Journal of Chromatography, 310 (1984) 379—385
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2194

Note

Analysis of vanillylmandelic acid, homovanillic acid and 5-hydroxyindoleacetic acid in human urine by high-performance liquid chromatography and fluorometry

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(First received December 28th, 1983; revised manuscript received May 16th, 1984)

Determination of 3-methoxy-4-hydroxymandelic acid (vanillylmandelic acid, VMA) in urine is a diagnostic aid for the detection of phaeochromocytoma. Probably the most well known and still widely used method was presented by Pisano et al. [1]. Recently, urinary 3-methoxy-4-hydroxyphenylglycol (MHPG) quantification has also been considered valuable either for detection of phaeochromocytoma or in the diagnosis of mental pathology and its treatment [2, 3]. Neuroblastomas and ganglioneuromas are generally accompanied [3] by increased urinary levels of 3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid, HVA). Intestinal tumours, which secrete large amounts of serotonin, are often discovered by the enhanced urinary excretion of 5-hydroxyindoleacetic acid (5-HIAA).

A number of papers have been devoted to the quantification of urinary VMA by high-performance liquid chromatography (HPLC), alone or in combination with HVA or other metabolites (see refs. 4—12, for example). Nearly all authors seem to agree on one aspect of the analysis: the complexity of the urine sample is best dealt with by solvent extraction of acidified urine (eventually followed by re-extraction) and by using the specificity of an electrochemical detector or a post-column or pre-column reaction. Anion-exchange extraction or solid—solvent extraction of VMA has been described and ultraviolet (UV) absorbance or fluorescence detection has been applied by a few authors.

However, measuring one or just a few components of the sample makes it impossible to obtain a "fingerprint" of the whole sample, which may be very valuable for screening purposes and for the elucidation of rare diseases. In a

0378-4347/84/$03.00

recent paper Thomasson et al. [13] compared the analytical performance of five methods for VMA analysis, including Pisano's method, gas—liquid chromatography (GLC) with flame ionization detection, and an HPLC method with electrochemical detection [9]. The HPLC method was the method of choice for VMA analysis, but the overall catecholamine metabolite profile was more informative with the GLC method.

We present an HPLC method that directly uses a purified urine sample, omitting laborious solvent extraction procedures. VMA or other components of interest are not isolated from the crude sample, but only those very lipophilic components that would disturb reversed-phase chromatography (due to their large capacity ratios) are removed. Hence, the "fingerprint" is hardly damaged.

MATERIALS AND METHODS

Urine samples (24 h) were collected in glass or polyethylene bottles containing 10 ml of 6 mol/l hydrochloric acid. Samples were stored at 4°C prior to analysis.

Standard solution was artificial urine (see below) containing weighed amounts of VMA (Sigma, St. Louis, MO, U.S.A.; Cat. No. H-0131), 5-HIAA (Sigma; Cat. No. H-8876) and HVA (Sigma; Cat. No. H-1252) at concentrations of 50 μmol/l, 25 μmol/l and 50 μmol/l, respectively. The pH of the standard solution was adjusted to 4.0 with 6 mol/l hydrochloric acid and 25-ml aliquots in polyethylene bottles were stored at −20°C in the dark.

Artificial urine was prepared by dissolving 20 g of urea, 2 g of creatinine, 10 g of sodium chloride, 1.7 g of potassium sulphate, 1.2 g of $MgSO_4 \cdot 7H_2O$, 2.3 g of $K_2HPO_4 \cdot 3H_2O$ and 1.4 g of KH_2PO_4 per litre of purified water. The pH was about 6.5 and the artificial urine was stored at −20°C.

Sample-purification aids were Sep-Pak C_{18} sample preparation cartridges (Waters Assoc., Milford, MA, U.S.A.) and disposable membrane filters, 25 mm in diameter, 0.45 μm pore size (SM 11306; Sartorius, Göttingen, F.R.G.). Cartridges were handled as suggested by the manufacturer.

Mobile phase A was an aqueous sodium citrate buffer pH 3.55, 0.05 mol/l (0.05 mol/l is the sum of citrate and citric acid concentrations). Mobile phase B was a mixture of acetonitrile—aqueous sodium citrate buffer pH 5.25, 0.05 mol/l (5:95, v/v). Mobile phase C was a mixture of acetonitrile—aqueous sodium citrate buffer pH 5.0, 0.05 mol/l (3:97, v/v).

HPLC-grade acetonitrile was from Rathburn Chemicals (Walkerburn, U.K.). Reagent-grade water was delivered by our Milli-Q deionization unit (Millipore, Bedford, MA, U.S.A.). Tris(hydroxymethyl)aminomethane, citric acid and trisodium citrate were obtained from E. Merck (Darmstadt, F.R.G.). Reagents not specified were of analytical grade.

