
| 5-Hydroxyindole-3-acetic acid | 17.8 | >0.05 |
| *m*-Hydroxyphenylacetic acid | 18.2 | >0.45 |
| Vanillic acid | 18.5 | >0.35 |
| 4-Hydroxy-3-methoxyphenyllactic acid | 19.5 | >0.20 |
| Homovanillic acid | 30.1 | >0.20 |

TABLE II

RETENTION TIMES AND DETECTION CHARACTERISTICS FOR POSSIBLE INTERFERENCES IN HVA ANALYSIS

For HVA mobile phase and HPLC conditions see Experimental. The flow-rate was 1.5 ml/min.

| Compound | Chromatographic retention time (min) | Potential required for oxidation (V) |
|---|---|---|
| *p*-Hydroxybenzoic acid | 1.7 | >0.40 |
| *p*-Hydroxyphenylpyruvic acid (I) | 2.0 | >0.45 |
| 1,3,7-Trimethyluric acid | 2.3 | >0.20 |
| 3-Hydroxyanthranilic acid | 2.3 | >0.05 |
| 3,4-Dihydroxyphenylacetic acid | 2.4 | 0.01 |
| 3-Indoxyl sulfate | 2.5 | >0.15 |
| *p*-Hydroxyphenyllactic acid | 2.7 | >0.35 |
| 5-Hydroxyindole-3-acetic acid | 2.8 | >0.05 |
| 4-Hydroxy-3-methoxyphenyllactic acid | 3.6 | >0.20 |
| Hydrocaffeic acid | 3.7 | 0.01 |
| *p*-Hydroxyphenylpyruvic acid (II) | 3.9 | >0.45 |
| *p*-Hydroxyphenylacetic acid | 3.9 | >0.35 |
| *m*-Hydroxybenzoic acid | 4.1 | >0.55 |
| 4-Hydroxy-3-methoxyphenylpyruvic acid (I) | 4.3 | >0.35 |
| Homovanillic acid (HVA) | 4.3 | >0.20 |
| Vanillic acid | 4.6 | >0.35 |
| 3,5-Dimethoxy-4-hydroxybenzoic acid | 4.7 | >0.20 |
| Caffeic acid | 4.9 | 0.01 |
| *m*-Hydroxyphenylacetic acid | 4.9 | >0.45 |
| iso-Vanillic acid (internal standard, I.S.) | 5.0 | >0.35 |
| iso-Homovanillic acid | 5.5 | >0.20 |
| *o*-Hydroxyphenylacetic acid | 5.6 | >0.40 |
| 4-Hydroxy-3-methoxyphenylpyruvic acid (II) | 5.6 | >0.35 |
| *o*-Hydroxyhippuric acid | 6.8 | >0.40 |
| *p*-Hydroxycinammic acid | 9.4 | >0.30 |
| 3-Indolelactic acid | 9.9 | >0.30 |
| 4-Hydroxy-3-methoxycinammic acid | 10.6 | >0.20 |
| Indole-3-acetic acid | 15.1 | >0.20 |
| 3-Indolepropionic acid | 35.2 | >0.35 |

urine concentrations of 1-methyluric acid and 1,3-dimethyluric acid over 100 mg/l [27, 28]. Complete elution of HVA requires 12 ml of this eluent; a small amount of VMA and iso-VMA is also eluted. For collection of hydrophobic compounds other than HVA, different elution volumes of acetic acid—ethanol could be used.

We studied the HPLC retention time of many organic acids in the two mobile phases which were used (Tables I and II). 7-Methyluric acid, the compound which is most likely to interfere with VMA measurement, is removed by acetic acid—ethanol during sample clean-up. Although some other compounds elute just behind VMA, they cannot be oxidized at 0.30 V. Dicarboxylic acids are not eluted from the anion-exchange clean-up column and were not examined; *o*-hydroxybenzoic acids such as gentisic acid and salicylic acid are also strongly retained. For HVA analysis, only *p*-OH-PAC and VA are observed in normal urines. The split HPLC peaks from keto and enol forms of 4-hydroxy-3-methoxyphenylpyruvic acid (VPA) have been observed previously

[29]. Small amounts of this compound might coelute with HVA in patients receiving L-3,4-dihydroxyphenylalanine, although HVA levels would be extremely elevated in this case. Normal urines do not contain measurable quantities of VPA [29]. *o*-OH-PAC is normally present in trace amounts but is elevated in phenylketonuria [30]. For metabolic studies of HVA/iso-HVA ratio, the analysis could be performed at lower oxidation potentials (0.18/0.30 V) to eliminate these potential interferences, but iso-VA could no longer be used as an internal standard.

