

TOTAL METANEPHRINES IN URINE - FAST – CODE Z13210
(by FLUORIMETRY)

BIOCHEMISTRY

Catecholamines are dihydroxyl organic compounds characterized by a phenolic ring (fig.1).

Epinephrine (E), Norepinephrine (NE), and Dopamine are the most important members of this family.

The biosynthetic pathway of catecholamines uses **L-tyrosine** as initial substratum. Chromaffin cells synthesize and store epinephrine in the adrenal medulla, while norepinephrine production occurs in the sympathetic nerve endings. Dopamine is above all a neurotransmitter in the CNS.

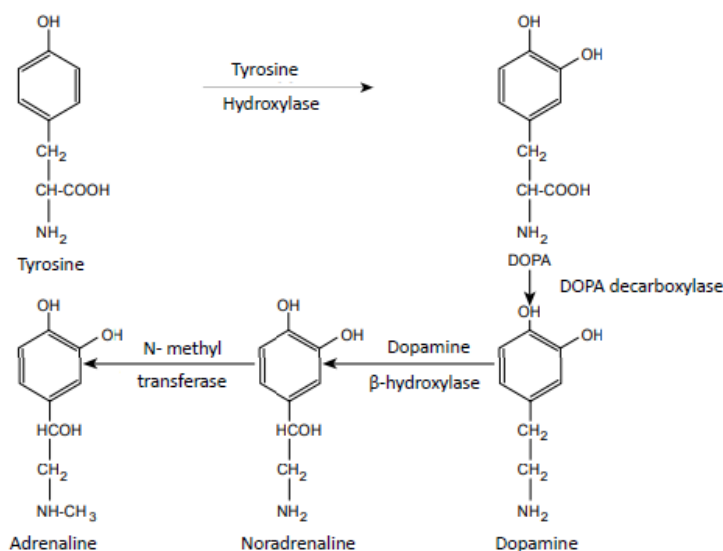


Fig 1. Biosynthesis of catecholamines

CATABOLISM

The biological effects of catecholamines terminate rapidly by uptake into the sympathetic nerve endings. The major changes that occur in these sites include their transformation into meta-O-methylated and deaminated metabolites due to **Catechol-O-methyltransferase (COMT)** and **monoamine Oxidase (MAO)** respectively and, finally, their conjugation with sulphate and glucuronide.

Homovanillic Acid is the major metabolite of **Dopamine** while **Vanillylmandelic Acid** is the main metabolite of norepinephrine and epinephrine. (fig. 2)

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This product fulfills all the requirements of Directive 98/79/EC on in vitro diagnostic medical devices (IVD). The declaration of conformity is available upon request.

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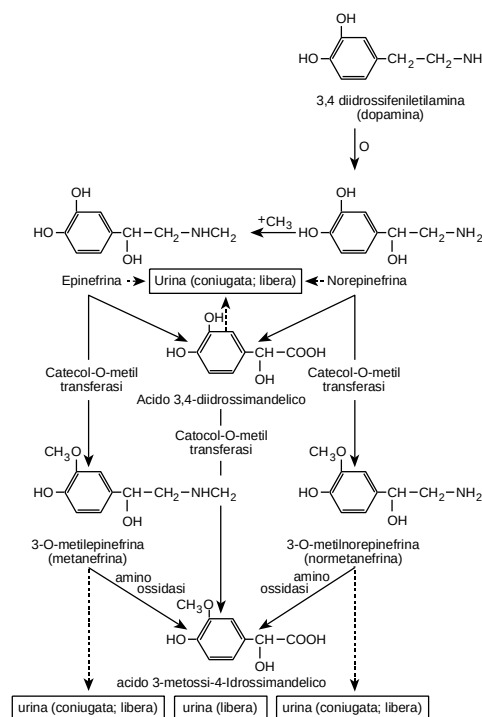


Figura 2: Metabolismo degli ormoni della midollare del surrene

Fig. 2: Metabolism of hormones of suprarenal medulla

CLINICAL BACKGROUND

Catecholamines are often determined in urine for neurological diagnosis and for monitoring the response to therapy in illnesses like **pheochromocytoma and neuroblastoma**.

