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ClinRep® kit for the analysis of Metanephrines in Urine

Introduction

Catecholamines exert numerous physiological actions in the cardio-vascular system and in the intermediary metabolism. Various tumors of neurogenic origin are responsible for a substantial rise in catecholamine production [1]. Catecholamines are primarily inactivated (up to 90 %) by the re-uptake into the adrenergic nerve endings. The remaining catecholamines are metabolised in the cells of the target organs or in the liver. Catecholamines are metabolised by:

- Methylation of the hydroxyl groups in the meta-position by catechol-o-methyltransferase
- Oxidative deamination by monoamine oxidase to the corresponding aldehyde and subsequent oxidation or reduction to acids (vanillylmandelic acid, homovanillic acid) or to alcohol (3-methoxy-4-hydroxy-phenyl-glycol).

Catecholamine metabolism by catechol-o-methyltransferase results in the formation of the methoxy analogues normetanephrine (N-Meta), metanephrine (Meta) and 3-methoxytyramine (3-Meth). The metabolites are released into the blood stream and excreted mainly by the kidney.

The amount of catecholamines and their metabolites is not only an index of the activity of the sympathetic nervous system but also a diagnostic aid in certain diseases [2-5]. The quantitative determination of the catecholamines and their metabolites is of great clinical significance for the diagnosis and treatment of neurogenic tumors [6-8].

Until now, the diagnostic procedure included first of all the measurement of the catecholamines and the acid metabolites vanillylmandelic acid and homovanillic acid in the urine [9, 10]. However, normal excretory rates of vanillylmandelic acid and homovanillic acid do not exclude the presence of a tumor of the sympathetic nervous system. The incidence of false negative results in the diagnosis of tumors of neurogenic origin is up to 9 % [11]. Further investigations are thus necessary for reliable diagnosis. Several studies showed that by including the excretory profiles of metanephrines in the diagnostic considerations the incidence of false negative results is reduced [12].

Various analytic procedures have been used for the quantitative determination of metanephrines in urine. Besides paper chromatography [13] and gas chromatography [14], the most widely used procedure has been photometric determination after oxidation of the extracted metanephrines to vanillin [15].

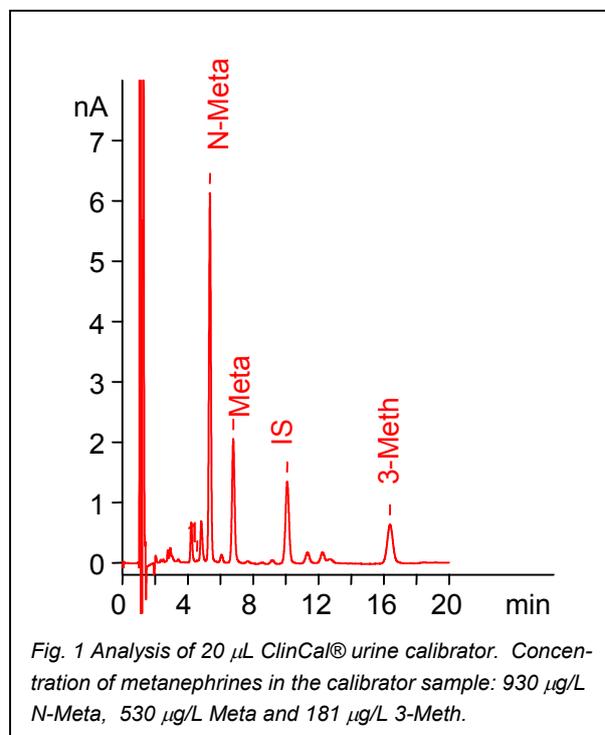


Fig. 1 Analysis of 20 µL ClinCal® urine calibrator. Concentration of metanephrines in the calibrator sample: 930 µg/L N-Meta, 530 µg/L Meta and 181 µg/L 3-Meth.

This method cannot, however, distinguish between metanephrine, normetanephrine and 3-methoxytyramine and, furthermore, detects related substances, such as the monohydroxy analogues tyramine, synephrine and octopamine which also can be present in urine.