A small amount of VMA is oxidized at the first electrode of the detector, and this amount is proportional to concentration. The first electrode potential should be set to achieve maximum oxidation of 1-methyluric acid (see Fig. 5A), while VMA response is limited to less than 8% of the signal at electrode two. The second electrode potential is set so that iso-VMA reaches near 50% of its maximum peak height. For both of these compounds, and for HVA and iso-VA as well, the maximum electrode response is observed at 0.50 V (see Fig. 5). For the analysis of HVA, a slightly lower potential (0.46 V) is used to avoid interference from *m*-OH-PAC. The small peak observed from VMA at the first electrode may be used for confirmation of VMA identity in abnormal urines, since elevated specimens show a proportional increase in response at both electrodes.

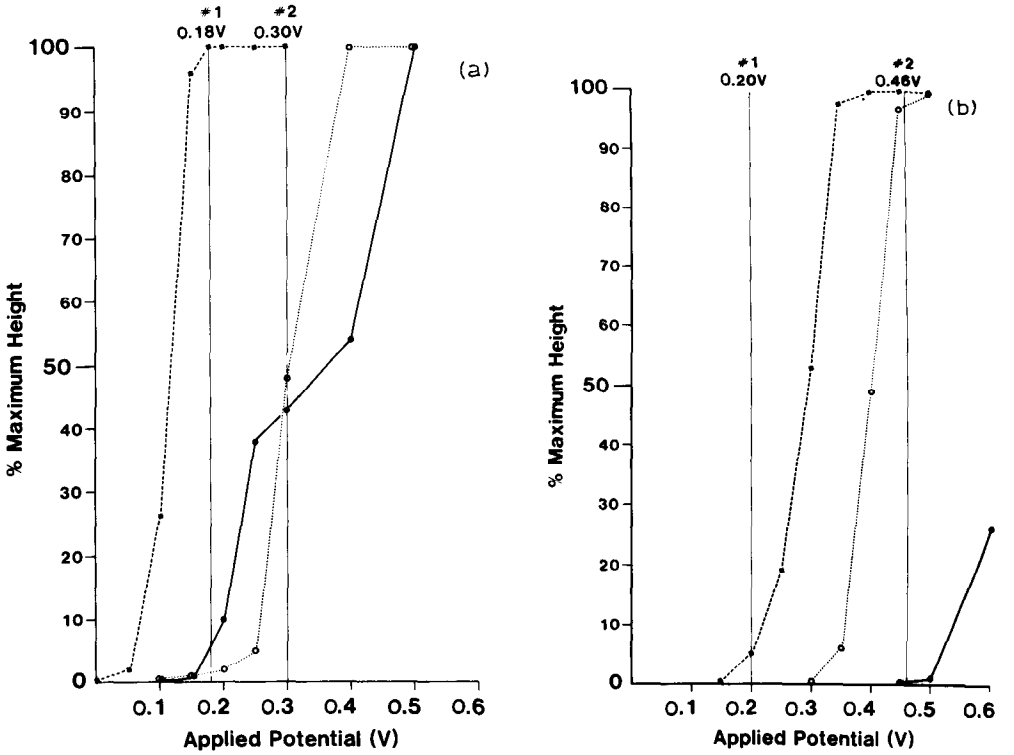


**Fig. 5. Normalized response of the ESA 5010 electrochemical cell at various voltage settings. (a) Compounds shown are vanilmandelic acid (•), iso-vanilmandelic acid (○) and 1-methyluric acid (▪). (b) Compounds shown are homovanillic acid (▪), iso-vanillic acid (○) and *m*-hydroxyphenylacetic acid (•).**

Elution of organic acids from anion-exchange resin is usually achieved by increasing the ionic strength of the buffer eluent without a change in pH. The elution of VMA in 0.5 *M* phosphoric acid does not suggest a traditional approach, as the eluent pH (1.2) is well below the $pK_a$ of VMA (3.4), and less than 1% of the VMA should be ionized. We compared different eluents (Fig. 6), and the results obtained with 0.5 *M* phosphoric acid (Fig. 6b) resemble the profile obtained when 3 *M* sodium chloride in phosphate buffer, pH 6.1, was used to elute VMA (Fig. 6a). Both of these elute HVA as well, but the recovery is typically low by this approach [16]. Several minor peaks, as well as 7-methyluric acid, are eluted by 0.5 *M* phosphoric acid when the acetic acid—ethanol step is omitted. Using an amperometric detector, peaks from 1-methyluric acid and 3,4-dihydroxyphenylacetic acid would be observed in Fig. 6a and b.