Pheochromocytoma is a catecholamine-producing tumor derived from adrenomedullary chromaffin cells. More than 90% appear to be benign. They are dangerous because of their capacity to store and release catecholamines in large amounts with subsequent production of alarming syndromes including sustained hypertension, resistant to conventional treatment, and hypertensive crisis with malignant hypertension and hypertensive encephalopathy. The diagnosis of pheochromocytoma is established by demonstration of increased urinary excretion of catecholamines or catecholamines metabolites, and their concentration is often determined in urine for monitoring the response to therapy. Correctly diagnosed and properly treated, pheochromocytoma is curable; misdiagnosed or improperly treated, it is fatal.

Neuroblastoma, the second most common solid tumor that occurs during childhood, may appear almost anywhere along the sympathetic nervous system chain. This tumor synthesizes and secretes catecholamines and metabolites like DOPA, dopamine, VMA, and homovanillic acid. Assessment of urinary and plasma catecholamines are useful in establishing a diagnosis and following the results of treatment.

ASSAY PRINCIPLES AND CHARACTERISTICS

This method allows to completely purify Metanephrines (NORMETANEPHRINE and METANEPHRINE) by an organic and specific complexant reagent. This way allows to have a complete extraction using a C-18 solid phase column and after derivatization you can read by a fluorimetric detector.

TECHNICAL FEATURES

Principle of the Method :

The Metanephtrines (NORMETANEPHRINE, METANEPHRINE and 3-METHOXYTYRAMINE), after purification with Clean up columns, are derivatized with 2 specific reagents and incubate at 70 °C for 15 minutes. The derivatized solution is injected in HPLC.

Recovery :

> 98 %

Sensitivity (LLOD) :

1,5 µg/l for each Analyte

Linearity :

5 - 8.000 µg/l for each Analyte

Minimum Concentration analyzable (LLOQ) :

5 µg/l for each Analyte

24 h URINE Normal Values :

NORMETANEPHRINE 88 – 444 µg/24 h

METANEPHRINE 52 – 341 µg/24 h

3-METHOXYTYRAMINE 55 – 247 µg/24 h

Accuracy intra serie (relative error %)

Metanephtrine :

Ci	Cs
40 µg/l	300 µg/l
8,61%	6,68%

Accuracy inter serie (relative error %)

Metanephtrine :

Ci	Cs
40 µg/l	300 µg/l
6,08%	4,68%

Reproducibility intra serie (coefficient of variation %)

Metanephtrine :

C LLOQ	Cm	CUP
5 µg/l	100 µg/l	600 µg/l
9,27%	3,97%	2,43%

Reproducibility inter serie (coefficient of variation %)

Metanephtrine:

C LLOQ	Cm	CUP
5 µg/l	100 µg/l	600 µg/l
9,98%	4,96%	2,58%

Coefficient of correlation R2 + Dev Std :

Metanephtrine 0,9995 ± 0,0003

Accuracy intra serie (relative error %)

Normetanephtrine :

Ci	Cs
40 µg/l	300 µg/l
6,14%	3,75%

Accuracy inter serie (relative error %)

Normetanephtrine :

Ci	Cs
40 µg/l	300 µg/l
6,70%	3,15%

Reproducibility intra serie (coefficient of variation %)

Normetanephtrine :

C LLOQ	Cm	Cs
5 µg/l	100 µg/l	600 µg/l
13,84%	3,01%	2,41%

Reproducibility inter serie (coefficient of variation %)

Normetanephtrine:

C LLOQ	Cm	Cs
5 µg/l	100 µg/l	600 µg/l
11,24%	5,16%	1,80%

Coefficient of correlation R2 + Dev Std :

Normetanephtrine 0,9991 ± 0,0008

Accuracy intra serie (relative error %)**3-Methoxytyramine :**

Ci	Cs
40 µg/l	300 µg/l
5,16%	2,46%

Accuracy inter serie (relative error %)**3-Methoxytyramine :**

Ci	Cs
40 µg/l	300 µg/l
6,83%	3,30%

Reproducibility intra serie (coefficient of variation %)**3-Methoxytyramine :**

C LLOQ	Cm	Cs
5 µg/l	100 µg/l	600 µg/l
12,63%	4,99%	2,08%

Reproducibility inter serie (coefficient of variation %)**3-Methoxytyramine:**

C LLOQ	Cm	Cs
5 µg/l	100 µg/l	600 µg/l
11,55%	3,78%	3,53%

Coefficient of correlation R2 + Dev Std :

