Journal of Chromatography, 225 (1981) 440—445
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 958

Note

High-performance liquid chromatographic determination of homovanillic acid in urine using Sephadex G-10 for isolation

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(First received February 19th, 1981; revised manuscript received May 4th, 1981)

It is well known that an increased amount of homovanillic acid (3-methoxy-4-hydroxyphenylacetic acid, HVA) in urine is usually associated with neuroblastoma. Even if the excretion of vanillomandelic acid (3-methoxy-4-hydroxymandelic acid, VMA) is also elevated in urine from such patients, HVA is found to be more consistently increased [1, 2].

Numerous techniques have been developed for the determination of HVA in urine. Colorimetric measurement is preceded by laborious solvent extractions [1]. Gas chromatographic procedures result in sensitive assays but necessitate tedious extractions and derivatization steps prior to analysis [2, 3]. A specific and highly sensitive method comprised solvent extractions, thin-layer chromatography and high-performance liquid chromatography (HPLC) with electrochemical detection [4]. However, gross substance losses (reported recovery: 63 ± 5% R.S.D.) are probably inevitable consequences of such multi-step procedures and especially the solvent extraction steps.

Recently, simple two-step chromatographic procedures were developed for the determination of 5-hydroxyindole-3-acetic acid in urine [5, 6]. The present paper describes a modification of the latter method [6] for the measurement of urinary HVA. The compound was isolated from acidified urine by elution on a small Sephadex G-10 column and was then separated and determined by reversed-phase HPLC with UV-absorbance detection. This technique was used to measure the HVA excretion from patients with neuroblastoma as well as from healthy adults and children.

EXPERIMENTAL

Apparatus

The previously described [6] chromatographic isolation equipment was

0378-4347/81/0000—0000/$02.50

used with some modification. To make the columns (12 cm × 4 mm I.D.) graduated 2-ml pipettes [5] were cut off leaving 17 cm and were marked at a height of 12 cm. The same HPLC—UV equipment [5, 6] was utilized, although provided with another LDC pump, ConstaMetric I (LDC, Riviera Beach, FL, U.S.A.). Furthermore, instead of PXS 1025, Partisil-10 ODS, a column (25 cm × 4.6 mm I.D. with ZDV fittings) was prepacked with Partisil-10 ODS-2 (Whatman, Clifton, NJ, U.S.A.). The same operation conditions as in the earlier studies [5, 6] were used.

Chemicals and reagents

The mobile phase (pH 2) consisted of 220 ml of acetonitrile, 780 ml of glass-distilled and degassed water, 0.4 ml of concentrated sulfuric acid and 100 mg of sodium lauryl sulfate. Check the accuracy of the stock solution [5, 6] of HVA (Sigma, St. Louis, MO, U.S.A.) in 0.1 *M* ammonium formate buffer (pH 3) (100 μg/ml) as described (diluting to 25 μg/ml resulted in A_{280} = 0.345 ± 0.010 S.D., n = 5, for each of the HVA lots 85C-5043 and 87C-5065). Store the stock solution at 5°C (stable for 6 months). Prepare the working standards, 25, 12.5, 6.25 and 3.125 μg/ml, by serial dilution [5, 6] from the stock solution and a 37.5 μg/ml standard from the 50 and 25 μg/ml standards. Pack the isolation columns with Sephadex G-10 (Pharmacia, Uppsala, Sweden) and equilibrate with 30 ml of 0.1 *M* ammonium formate buffer (pH 3) (flow-rate 3 ml/h). Other chemicals (Sigma) were VMA, 3-hydroxyphenylacetic acid (3-HPAA), 4-hydroxyphenylacetic acid (4-HPAA), acetylsalicylic acid (ASA), salicyluric acid (SUA), 1,3,7-trimethylxantine (caffeine) and bovine serum albumin.