Today, LC-EC has been established as a fast and reliable method for the determination of catecholamines and metabolites in plasma and urine [16,17]. The ClinRep® complete kit for metanephrines in urine of Recipe GmbH (Munich, Germany) is a standardised kit for the sample preparation and routine analysis of urinary metanephrines [18]. In this application note the results are reported of the analysis of urine samples using a ClinRep® kit with an ALEXYS 110 LC-EC system.

Method

One Recipe ClinRep® complete kit contains all the necessary chemicals and (calibration) materials for sample preparation. Prior to analysis the urine samples are first acid hydrolysed to free the conjugated metanephrines followed by a sample clean-up step on an extraction column. The sample preparation procedure can be summarized as follows:

- Acidified urine is mixed with 20 µL internal standard (IS) and hydrolyzed for 30 minutes.

- After hydrolyses the sample is diluted and applied to a ClinRep® sample preparation column to trap the unconjugated methanephrines.
- The column is then washed followed by elution of the metanephrines and 20 µL injection in the LC system.

The quantification of the metanephrines in the urine samples is performed by means of a single-point calibration method using a urine calibrator. The ClinCal® urine calibrator supplied in the ClinRep® kit is a lyophilised urine sample with a known amount of metanephrines. The urine calibrator should be processed via the same sample preparation method as the urine samples. An example chromatogram of a urine calibrator analysis is shown in figure 1.

An IS is used to compensate for recovery losses during the sample preparation step. The IS response of the samples is compared to that of a directly injected standard solution (ClinTest® standard) to determine the recovery. The sample response is then interpolated to 100% recovery to establish the real metanephrine concentration in the urine samples.

Set-up

HPLC	ALEXYS 110 LC-EC system with DECADE II SDC (p/n 190.0035)
Flow cell	GC type flow cell with Ag/AgCl saltbridge REF
Column	ClinRep® Analytical column for metanephrines in urine

Furthermore, for sample preparation (hydrolysis and extraction) a water bath, pH meter, vortex mixer, hydrolysis tubes and column rack are required.

LC-EC conditions

Flow rate	1.0 mL/min
Sample	20 µl, extracted with ClinRep® sample preparation columns
Mobile phase	ClinRep® catecholamine buffer [#]
Temperature*	T _{D2 SDC} 30°C (separation & detection), T _{AS110} 4°C (sample cooling)
E-cell	720 mV (vs. Ag/AgCl sat'd)
Range	50 nA/V
I-cell	0.1 – 3.0 nA
ADF	0.1 Hz
Analysis time	20 minutes

[#]) mobile phase was recycled during experiments. ^{*}) minimum actual oven & tray temperature which can be reached is dependent on ambient conditions.

Analysis of ClinChek® controls

For quality control of the analytical determination Recipe ClinChek® urine controls have been used in both the normal (level I) and the pathological range (level II).

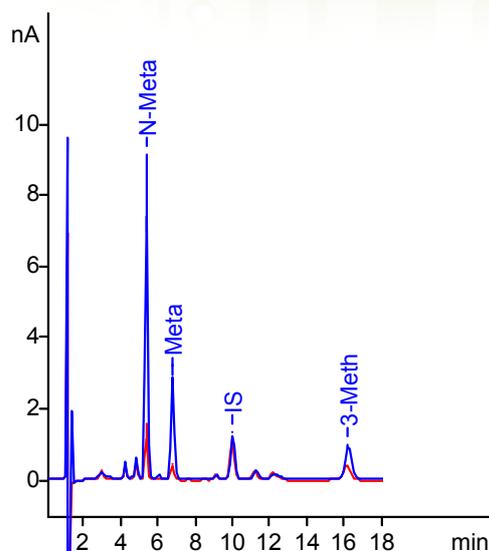


Fig. 2. Overlay of chromatograms of 20µL injections of ClinChek® control level I and II.

Table I. Calculated concentration of urine controls level I and II (n=4). Concentration range specified by Recipe is given for reference (source: data sheet supplied with controls).

