DRG® HVA ELISA (EIA-1573)

Revised 18 July 2012 rm (Vers. 2.1) **IVD**

NAME AND INTENDED USE

The HVA KIT is designed for in vitro quantitative measurement of homovanillic acid (HVA) concentration in patients' urine.

BACKGROUND

Historically, colorimetric analysis of HVA utilized the reaction of 1-nitroso-2-naphthol with biogenic amines (1), then the method was improved by substituting 1-nitroso-2-naphthol-4-sulfonic acid (2). However, the colorimetric methods are not specific for HVA due to known interference by many compounds.

Thin layer chromatography (TLC) on silica gel was another approach to determine HVA (3). This method is slow and requires special equipment. Using a flame-ionization detector and electron-capture detection, a gas chromatographic (GC) method was also developed (4,5), but has not been widely adapted due to the relatively poor sensitivity. Furthermore, gas chromatography-mass spectrometry was also used for quantitating of HVA in both serum and urine (6).

Finally high-performance liquid chromatography (HPLC) methods involving initial separation of the biogenic amines by anion-exchange chromatography, and final separation by reverse-phase (C18) chromatography have become most common, and preferred methods (7-12). These methods are reasonably rapid (although requires sample pretreatment), have excellent sensitivity and little interference from other endogenous compounds or exogenous drugs and foods.

SUMMARY AND EXPLANATIONS OF TEST

Pheochromocytomas are rare catecholamine-producing tumors. Neuroblastoma is one of the most common malignant tumors in infancy and childhood.

Biochemical examinations may be extremely important in both the diagnosis and the monitoring of therapy in these conditions. However, the urinary excretion pattern of the catecholamines are, unfortunately very variable in patients, so that single determination cannot be relied on to rule the diagnosis in or out. Most studies indicate that 2-10 times the normal range of vanillylmandelic acid (VMA) is excreted in approximately 80% of patients with neuroblastoma.

In smaller percentage, but clearly significant numbers, an excessive excretion of homovanillic acid (HVA) has also been found.

PRINCIPLE OF THE ASSAY

HVA KIT is a solid phase enzyme-linked immunosorbent assay (ELISA) based on the competition between HVA coated on a microtiter well and that in urine for the monoclonal antibody. Outlined steps are:

1. Sampling and reaction: The samples are incubated in the wells with horseradish peroxidase conjugated anti-HVA monoclonal antibody.
2. Washing: Unbound HVA and the antibody bound to urinary HVA are removed by washing with 0.9% NaCI solution.
3. Enzyme Reaction (Color Development): The amount of bound peroxidase is inversely proportional to the concentration of the HVA present in the urine sample. Upon addition of the substrate (TMB), a blue color is developed, then it is changed to yellow by adding Stopping Solution. The intensity of this is inversely proportional to the concentration of HVA in the Calibrators or urine samples.
4. Absorbance Detection: After addition of Stopping Solution, absorbance is measured at 450 nm. And the readings are converted into the concentrations from the Calibration curve.

Refer to the schematic description of Fig. 1.

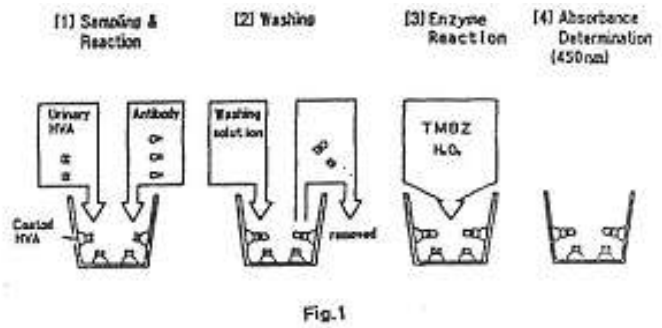


Fig.1

MATERIALS PROVIDED IN THE HVA KIT

1. HVA Coated Microwell Plate: 1 HVA coated 96-microwell plate.
2. Anti-HVA-Enzyme Conjugate: Horseradish peroxidase conjugated to an anti-HVA monoclonal antibody, 0.3 mL.
3. VMA/HVA Conjugate Diluent Solution (12 mL)
4. VMA/HVA Color Developing Reagent: Tetramethylbenzidine (TMB) solution (20 ml)
5. VMA/HVA Stopping Solution: Mixture of Sulfuric acid and Hydrochloric acid (20 ml)
6. HVA Calibrator Set: HVA solutions of 0, 0.125, 0.25, 0.5, 2, 4, 8 and 16 µg/ml in phosphate buffered saline, 0.01M, pH 7.4 (1.0 ml each).

