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Evaluation of sample work-up methods and internal standards for the determination of catecholamines in urine by HPLC with electrochemical detection*

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Abstract: Two sample work-up methods: (I) one consisting of adsorption of the catecholamines onto alumina followed by ion pair extraction and (II) another consisting of isolation by cation exchange and subsequent adsorption onto alumina, have been evaluated for the assay of urinary catecholamines by means of HPLC with electrochemical detection. With the aim of achieving high precision, two internal standards, i.e. dihydroxybenzylamine and epinine, have been compared. The results indicate that clean HPLC chromatograms are obtained with both work-up methods and that the highest precision (RSD <4%) is achieved with method II and with epinine as internal standard, whereas the lowest precision is obtained with method I and with dihydroxybenzylamine.

Keywords: *Catecholamines; urine; high-performance liquid chromatography; electrochemical detection.*

Introduction

In many laboratories, ion-pair reversed-phase HPLC in combination with electrochemical detection (ED) has become the technique of choice for the analysis of the catecholamines, noradrenaline (NA), adrenaline (A) and dopamine (DA) in urine. These catecholamines play an important role in the central nervous system and in the regulation of blood pressure and are clinically of interest in the detection of certain tumours that secrete these compounds, e.g. neuroblastoma and pheochromocytoma. A variety of work-up procedures for isolation of catecholamines from urine has been reported and different internal standards have been employed for quantitative analysis. Most methods for isolation from urine, which is a complex biological matrix, are two-step procedures consisting of a combination of two of the following isolation methods:

(i) adsorption onto aluminum oxide [1, 2];
(ii) adsorption onto boric acid gel [3, 4];

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(iii) cation exchange [5, 6];
(iv) ion-pair extraction [7].

By combining methods based on different principles, it is possible to eliminate interferences experienced during the assay of urine. In an effort to develop a specific method, two sample work-up methods previously reported in the literature have been evaluated, namely one consisting of adsorption of the catecholamines onto aluminum oxide followed by ion-pair extraction [8], and another combining isolation by cation exchange and subsequent adsorption onto aluminum oxide [9]. With the aim of achieving higher precision, two internal standards, viz., dihydroxybenzylamine (DHBA) and epinine (E), both of which are structurally related to the catecholamines to be assayed have been evaluated in order to compensate for losses during the processing of samples.

Experimental

Reagents and Standards

Adrenaline bitartrate and tris(hydroxymethyl)aminomethane (Tris) were obtained from Sigma (St. Louis, MI, U.S.A.). Dopamine hydrochloride, noradrenaline-L-hydrogentartrate and tetraoctylammoniumbromide were obtained from Fluka (Buchs, G.D.R.). Dihydroxybenzylamine, diphenylboric acid ethanolamine complex, epinine, *n*-heptane, 1-heptanesulphonic acid sodium salt and *n*-octanol were purchased from Janssen Chimica (Beerse, Belgium). Aluminum oxide was obtained from Woelm Pharma (Eschwege, G.D.R.) and was activated as described by Anton and Sayre [1]. Plastic isolation columns containing Biorex 70 cation exchange resin were purchased from Biorad (München, G.D.R.). Inorganic salts and the other organic solvents used were of analytical grade and were obtained from Merck (Darmstadt, G.D.R.).

Concentrated stock solutions of NA, A and DA and of the internal standards DHBA and E were prepared in 0.025 M hydrochloric acid and stored at −20°C. These solutions were diluted 200-fold with 0.025 M HCl to prepare the standard and internal standard solutions and were prepared immediately before use.

Work-up procedures

Method I. This consisted of adsorption onto aluminum oxide and ion-pair extraction and has been adapted from Moerman and De Schaepdryver [8]. A urine aliquot (5 ml) or aqueous standards covering the concentration range of interest were processed. After addition of 200 mg Na_2EDTA and 1 ml of internal standard solution containing 110 ng DHBA and 161 ng E, 500 mg aluminum oxide was added. Following pH adjustment to 8.5 with 0.1 M NaOH by means of an automatic titrator, the samples were transferred to a glass column (i.d. = 5 mm) and washed with 3 ml water containing 0.01% Triton X-100. Subsequently, the catecholamines together with the internal standards were eluted with 2 ml 0.2 M aqueous acetic acid containing 0.01% Triton X-100. An aliquot (0.5 ml) was used for the ion-pair extraction as described by Smedes *et al.* (7). After addition of 1 ml of 2.0 M NH_4Cl buffer (pH 8.5) containing 0.2% diphenylborate-ethanolamine complex and 0.5% Na_2EDTA, the pH of the samples was readjusted to 8.5 with 40 µl 0.1 M NaOH. Subsequently, 4 ml of ion-pairing solvent consisting of *n*-heptane containing 1% octanol and 0.25% tetraoctylammonium bromide, was added. The samples were shaken for 1 min and centrifuged for 5 min at 1000 **g**. The organic layer was transferred to a conical tube and after addition of 2 ml *n*-octanol and 0.4 ml 0.08 M aqueous acetic acid, the samples were shaken for 1 min and centrifuged for 5 min at

1000 g. The upper layer was removed by aspiration and an aliquot of the lower layer was analysed by HPLC–ED. The recoveries for the catecholamines was between 60 and 70%.

