

# ANTEC LEYDEN

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## ClinRep® kit for the analysis of HVA, VMA and 5-HIAA in Urine

### Introduction

The catecholamines norepinephrine, epinephrine and dopamine exercise a number of important functions within the central and peripheral nervous system [1]. The degradation pathway of catecholamines has a number of steps. Vanillylmandelic acid (VMA) is the major end product of catecholamine metabolism; homovanillic acid (HVA) is the analogue end product of dopamine. The analysis of plasma and urinary catecholamines and their metabolites is crucial for the detection and diagnosis of chromaffin cell tumours and a number of other diseases [3-6]. In case of tumours of the central nervous system, there is an extremely high production of catecholamines and consequently high excretion of the corresponding metabolites in urine. Pheochromocytoma, neuroblastoma and ganglioneuroblastoma belong to this group of diseases [7-11]. Serotonin, another biogenic amine, is mainly located in the enterochromaffine cells of the small intestine. Biochemically, serotonin is degraded by the enzymes monoaminooxidase (MAO) and aldehydedehydrogenase (Ald-DH) to 5-hydroxyindoleacetic acid (5-HIAA). Malignant growth in the enterochromaffine cells of the intestine results in increased production of serotonin and hence an increased excretion of 5-HIAA.

LC-EC has been established as a fast and reliable method for the determination of catecholamines and metabolites in plasma and urine [12,13]. In this application note the optimised system settings for the ALEXYS 110 are reported for the analysis of HVA, VMA and 5-HIAA in urine using the ClinRep kit of Recipe GmbH (Munich, Germany).

### Method

One Recipe ClinRep® complete kit contains all the necessary chemicals and (calibration) materials for sample preparation and analysis of 100 assays [14]. Prior to analysis a sample clean-up step is applied to the urine samples using a ClinRep® sample preparation column. The sample preparation procedure consists of the following steps:

- 250 µL urine sample is mixed with 50 µL internal standard (IS) and diluted to a volume of 5 mL.
- 1 mL of the diluted urine is applied to the sample preparation column to trap the acidic metabolites.
- The column is subsequently washed with an ammonium solution, followed by two washing steps with a boric acid solution.
- Finally 2 mL of eluting agent is applied to the sample preparation column and 20 µL of the eluate is injected into the LC system for analysis.

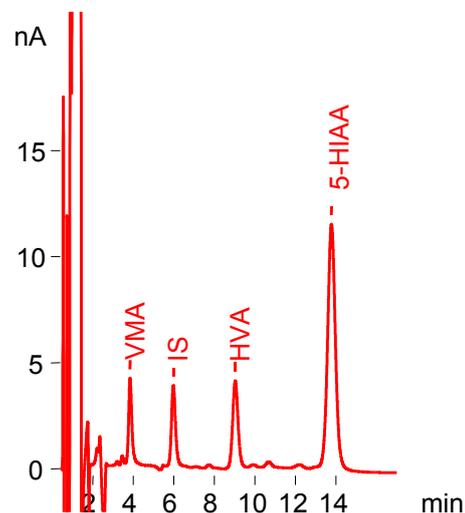


Fig. 1 Analysis of 20 µL ClinCal® urine calibrator reconstituted in 0.2M HCl. Concentration of acidic metabolites in the sample: 11 mg/L VMA, 10.2 mg/L HVA and 16.7 mg/L 5-HIAA.

The quantification of the acidic metabolites 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 lyophilized urine sample with a known amount of HVA, VMA and 5-HIAA. 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. The lyophilized calibrator was reconstituted in 0.2 M HCl.

An internal standard (IS), iso-VMA, 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 concentration of HVA, VMA and 5-HIAA 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 HVA, VMA and 5-HIAA in urine



Furthermore, for sample preparation a pH meter, vortex mixer, and centrifuge (800 x g) are required.

### LC-EC conditions

Flow rate	0.9 mL/min
Sample	20 µl, extracted with ClinRep® sample preparation columns
Mobile phase	ClinRep® HVA, VMA and 5-HIAA in urine buffer <sup>#</sup>
Temperature*	T <sub>D2 SDC</sub> 30°C (separation & detection), T <sub>AS110</sub> 4°C (sample cooling)
E-cell	850 mV (vs. Ag/AgCl sat'd)
Range	50 nA/V
I-cell	2 – 20 nA
ADF	0.1 Hz
Analysis time	18 minutes

<sup>#</sup>) 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).