MATERIALS REQUIRED BUT NOT PROVIDED

1. Plate reader with 450 nm filter
2. pH meter of pH paper with the range of 5.0-10.0
3. Pipettor with tips for 10, 50, and 100 µl
4. Pipettor with tips for 50 and 100 µl
5. Volumetric cylinders, 10 and 100 ml
6. Volumetric and serological pipettes, 10 ml
7. Disposable test tubes or vials
8. 5N NaOH solution
9. 5N HCI solution
10. Phophate buffered saline, 0.01 M, pH 7.4
11. Plate washer (optional)
12. Plate shaker (optional)
13. NaCl, or Saline buffer
14. 0.01 M phosphate buffered saline, pH 7.4

PREPARATION FOR THE ASSAY

1. Prepare 0.01 M phosphate buffered saline, pH 7.4.
   This solution is used to dilute all unknown urine samples prior to analysis.
2. Before beginning the test, bring all urine samples and reagents to room temperature (15-30°C) and mix well.
3. Set all reagents and urine samples ready before the assay. The entire test procedure must be performed without any interruption in order to get the most reliable and consistent results.

Revised 18 July 2012 rm (Vers. 2.1) IVD

WARNING AND PRECAUTION
- HVA KIT is designed for in vitro only.
- The components in this kit are intended for use as an integral unit.
- The components of different lots should not be mixed.
- Do not use the HVA Calibrators in this kit for other purposes (e.g. HPLC).
- Use a new pipette tip for each Calibrator or urine sample to avoid cross-contamination.

STORAGE AND STABILITY
- Store the kits at 2-8°C in refrigerator.
- Keep microwell plates in dry bag with desiccants. Open the bag only when needed.
- Expiration dates of the reagents are stated on their labels.
- Color Developing Reagent should be colorless.
- Protect the reagents and reaction mixture from exposure to direct sunlight.

SPECIMEN COLLECTION AND HANDLING
- 24-hour urine specimen should be collected with 10 ml of 6 M HCI as a preservative.
- Overnight or randomly collected urine should be acidified to a pH between 2 and 3 immediately after collection. Record the total volume and save 1-5 ml for the analysis of HVA and total creatinine.
- All samples should be refrigerated until tested. Centrifuge turbid urine samples containing crystals or sediment.

ASSAY PROCEDURE

A. Preparation of Reagents

1. **Enzyme Conjugate Solution:**
   Predilute Enzyme Conjugate by: Adding 50 uL of Anti-HVA Enzyme Conjugate to 5.5 mL of VMA/HVA Conjugate Diluent.
2. **Washing Solution**:
   Dissolve 9 grams of NaCl in 1 liter of deinoized distilled water. Commercially available normal saline can also be used.

B. Preparation of Samples

1. Take 1.0 ml of acidified urine and transfer to a disposable tube in which the pH of urine sample can be readjusted.
2. Bring pH of all samples within the range of 6 and 9 by stepwise addition of small amounts of 5N NaOH (e.g. 5 µl) while monitoring the pH either with a pH meter or using pH paper.
3. Dilute pH re-adjusted samples at a 1:10 ratio with phosphate buffered saline. The pH for diluted samples should be between 7.0 and 8.0.

