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Automated analysis of urinary catecholamines using On-line SPE-HPLC

Prospekt **Clinical**

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Introduction

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 most suitable features. Diphenylborate forms a negatively charged complex with the diol groups of catecholamines. The complex is strongly retained on PLRP-s and PRP-1 cartridges in alkaline medium (pH 8.5). On-line desorbtion and separation was performed by means of ion-paired reversed phase HPLC, with sodium heptane sulfate as counter-ion, and electrochemical detection.

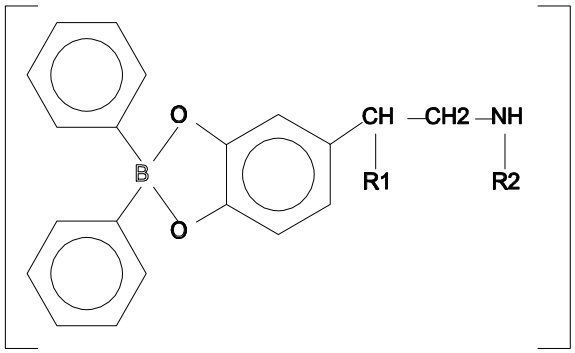

Figure 1: The complex between catecholamines and diphenylboronic acid in alkaline medium (pH 8.5).

Experimental

Instrumentation

Samples were introduced by a Spark Holland MARATHON autosampler. A Solvent Delivery Unit (SDU) was used for sample clean up. Automated on-line solid-phase extraction was performed with a Spark Holland PROSPEKT system which controlled the autosampler, the SDU and all switching valves.
The chromatographic system consisted of a Model 126 solvent delivery module "System Gold", programmed by a Model 424 system controller (Beckman Instruments); a dual electrode coulometric ESA detector Model 5100A equipped with a 5021 Conditioning Cell and a 5011 High-Sensitivity Cell. An integrator Model C-R3A (Shimadzu) was used for data acquisition.

Chromatographic conditions

Anal. column 1:	*C18, 30 x 4.6 mm, 5 µm (Brownlee Labs, Applied Biosystems)*
Anal. Column 2:	*C18, 250 x 4.6 mm, 5 µm (Beckman Instruments)*
Mobile phase:	*50 mMol potassium dihydrogen phosphate, 1 mMol sodium heptane sulfate and 0.07 mMol EDTA solution / methanol / acetonitril (v/v 100:8:15). Final pH was adjusted to 3.2 with 1.5 M orthophosporic acid.*
Flow rate:	*0.8 mL/min*
Column temp.:	*ambient*
SPE cartridge:	*PLRP-s, 15-25 µm, 10 x 2 mm ID (Spark Holland)*
Detection:	*Coulometric detector*
	Oxidizing electrode: +350 mV
	Screen electrode: +100 mV
	Quantifying electrode: !300 mV
	Response time: 4 sec.

Preparation of standards

Stock solutions of Noradrinalin (NA, 1 g/L), Adrenalin (A, 1 g/L), Dopamine (DA, 4 g/L) and the internal standard Dihydroxybenzylamine (DBHA, 1.26 mg/L) were prepared in 0.1 M HCl and were kept at -20EC. Work solutions were obtained adding to an acidified normal urine 0, 10, 50, 100, 200 µg/L of NA and A and 0, 125, 250, 500, 1000 µg/L DA. These standards were prepared daily.

Sample preparation

Urine specimen (24 hours) were collected in dark polyethylene bottles containing 10 mL of 6 M HCL (final pH 1-3) and stored frozen if not assayed within 24 hours. Before analysis, sample was diluted in two-fold with 2 M ammonia-ammonium chloride buffer pH 8.5, containing 0.5% EDTA (solvent A). Then it was

complexed with an equal volume of 2 M ammonia-ammonium chloride buffer pH 8.5, containing 0.5% EDTA and 0.2% diphenylborate (PBA). To 1.2 mL of this mixture 20 µL internal standard solution (1.26 g/L dihydroxybenzylamine) was added.

200 µL of this mixture was injected on the SPE cartridge.

The following sample preparation program was used:

PROSPEKT Sample Preparation program

All flowrates 1 mL/min

1. *Activate cartridge with 1 mL methanol.*
2. *Condition cartridge with 0.5 mL water and 1.5 mL of 10 times diluted solvent A (0.2 M) (solvent B).*
3. *Inject sample (200 µL flushed loop)*
4. *Load cartridge with 1 mL solvent B.*
5. *Wash sample with 1 mL solvent B /methanol (v/v 80:20) and 2.15 mL water.*
6. *Elute sample with mobile phase on columns 1 and 2 during 0.5 min.*
7. *Switch column 1 during 10 min to waste, 1 min 15 sec. after the elution starts.*