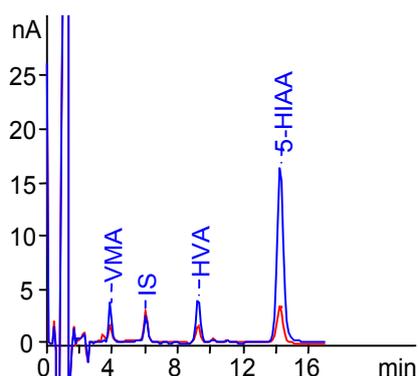


Fig. 2. Overlay of chromatograms of 20µL injections of ClinChek® control level I (red curve) and II (blue curve).

Table I. Calculated concentration of urine controls level I and II (n=2). Concentration range specified by Recipe is given for reference (source: data sheet supplied with controls).

Component	Specified conc (mg/l)		Calculated conc (mg/l)	RSD (%)
	Min	Max		
<i>Control level I</i>				
VMA	4.4	6.6	6.5	0.1
HVA	4.0	6.1	4.6	0.1
5-HIAA	4.1	6.9	5.0	0.1
<i>Control level II</i>				
VMA	13.2	19.8	16.4	0.2
HVA	12.2	18.2	14.1	0.2
5-HIAA	21.0	31.4	28.9	0.6

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 reconstituted in 0.2 M HCl, analysed and the analyte concentrations quantified using the ClinCal urine calibrator. For both urine controls level I and II the determined concentrations of VMA, HVA and 5-HIAA 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 and intra-assay precision of the method. 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) x 2 (duplicate injections) for two days.

Component	RSD (%)	Conc. (mg/l)
<i>Day 1</i>		
VMA	8.3	5.5
HVA	3.5	4.9
5-HIAA	2.1	8.5
<i>Day 2</i>		
VMA	4.6	6.2
HVA	3.3	5.4
5-HIAA	2.7	7.7

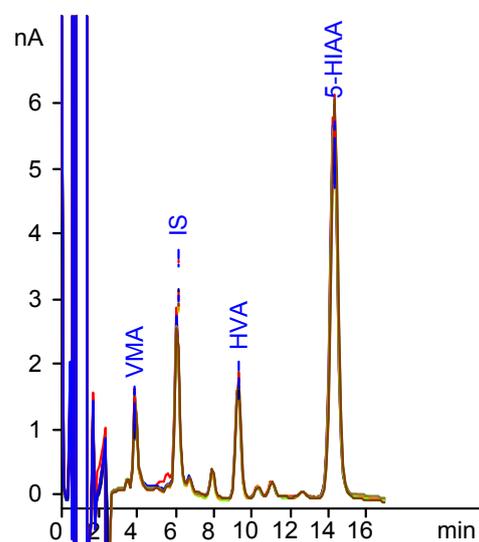


Fig. 3. Overlay of 6 chromatograms of 20µL injections of urine sample A on day 2.

The intra-assay RSD's for HVA and 5-HIAA were typically smaller than 4%. The RSD for VMA was larger on day 1. This due to an interfering peak in this urine sample, which complicated peak integration.

For all urine samples, controls and calibrator recoveries typically in the range of 55 – 75% were found, compared to a directly injected standard. The concentration limit of detection ( $C_{LOD}$ ) for the method was approximately 20  $\mu\text{g/L}$  for all metabolites. The  $C_{LOD}$  here is based on a 20  $\mu\text{L}$  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 HVA, VMA and 5-HIAA in the concentration range from 0.1 – 300  $\text{mg/L}$  [from ref. 18].

## Conclusion

The ClinRep® complete kit for HVA, VMA and 5-HIAA in urine provides a standardised method for the sample preparation and analysis of these urinary metabolites using LC-EC.

### Parts and configuration used

190.0035 <sup>#</sup>	ALEXYS 110 LC-EC system with DECADE II SDC
3000*	ClinRep® complete kit , HVA, VMA and 5-HIAA in urine
3030*	ClinRep® Analytical column for HVA, VMA and 5-HIAA in urine
8021*	ClinChek® urine control, level I
8022*	ClinChek® urine control, level II

<sup>#</sup>) 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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