On-line SPE-LC-ECD Analysis of Catecholamines in Urine and Plasma

In order to guarantee the basic requirement for a clinical-chemical analysis or diagnosis being :
c (Analyte)$_{in\text{-}vitro}$ = c (Analyte)$_{in\text{-}vivo}$
we investigated and standardized the stabilization of urinary marker molecules

a) after renal excretion, i.e. during a 24 hour urine collection

b) after venipuncture in blood plasma (catecholamines)

c) during transport to and storage in the laboratory at RT until analysis.

In order to achieve and to fully automate the analysis of catecholamines in different biofluids, we developed and validated a multidimensional HPLC-method with electrochemical detection (ECD). The procedure relies on SPE – LC column switching and allows – for the first time – the direct injection and subsequent on-line separation and quantitation of catecholamines in raw urine or plasma samples.

Preanalytics of Marker Molecules: Standardization

A) *Urine*

Figure 1 shows the instability of Dopamine in urine at physiological pH and room temperature. The stabilization and recovery of the following marker molecules in 24-hour urine at pH 2,2 has been investigated (Fig. 1):

- Norepinephrine (NE)
- Epinephrine (E)
- Dopamine (DA)
- Normetanephrine (NME)
- Metanephrine (ME)
- Vanillylmandelic acid (VMA)
- Homovanillic acid (HVA)
- 5-Hydroxyindole acetic acid (5-HIAA)
- Hydroxyproline (HYP)

These measurements were performed using the "all-in-one" collection-set for 24-hour urine : UriSet 24, developed in cooperation with Sarstedt AG, Nümbrecht, Germany. It allows a standardized preanalytical stabilization of urinary marker molecules for at least five days at RT, i.e. during collection, transport and storage in the laboratory. The volume of the collecting container is 3000 ml (Fig. 2).

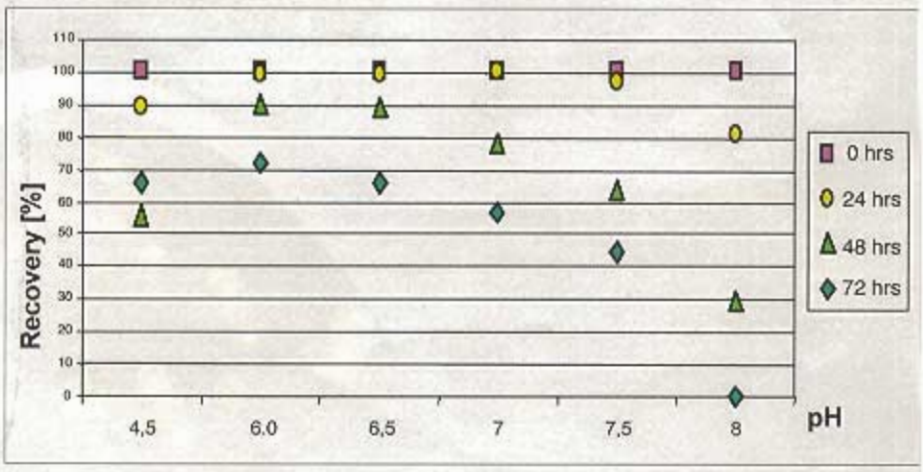


Fig. 1: Instability at physiological pH, Example: Dopamine

B) *Plasma*

For the analysis of catecholamines in blood plasma one has to take care of:

1. Stabilization of catecholamines for 24 hours at 4 °C by addition of 60 µmol glutathione and 30 µmol EGTA to 10 ml of whole blood
2. Storage at –20 °C if > 24 hrs
3. Not to use sample vials made of glass.

Fig. 4 illustrates the different steps of the Affinity (AC) – and Size Exclusion Chro-

Fig. 2: Uriset 24 (Sarstedt AG)

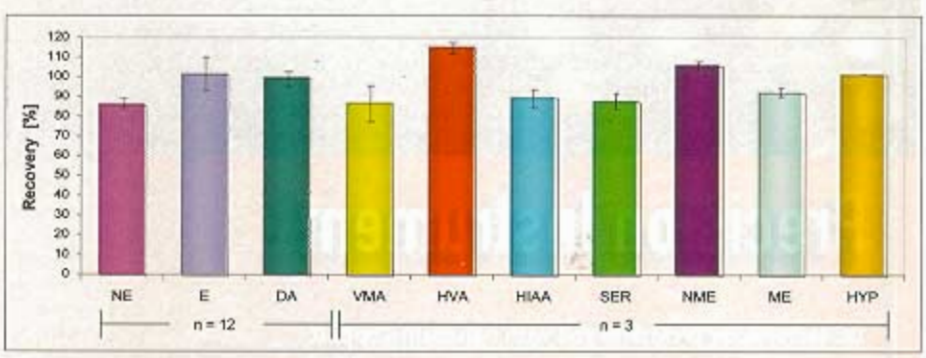


Fig. 3: Stabilization and Recovery of Marker Molecules in 24 hour Urine at pH 2,2