The precision of both methods was adequate for clinical purposes (see Table III). For normal concentrations, within-run coefficients of variation (C.V.) were less than 2% and between-run C.V. values were less than 5%.

We measured HVA in 30 normal adults and found a mean level of 2.3 mg HVA per g creatinine with ratios ranging from 1.1 to 4.3. These results agree with previously reported findings [31, 32]. Creatinine was determined by a modified Jaffe method [33].

Analysis of HVA for neuroblastoma rarely produces borderline results, as elevations are usually 5—100 times normal concentrations [31]. With VMA, marginal elevations (two to three times of normal) may indicate pheochromocytoma [1]. For this reason, we completed a larger normal study for VMA on 24-h collections from 110 healthy adults, with ages from 16 to 70 years.

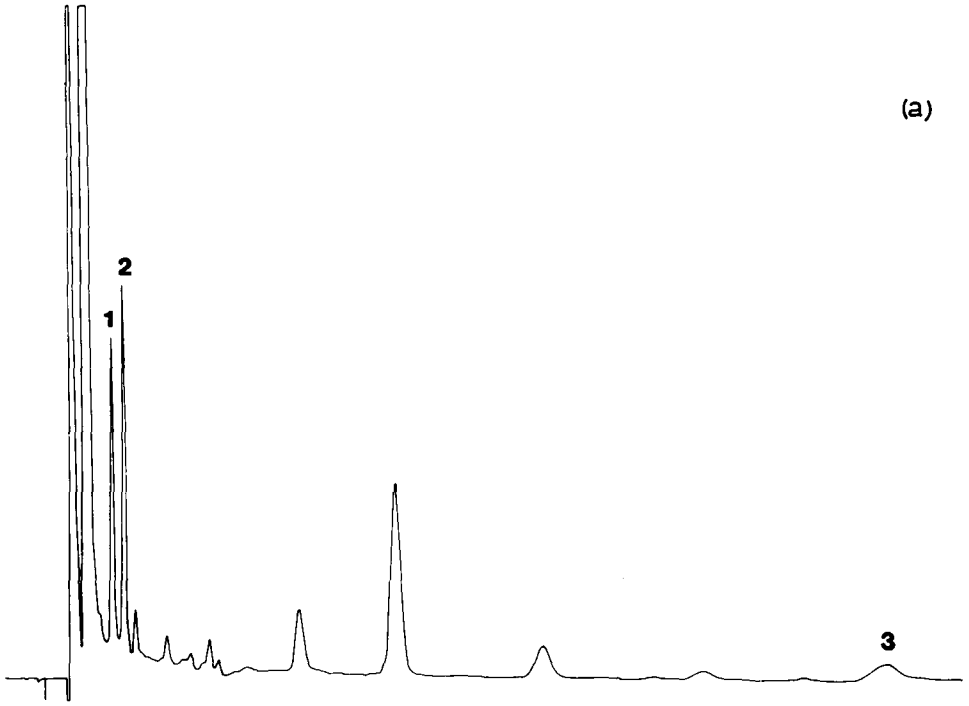


Fig. 6.