C. Standard Procedure for the Assay

1. Make worksheet with Calibrators and sample identification (Fig. 2).

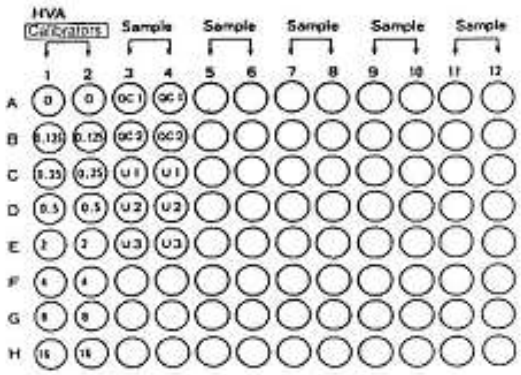


Fig.2 Example of work sheet

2. Sampling:

QC: Quality control sample U: Unknown sample

   a. Dispense 50 µl of HVA Calibrators into appropriately designated wells.
   b. Dispense 50 µl diluted (1:10) controls or samples to respective wells.
3. Addition of the Anti-HVA-Enzyme Conjugate:
   Dispense 50 µl of **prediluted** Enzyme Conjugate to each well, using a pipettor.
4. Antigen-Antibody Reaction:
   Mix the plate by moving it back and forth slow horizontal movements for a minute. A plate shaker can be used for this purpose also. Allow the plate to stand at 15-30°C, room temperature for 1 hour.
5. Washing:
   Wash only once. Removing incubation mixture by decanting the plate into a sink and blotting the plate on absorbant paper.
   Dispense 300 µl of normal saline to each well. Remove saline by decanting the plate and blotting it on absorbent paper. **Washing can also be done on a plate washer**.
6. Enzyme Reaction:
   Dispense 100 µl of VMA/HVA Color Developing Reagent to the well and allow it to stand at 15-30°C, room temperature for 25 minutes.
7. Stopping Color Development:
   Dispense 100 µl of VMA/HVA Stopping Solution to the wells.
8. Absorbance Measurement:
   Any microwell reader capable of detecting absorbance at 450nm may be used.

D. Calculation of Results

1. Using semi-log linear paper (Fig. 3) or log-logit paper (fig. 4) the Calibration curve is generated by plotting HVA concentrations on the abscissa and the absorbance on the ordinate. HVA concentration for each unknown sample is obtained from the Calibration curve.

For example:

Description	Absorbance (450 nm)		Average of B/Bo(%)	HVA (µg/ml)
HVA Calibrator (µg/ml)				
0	1.092	1.096	100.0	
0.125	0.900	0.881	81.4	
0.25	0.780	0.792	71.8	
0.5	0.648	0.631	58.5	
2	0.335	0.324	30.1	
4	0.232	0.220	20.7	
8	0.169	0.114	12.9	
16	0.108	0.108	9.9	
Sample A	0.966	0.950	87.6	0.01 x 10
Sample B	0.225	0.222	20.4	4.70 x 10
Sample C	0.555	0.518	49.0	0.97 x 10

The results obtained above indicate HVA concentration in µg/mL. When the total HVA in 24-hour urine sample is required;

$$\text{HVA (µg/ml)} \times \frac{\text{urine volume (ml)}}{1000} = \text{HVA mg/24 hours}$$

or, when HVA/Creatinine value is required;

$$\text{HVA (µg/ml)} \div \frac{\text{Creatinine (mg/dl)}}{100} = \text{HVA µg/ml Creatinine or}$$
$$= \text{HVA mg/g Creatinine}$$