3-Methoxytyramine 0,9983 ± 0,0017

Components of the kit :**Reagent A** – Conditioning Solution N° 1, **1 x 120 ml****Reagent B** – Conditioning Solution N° 2, **1 x 110 ml****Reagent C** – Conditioning Solution N° 3, **1 x 120 ml****Reagent D** – Diluting Solution, **1 x 120 ml****Reagent E** – Wash Solution N° 1, **1 x 120 ml****Reagent N** – Wash Solution N° 2, **1 x 220 ml****Reagent F** – Eluting Solution, **1 x 80 ml****Reagent G** – Test Solution, **1 x 5 ml****Reagent H** – Internal Standard Solution, **1 x 5 ml****Reagent I** – Hydrolysis Solution, **1 x 10 ml****Reagent K** – Buffer Solution, **1 x 50 ml****Reagent J** – Derivatization Solution, **1 x 10 ml****Reagent L** – Starter Solution, **1 x 10 ml****Reagent P** – Indicator Solution, **1 x 1 ml****Reagent Q** – Stabilization Solution, **1 x 10 ml****Reagent M** – Mobile Phase, **5 x 500 ml****Urine Calibrator lyophil.**, **2 x 5 ml****Clean-up Columns**, **100 pcs**

All the components are ready-to-use. Stability: 3 years at room temperature ($\leq 20\text{ }^{\circ}\text{C}$), except for the Urine Calibrator lyophil, see Warnings for stability and storage.

Store at 2-8 °C**Dilute 1:10 before use with Water HPLC grade****packed separately****Code Z12516 (packed separately – see data sheets)****Minimum Instrumental equipment required:**

Isocratic HPLC System with loop of 20µl
Spectrofluorimetric Detector $\lambda_{\text{EX}}=275\text{ }\lambda_{\text{EM}}=325$
Chromatograms Recorder

Optional Equipment:

Autosampler
Sample Automatic Preparator

24 h Urine Collection Procedure:

24-hour urine must be collected into a container with 5 ml (Child) or 10 ml (Adult) of HCl 5 M for each urine litre. After collection, 10 ml of urine should be delivered to the lab with the indication of the total diuresis. **Laboratory should verify that the delivered urine has a pH between 2,0 and 4,0. *If the pH is > 7 the sample may not be suitable for testing.*** Delayed analyses require sample freezing at –20°C or less. Stable over 2 months.

PREANALYTICAL PROCEDURE

Pipette in a tube :

- 100 µl of **Reagent G – Test Solution**
- 100 µl of Water HPLC grade

INJECTION :

- Inject 20 µl of this solution in the chromatographic system.

Verify that the Test Solution has retention time similar to fig. 3. If the Test is all right you can start with the analytical procedure; if not, check the functionality of the analytical system

Important : Don't use this solution to calibrate!

ANALYTICAL PROCEDURE

SAMPLE HYDROLYSIS

1. Pipette in 10 ml glass tube with Teflon screw cap:
 - 1 ml of Samples or Calibrator or Controls
 - 50 µl of **Reagent H – Internal Standard Solution** **diluted 1:10 before use with Water HPLC grade**
2. Add 500 µl of Water HPLC grade
3. Add drop by drop about 100 µl of **Reagent I – Hydrolysis Solution**
4. Close hermetically the caps and make sure of their seal.
5. Incubate at 90 °C for 30 min.
6. Keep cooling; then add 10 µl of **Reagent P – Indicator Solution**
7. Add 100 µl of **Reagent Q – Stabilization Solution**
8. Add 500 µl of **Reagent K – Buffer Solution**

Check that the solution turns blue/green. If this does not happen, make sure that the pH of the solution has reached a value between **4.5 and 6.0**; if the pH has not reached the value of the range adjust it with NaOH 2M or HCl 1M.

