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# Determination of Urinary Vanilmandelic Acid and Homovanillic Acid by High Performance Liquid Chromatography with Amperometric Detection

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**Summary:** A method was developed for the simultaneous determination of urinary vanilmandelic acid and homovanillic acid, which included a two step prepurification and a reversed-phase high-performance liquid chromatography with amperometric detection. Conditions were evaluated for performing measurements with the amperometric detector free from electric interference.

The method was linear between 2.5 and 100 μmol/l vanilmandelic acid and homovanillic acid with good precision (CV always less than 10%).

The correlation between the present determination of vanilmandelic acid and the procedure of *Pisano* et al. ((1962) Clin. Chim. Acta 7, 285–291) was very good (r = 0.931). No interfering substances could be detected.

*Bestimmung von Vanillinmandelsäure und Homovanillinsäure im Urin mit Hochdruckflüssigkeitschromatographie und amperometrischer Detektion*

**Zusammenfassung:** Eine neue Methode zur gleichzeitigen Bestimmung von Vanillinmandelsäure und Homovanillinsäure im Urin wurde erarbeitet. Sie basiert auf der Kombination von HPLC und amperometrischer Detektion sowie einer zweistufigen Vortrennung. Es wurden die Versuchsbedingungen untersucht, die eine störungsfreie Messung mit dem amperometrischen Detektor ermöglichen.

Das Verfahren ist von 2,5 bis 100 μmol/l Vanillinmandelsäure und Homovanillinsäure linear, wobei der Variationskoeffizient über den gesamten Bereich kleiner als 10% ist. Die Korrelation der Bestimmung der Vanillinmandelsäure mit der Methode von *Pisano* et al. ((1962) Clin. Chim. Acta 7, 285–291) ist hoch (r = 0,931). Störende Substanzen konnten nicht festgestellt werden.

## Introduction

The photometric determination of 3-methoxy-4-hydroxymandelic acid (vanilmandelic acid) by the procedure of *Pisano* (1, 2) is very time consuming and necessitates a large number of extraction steps. Therefore, in the last few years several methods have been developed to improve the vanilmandelic acid determination. Some of these methods were based on high-performance liquid chromatography with amperometric detection. *W. Rich* et al. (3) used equipment comprising two pumps, two conductivity detectors and five columns with an overall length of 800 mm. By using this expensive equipment, the authors were able to measure urinary vanilmandelic acid without any prepurification.

An equally simple procedure, using a less expensive equipment was described by *Morrisey & Shihabi* (4). But it was not possible to reproduce their experiments. A lot of unknown substances interfered with the measurement of vanilmandelic acid and some substances were not removed from the column until several hours after the injection of the urine samples.

The present method for vanilmandelic acid determinations is simple and reliable. The equipment for high-performance liquid chromatography consists only of one pump, one column and one detector. The application of the amperometric detector proved to be simpler than the fluorometric detection. But some special provisions had to be taken to get good measurements with a high signal-to-noise ratio.

The prepurification (ethyl acetate extraction followed by small column chromatography on diethylaminoethyl-cellulose) was absolutely necessary. This could be done in such a way, that vanilmandelic acid and 3-methoxy-4-hydroxyphenyl acetic acid (homovanillic acid), could be eluted in the same fraction and subsequently determined simultaneously by HPLC. The simultaneous determination of vanilmandelic acid and homovanillic acid is desirable as could be shown by *La Brosse* et al. (5) in a

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study of 258 patients with neuroblastoma. They found a diagnostic sensitivity of 76.4% for homovanillic acid, 69.4% for vanilmandelic acid and 81.8% for the combination of both determinations. It was evident that the diagnostic sensitivity had clearly increased by the simultaneous determination of both acids. In the method described here, this improvement in diagnostic sensitivity can be achieved by using one single analytical procedure.

## Materials and Methods

### Equipment

*High-performance liquid chromatography*

A Model 6000 A pump (Waters Associates Inc., Königstein, Taunus) was used to deliver the solvent through a 300 × 3.9 mm (i.d.) column of μBondapak C18, 10 μm average particle size (Waters Associates Inc., Königstein, Taunus). The samples were introduced through a 20 μl loop injector (Model 7125, Rheodyne Inc., Berkeley, California).