2. Calculation can be made with a computer set so as to draw calibration curves based on 4 coefficient log-logit.

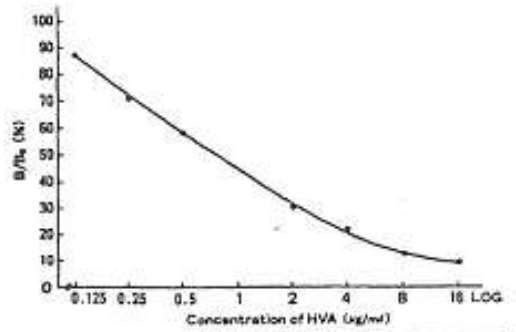


Fig.3 Calibration Curve of HVA using semi-log linear paper

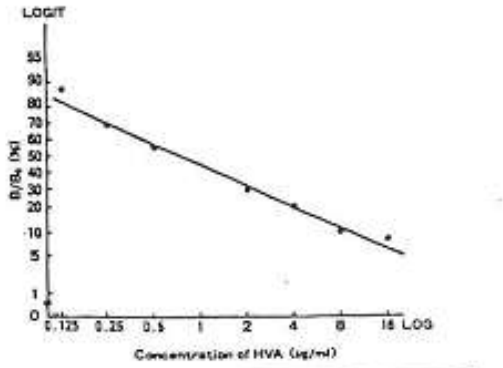


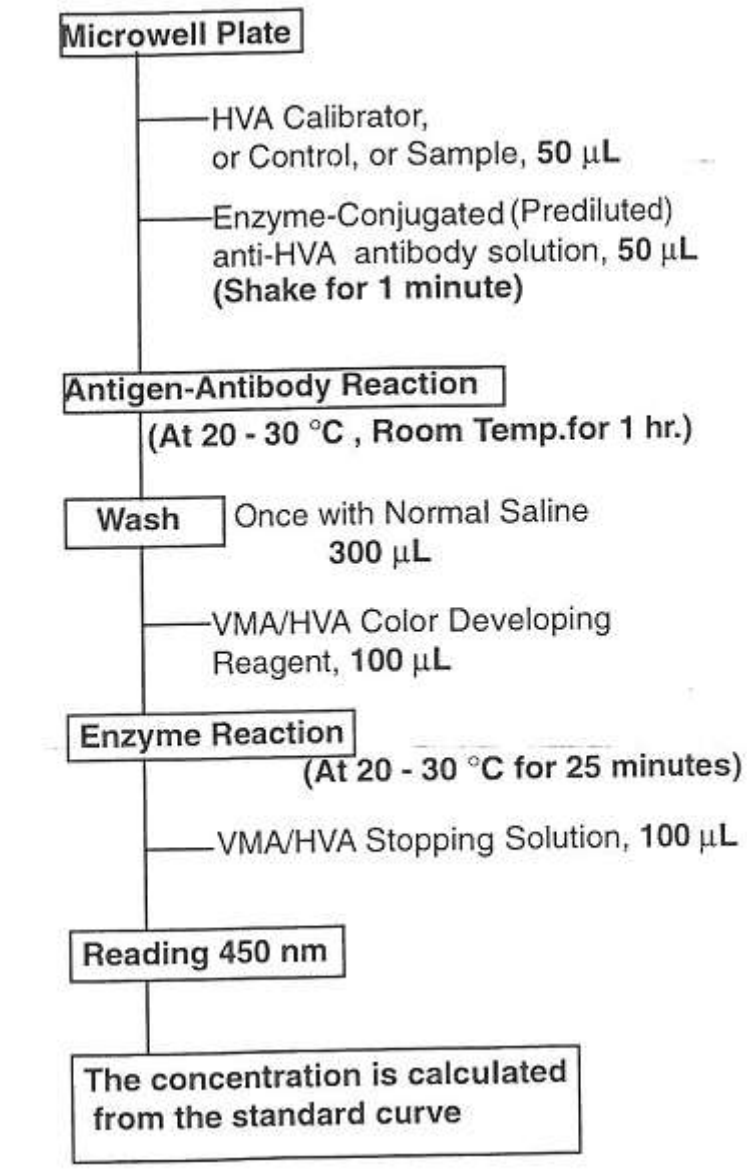
Fig.4 Calibration Curve of HVA using log-logit paper

THIS IS A REPRESENTATIVE CURVE AND SHOULD NOT BE USED TO CALCULATE RESULTS FOR UNKNOWN SAMPLES.

PROCEDURAL NOTE

1. It is very important to wash the microwells thoroughly, yet uniformly and remove any residual liquid from the wells to achieve optimal results.
2. Pipette Calibrators or urine samples into the bottom of each well. Vortex-mixing or shaking of wells after each pipetting is not required.
3. Absorbance is a function of the incubation time and temperature. It is, therefore, recommended to ensure the equally elapsed time for each pipetting without interruption.

Procedure Summary Flow Diagram



LIMITATIONS

1. This HVA Kit is designed for the quantitative determination of HVA in urine only.
2. All samples with HVA concentrations greater than 16 µl/ml should be repeated on much larger dilution(s), e.g. 1:20 or more.
3. Interference by Sodium Azide: As Sodium Azide inhibits the enzyme reaction, urine or any buffer used to dilute urine samples containing sodium azide as an antiseptic cannot be used.

QUALITY CONTROL

In order to monitor precision of the analytical performance, it is recommended that commercially available urine control samples be included in every run.

EXPECTED VALUES

Each laboratory should determine a normal range to conform with the characteristics of the population being tested. The range given here was determined from 24-hour urine collections on 280 subjects.*
Urinary creatinine was measured on Astra to assess the completeness of each collection, and mg HVA per gram creatinine was calculated.
*All urine specimens were obtained in USA.