SAMPLES, CALIBRATOR AND CONTROLS PURIFICATION

STEP 1: Pipette in a tube:

	Calibrator	Samples	Controls
Reagent D – Diluting Solution	1000 µl	1000 µl	1000 µl
Reagent U – Hydrolyzed Calibrator	1000 µl	/	/
Hydrolyzed Samples	/	1000 µl	/
Hydrolyzed Controls	/	/	1000 µl

STEP 2: Clean-up Column Preparation :

Place the Clean-up Columns, for Calibrator + Samples + Controls, on Vacuum Manifold or in a suitable system of percolation and identify them with a marking pen.

STEP 3: Clean-up Column Conditioning :

Pipette in Clean-up Columns:

- 1,0 ml of **Reagent A – Conditioning Sol. N° 1** and discharge the percolated liquid.
Don't let the columns be dry!
- 1,0 ml of **Reagent B – Conditioning Sol. N° 2** and discharge the percolated liquid.
Don't let the columns be dry!
- 1,0 ml of **Reagent C – Conditioning Sol. N°3** and discharge the percolated liquid.
Don't let the columns be dry!

WARNING! ELUTE SLOWLY FOR ABOUT 1 MINUTE!

STEP 4: Clean-up Columns Samples loading

Pipette in Clean-up Columns the Sample, the Calibrator and the Controls prepared at **STEP 1:**

- 2,0 ml of **Calibrator**,
 - 2,0 ml of **Samples**,
 - 2,0 ml of **Controls** ,
- Discharge the percolated liquid

STEP 5: Clean-up Columns Washing

Pipette in Clean-up Columns:

- 1,0 ml of **Reagent E – Wash Solution N° 1** and discharge the percolated liquid.
Let dry with a light flux of air!
- 2,0 ml of **Reagent N – Wash Solution N° 2** and discharge the percolated liquid.
Let dry with a light flux of air!

STEP 6: Elution

Place the Clean-up Columns in clean tubes. Pipette in Clean-up Columns:

- 500 µl of **Reagent F – Eluting Sol.** and collect the eluate.

STEP 7: Add:

- 20 µl of **Reagent J – Derivatization Solution**
- 20 µl of **Reagent L – Starter Solution**

Vortex for 10 seconds

Incubate at **70 °C** for **15 minutes**.

- Cooling and add 400 µl of Water HPLC grade

Vortex for 10 seconds

N.B.: at this step, the sample is stable 2 days at 2-8 °C

INJECTION :

- Inject 20 µl of this solution in the chromatographic system.

TOTAL METANEPHRINES in URINE FAST – Warnings

REAGENT G: TEST SOLUTION

NORMETANEPHRINE	About 1300 µg/l
METANEPHRINE	About 700 µg/l
3-METHOXYTYRAMINE	About 160 µg/l

SPECTROFLUORIMETRIC DETECTOR

λ _{EX}	275 nm
λ _{EM}	325 nm
GAIN	MEDIUM or HIGH
INTEGRATION TIME	4 or 5

HPLC COLUMN PROTECTION

To save the analytical column Reverse Phase POROSHELL PFP (100 x 4,6 mm – 2,7 µm), the use of Javelin Prefilters (1 x 10 pcs), code S90199511 is obligatory.

HPLC COLUMN CONDITIONING

Install a new analytical column Reverse Phase POROSHELL PFP (100 x 4,6 mm – 2,7 µm). Disconnect the detector and flux a solution of H₂O : Acetonitrile (20 : 80 v/v) set flow at 1 ml / min. **Don't recycle the washing solutions.** Flux a solution of H₂O : Acetonitrile (20 : 80 v/v) set flow at 1 ml / min. **Don't recycle the washing solutions.** To achieve shorter retention times, the thermostat of the 45 °C column is optional. Condition further the column with the mobile phase at a flow of 1 ml / min. for 30 min. And discharge first 60 ml. Finally inject the derivatized Chemical Standard and verify the quality of the HPLC run.

For Metanephrines it is NOT possible to make analysis at recycling phase. If room temperature is > 20 °C store the Mobile Phase at 2-8 °C between an analytical session and another.