*Electrochemical detection*

The effluent was monitored with a glassy carbon electrode cell, a Ag/AgCl-reference electrode and an amplifier LC 4 (Bioanalytical Systems Inc., West Lafayette, In.). The chromatograms were plotted with a Servogor 210 recorder (BBC Goerz, Wien).

*Electrical interference suppression*

The electrochemical detection was realized in a *Faraday* cage by a flowing potential measurement. The cage surrounded both the detection cell and the amplifier. The connection wire to the recorder was covered with a tube produced from a metal fabric. Remaining interferences were eliminated by a double-L low-pass-filter (100 kΩ, 3.3 μF) near the input of the recorder. Moreover a line filter is required, constructed with VDR-varistors (Type 2322 594 13912 Valvo, Hamburg) and two wide-band filters (Type 9764 Valvo, Hamburg) connected in series.

*Prepurification*

Ethyl acetate extraction was carried out in 20 ml glass tubes and column chromatography in small polyethylene columns (9 mm i.d.) (QS-Q, Isolab Inc., Akron, Ohio). The filling height of diethyl-aminoethyl-cellulose was 30 mm.

*Mechanization*

Programmed sample injection and electrical evaluation was done with a WISP 710 B, and automatic evaluation was performed with Data Module (Waters Association, Königstein, Taunus).

### Chemicals and reagents

All reagents were of analytical grade and purchased from Merck AG. Darmstadt, unless otherwise stated.

*Ethyl acetate extraction*

6 mol/l hydrochloric acid, sodium chloride, ethyl acetate, 0.2 mol/l tris(hydroxymethyl)aminomethane solution (solution A).

*Column chromatography*

Diethylaminoethyl-cellulose (Servacel DEAE 32, Serva, Heidelberg), 50 mmol/l tris-buffer pH 8.1 (solution B), 0.3 mol/l sodium chloride in 10 mmol/l hydrochloric acid (solution C).

*Regeneration of diethylaminoethyl-cellulose*

3 mol/l sodium chloride in 10 mmol/l sodium hydroxide solution (solution D).

*Mobile phase*

200 ml of 50 mmol/l citric acid and 800 ml of 50 mmol/l disodium phosphate were mixed, filtered (Millipore HA, 0.45 μm pore size, Millipore Corp., Bedford, Massachusetts) and degassed for 10 min. Then 20 to 60 ml acetonitrile were added and again degassed for 3 min. 60 ml of acetonitrile were added, if new columns were used, resulting in a retention time of 8 min for homovanillic acid. The retention time decreased with increasing age of the column. This could satisfactorily be compensated by the reduction of the acetonitrile concentration to 20 ml/l eluent. Thus the retention time of 8 min for homovanillic acid could be maintained.

*Standard solution* (solution E)

A solution of 20 μmol/l vanilmandelic acid (Serva, Heidelberg) and homovanillic acid (Fluka AG, Neu-Ulm) was prepared by dilution of a stock solution with distilled water.

*Stock solution*

0.1 mmol vanilmandelic acid and 0.1 mmol homovanillic acid were dissolved in 80 ml water, acidified with 1 ml of 6 mol/l hydrochloric acid and filled up to 100 ml with water. This solution was stable for several months if stored at – 14 °C.

*Urine pool*

A large amount of urine was acidified with 10 ml of 6 mol/l hydrochloric acid to 1 l of urine, thoroughly mixed, divided in portions of 50 ml and stored at – 14 °C.

### Methodology

Standard solution (E) and urine samples (F) were measured according to the following procedure: ca. 300 mg of sodium chloride and 100 μl of 6 mol/l hydrochloric acid were added to 1 ml of a sample (E or F) in 20 ml glass tubes, and mixed for a short time. After adding 8 ml of ethyl acetate the mixture was shaken for about 30 s on a Vortex-mixer and centrifuged at 200 *g* for 5 min. 6 ml of the organic upper layer were transferred into a second glass tube and extracted with 3 ml of tris solution (A). Shaking and centrifugation was performed as described before. Now the organic upper layer was aspirated and discarded.