Sample and standard preparation

The urine sample or standard solution was adjusted to pH 6.0 with 3 mol/l Tris. A portion of ca. 5 ml was gently passed through a C_{18} cartridge and a membrane filter, yielding a clear, colourless or nearly colourless solution. The first few millilitres of eluate and filtrate were discarded. The filtrate was brought to pH 4.0 with 2.0 mol/l citric acid. Samples prepared this way were either directly analysed or stored at −20°C.

For quantitative analysis of 5-HIAA and HVA, the urine was adjusted to pH 5.0 and passed through the membrane filter. The filtrate is either directly analysed or stored at −20° C.

Chromatographic analysis

Two mobile phases were employed at a total flow-rate of 2.0 ml/min. The pump system consisted of a Model 6000A pump, a Model 45 pump and a Model 680 Gradient Controller (all from Waters Assoc.). Instead of running the programme depicted in Fig. 1, equivalent results could be obtained when solvent switching was carried out manually using only the 6000A pump.

A 20-μl sample was injected using a Model 7125 valve (Rheodyne, Cotati, CA, U.S.A.). The column was a Radial-Pak C_{18} (10-μm particles, 10 cm × 8 mm; Waters Assoc.) pressurized by a so-called Z-module (Waters Assoc.). A corresponding guard column was inserted. Detection was performed by a Model SFM 23/B spectrofluorometer (Kontron, Zürich, Switzerland) equipped with a 20-μl flow-through cell thermostated at 25° C. Excitation was set at 285 nm, emission was detected at 315 nm. The resulting signal was displayed on a Kipp BD 40 recorder (Kipp & Zonen, Delft, The Netherlands). Peak areas and retention times were calculated by a Minigrator computing integrator (Spectra Physics, Mountain View, CA, U.S.A.).

A complete run took about 16 min for VMA quantification and 5-HIAA and HVA screening, and about 24 min for quantification of 5-HIAA and HVA. For the analysis of hippuric acid and *m*-methylhippuric acid, a variable-wavelength UV detector (Model 450; Waters Assoc.) was coupled after the fluorometric detector.

Concentrations were calculated by the well known external standard method. Minor differences in volume expansion of sample and standard caused by the sample preparation procedure could be neglected. A suitable internal standard method is under investigation.

EXPERIMENTS AND DISCUSSION

Method development

Several authors have described isocratic reversed-phase chromatography at low pH or gradient elution with decreasing eluent polarity for VMA analysis in urinary extracts (see, for example, refs. 2, 5, 8, 9). For the simultaneous determination of VMA and other metabolites more sophisticated chromatographic principles have been used. Breebaart and Grave [12] employed reversed-phase ion-pair chromatography plus a methanol gradient with direct injection of filtered urine samples. We have tested several of these approaches and found either a limited resolving power for the whole urine fingerprint or an excessive time for regeneration needed after each run. The latter holds especially for complex chromatographic systems based upon ion-pair formation with, for example, tetraalkylammoniumbromide and a gradient in the eluent's polarity. The operational system we selected is a biphasic approach based upon the pK_a values of VMA, 5-HIAA and HVA. As a result VMA is eluted at pH 3.6 while 5-HIAA and HVA are eluted at pH 5.2 and in the presence of 5% (v/v) acetonitrile. Adaptation of the column to a shift in pH and/or organic solvent

content occurs obviously much faster than the equilibration with, for example, tetrabutylammonium bromide.

In order to isolate VMA, HVA and 5-HIAA from urine, we tested several approaches of solvent—solvent or solvent—solid extraction and the use of ion-exchange procedures. Instead of this, it was found much more convenient to use Sep-Pak C_{18} cartridges, not for isolation of components of interest, but to get rid of those strongly lipophilic sample constituents that would demand rigid column-cleaning procedures after each direct injection of filtered urine using reversed-phase chromatography. Since we use these cartridges only to capture disturbing lipophilic components, the sample injected still contains the majority of the original anions, cations and other polar components. This approach is obviously advantageous for screening purposes.