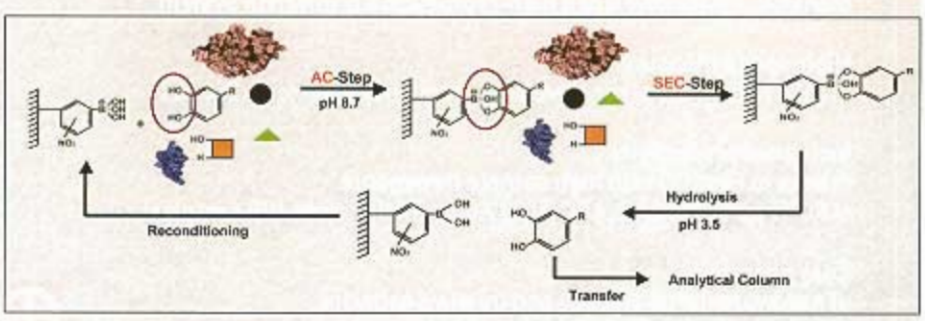


Fig. 4: HPLC-integrated Sample Clean-up

matography (SEC) based Solid Phase Extraction (SPE) of catecholamines.
Conditions for HPLC-integrated Sample Clean-up:

Basis material: Hydrophylic porous Copolymer;
size exclusion limit: approx. 10^4 Da
Ligand: Nitrophenylboronic acid
SPE-column size: 30 x 4 mm (Recipe GmbH, Munich, Germany)

On-line SPE-LC-ECD Analysis of Catecholamines

Fig. 5 shows the system set-up for the on-line SPE-LC-ECD analysis of catecholamines. The different valve positions cycles of the on-line SPE-LC-ECD system are illustrated in Fig. 6.

On-line analysis of catecholamines in raw human biofluids was performed under the following conditions:

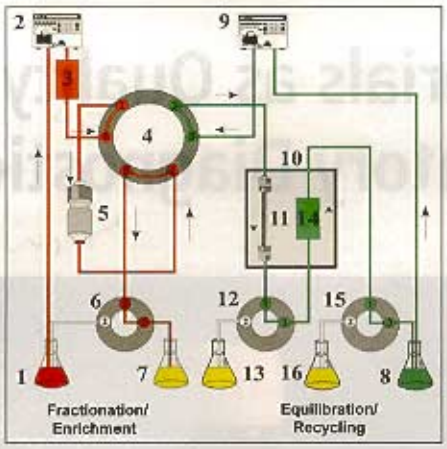
- **Fractionation:**
  Mobile Phase: 0,2 M $(NH_4)_2HPO_4$; 10 mM EDTA
  Flow rate: 2 ml/min for 2 min
- **Separation:**
  Analytical Column: RP-18; 150 x 4,6 mm; 30 °C
  Mobile Phase: 50 mM KH_2PO_4; 0,27 mM EDTA; 2,5 mM sodiumoctylsulfonate
  Flow rate: 1,2 ml/min
  Heart-cut: 2.0–5,5 min (1,8 ml/min)
  Detection: Electrochemical; 30 °C; Working potential: 500 mV; Working range: ±10 nA

Chromatograms of the analysis of catecholamines – Norepinephrine (NE), Epinephrine (E) and Dopamine (DA) – in urine and blood plasma are presented in Fig. 7. Validation parameters of the method are listed in Table 1.

Conclusion

The standardization of the preanalytical phase combined with a fully automated HPLC analysis of the target compounds guarantees a high quality of the over-all analytical process.