	HVA mg/day	HVA mg/g Creatinine
Number of samples (n)	277	277
Mean Value (x)	4.35	3.63
±2 S.D. range	0.37 – 8.33	0 – 7.51
Reference Range	up to 8.30 (Fig. 5)	up to 7.50 (Fig. 6)

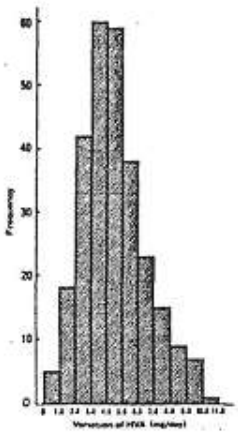


Fig.5 Distribution of total HVA excretion per day in 277 cases of normal controls

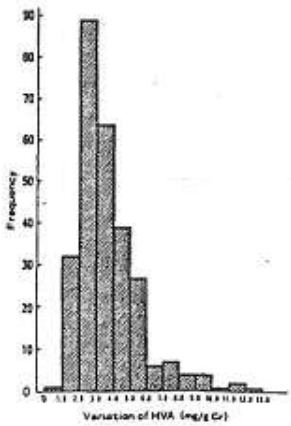


Fig.6 Distribution of HVA concentration in 277 cases of normal controls

PERFORMANCE CHARACTERISTICS

ACCURACY

a. Recovery studies were performed in urine samples from four healthy adults, to which HVA was added at various concentrations. Recovery was determined by dividing observed value by expected value for each prepared sample.

Sample	Baseline (µg/ml)	Added (µg/ml)	Expected Value (µg/ml)	Observed Value (µg/ml)	Recovery (%)
A	1.58	0.00			
		8.00	8.79	9.20	104
		4.00	5.19	5.53	106
		2.00	3.38	3.18	94
		1.00	2.48	2.34	94
B	1.05	0.00			
		8.00	8.52	7.85	90
		4.00	4.78	4.45	93
		2.00	2.91	2.84	93
		1.00	1.98	1.90	96
C	0.90	0.00			
		8.00	8.45	9.32	110
		4.00	4.67	4.38	94
		2.00	2.78	2.88	103
		1.00	1.84	1.87	101
D	3.78	0.00			
		8.00	9.88	10.96	110
		4.00	6.37	6.62	97
		2.00	5.29	5.85	110
		1.00	4.52	4.58	101

b. Linearity and Parallelism: Four urine samples and two commercial urine controls (Lypochek 1 and 2, Bio-Rad) were serially diluted with phosphate buffered saline.

c. The ratio (B/B_0) of absorbance for each dilution (B) to the absorbance of 0 µg/ml Calibrator (B_0) was calculated and plotted on log-logit paper (Fig. 7).

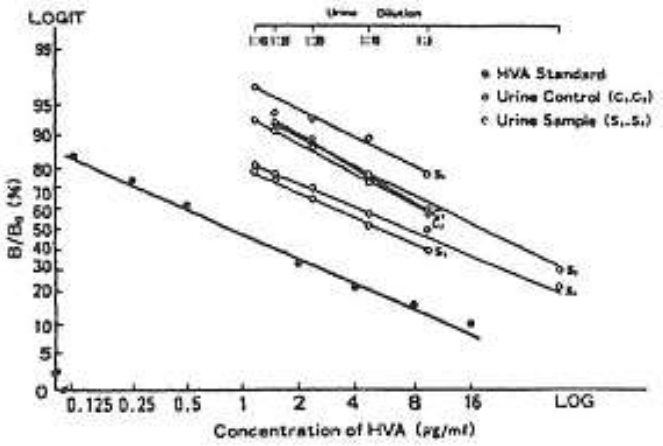


Fig.7 Linearity and parallelism test

PRECISION

a. Intra-assay coefficient of variation was evaluated in three urine samples at different HVA concentrations.

Intra-Assay	Sample A	Sample B	Sample C
N	12	26	14
Mean (µg/ml)	0.91	2.89	6.99
S.D. (µg/ml)	0.06	0.17	0.37
C.V. (%)	7.0	6.0	5.0

b. Inter-assay coefficient of variation was evaluated at three different concentrations, by analyzing the samples in 16 to 19 separate occasions.

Inter-Assay	HV1	HVA 2
N	19	16
Mean (µg/ml)	1.612	6.969
S.D (µg/ml)	0.133	0.555
C.V. (%)	8.2	8.0

SPECIFICITY

The following substances were tested for cross-reactivity of the assay. Cross-reactivity is expressed in terms of percentage of the concentration of each substance that produced 50% displacement to the HVA concentration resulting in 50% displacement.