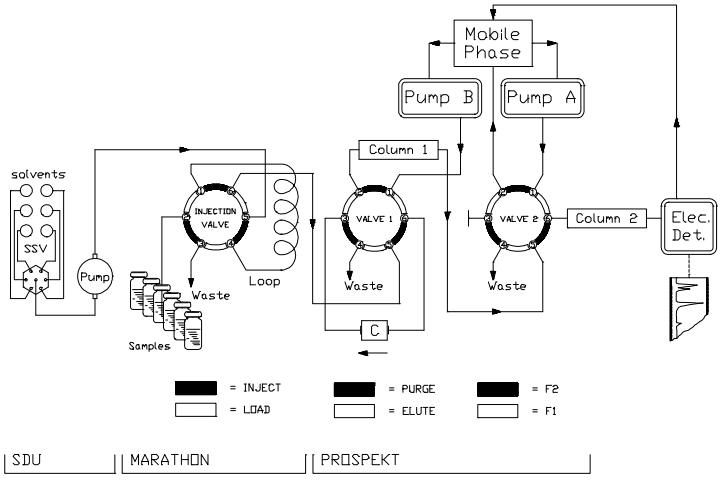

Figure 2: System switching diagram.

Sample Preparation Program									***Application Info 42***
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Time *H:MM:SS*	*Solv.*	*Purge flow* *mL/min*	*Injection valve* *F1: Load* *F2: Inject*	*Change cartridge* *F1: Execute*	*Valve 1* *F1: Elute* *F2: Purge*	*Valve 2* *F1: 1-6* *F2: 1-2*	*Aux* *1*	*END TIME*	*Comment*
0:00	*1*	*1.0*	*F1*		*F2*				*Activate: solvent 1*
1:00	*2*								*Condition: solvent 2*
1:30	*3*								*Condition: solvent 3*
3:00			*F2*						*Load sample*
4:00	*4*								*Wash: solvent 4*
4:15	*2*								*Wash: solvent 1*
6:30			*F1*		*F1*		*On*		*Start Data,* *Elute to column 1+2 ①*
6:31							*Off*		
7:00	*1*				*F2*				
7:45						*F1*			*Clean column 1 to waste ①*
9:00		*0.0*							
17:45									*Equil. column 1 + 2 ①*
18:30						*F2*		*END*	*Next cycle*

Solvents: *1: Methanol*
2: Water
3: 0.2 M buffer
4: 0.2 M buffer / MeOH 4:1

Note ①: with mobile phase, flow 0.8 mL/min, pump B.

Chromatograms

Chromatogram A: Standard mixture contains 100 µg/L NA, 100 µg/L A, 180 µg/L DHBA, 323 µg/L DA.
Chromatogram B: Normal urine contains 29 µg/L NA, 13 µg/L A, 79 µg/L DA.
Chromatogram C: Abnormal urine contains 142 µg/L NA, 72 µg/L A, 640 µg/L DA.