Procedure

Calibration process. Place sets of three equilibrated G-10 columns on the fraction collector over sets of 20 small test tubes, marked for 0.5 ml. Run 200 μl of a 25 μg/ml standard solution as described [6]. Inject 20 μl from each 0.5-ml fraction on to the reversed-phase column (detector sensitivity: 0.02 a.u.f.s.) and establish the elution range as well as the elution volume.

Sample preparation. Obtain morning urine (accumulated from midnight) voided after fasting overnight. Acidify with glacial acetic acid (0.03 ml per ml of urine), centrifuge and analyze.

Determination of HVA in urine. Carry out the determination as described in Calibration process with the following modifications. Place a set of one to ten calibrated columns over one to ten sets of two test tubes, graduated for 10 ml. Run 200 μl of the sample. Collect 4 ml and discard. Collect 3 ml in the second test tube and keep for separation and quantitative determination on the HPLC—UV system, checked daily with an HVA stock solution (average peak height for a 100 μg/ml standard at 0.64 a.u.f.s. is 46 ± 2 mm). Obtain the concentration by means of a calibration curve (peak height in mm versus concentration in μg/ml) constructed using working standards, run in the same way as the samples.

Column maintenance. After using, regenerate the G-10 columns by elution with about 30 ml of 0.1 *M* ammonium formate buffer (pH 3) and store tightly capped at 5°C (see also ref. 6, p. 462). Flush the ODS-2 column daily

with about 30 ml of methanol and store in methanol at 5°C. It may be added that the following test for albumin was performed. Proteins, if present in large amounts in urine, may be carried over into the second test tube and cause contamination of the ODS-2 column, especially with the mobile-phase composition used here. A 5 mg/ml solution in the buffer was chromatographed on G-10 as described in Calibration process and the UV absorbance (A_{280}) of the individual fraction was monitored on the spectrophotometer. The compound was eluted in the first half of the 4-ml fraction to be discarded, indicating the probability of protein-free injections all through.

RESULTS AND DISCUSSION

HVA was determined in morning-urine specimens obtained from 23 healthy individuals, ranging from 1 to 63 years of age. The HVA content ranged, without any age dependency, from 2 to 17 μg/ml, corresponding to a total output of 0.2—5 mg. An amount of 37.5 μg/ml was found to be excreted in urine from a neuroblastoma patient (Fig. 1B). No reports concerning normal HVA levels in morning urine could be found in the literature. As normal values for 24-h urine, the following data were available (mg per 24 h): 5.4 ± 1.4 [7], 9.4 ± 2.5 [8] and 4.9 ± 1.3 [9]. Otherwise, the amount of HVA in urine was generally reported [1, 4, 10] as related to the creatinine content (μg/mg or mg/g) only. Expressed in this way, an age dependency was observed [1, 10], which could be misleading. As was earlier suggested [11], the excretion

