# Measurement of Urinary Free Metanephrines

## Using Automated On-Line Solid-Phase Extraction-High Performance Liquid Chromatography-Tandem Mass Spectrometry

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## Introduction

Usually, in urine total (free + conjugated) fractionated metanephrine (MN) and normetanephrine (NMN) are measured for the diagnosis of pheochromocytoma (Fig. 1), a catecholamine-pruducing tumor of the adrenal medulla.

However, this total fraction is influenced by several exogenous factors, such as diet[1], and needs a laborious and uncontrolled hydrolysis step in sample preparation, when compared to urinary free metanephrines. Also the measurement of the free fraction is recommended.[2]

The aim of the current study was to develop a method for the quantification of urinary free metanephrines, using automated on-line solid phase extraction coupled to liquid chromatography with tandem mass spectrometric detection (XLC-MS/MS) in order to replace urinary total (deconjugated) metanephrines or be an alternative for plasma free metanephrines.[3]

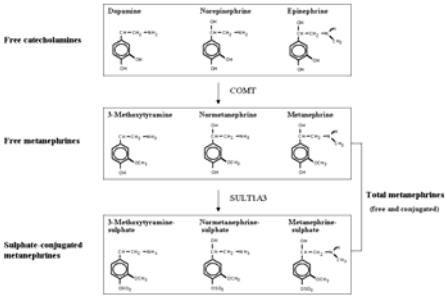


**Figure 1:** Nomenclature of metanephrine fractions occurring in urine and plasma after 3-O-methylation and sulphate-conjugation.

## Methods

**Working procedure:**

**Dilute sample**

**Add internal standard**

**Analyze (automated)**

**Calculate**

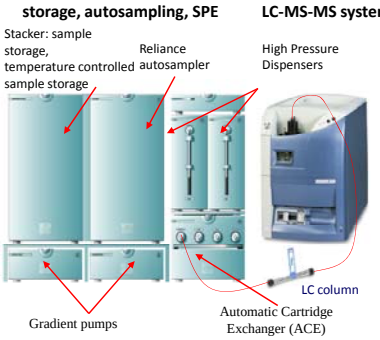


**Fig. 2: Schematic view of the instrument arrangement**

**On-line Solid-Phase Extraction (Fig. 2)**
Instrumentation: Symbiosis™ Pharma
Sample volume: 100 µL (= 5 µL urine)
Cartridge: 10x1 mm Oasis WCX® (Waters)
Weak cation exchange
Elution by 5 mM ammonium formate @ pH 3 in AcN
Column: Atlantis HILIC (Waters)
2.1 mm x 50mm; 3.0 µm
Total XLC-MS/MS cycle time per sample : 8 min (Fig. 3)

**LC Conditions**
Mobile Phase A: 100 mM Amm form @pH3
Mobile Phase B: Acetonitrile
Gradient from 95% B - 80% B
Flow rate: 0.400 ml/min

**Mass Spectrometry**
Instrumentation:
Quattro premier™ MS/MS (Waters);
Detection: positive electrospray ionisation (ESI+) in MRM. Protonation of MN, NMN and 3-MT and Characteristic fragmentation upon collision induced dissociation (CID) to daughter ions (Table 1).

**Table 1: MRM transitions MN, NMN and 3-MT**

| | Protonation [M+H⁺ | Transition 1 Quantifier | Transition 2: Qualifier |
|---|---|---|---|
| MN | 198 | 180→148 | 198→180 |
| MN-d3 | 201 | 183→151 | 201→183 |
| NMN | 184 | 166→134 | 166→106 |
| NMN-d3 | 187 | 169→137 | 169→109 |
| 3-MT | 169 | 151→91 | 151→119 |
| 3-MT-d4 | 173 | 155→95 | 155→123 |

## Results

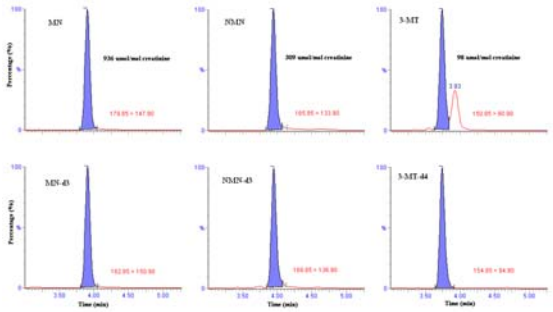


**Fig. 3: Mass specific chromatograms of urine from a pheochromocytoma patient. Chromatograms of MN, NMN and 3-MT and their deuterated internal standards were obtained in multiple reaction monitoring (MRM) mode.**

- Intra-assay analytical variations (n=20): < 6%.
- Inter-assay analytical variation (n=20): < 16%
- Linearity (R>0.99): LOQ - 4000 nmol/L.
- Recoveries: 70% (MN), 50% (NMN), and 90% (3-MT).
- Limits of quantitation: 1.6 nmol/L (MN), 1.3 nmol/L (NMN) and 3.2 nmol/L (3-MT).
- Sample stability up to 7 days at 5°C, up to 24 h at RT and up tp 3 freeze-thaw cycles.
- Reference intervals (n=120): 4.6-20.4 µmol/mol creatinine (MN), 6.9-29.0 µmol/mol creatinine (NMN) and 12.5-46.4 µmol/mol creatinine (3-MT).
- Urinary free metanephrines should be measured in 24 hr urine samples due to diurnal variation (Fig. 4). This biological variation especially important for NMN.

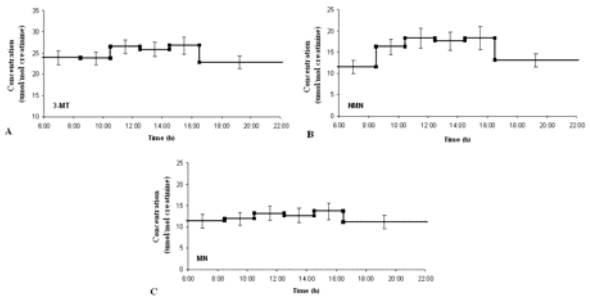


**Fig. 4: Biological intraday variation of urinary free MN, NMN and 3-MT in 26 healthy subjects.** Concentrations (µmol/mol creatinine) are given as mean concentrations with ± 95% confidence interval at five time intervals during one day.

## Discussion

Metanephrines are excreted in urine as both free amines and conjugates, with the free levels representing < 3% of the total urinary MNs excretion.[4] The conjugated fraction has a lower specificity than the free fraction due to exogenous influences as diet,[5] because the gastrointestinal tract provides a major site for conjugation. In addition, free metanephrines, in either plasma or urine are the recommended markers for the biochemical diagnosis of pheochromocytoma. The analysis of plasma free metanephrines requires advanced equipment due to concentrations in the nmol/L range.[3]
The measurement of urinary free metanephrines should however be a useful alternative for both plasma free and urinary total metanephrines. With respect to plasma, urinary concentrations are higher which implies less advanced equipment. In comparison with urinary total metanephrines, the poorly controlled hydrolysis step for deconjugation can be eliminated from the protocol. Our method on advanced equipment (XLC-MS/MS) should therefore easily be transferred to a simpler HPLC method.

## Conclusions

This study shows that from an analytical perspective the measurement of urinary total metanephrines can be replaced by that of the free fraction, although diagnostic sensitivity and specificity remain to be investigated. This results in less sample preparation (no hydrolysis) and lower imprecision and time per analysis (high-throughput) in comparison with the measurement of total MNs.

## References

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