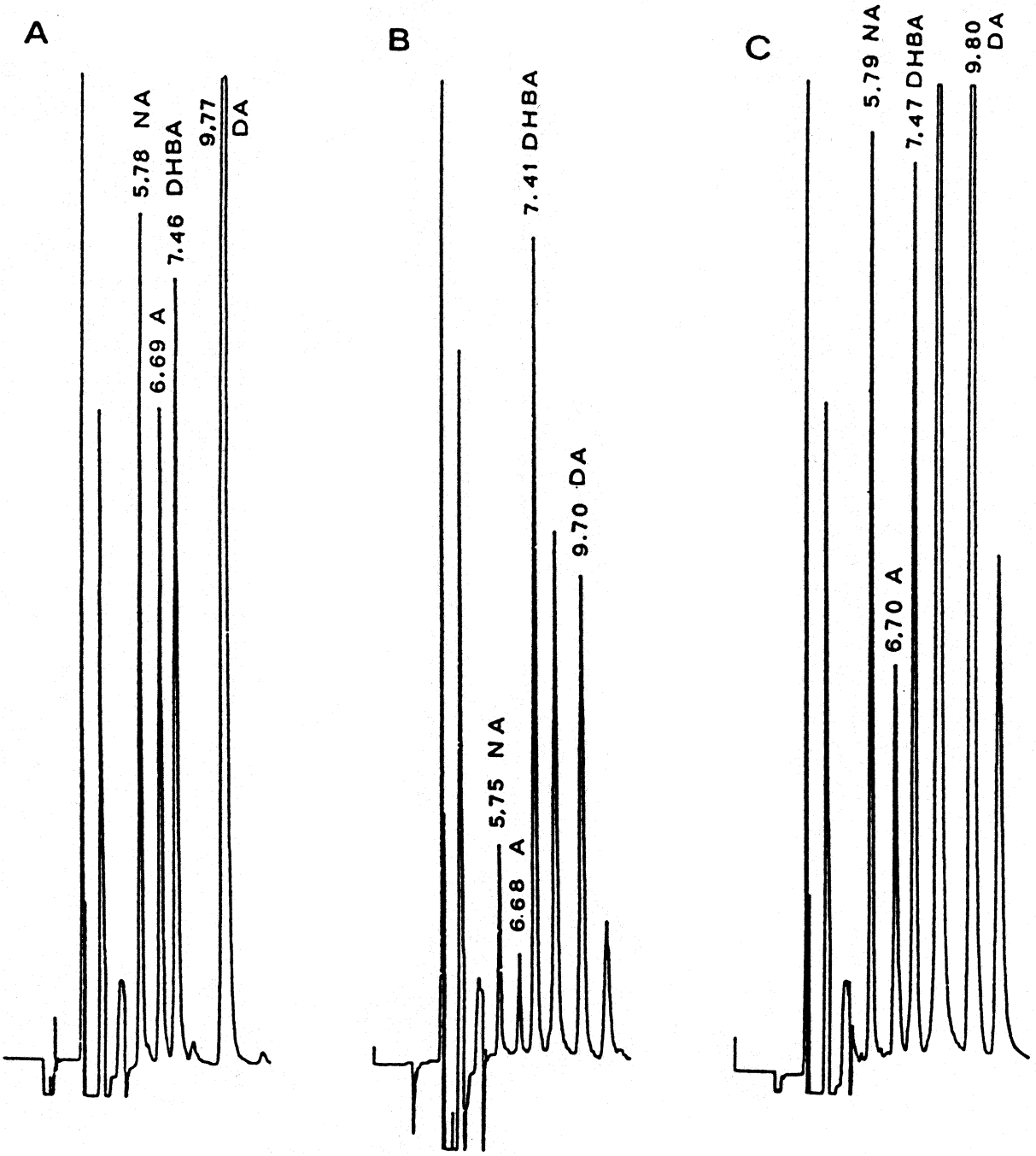

Figure 3: Chromatograms from a standard mixture (A), a normal urine (B) and a abnormal urine (C) sample.
Peaks: Noradrenalin (NA), Adrenalin (A), Dihydroxylbenzilamine (DHBA, internal standard), Dopamine (DA).

Results and discussion

On-line SPE is used to retain the complex and to remove the matrix components.
Column switching is used to eliminate interfering peaks produced by complexing agent in excess, which follow the catecholamine peaks and increase the turnover time between analysis. To realize this two on-line analytical columns with the same packing have been used. After elution of the catecholamines from the short column 1 into the longer column 2, valve 2 switches, permitting the elution of interferences from the first column to waste while the catecholamines elute from the second column to the detector. Two isocratic pumps supply the mobile phase. Before the column-switching pump B pumps mobile phase

through both columns. After the column-switching pump B pumps through column 1 and pump A through column 2 (see figure 2).

Five types of cartridges where tested, PLRP-s, PRP-1, C18, C18OH low hydrocarbon and C18 High Hydrophobic. The capacity of the latter 3 phases, with obtained recoveries less than 50%, was insufficient. The compositions of complexing reagent and mobile phase were achieved after optimization of the concentration levels for the individual compounds. Chromatograms obtained from standard and urine samples are shown in figure 3. The analysis time is 10 min and the total process requires 18 min. Recovery and reproducibility obtained using Lyphochek Normal and Abnormal urine controls are presented in Tables 1 and 2. Calibration graphs are linear between 0-300 µg/L for noradrenalin and adrenalin and between 0-1200 µg/L for dopamine. A correlation study between manual extraction on BioRex 70 cation exchange resin and the developed method was performed on 30 urine samples giving the results shown in Table 3. Based on a signal-to-noise ratio of 3, the limits of catecholamine detection are 1.3, 2.0, 3.0 mg/L for noradrenalin, adrenalin and dopamine respectively. In urine, the catecholamine-diphenylborate complex is stable for more than 6 hours at room temperature and at least 24 hours at 4EC.

Table 1: Absolute recoveries (n=10)

	Amount added (µg/L)	Recovery (%)
NA	200	97 ± 2.9
A	100	99 ± 2.0
DHBA	180	99 ± 2.0
DA	500	98 ± 2.5

Table 2: Catecholamines automated analysis precision.

	Within days		Between days	
	Mean value (µg/L)	CV (%)	Mean value (µg/L)	CV (%)
Normal urine (n=24)				
NA	34	2.1	33	3.5
A	13	1.8	14	3.8
DA	70	2.2	73	4.4
Abnormal urine (n=24)				
NA	166	3.6	163	4.6
A	78	1.1	82	3.6
DA	430	2.0	425	2.7

Table 3: Urinary catecholamines correlation (n=30)

	X = manual extraction Y = column switching extraction
NA	Y = 0.996 X + 1.116
A	Y = 0.990 X + 0.103
DA	Y = 1.015 X – 0.891

Conclusions

A reliable automated on-line SPE-HPLC application has been developed for the analysis of catecholamines in urine. The method gives almost quantitative recoveries for noradrenalin, adrenalin and dopamine. A good correlation is achieved with a previously used manually method based on cation-exchange extraction. The stability of the catecholamine-diphenylborate complex and the reasonable short turnover time allows the analysis of a large batch of 70 urine samples in clinical laboratories, in 24 hours, including sample preparation and HPLC analysis.

References

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