

Application Note

► Online SPE-LC for the determination of catecholamines in urine with fluorescence detection

Category	Clinical
Matrix	Urine
Method	Online SPE-HPLC
Keywords	Online SPE-HPLC, catecholamines, biogenic amines, tumor diseases
Analytes	norephedrine, epinephrine, dopamine
ID	VCL2, January 2008



Summary

With this HPLC application, the determination of catecholamines is performed from urine. A fully automated method allows the injection of urine samples directly to an Online SPE-HPLC system. The sample preparation is realized by Online SPE technique with a highly selective SPE column. The present complete kit allows a quick and safe quantification of the catecholamines and excels in an easy sample preparation and an extraordinary good lower detection limit.

Introduction

Catecholamines are important marker for the diagnosis and management of tumor diseases of the sympathoadrenal system. These tumors, pheochromocytoma, are causing an elevated biosynthesis within the affected tissue. Catecholamines are small molecules made by nerve tissue (including the brain) and the adrenal gland. The major catecholamines are dopamine, norepinephrine, and epinephrine. These substances break down into other compounds, which leave your body through your urine. A urine test can be done to measure the level of catecholamines in the human body. A sensitive and rugged method for determination of catecholamines is HPLC in combination with electrochemical or fluorescence detection [1,2]. Various separation methods have been used for the clean up of catecholamines in biological fluids: solvent extraction, adsorption on alumina, ion-exchange and solid phase extraction of a diphenylboronic acid-catecholamine complex. From the point of simplicity reproducibility and automation the last method shows the most suitable features. Diphenylborate forms a negatively charged complex with the diol groups of catecholamines. The complex is strongly retained on a polystyrene-divinylbenzene cartridge in alkaline medium (pH 8.5). After elution from the SPE column the analytes will be transported to the reversed phase column to separate the analytes.

Experimental Sample Preparation

The collected urine samples should be stabilized with 10 ml/l concentrated hydrochloric acid (32%). For longer storage of the urine sample the pH value should be adjusted to pH 1-2 and the sample can be stored at -20°C. The stability of this acidic, refrigerated urine sample is up to 1 year. The durability of the analytes in the pretreated urine sample at room temperature is limited by 18 hours. It can be enlarged by 4°C storage to 5 days. To verifying the relative recovery it is recommendable to dope the sample with internal standard (IS).

1 ml Urine sample + 20 µl IS solution

Before injection 50 µl of the doped urine sample should be centrifuged at 10.000 rpm for 5 min.

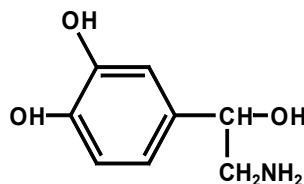
Experimental Preparation of Standard Solution

The preparation of the standard solution is quite easy. For calibration of the Online SPE-HPLC system we recommend the ClinChek® urine controls. These controls are available for the normal range as well as for the pathological range. The injection volume of the calibration run is 50 µl. Add exactly 5 ml of 0.1 M HCl to the vial and mix for 15 min. When all material is dissolved, the solution is ready to use. The concentration of the analytes is listed in Table 1.

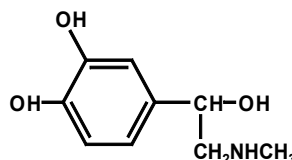
Experimental Passivation of HPLC System

If several applications are running it is recommendable to passivate the HPLC system every six month. It is necessary that all parts of the Online SPE-HPLC system which comes directly in contact with the eluent are passivated with exclusion of the SPE and analytical column. The passivation can be done with half concentrated nitric acid. Before starting the passivation process replace the SPE and analytical column with a connector. Flush the system with distilled water (15 min), with 2-propanol (10 min), and again with distilled water (10 min). After this procedure the passivation with nitric acid solution can be run. To passivate also the injection system it is recommendable to inject nitric acid solution (6 N HNO₃). The whole system can be flushed with pure water. Replace the water reservoir at minimum three times for avoiding pH lowering by contamination of nitric acid. To control the pH value be sure that after sufficient flushing with water at the end of the detector capillary no acidic pH value can be observed. The system can be flushed with the SPE eluent and the mobile phase. Replace the connectors with the SPE column and the analytical column. Equilibrate the SPE column with SPE eluent at 0.5 ml/min flow rate for 15 min. The analytical column should be equilibrated with the mobile phase starting with low flow rate at 0.2 ml/min and carefully updating to 1 ml/min for 15 min. A 30 min baseline check can be helpful to recognize if the Online SPE-HPLC system is ready for injection.