COLUMN CLEANING

Disconnect the detector. Turn the column upside down and flush 15 ml of H₂O: Acetonitrile (80: 20 v / v) to flow of 1.0 ml / minute and discharge. Flux a solution of H₂O: Acetonitrile (30: 70 v / v) for 30 minutes by discharging it. **It is recommended to wash the Analytical Column at the end of each analytical session.** The column should be stored in a solution of H₂O: Acetonitrile (30: 70 v / v).

INJECTION NEEDLE WASHING

Water / Ethanol or Methanol or Acetonitrile (80 : 20 v/v).

HPLC PARAMETERS

LOOP	20 µl
FLOW	0,9 ml/min.
PRESSURE	About 189 bar
COLUMN THERMOSTATATION	45°C

ACCESSORIES AND CONSUMABLES

CODE	DESCRIPTION	PACKAGING
Z12516	Calibrator in urine for Metanephrines	4 x 5 ml
Z10559	Control in urine for Biogenic Amines - Levels 1 and 2	2 x 5 x 5 ml
Z13211	Mobile Phase	4 x 500 ml
Z695975408	Poroshell 120, PFP (100 x 4,6 mm - 2,7 µm) Analytical Column	1 Pc
S90199511	Javelin Prefilters	1 x 10 Pcs
S29057U	Standard glass vials of 2 ml with screw cap	1 x 100 Pcs



TOTAL METANEPHRINES IN URINE FAST (Reference Chromatograms)

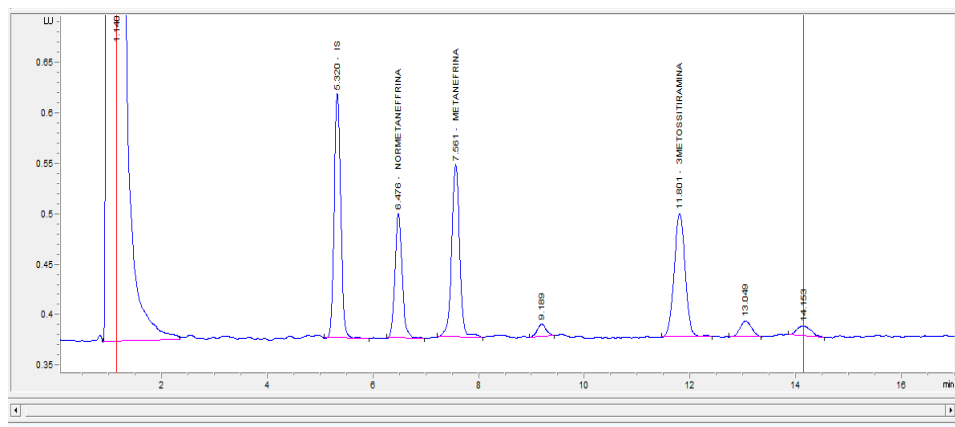


Fig. 1:	Urine Calibrator		
	R.T. 5.32	Internal Standard	
	R.T. 6.47	Normetanephrine	422,3 µg/l
	R.T. 7.51	Metanephrine	372,7 µg/l
	R.T. 11.80	3-Methoxytyramine	218,1 µg/l

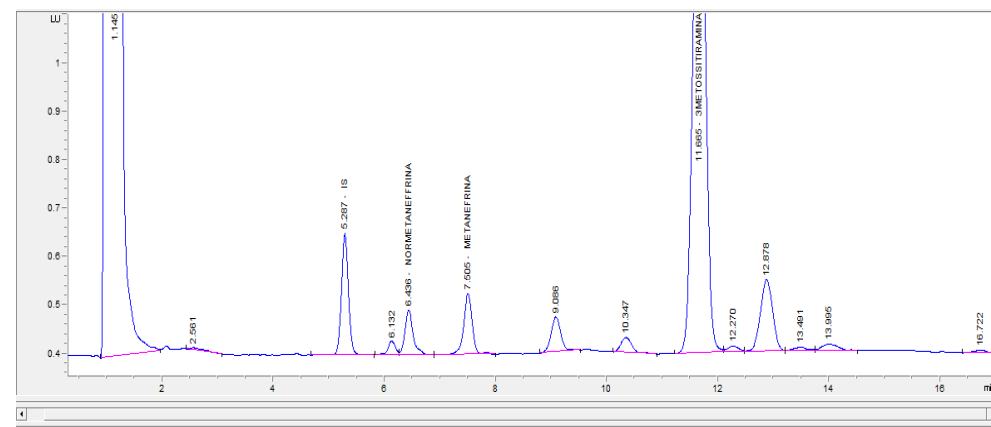


Fig. 2:	Urine Sample		
	R.T. 5.28	Internal Standard	
	R.T. 6.43	Normetanephrine	
	R.T. 7.50	Metanephrine	
	R.T. 11.66	3-Methoxytyramine	