Component	Specified conc (µg/l)		Calculated conc (µg/l)	RSD (%)
	Min	Max		
<i>Control level I</i>				
Nor-Meta	238	358	269	0.8
Meta	112	168	137	0.5
3-Meth	110	164	132	2.0
<i>Control level II</i>				
Nor-Meta	1222	1832	1414	0.1
Meta	722	1084	839	0.3
3-Meth	222	332	275	1.4

The control samples are lyophilised urine samples which have to be processed in the same way as the urine samples. Both Control I and Control II were analysed and the analyte concentrations quantified using the ClinCal urine calibrator. For both urine controls level I and II the determined metanephrine concentrations were within the concentration ranges specified by Recipe on the urine control data sheet (see table I).

Analysis of urine samples

A urine sample (A) was collected from an apparently healthy volunteer and analysed multiple times to determine the recoveries, LOD, intra- and inter-assay precision of the method. The intra-assay precision of the method was determined using urine sample A. The urine sample was worked-up 5 times on two different days and duplicate analysis were performed to determine the relative standard deviation (RSD, %).

Table II. Intra-assay precision of urine sample A,

$n=5$ (samples) \times 2 (duplicate injections).

Component	RSD (%)	Conc. ($\mu\text{g/l}$)
<i>Day 1</i>		
Nor-Meta	3.0	451
Meta	3.9	201
3-Meth	4.5	220
<i>Day 2</i>		
Nor-Meta	2.4	502
Meta	4.1	231
3-Meth	4.1	216

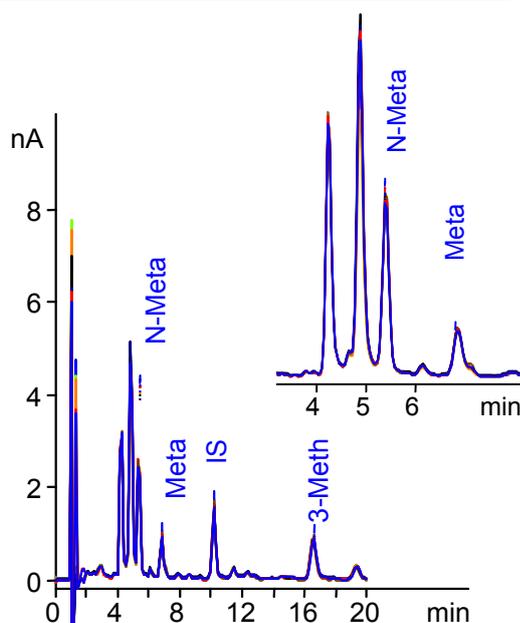


Fig. 3. Overlay of 10 chromatograms of 20uL injections of urine sample A. Top-right: zoom in on N-Meta and Meta A peaks.

The intra-assay RSD's for all components were typically smaller than 5%. For all urine samples, controls and calibrator recoveries typically in the range of 50 – 80% were found, compared to a directly injected standard. The concentration limit of detection (C_{LOD}) for the method was approximately 0.5 $\mu\text{g/L}$ for all metanephrines. The C_{LOD} here is based on a 20 μL injection and defined as the concentration that gives a signal that is three times the peak-to-peak noise. The method is linear for the determination of the metanephrines in the concentration range from 5 – 7500 $\mu\text{g/L}$ [18]. To determine the inter-assay RSD's the results of two days were averaged for sample A, see table III.

Table III. Inter-assay precision of urine sample A, $n=5$ (samples) \times 2 (duplicate injections) \times 2 (days).

Component	RSD (%)	Conc. ($\mu\text{g/l}$)
<i>Sample A</i>		
Nor-Meta	6.1	476
Meta	7.9	216
3-Meth	3.4	217

The inter-assay RSD's for the metanephrines were typically smaller than 8%.

Conclusion

The ClinRep® complete kit for metanephrines in urine provides a standardised method for the sample preparation and analysis of urinary metanephrines using LC-EC.

Parts and configuration used

190.0035 [#]	ALEXYS 110 LC-EC system with DECADE II SDC
4000*	ClinRep® complete kit, Metanephrines in urine
4030*	ClinRep® Analytical column for Metanephrines in urine
8021*	ClinChek® urine control, level I
8022*	ClinChek® urine control, level II

[#]) A GC-type flow cell with Ag/AgCl saltbridge REF should be ordered separately.

*) Parts from Recipe GmbH, Sandstrasse 37-39, D80335 Munich, Germany.

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