**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.

![Biosynthesis of catecholamines](image)

**CATABOLISM**

The biological effects of catecholamines terminate rapidly by uptake into the symphathetic nerve endings. The major changes that occur in these sites include their transformation into meta-O-methylated and deaminated metabolites due to **Cathechol-O-methyltransferase (COMT) and monoamine Oxidase (MAO)** respectively and, finally, their conjugation with sulfate and glucuronide. **Homovanillic Acid** is the major metabolite of **Dopamine** while **Vanillylmandelic Acid** is the main metabolite of norepinephrine and epinephrine. (fig. 2)
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. Assays of urinary and plasma catecholamines are useful in establishing a diagnosis and following the results of treatment.

ASSAY PRINCIPLES AND CHARACTERISTICS

The present method allows to analyse Catecholamines in urine without Clean-up procedure. After derivarization 50 µl of the eluate are injected and read by a Fluorimetric Detector.
**TECHNICAL FEATURES**

**Principle of the Method:**

Catecholamines (Epinephrine, Norepinephrine, and Dopamine) after purification with a specific resin are treated with 2 specific reagents and incubated at 37°C for 30 min. or at 70 °C for 15 min. The eluate is added with 300 µl of H₂O HPLC grade and directly injected into the HPLC System.

**Recovery:**

> 98 %

**Sensitivity:**

< 0,02 µg/l

**Linearity:**

0,02 - 2.000 µg/l

**Normal Urine Values in 24 h:**

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>EPINEPHRINE</td>
<td>2 - 22 µg/24 h</td>
<td></td>
</tr>
<tr>
<td>NOREPINEPHRINE</td>
<td>20 - 81 µg/24 h</td>
<td></td>
</tr>
<tr>
<td>DOPAMINE</td>
<td>40 - 400 µg/24 h</td>
<td></td>
</tr>
</tbody>
</table>

**Components of the kit:**

| Reagent A – Purification Resin, 1 x 50 ml | Store at 2-8 °C |
| Reagent B – Buffer Solution, 1 x 100 ml | Store at 2-8 °C |
| Reagent B1 – Purification Buffer, 1 x 50 ml | Dilute 1:10 before use |
| Reagent C – Eluting Solution, 1 x 200 ml | See Warnings |
| Reagent D – Starter Solution, 1 x 2 ml |          |
| Reagent E – Derivatization Solution, 1 x 2 ml |          |
| Reagent G – Test Solution, 1 x 5 ml |          |
| Reagent H – Internal Standard Solution, 1 x 5 ml |          |
| Reagent U – Urine Calibrator Lyophil., 1 x 5 ml |          |
| Reagent M – Mobile Phase, 2 x 500 ml |          |

**Minimum Instrumental equipment required:**

Isocratic HPLC System with loop of 50µl
Fluorimetric Detector \( \lambda_{EX}=360 \text{ nm} \) \( \lambda_{EM}=490 \text{ nm} \)
Chromatograms Recorder

**Optional Equipment:**

Autosampler
Operational Computer

**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 2.5 and 3.5. 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 1,5 ml vial with cap:
- 400 µl of **Reagent C** – Eluting Solution
- 50 µl of **Reagent G** – Test Solution
- 50 µl of **Reagent H** – Internal Standard Solution (diluted 1:10 with H₂O HPLC grade)
- 20 µl of **Reagent D** – Starter Solution
- 20 µl of **Reagent E** – Derivatization Sol.
- Incubate at 70 °C for 15 min.  
  *Cooling at Room Temperature*

**INJECTION**:

- Inject 50 µl of the solution into 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**

**STEP 1**: Verify of pH

Pipette in a plastic or glass tube of 10 ml:
- 1 ml of **Reagent B** – Buffer Solution
- 1 ml of Sample or Calibrator or Control  
  *Vortex for 20 sec.*

- Verify that the pH of the Samples, Calibrators and Controls is between 7 and 8; eventually correct with a little bit of NaOH 2N.
- Shake well by inversion the Reagente A  
  *(Repeat the operation every 10 samples)*

**STEP 2**: Samples Purification

Pipette in a plastic or glass tube of 10 ml:
- 500 µl of **Reagent B1** - Purification Buffer
- 500 µl of **Reagent A** – Purification Resin
- 1000 µl of Samples, Calibrators or Controls prepared in the **STEP 1**  
  *Vortex for 20 sec.*

**STEP 3**: Centrifuge at 4.000 rpm for 2 minutes

**STEP 4**: Take the surrnatant and drop it away

**STEP 5**: Elution

Pipette in the tube with the precipitate:
- 2 ml of **Reagent C** - Eluting Sol.  
  *Vortex for 20 sec.*

**STEP 6**: Centrifuge at 4.000 rpm for 2 minutes

**STEP 7**: Derivatization

Pipette in plastic tube of 1,5 ml with cap:
- 200 µl of Sample from Purification procedure
- 20 µl of **Reagent D** – Starter Solution
- 20 µl of **Reagent E** – Derivatization Sol.  
  *Vortex for 20 sec.*

**STEP 8**: Incubate at 37 °C for 30 minutes or at 70 °C for 15 minutes.

**STEP 9**: Add 300 µl of H₂O HPLC grade  
  *Vortex for 20 sec.*

**INJECTION**:

- Inject 50 µl of this solution in the chromatographic system.