TOTAL METANEPHRINES IN URINE FAST (Reference Chromatograms)

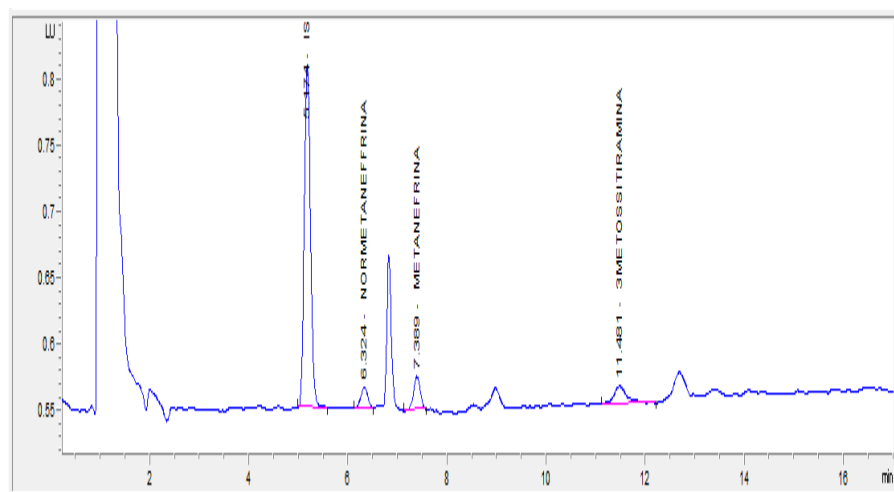


Fig. 3:	Urine Low Control		
	R.T. 5.17	Internal Standard	
	R.T. 6.32	Normetanephrine	57,0 µg/l
	R.T. 7.38	Metanephrine	42,0 µg/l
	R.T. 11.48	3-Methoxytyramine	29,0 µg/l

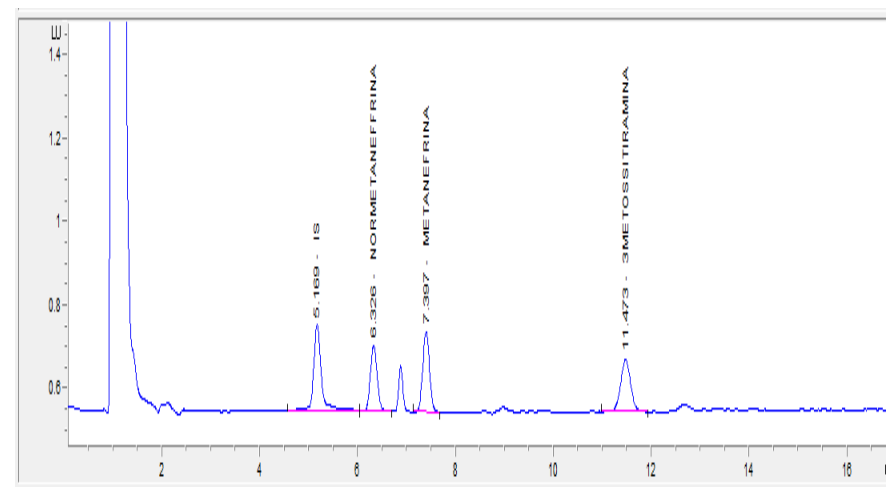


Fig. 4:	Urine High Control		
	R.T. 5.18	Internal Standard	
	R.T. 6.32	Normetanephrine	809,0 µg/l
	R.T. 7.39	Metanephrine	684,0 µg/l
	R.T. 11.47	3-Methoxytyramine	412,0 µg/l