Chromatographic experiences

The chromatograms shown in Fig. 1 represent typical urine samples. The conditions selected allow VMA quantification and a semiquantitative determination of 5-HIAA and HVA amongst others. The urinary constituents nominated were identified by means of their retention times and their

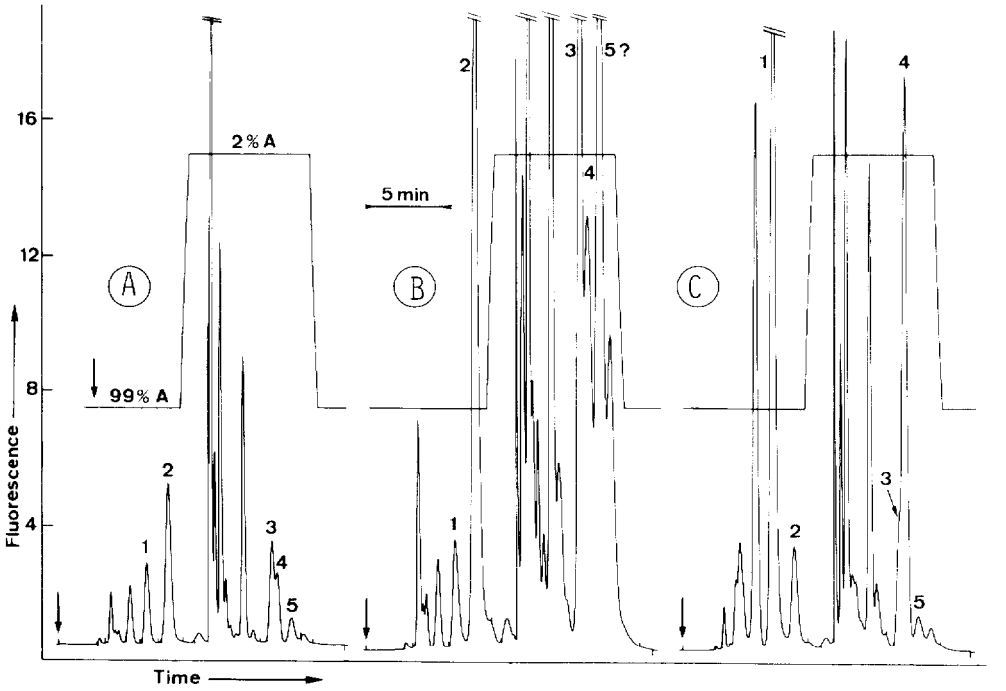

Fig. 1. (A) Chromatogram obtained from a "normal" urine sample (1830 ml per 24 h) containing 14.4 μmol/l VMA (1), tyrosine (2), 5-HIAA (3), HVA (4) and tryptophan (5). The biphasic elution is visualized by the jump from 99% mobile phase A—1% mobile phase B to 2% mobile phase A—98% mobile phase B. The time of injection is indicated by an arrow. Fluorescence intensity is given in arbitrary units. (B and C) Chromatograms from two pathological urine samples. Peaks are identified as in A. (B) Urine sample (1020 ml per 24 h) containing a normal VMA concentration of 22.5 μmol/l, but elevated levels of tyrosine, 5-HIAA and HVA. (C) Urine of a female child (360 ml per 24 h) containing a largely increased VMA level of 154 μmol/l plus an increased HVA excretion.

coelution with added pure components in at least two different chromatographic systems. For a fast (less than 12 min per analysis) VMA quantification it is adequate to increase the acetonitrile content of mobile phase B to 30% (v/v). A 3-min wash with mobile phase B immediately after the elution of VMA effectively cleans the column. However, we prefer the type of analysis represented in Fig. 1A, allowing a screening for 5-HIAA and HVA excretion. The occasional samples containing an increased amount of HVA and/or 5-HIAA and the rare urine samples submitted specially for quantitative analysis of HVA and/or 5-HIAA were subjected to analysis under different chromatographic conditions. Combining VMA, HVA and 5-HIAA into one analysis was possible by selecting an appropriate gradient. However, we found it a waste of time to run such extended programme for each VMA analysis requested.

The analysis of 5-HIAA and HVA in urine is presented in Fig. 2. Mobile phase C was used at a flow-rate of 2 ml/min. An increase to 30% (v/v) of the acetonitrile content for mobile phase C was useful for rapid cleaning of the column between analyses. For some samples the use of a modified mobile phase C, at pH 5.25 and containing 2% (v/v) acetonitrile, was found advantageous.

Preliminary experiments have demonstrated the applicability of this VMA analysis for the simultaneous determination of hippuric acid and *m*-methylhippuric acid. Optimization of such an analysis could start from the use of mobile phase B and UV absorbance measurements near 230 nm.