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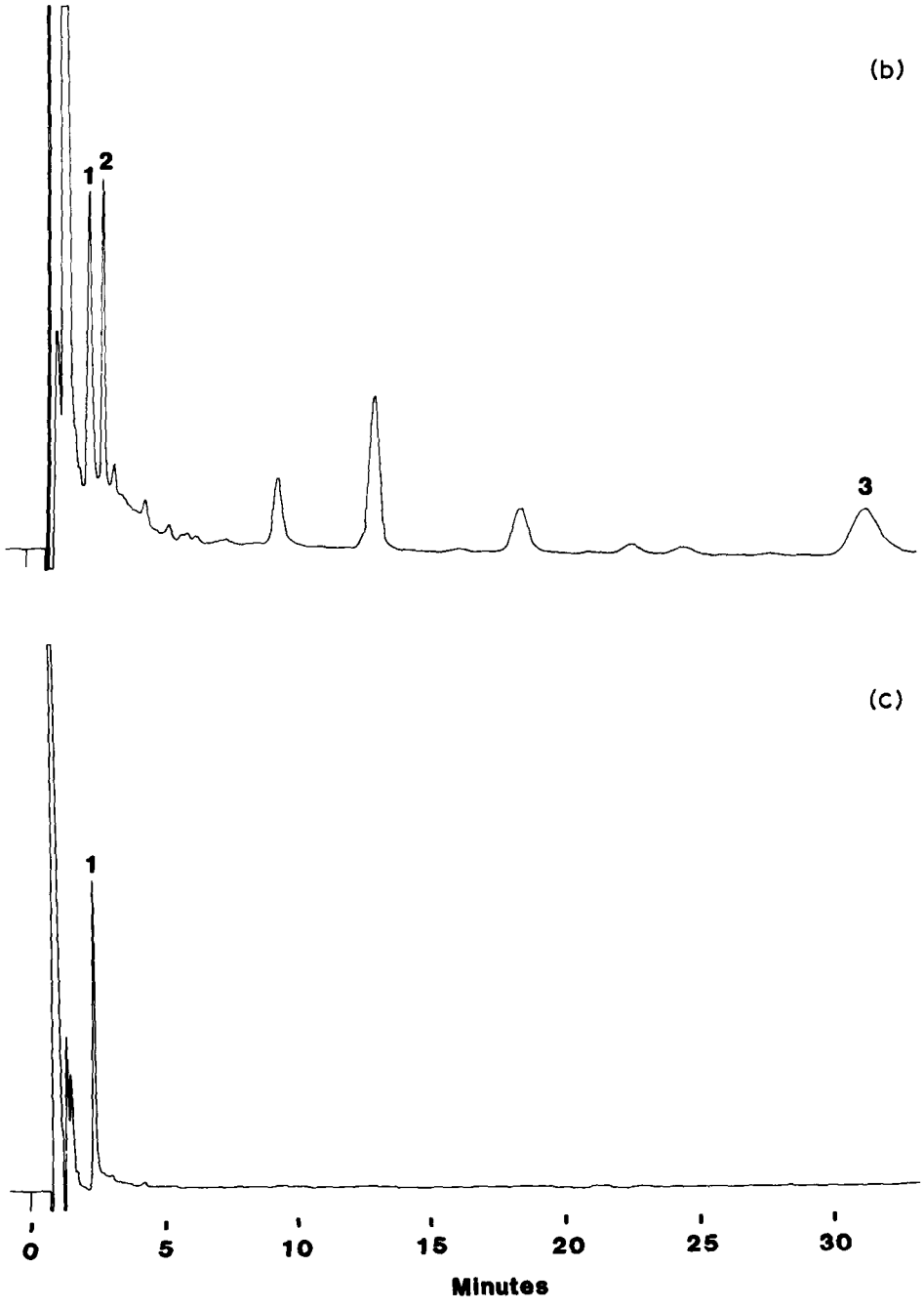


Fig. 6. Chromatograms of a normal urine after three different elution protocols: (a) 12 ml of 3.0 *M* sodium chloride in phosphate buffer, (b) 12 ml of 0.5 *M* phosphoric acid, (c) 12 ml of 0.5 *M* phosphoric acid after a prerinse with 12 ml of 1.0 *M* acetic acid—ethanol. Mobile phase and conditions are as in Fig. 1A; ESA detector gain is 40 × 1. Peaks: 1 = vanilmandelic acid; 2 = 7-methyluric acid; 3 = homovanillic acid.

TABLE III

PRECISION OF VMA AND HVA MEASUREMENT

| | VMA | | HVA | |
|---|---|---|---|---|
| | 3.7 mg/l | 15.0 mg/l | 2.6 mg/l | 15.0 mg/l |
| Within-run C.V. (%) ($n$ = 6) | 1.5 | 0.8 | 0.9 | 1.3 |
| Between-run C.V. (%) ($n$ = 20, 5 runs) | 3.9 | 3.0 | 4.6 | 3.4 |

TABLE IV

NORMAL-RANGE STUDIES FOR URINARY VMA AND HVA

| | VMA | | HVA | |
|---|---|---|---|---|
| | mg per day | mg per g creatinine | mg per day | mg per g creatinine |
| Number of subjects | 110 | 110 | 30 | 30 |
| Range of values | 1.60—7.27 | 1.14—5.18 | 0.67—7.78 | 1.13—4.29 |
| 95% Range (non-parametric) | 1.77—6.74 | 1.56—4.20 | — | — |
| Mean ± S.D. | 3.86 ± 1.33 | 2.65 ± 0.79 | 3.14 ± 1.55 | 2.27 ± 0.78 |