Rosa Morello
Karl-Siegfried Boos
Dietrich Seidel
Institute of Clinical Chemistry
University Hospital Grosshadern
81366 Munich, Germany
rosa.morello@med.uni-muenchen.de



1. Mobile Phase (Fractionation)
2. Isocratic Pump A
3. Autosampler
4. High Pressure Valve (6-Way)
5. SPE-column
6. Low Pressure Valve (3-Way)
7. Waste
8. Mobile Phase (Separation)
9. Isocratic Pump B
10. Thermostate (analytical column and electrochemical cell)
11. Analytical Column
12. Low Pressure Valve (3-Way)
13. Waste
14. Electrochemical Cell
15. Low Pressure Valve (3-Way)
16. Waste

Fig. 5: System Set-up for On-line SPE-LC-ECD Analysis of Catecholamines

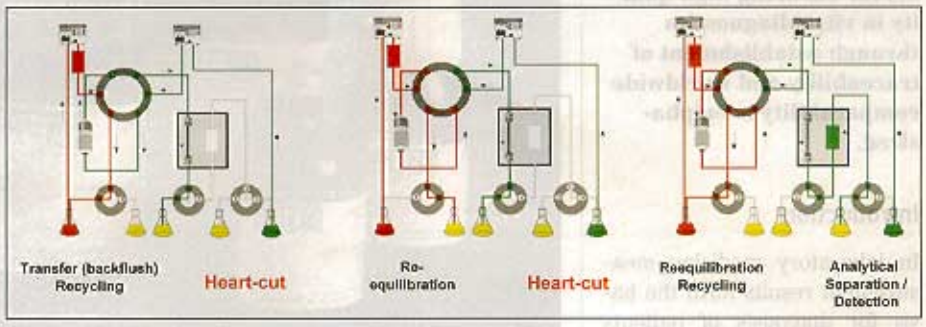


Fig. 6: Column-switching

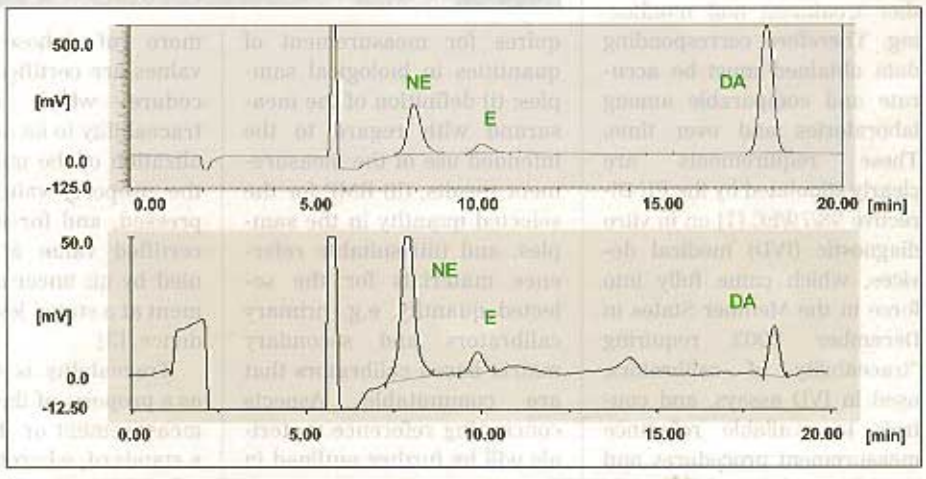


Fig. 7: On-line SPE– LC / ECD of Catecholamines in raw Human Biofluids. Top: 20 µl Urine (NE: 892 nmol/l, E: 175 nmol/l, DA: 1484 nmol/l); bottom: 500 µl Plasma (NE: 7116 pmol/l, E: 1009 pmol/l, DA: 1006 pmol/l)

Linearity		NE	E	DA	nmol / L
	Urine	5.91 - 5910	5.15 - 5150	3.26 - 6514	r ≥ 0.99
	Plasma	0.15 - 29.6	0.15 - 25.8	0.06 - 32.6	r ≥ 0.98
Precision					CV (%)
Intra-Assay					n = 10
	Urine	5.3	5.8	4.7	
	Plasma	6.0	6.3	5.8	
Inter-Assay					n = 6
	Urine	6.3	6.1	5.3	
	Plasma	7.0	7.0	6.2	
LOD					nmol / L
	Urine	3.55	3.09	1.95	
	Plasma	0.09	0.10	0.03	
LOQ					nmol / L
	Urine	5.91	5.15	3.26	
	Plasma	0.15	0.15	0.06	
Recovery		NE, E, DA from urine or plasma: 95-103%			
Robustness		"Life-time" SPE-column, i.e. # of injections / analysis cycle			
	Urine	up to 3000 each of 20 µl			
	Plasma	up to 1000 each of 500 µl			

Fig. 8: Validation (data in part courtesy of Recipe, Munich)