Substance	Cross-reactivity (%) HVA
Homovanillic Acid	100
Vanillylmadelic Acid	<0.01
DL-3,4-Dihydroxymandelic Acid	<0.01
3,4-Dihydroxyphenylacetic Acid	<0.01
Metanephrine	<0.01
Vanillylpyruvic Acid	<0.01
Vanillic acid	<0.01
Dopamine	<0.01
5-Hydroxy-3-indolacetic Acid	<0.01
Vanillyllactic Acid	<0.01
3-methoxy-4-hydroxyphenyl Glycol	<0.01

SENSITIVITY

The sensitivity of this test is higher than 0.125 µg/ml. The minimal concentration of HVA is estimated to be 0.035 µg/ml. The minimal detectable concentration is defined as the concentration of HVA which corresponds to the absorbance that is two standard deviations from the mean absorbance of 20 determinations of zero dose HVA.

SAMPLE STABILITY

Sample stability was studied in two different urine samples at 4°C and -20°C for up to 5 days and 50 days, respectively. The results confirm that HVA is stable at 4°C storage at least for 5 days tested, and for up to 50 days tested when stored at -20°C.

REFERENCES

1 Goldenberg, H.: *Specificity of the nitrosonaphthol reaction: Detection of metanephrine and other guaiacol derivatives*. Clin. Chem. 13: 698, 1967.

2. Knight, J.A. and Haymond, R.E.: *Improved colorimetry of urinary 3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid).* Clin Chem. 23;2007, 1977.
3. Huck, H. and Dworzak, E.: *Quantitative analysis of catecholamine and serotonin metabolites on thin-layer plates. II, Reflectance measurements after a specific photochemical reaction.* J. Chromagogr. 74:303, 1972.
4. Brewster, N.A., Berry, D.H. and Moriarty, M.: *Urinary 3-methoxy-4-hydroxyphenylacetic (homovanillic and 3-methoxy-4-hydroxymandelic (vanillylmandelic) acids: gas-liquid chromatographic methods and experience with 13 cases of neuroblastoma.* Clin. Chem. 23: 2247, 1977.
5. Cahuhan, J. and Darbre, A.: *Determination of homovanillic, iso-homovanillic and vanillylmandelic acids in human urine by means of glass capillary gas-liquid chromatography with temperature programmed electron-capture detection.* J. Chromatogr. 813: 391, 1980.
6. Takahashi, S., Yoshhioka, M., Yoshiue, S. and Tamura, Z.: *Mass fragmentographic determination of vanilmandelic acid, homovanillic acid and isohomovanillic acid in human body fluids.* J. Chromatogr. 145: 1, 1978.
7. Soldin, S.J., Lam, G., Pollard, A., et al.: *High performance liquid chromatographic analysis of urinary catecholamines employing amperometric detection: reference values and use in laboratory diagnosis of neural crest tumors.* Clin. Biochem. 13: 285, 1980.

8. Krstulovic, A.M., Zakaria, H., Lohse L. and Bertani-Dziedzic, L.: *Diagnosis of neural crest tumors by reversephase high-performance liquid chromatographic determination of urinary catecholamine metabolites.* J. Chromatogr. 186: 733, 1979.
9. Frattini, P., Santagostine, G., Schinelli, S., et al.: *Assay of urinary vanilmandelic, homovanillic and 5-hydroxy-indole acetic acids by liquid chromatography with electrochemical detection.* J. Pharmacol. Methods 10: 193, 1983.
10. Yoshida, A., Yoshioka, M., Tanimura, T. and Tamura, Z.: *Determination of vanilmandelic acid and homovanillic acid in urine by high speed liquid chromatography*. J. Chromatogr. 116: 240, 1976.
11. Morrisey, J.L. and Shihabi, Z.K.: *Assay of 4-hydroxi-3-methoxyphenylacetic (homovanillic)acid by liquid chromatography with electrochemical detection.* Clin. Chem. 25: 2045, 1979.
12 Baursfeld, W., Diener, U., Knoll E., et al.: *Determination of urinary vanilmandelic acid and homovanillic acid by high performance liquid chromatography with amperometric detection.* J. Clin. Chem. Clin. Biochem. 20: 217, 1982.

Version_0809_RM