*Column chromatography*

2 ml of the lower aqueous layer were applied on the top of the bed of a diethylaminoethyl-cellulose column. Then the gel was washed in two steps. The first washing was carried out with 2 ml of tris buffer (B) and the second with 1.5 ml of sodium chloride solution (C). Subsequently vanilmandelic acid and homovanillic acid were eluted with 2 ml of sodium chloride solution (C). The eluate can be stored at – 14 °C until quantitative determination by high-performance liquid chromatography and amperometric detection. It is stabile for several weeks under these conditions.

*High-performance liquid chromatography and amperometric detection*

The fraction containing vanilmandelic acid and homovanillic acid was diluted 1:2 with the mobile phase used for the high-performance liquid chromatography. 20 μl of these dilutions were injected every 30 min. In most urines interferring substances were eluted after 18 min. However, in two urine samples one peak was observed after 28 min. So it was necessary to wait 30 min for the next injection. But it was possible to inject the standard solution in the last 10 min of each chromatogram to test the sensitivity of the detector. The flow rate of the eluent was 0.9 ml/min. The amperometric detector was set to an oxidizing potential of + 650 mV, a sensitivity of 10 nA/V, a filtering of 0.5 s and an offset between 3 and 20 nA.

*Evaluation*

The concentration of vanilmandelic acid and homovanillic acid was calculated by comparison with the peak height obtained for the standard solution.

*Regeneration of the diethylaminoethyl-cellulose*

With the aid of 50 ml pin up funnels the diethylaminoethyl-cellulose was washed successively with 50 ml of alkaline sodium chloride solution (D), 25 ml of acidic sodium chloride solution (C) and 50 ml tris-buffer (B).

*Working period*

If the oxidizing potential was switched off and the flow rate of the eluent set to 0.5 ml/min through the night, polishing of the electrode was only necessary in intervals of more than three days. If the column was washed for 2 h with 300 ml/l aqueous methanol after each series of measurements, it could be used for more than half a year.

## Results and Discussion

### Improvement of measuring system

*Baseline noise*

If pump and injection system was rendered passive (10 min with 3 mol/l nitric acid) and mobile phase was thoroughly degassed and kept at 40 °C in a water bath, a signal-to-noise ratio of more than 100:1 (40 dB) could be achieved. Thus no electric interferences could be detected.

*Relative peak height*

In the course of the preseparation the original urine was diluted by 1:8. If dilution was lower, peak height increased, but stability of peak height was affected. If dilution was higher, the signal-to-noise ratio decreased.

*Prepurification*

The column chromatography on diethylaminoethyl-cellulose is necessary in order to achieve a satisfactory specificity of the method. Especially the 5-hydroxy-indole-3-acetic acid would interfere with homovanillic acid if this separation step were omitted. Homovanillic acid and 5-hydroxyindole-3-acetic acid separation improved with increasing salt concentrations. However, if the concentration of salt was greater than 0.3 mol/l the separation could not be done with good reproducibility, because increasing concentrations of salt result in a decrease of retention.

*Mechanization*

The measuring system was suitable for programmed sample injection combined with electronic evaluation. The fluctuations of pressure following automatic sample injection caused some peaks in the first few minutes after sample injection. But these peaks did not interfere with the detection of vanilmandelic acid and homovanillic acid.

### Reliability criteria of the method

*Precision*

The reproducibility of the method was evaluated by multiple determinations of a pooled urine (E), containing 23.1 μmol/l vanilmandelic acid and 24.4 μmol/l homovanillic acid. The within-run precision was estimated by n = 8 measurements to s = 0.9 μmol/l vanilmandelic acid and s = 2.5 μmol/l homovanillic acid (tab. 1). The between-run precision – measured at 7 days – resulted in s = 1.9 μmol/l vanilmandelic acid and s = 2.5 μmol/l homovanillic acid.