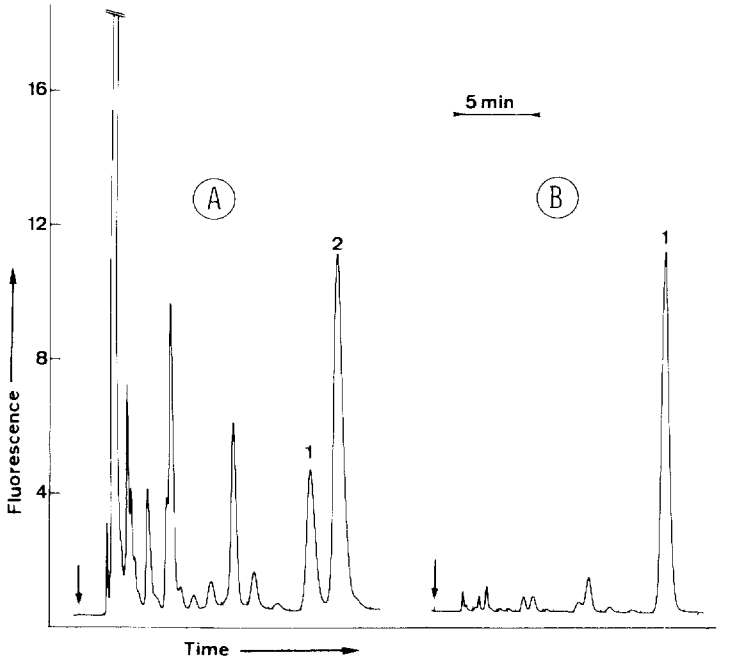

Fig. 2. (A) Quantitative analysis of 5-HIAA and HVA in the urine analysed in Fig. 1C. The urine of this 3-year-old girl (360 ml per 24 h) contained a normal 5-HIAA level (peak 1) of 25.6 μmol/l and a largely increased concentration of HVA (2) of 140.5 μmol/l. A case of ganglioneuroma was finally diagnosed. (B) Chromatogram of a diluted (1:39, v/v) urine of an adult male (940 ml per 24 h) excreting large amounts of 5-HIAA (1). In the diluted sample a concentration of 62.7 μmol/l was measured, hence the daily excretion was about 2668 μmol.

Analytical performance

On comparing the results for four samples prepared for VMA analysis as described in Materials and methods with the results obtained by directly injecting the same fresh, untreated urine samples, we found recoveries of approximately 98%, 87% and 50% for 12 μmol/l VMA, 18 μmol/l 5-HIAA and 56 μmol/l HVA, respectively. The recoveries for 5-HIAA and HVA using the procedure especially developed for their quantification was about 97% for both components. For VMA analysis the inter-assay coefficient of variation was 5.5%, calculated from the analysis of the same urine sample in ten consecutive batches. The intra-assay coefficient of variation for 5-HIAA and HVA analysis was found to be 3.2% and 2.9%, respectively (n = 5). Linearity was excellent for VMA, HVA and 5-HIAA in the concentration range tested (up to 200 μmol/l).

Comparison with Pisano's VMA analysis

We compared our HPLC method with the method of Pisano et al. [1] using urine blanks. For 27 urine samples the HPLC method found 26 ± 17% less than the concentration determined by Pisano's method. However, it is well known that several components interfere with Pisano's spectrophotometric method, apparently increasing the VMA concentration (see ref. 13, for example). The largest differences were in general associated with the most "crowded" chromatograms, suggesting such interferences for Pisano's method. An artificial increase in the HVA concentration of a urine sample by 28 μmol/l caused a 65% increase in the absorbance of the urine blank but no increase in the VMA concentration, as measured by Pisano's method. However, an increase of 15 μmol/l for MHPG (still a physiological amount) raised the VMA concentration (Pisano's method) from 16 to 34 μmol/l. The inter-assay coefficient of variation for Pisano's method was 9.1% (n = 10). Our HPLC method did not suffer from positive or negative interferences due to the presence of, for example, HVA, 5-HIAA, MHPG and vanillin in the urine.

Exploration of the fingerprint

This part of our study is still receiving much attention. Constituents of the urine samples that have been identified (in order of increasing elution time) are VMA, tyrosine, 5-HIAA, HVA and tryptophan. Physiological concentrations of MHPG can be quantified as well but require another sample preparation procedure. In due time we will expand the diagnostic usefulness of our approach.

CONCLUSION

An HPLC method for quantification of urinary VMA, HVA and 5-HIAA is presented, which includes a fast sample preparation method without extraction procedures and the ability to screen a number of physiologically important metabolites in one and the same analysis.

ACKNOWLEDGEMENTS

We thank Mr. H.J.G. Groothuyzen, Mr. L.M.L. Habets and Mr. Th.J.M. Gransier for their technical assistance and valuable practical suggestions.

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