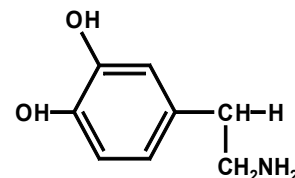
Chemical Structures



norepinephrine



epinephrine



dopamine

Table 1

norepinephrine 10 µg/l

epinephrine 6 µg/l

dopamine 40 µg/l

Method Parameters

HPLC Column	Eurospher 100-5 C18 100 x 4 mm, with precolumn
SPE Column	SPE column for catecholamines
HPLC Eluent	Mobile phase for catecholamines
SPE Eluent	SPE buffer for catecholamines
Flow HPLC Eluent	1.2 ml/min
Injection volume	50 µl urine calibrator and sample/ 100 µl standard solution
Column temperature	30 °C
System pressure	approx. 50 bar
Detection	Fluorescence: Ex 275 nm Em 330 nm
Run time	20 min (incl. regeneration)

Method Parameters

SPE Unit	Time (min)	Flow Rate	SPE Column (Valve Position)	SPE Column
	0.0	0.5	Loading (Pos.1: LOAD)	Equilibration
	0.1	2.0	Loading (Pos.1: LOAD)	Equilibration
	3.0	2.0	Elution (Pos.2: INJECT)	Loading
	3.1	0.5	Elution (Pos.2: INJECT)	Loading
	5.0	0.5	Equilibration (Pos.1: LOAD)	Separation
	20.0	0.5	Equilibration (Pos.1: LOAD)	Separation

Method Recommendations

Due to the high buffer content in SPE eluent and mobile phase it is highly recommended that the filter and sieve combinations, the eluent filter and the precolumn be replaced after 250 injections. The system pressure without SPE and analytical column should not exceed 20 bar. The first column inlet filter unit should be installed between autosampler and switching valve of Online SPE unit. A second inline filter unit should be placed on the outlet of the HPLC pump. If the Online SPE-HPLC system is not used for one day the pumps should not be switched off! A lowering of the flow rate (SPE pump and HPLC pump) to 0.1 ml/min is recommendable. If the system is not used for a longer time period, disconnect the columns and replace the buffer solutions. Flush the whole system intensively with distilled water and finish the switch off with methanol/water mixture (50:50, v/v). **Don't switch off the pumps for a longer time if buffer solutions are used!** Crystallization processes of the buffer can irreversible destroy the pump head. All samples should originally closed and stored at 4°C. After reconstitution the stability of the analytes is at least 18 hours at 25°C and at least 5 days at 4°C.