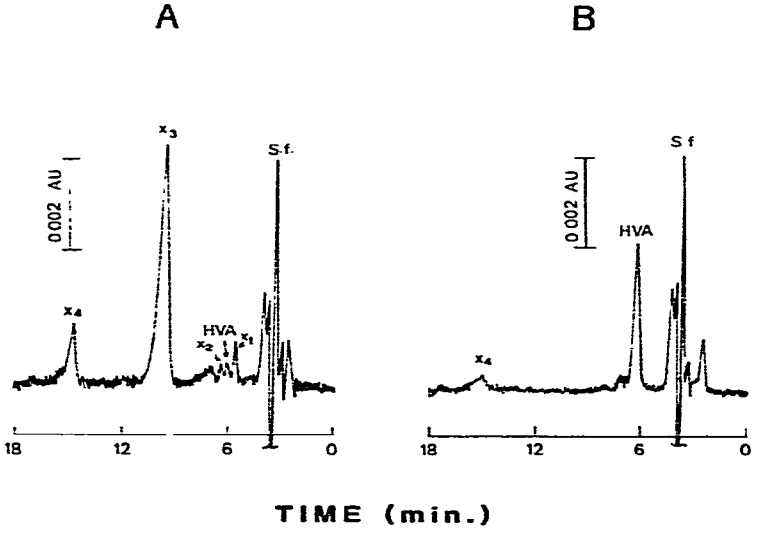

Fig. 1. Typical chromatograms of HVA in urine obtained by the present HPLC method. (A) A morning urine specimen from a healthy subject (18 months old) containing 4.1 μg/ml; and (B) from a neuroblastoma patient (1 year old) containing 37.5 μg/ml. Referring to Tables I and II and Fig. 2, the peaks x_1—x_3 were identified as follows: x_1 (t_R = 5.6 min) = 4-HPAA; x_2 (t_R = 6.6 min) = 3-HPAA; and x_3 (t_R = 9.1 min) = SUA. (t_R of x_4 was 14.1 min.)

TABLE I

RETENTION ON SEPHADEX G-10 OF SOME AROMATIC REFERENCE SUBSTANCES

Volumes of 200 μl of a 25 μg/ml* solution of each respective compound in 0.1 *M* ammonium formate buffer (pH 3)** were chromatographed on a 12 cm × 4 mm I.D. column and fractions of 0.5 ml monitored by HPLC—UV (see Calibration process). V_e = elution volume, and V_t = total volume of the mobile phase.

Compound	Elution range as Nos. of UV-positive fractions	Total volume of UV-positive fractions (ml)	V_e/V_t
HVA	9—14	3.0	3.40—3.66
VMA	6— 7	1.0	1.73—2.00
3-HPAA	12—18	3.5	3.73—4.00
4-HPAA	11—17	3.5	3.73—4.00
ASA	5—10	3.0	2.06—2.33
SUA	11—15	2.5	4.40—4.66
Caffeine***	2— 4	1.5	0.73—1.00

*ASA: 100 μg/ml.
**ASA, respective SUA, in distilled water.
***When 200 μl of a 10 mg/ml solution was chromatographed, the caffeine was eluted in fraction Nos. 1—5, corresponding to 2.5 ml of UV-positive eluate.

of most metabolites relative to creatinine appears to be very high in the first few years of life, owing to the fact that the creatinine output in that period is much lower than in later life.

In order to investigate any possible interference with HVA by some structurally related urine compounds and other aromatic compounds frequently found in urine, the corresponding reference solutions were run on G-10 and on ODS-2, respectively (see Table I and Fig. 2). The elution range on G-10 of some of the tested compounds was found to overlap the elution range of HVA. However, none of these proved to be any interference risk with regard to the analysis procedure as a whole, since those compounds from the G-10

TABLE II

RETENTION ON SEPHADEX G-10 OF THE COMPOUNDS x_1—x_4 IN FIG. 1

A 200-μl aliquot of morning urine* from a healthy subject was chromatographed on a 12 cm × 4 mm I.D. column and fractions of 0.5 ml were collected (see Calibration process). Fractions between Nos. 5 and 20 were monitored by HPLC—UV (operation as in Apparatus).

Compound	V_e/V_t **
x_1	3.73—4.00
x_2	3.73—4.00
x_3	4.40—4.66
x_4	4.40—4.66

*Drug-free (x_1, x_2 and x_4) and after intake of aspirin (x_1—x_4).
**V_e/V_t of the endogenous HVA was 3.40—3.66.