Tab. 1. Precision.

| | | Vanil-mandelic acid | Homo-vanillic acid |
|---|---|---|---|
| Within-run | n | 8 | 8 |
| | $\bar{x}$ (μmol/l) | 23.1 | 24.4 |
| | s (μmol/l) | 0.9 | 2.5 |
| | CV (%) | 3.9 | 10.1 |
| Between run | n | 7 | 7 |
| | $\bar{x}$ (μmol/l) | 21.2 | 21.2 |
| | s (μmol/l) | 1.9 | 2.5 |
| | CV (%) | 8.8 | 11.6 |

*Recovery*

The recovery was determined by adding known amounts (20.7 μmol/l vanilmandelic acid and 32.7 μmol/l homovanillic acid) to a urine pool. An average recovery (n = 12) of 99.5% (s = 5.7%) and 102.3% (s = 6.3%) was found for vanilmandelic acid and homovanillic acid, respectively.

*Linearity*

The calibration curve of the assay was linear between 2.5 and 100 μmol/l. The correlation coefficient between the calculated and the measured concentrations was determined in twelve measurements. The values of vanilmandelic acid and homovanillic acid were r = 0.995 and r = 0.993.

*Specificity*

Specificity was tested by adding potential interferants to a urine pool. No interferences could be seen with any of the tested compounds. The elimination of various interferants during the prepurification was studied by high performance liquid chromatographic measurements of their concentrations in all fractions.

Dopa (≤ 4.0 μmol/l), dopamine (≤ 8.3 μmol/l), metanephrine (≤ 3.8 μmol/l) and normetanephrine (≤ 4.8 μmol/l) were well separated by ethyl acetate extraction and did not interfere with the determination

of vanilmandelic acid or homovanillic acid. Phenolic alcohols like 3-methoxy-4-hydroxy-phenylglycol (⩽ 31.7 μmol/l) were extracted and reextracted in small amounts, but they did not adsorb on diethylaminoethyl-cellulose and therefore passed through the column before vanilmandelic acid was eluted. Heterocyclic acids like 5-hydroxyindole-3-acetic acid (⩽ 72.6 μmol/l) were well extracted and reextracted, but they were eluted from diethylaminoethyl-cellulose after vanilmandelic acid and homovanillic acid. Phenolic acids like 3,4-dihydroxymandelic acid (⩽ 6.9 μmol/l) and 3,4-dihydroxyphenylacetic acid were not eliminated by the preseparation, but they did not interfere, because they were well separated by high-performance liquid chromatography.

*Method comparison*

The comparison of the present method vs. the procedure of *Pisano* (1, 2) resulted in a good correlation (r = 0.931). The regression line: y = 0.992x + 0.072 (abscissa = photometric determination) was calculated from 20 measurements (fig. 1).

*Sensitivity*

The detection limit is based on the reproducibility of the peak height at low concentrations of vanilmandelic acid or homovanillic acid. For vanilmandelic acid or homovanillic acid concentrations lower than 2.5 or 2.6 μmol/l, resp. (≙ 0.5 mg/l) the coefficient of variation was lower than 10% (n = 8). At lower levels a precise evaluation was not possible, because the peak-height became smaller than 1 cm. In 4 samples containing 1.1 μmol/l (≙ 0.21 mg/l) vanilmandelic acid and 0.9 μmol/l (≙ 0.17 mg/l) homovanillic acid coefficients of variation of 10.9% and 13.3% were found.