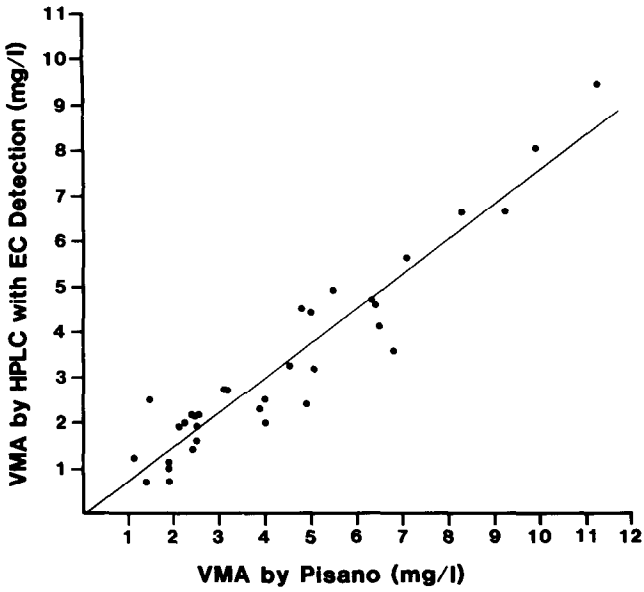


Fig. 7. Comparison of vanilmandelic acid results obtained by a Pisano [19] method ($X$) to results obtained by this HPLC technique ($Y$). For $n$ = 34, $Y = 0.760X - 0.074$, $r = 0.9512$.

In this group we found VMA levels ranging from 1.6 to 7.3 mg per day (see Table IV). When corrected for creatinine, the ratios obtained ranged from 1.1 to 4.3 mg VMA per g creatinine with one outlier at 5.2. These results show

good agreement with the cut-off value of 4 mg VMA per g creatinine recommended by Gitlow [1]. For comparison, 34 urines assayed by a Pisano method [19] were run by this HPLC technique. Results were about 25% lower and the correlation was 0.951 (see Fig. 7).

Recovery of both compounds was reproducible. For VMA, absolute recovery was 75—85% and analytical recovery (after correction for the internal standard) was 97—103%. For HVA, absolute recovery was 90—102% and analytical recovery was 98—102%.

## DISCUSSION

Many laboratories lack gradient HPLC systems; also the use of gradients with electrochemical detectors may cause baseline drift and require frequent recalibration. Specific HPLC methods have been developed using post-column reaction systems [20, 34, 35], but this apparatus is rarely found in clinical laboratories. For these reasons, we have developed two isocratic mobile phases for the HPLC analysis of VMA and HVA by electrochemical detection. The step-gradient which was used by Joseph et al. [36] for VMA/HVA analysis embodies the same approach. Other isocratic systems and ion-pairing conditions have been employed which allow simultaneous measurement of VMA and HVA, but no internal standards were used. Also, many methods do not indicate whether iso-HVA is resolved from HVA.

Recently, methods have been published which demonstrate the electrochemical measurement of VMA or HVA in urine with no clean-up step [37, 38]. Although this approach may succeed with urines from healthy persons, hospitalized patients often excrete large amounts of phenolic acids as well as basic compounds, which will decrease electrochemical response in subsequent samples. Under the best conditions, there is still some drift in response with time, which has led to the use of the standard/sample/standard sequence for quantitative analysis [39, 40]. The use of purified samples and appropriate internal standards, in combination with the high capacity of the porous graphite cell, yields conditions where drift due to sample injection is negligible in an equilibrated HPLC system.

The recovery of HVA is less than 70% when ethyl acetate followed by phosphate buffer is used for extraction [41]. Higher recoveries have been obtained using a back-extraction into potassium carbonate [5], but VMA is not stable in basic buffers [42]. An HVA recovery of 97% was recently reported from an AG® 1-X4 formate column using 4 *M* formic acid—methanol (20:80) for elution [43], so it appears that ion-moderated partition is a good choice for the extraction of HVA from urine. Iso-VMA as an internal standard has been used previously in the HPLC measurement of VMA with a post-column reaction system [20]. Endogenous levels have been quantitated by gas chromatography—mass spectrometry and typically represent 0.7% of VMA levels [44], or about 0.03 mg/l. Since the iso-VMA added represents 30 mg/l, endogenous concentrations do not affect measurement. Endogenous iso-VA has not been reported or measured to our knowledge.

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