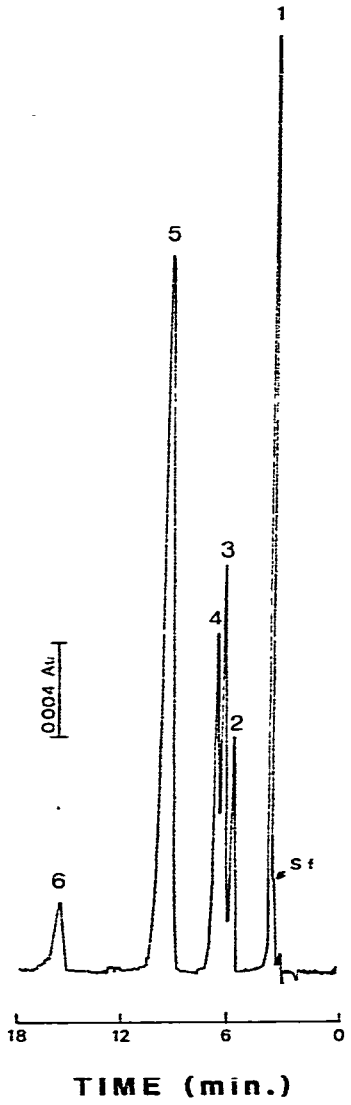

Fig. 2. Chromatogram of a pool of equal volumes of 100 μg/ml reference-substance solutions. Respective peaks were identified by injection of each stock solution separately (operating as under Apparatus): 1 (t_R = 3.5 min) = VMA; 2 (t_R = 5.6 min) = 4-HPAA; 3 (t_R = 6.1 min) = HVA; 4 (t_R = 6.6 min) = 3-HPAA; 5 (t_R = 9.2 min) = SUA and caffeine; and 6 (t_R = 15.1 min) = ASA.

column would be adequately resolved from the HVA by separation on the ODS-2 column with the mobile phase used. Moreover, the retention data obtained in this way (see also Table II) could be utilized for identification purposes also.

The HVA calibration curve is linear from 3.125 to 37.5 μg/ml, passing through the origin by extrapolation. Higher concentrations will require dilution of the urine samples. For quantities less than about 3 μg/ml the second

fractions are concentrated [6]. The lowest detectable amount of HVA in urine is 0.5 μg/ml, corresponding to 10 ng injected.

The same set of G-10 columns was used repetitively for hundreds of urine analyses required for the present work, without any reduction in capacity. By checking with a 25 μg/ml standard, 99.1% (0.6% R.S.D., $n = 5$) of HVA was recovered. The ODS-2 column retained its performance after being utilized extensively for this and other investigations during a two-year period.

The specificity, the simplicity and the quantitative recovery make the new method useful for diagnostic purposes. Furthermore, the data presented show that the general approach [5, 6] may also be utilized as a basis for simple methods for the determination of some other aromatic urine components.

ACKNOWLEDGEMENTS

This work was supported by the Swedish Natural Science Research Council (Grant No. 3953-100). The author is indebted to Professor Carl-Henrik de Verdier for being permitted to use HPLC—UV equipment and patient specimens at the Department of Clinical Chemistry, University Hospital, Uppsala, Sweden.

REFERENCES

1 J.A. Knight and R.E. Haymond, Clin. Chem., 23 (1977) 2007.
2 M.A. Brewster, D.H. Berry and M. Moriarty, Clin. Chem., 23 (1977) 2247.
3 M.S. Roginsky, R.D. Gordon and M.J. Bennet, Clin. Chim. Acta, 56 (1974) 261.
4 L.J. Felice and P.T. Kissinger, Anal. Chem., 48 (1976) 794.
5 N. Fornstedt, Anal. Chem., 50 (1978) 1342.
6 N. Fornstedt, J. Chromatogr., 181 (1980) 456.
7 T.L. Sato, J. Lab. Clin. Med., 66 (1965) 517.
8 F.S. Messiha, E. Bakutis and V. Frankos, Clin. Chim. Acta, 45 (1973) 159.
9 A.W. Stolt, J.R. Lindsay, S.P. Hansson and R. Robinson, Clin. Chim. Acta, 63 (1975) 7.
10 F.A.J. Muskiet, D.C. Fremouw-Ottevangers, B.G. Wolthers and J.A. de Vries, Clin. Chem., 23 (1977) 863.
11 D.A. Applegarth and P.M. Ross, Clin. Chim. Acta, 64 (1975) 83.