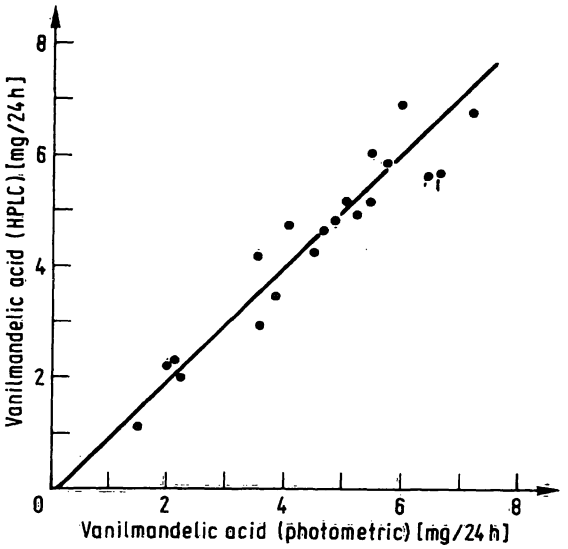


Fig. 1. Comparison of the HPLC-method and the photometric procedure; y = 0.992 x + 0.072, r = 0.931, n = 20.

## Application of the Method

Figure 2 shows a chromatogram of urine samples of (a) a healthy man and (b) a patient with a pheochromocytoma (diluted 1:10). The urinary excretion of vanilmandelic acid – determined with the procedure of *Pisano* – was 23.2 μmol/24 h (≙ 4.6 mg/24 h) for the healthy man and 326.5 μmol/24 h (≙ 64.7 mg/24 h) for the patient with pheochromocytoma. The corresponding values – determined by HPLC and amperometric detection – were 23.2 μmol/24 h (≙ 4.6 mg/24 h) and 284.1 μmol/24 h (≙ 56.3 mg/24 h) for the vanilmandelic acid and 25.3 μmol/24 h (≙ 4.6 mg/24 h) and 70.8 μmol/24 h (≙ 12.9 mg/24 h) for the homovanillic acid.

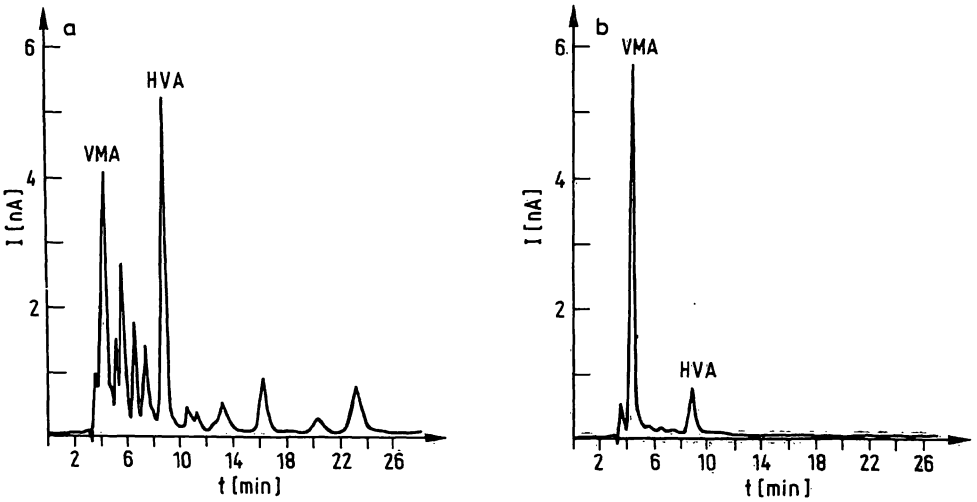


Fig. 2. Chromatogram of urine of a healthy man (a) and of a patient with pheochromocytoma (diluted 1:10, b). VMA = Vanilmandelic acid HVA = Homovanillic acid

## References

1. Pisano, J. J., Crout, J. & Abraham, D. (1962) Clin. Chim. Acta *7*, 285–291.
2. Wisser, H. & Stamm, D. (1970) this J. *8*, 21–26.
3. Rich, W., Johnson, E., Lois, L., Kabra, P., Stafford, B. & Marton, L. (1980) Clin. Chem. *26*, 1492–1498.
4. Morrisey, J. L. & Shihabi, Z. K. (1979) Clin. Chem. *25*, 2043–2045.
5. LaBrosse, E. H., Com-Nougue, Catherin, Zucker, J.-M., Comoy, E., Bohuon, L., Lemerle, J. & Schweisguth, Odice (1980) Cancer Res. *40*, 1995–2001.

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