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**Rev. N° 024**  |  Free Catecholamines in urine with resin by Fluorimetry  |  March 2010
**FREE CATECHOLAMINES in URINE - Warnings**

**REAGENT G : TEST SOLUTION**

<table>
<thead>
<tr>
<th>Catecholamine</th>
<th>Concentration (µg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOREPINEPHRINE</td>
<td>about 90.75</td>
</tr>
<tr>
<td>EPINEPHRINE</td>
<td>about 28.80</td>
</tr>
<tr>
<td>DOPAMINE</td>
<td>about 545.25</td>
</tr>
</tbody>
</table>

**REAGENT U : URINE CALIBRATOR LYOPHIL. - Lot n° 001**

<table>
<thead>
<tr>
<th>Catecholamine</th>
<th>Concentration (µg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOREPINEPHRINE</td>
<td>88.02</td>
</tr>
<tr>
<td>EPINEPHRINE</td>
<td>26.86</td>
</tr>
<tr>
<td>DOPAMINE</td>
<td>690.80</td>
</tr>
</tbody>
</table>

**Use and Reconstitution:** The calibrator must be used to calibrate the HPLC system. It must be treated as a urine sample adding exactly 5 ml of HPLC grade H₂O, than mixing upside down. Wait 15 minutes before use.

**Stability:** 36 months if stored at 2-8°C. After reconstitution is stable 12 hours at room temperature, 7 days at 2-8°C and 1 month at -20°C. Don’t use after expiry date.

**Packaging:** 1 x 5 ml

**Warnings:** The calibrator derives from human urine, so it could be potentially infected. It must be handled with care.

**FLUORIMETRIC DETECTOR PARAMETERS (Es. : Jasco Fluorimeter 821 FP)**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>λ of EXCITATION</td>
<td>360 nm</td>
</tr>
<tr>
<td>λ of EMISSION</td>
<td>490 nm</td>
</tr>
<tr>
<td>GAIN</td>
<td>1 x 100</td>
</tr>
<tr>
<td>INTEGRATION PARAMETERS</td>
<td>5 sec.</td>
</tr>
</tbody>
</table>

**HPLC COLUMN PROTECTION**

To save the analytical column Reverse Phase GENESIS 4,6 x 150 mm, 4 µ, the use of Metasaver PreColumn Filter 0.5 um (1 x 10 pcs.) cod. ZA6005 is obligatory.

**HPLC COLUMN CONDITIONING**

Install a new analytical C₁₈ Reversed-Phase column (GENESIS 4.6 mm x 150 mm, 4 µ). Disconnect the detector and flux 30 ml of H₂O : Acetonitrile (20 : 80 v/v) solution and subsequently 30 ml of H₂O per HPLC, set flow at 1.2 ml / min. Don’t recycle the washing solutions. Filter the mobile phase with a vacuum system and a suitable filter of 0.22 µ or 0.45µ. Condition the column with the mobile phase at a flow of 1.2 ml / min. and discharge the first 30 ml. Condition further on the column for 30 min. also at recycling phase. It is possible to make analysis at recycling phase, providing that you filter the same mobile phase with a filter of 0.22 µ before any analytical run. 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. Flux 30 ml of water and discharge. Flux a solution of H₂O HPLC grade : Acetonitrile (20:80 v/v) for 30 min. Discharge. A new use of the column needs a flow of 30 ml of H₂O before a new conditioning with the mobile phase.

**HPLC PARAMETERS**

<table>
<thead>
<tr>
<th>Parameter</th>
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<tbody>
<tr>
<td>LOOP</td>
<td>50 µl</td>
</tr>
<tr>
<td>FLOW</td>
<td>1.2 ml/min</td>
</tr>
<tr>
<td>PRESSURE</td>
<td>About 100 bar</td>
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**INTEGRATOR HP – 3394 / 3395 / 3396 PARAMETERS**

<table>
<thead>
<tr>
<th>Parameter</th>
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<tbody>
<tr>
<td>ATTENUATION</td>
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**OPERATIONAL COMPUTER PARAMETERS**

IN CONFORMITY WITH THE SPECIFICATION OF COMPUTER SOFTWARE
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<th>CODE</th>
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<tbody>
<tr>
<td>Z10116</td>
<td>Urine Calibrator lyophil. for Catecholamines</td>
<td>4 x 5 ml</td>
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<tr>
<td>Z10557</td>
<td>Urine Control lyophil. for Biogenic Amines, Level 1</td>
<td>5 x 5 ml</td>
</tr>
<tr>
<td>Z10558</td>
<td>Urine Control lyophil. for Biogenic Amines, Level 2</td>
<td>5 x 5 ml</td>
</tr>
<tr>
<td>Z10559</td>
<td>Urine Control lyophil. for Biogenic Amines, Levels 1 and 2</td>
<td>2 x 5 x 5 ml</td>
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<tr>
<td>ZFM15960E</td>
<td>Analytical Genesis C18 (150 x 4,6mm -4 u)</td>
<td>1 PK</td>
</tr>
<tr>
<td>ZA6005</td>
<td>Metasaver Precolumn Filter 0.5 um</td>
<td>1 x 10 PK</td>
</tr>
<tr>
<td>S29057U</td>
<td>Standard glass vials of 2 ml with screw cap</td>
<td>1 x 100 PK</td>
</tr>
</tbody>
</table>
FREE CATECHOLAMINES IN URINE BY FLUORESCENCE
(Reference Chromatograms)

**Fig. 3: Test Solution**
- R.T. 4.739 Norepinephrine
- R.T. 6.833 Internal Standard
- R.T. 11.635 Epinephrine
- R.T. 17.243 Dopamine

**Fig. 4: Lyphocheck Control Level 1**
- R.T. 4.665 Norepinephrine 46 µg/l
- R.T. 6.633 Internal Standard
- R.T. 11.174 Epinephrine 14 µg/l
- R.T. 16.533 Dopamine 82 µg/l

**Fig. 5: Lyphocheck Control Level 2**
- R.T. 4.670 Norepinephrine 195 µg/l
- R.T. 6.633 Internal Standard
- R.T. 11.189 Epinephrine 81 µg/l
- R.T. 16.551 Dopamine 488 µg/l