Results

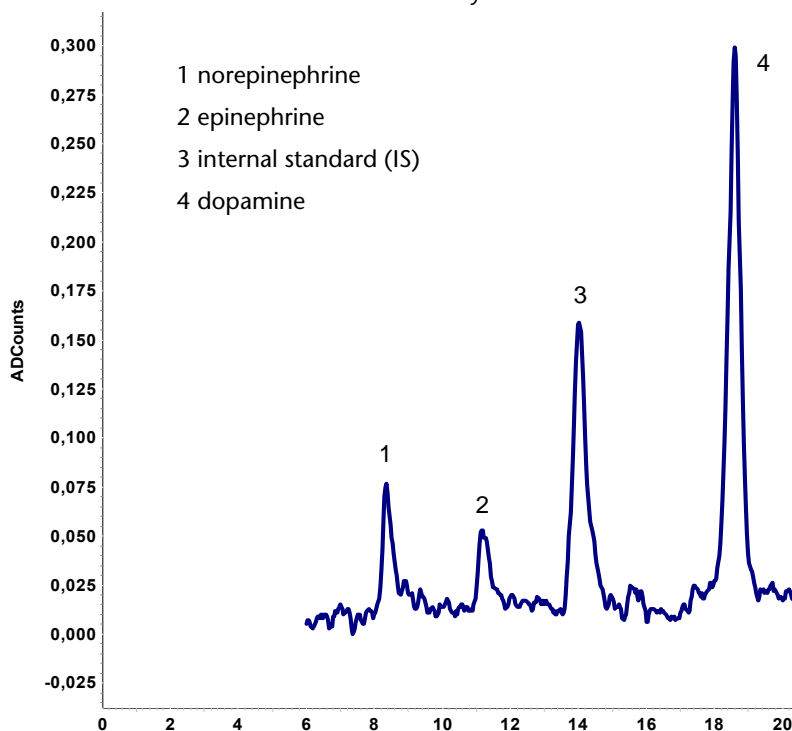


Fig. 1
Catecholamines Standard

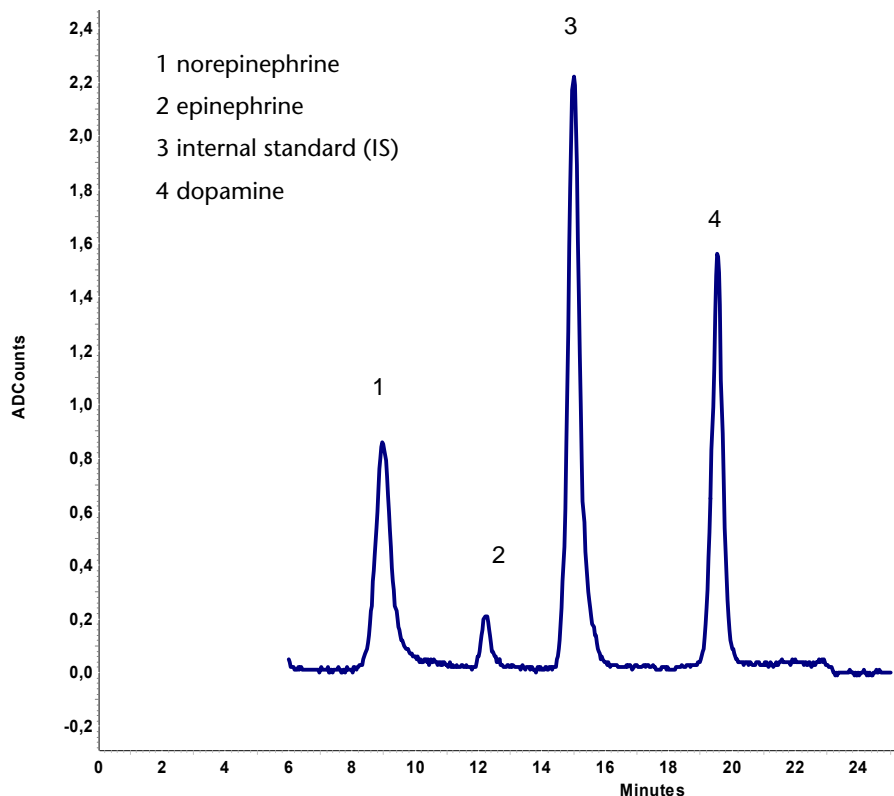


Fig. 2
Urine Calibrator + 20 µl IS

Substance	t_r (min)	Concentration [µg/l]	Recovery
Norepinephrine	19.33	107	70 - 100
Epinephrine	14.80	31.4	70 - 100
Internal Standard	12.10	-	-
Dopamine	8.77	246	70 - 100

Method Performance

Limit of detection	1 µg/l (S/N = 3)
Linearity (r^2)	1.0 (one point calibration through zero)
Linearity range	3 µg/l – 1500 µg/l
Retention time precision*	< 1 % RSD
Peak area precision*	< 1-8 % RSD

*repeatability calculated over 36 replicate runs

For quality control of the analytical determinations we recommend using the ClinChek® urine controls. These controls are available for the normal range as well as for the pathological range.

Conclusion

The Online SPE Sample Preparation Unit 6300 dramatically streamlines the urine analysis for the determination of catecholamines. Automated sample handling, proprietary column chemistry and sensitive fluorescence detection have been combined. The system requires only a fraction of a typical sample volume. This Online SPE-HPLC solution increases speed, improves accuracy, precision, and reduces cost.

References

- [1] T.G. Rosanjo, T.A. Swift, L.W. Hayes: Advances in catecholamine and metabolite measurements for diagnosis of pheochromocytoma, Clin. Chem. 37 (10/2) 1991 1854-1867
- [2] H. Weicker: Determination of free and sulfoconjugated catecholamines in plasma and urine by high performance liquid chromatography, Int. J. Sports Med. 9 (1988) 68-74
- [3] D.J. Wang, Y. Qu, P.Hu and P.L. Zhu: Determination of free catecholamines in urine by direct injection onto a shielded hydrophobic phase column, Chromatographia Volume 31, Numbers 3-4 / February 1991

Physical Properties of recommended Column



Eurospher 100 C18 material can be universally used in different application areas. The stationary phase is stable in a pH range 2 – 8.5.

Stationary phase	Eurospher 100-5 C18
USP code	L1
Pore size	100 Å
Pore volume	0.9 ml/g
Specific surface area	350 m ² /g
Particle size	5 µm
Form	spherical
Surface area	350 m ² /g
% C	15
Endcapping	yes
Dimensions	100 x 4 mm
Order number	10WF181ESJ (with integrated precolumn)
Precolumn 5 x 4 mm	P5DE181ESJ

Recommended Instrumentation



This application requires an Online SPE-HPLC system equipped with degasser, cooled autosampler, column oven, and fluorescence detector. Other configurations are also available. Please contact KNAUER to configure a system that's perfect for your needs.

Description	Order No.
Sample Preparation Unit 6300	A5003V10
Smartline Pump 1000, incl. 10 ml pump head	A50303
Autosampler 3900 Cool	A15081
Smartline Column Oven 4050	A5300
Fluorescence Detector RF-10 AXL	A0815
ChromGate Software	A1493
Column Inlet Filter	A0109 (2 x)
ClinRep® Complete Kit (500 assays)	Y1181
Mobile Phase Catecholamines 2500 ml	Y1181-1
SPE Buffer Catecholamines 2500 ml	Y1181-2
ClinTest® Standard Solution (10 ml)	Y1181-3
ClinCal® Urine Calibrator (5 x 5 ml)	Y1181-4
ClinChek® Urine Control Level I+II (2 x 5 x 5ml)	Y1181-5
ClinRep® IS Internal Standard (3 ml)	Y1181-6
SPE Column Catecholamines	Y1181-7

Authors

Corinna Pfeiffer, Product Management, KNAUER
Silvia Marten, Head of Columns and Applications Department, KNAUER

Contact Information

Wissenschaftliche Gerätebau
 Dr. Ing. Herbert Knauer GmbH
 Hegauer Weg 38 ·
 14163 Berlin, Germany

Tel: +49 (0)30 / 809727-0
 Fax: +49 (0)30 / 8015010
 E-Mail: info@knauer.net
 Internet: www.knauer.net