Method II. This method consisted of cation exchange on Biorex 70 resin followed by adsorption onto aluminum oxide and was adapted from the procedure described in Bioanalytical Systems application note [15]. A urine aliquot (5 ml) or aqueous standards covering the concentration range of interest were processed. After addition of 1 ml internal standard solution containing 110 ng DHBA and 161 ng E and 15 ml 0.1 M phosphate buffer (pH 7), the pH of the samples was checked; if the pH was below 6, the pH was adjusted to 6 with 1 M NaOH. The samples were applied onto commercial Biorad columns filled with Biorex 70 resin and washed with 10 ml water. The catecholamines were eluted with 1 ml 0.7 M H_2SO_4 and 4 ml 2 M $(NH_4)_2SO_4$. In preliminary experiments, elution was started after acidifying the column with 1 ml 0.7 M H_2SO_4, but we found that this resulted in a partial loss of dopamine. Subsequently, the eluate was further purified and the catecholamines together with the internal standards concentrated by adsorption onto 100 mg aluminum oxide. The pH was adjusted by adding 0.7 ml 3 M Tris/EDTA buffer of pH 8.6. After shaking for 10 min, the samples were transferred to glass columns (i.d. = 4 mm) and the adsorbent was washed three times with 4 ml water. Subsequently, the columns were acidified with 0.2 ml 0.2 M HCl and the catecholamines together with the internal standards eluted with 0.2 ml of the same solution. After addition of 0.2 ml water, an aliquot was analysed by HPLC–ED. The recoveries for the catecholamines was between 60 and 65%.

Instrumental conditions

The HPLC analyses were performed with a Model 45 M solvent delivery system (Waters Associates, Milford, MA, U.S.A.) equipped with a Model U6K universal sample injector (Waters Associates) and with an amperometric detector (Model LC4, Bioanalytical Systems, West Lafayette, IN, U.S.A.) with a glassy carbon working electrode. The electrochemical detector was operated at a potential of +0.7 V. In some experiments, detection was carried out with a Model 5100 A dual cell coulometric detector (E.S.A., Bedford, MA, U.S.A.) with the first porous graphite cell operated at +0.25 V in the oxidation mode and the second one operated at −0.15 V in the reduction mode. The chromatographic traces were registered by a potentiometric recorder.

A reversed-phase column (μm Bondapak C18, 250 × 4.6 mm i.d., 10 μm particles, Chrompack, Middelburg, The Netherlands) was used. The eluent consisted of a 0.07 M NaH_2PO_4 buffer containing 0.1 M Na_2EDTA, 1 mM *n*-heptanesulphonic acid and methanol (90:10, v/v). The pH of the eluent was adjusted to 3.9. The solvent flow was 1 ml min^{-1}. The eluent was filtered through a Millipore-type RA 1.2 μm filter and degassed before use.

Quantitative analysis and evaluation of precision

Standard curves were obtained using six standard solutions, each containing known amounts of the catecholamines and covering the concentration ranges of interest. Exactly the same amount of the internal standards was added to the aqueous standard solutions and to urine samples, and all samples were carried through the complete work-up procedure or, for precision evaluation purposes, through the partial procedure. Quantitative analysis was based on peak height ratios of the catecholamines versus the

internal standards, and unweighted least-squares linear regression analysis was performed. Using the regression parameters of the calibration curve, the amounts of endogenous catecholamines in urine were calculated.

Results and Discussion

The initial experiments were designed to test whether or not a one-step work-up procedure, i.e. ion-pair extraction and adsorption onto aluminum oxide, for determining urinary catecholamines by ion-pair reversed-phase HPLC in combination with dual cell coulometric detection, would result in clean chromatograms. However, the chromatographic traces obtained in the reduction mode, which were more interference-free than the traces obtained in the oxidation mode did not result in sufficient selectivity and prompted the evaluation of two-step procedures. The two methods selected resulted in virtually interference-free chromatograms in the oxidation mode (Fig. 1). In further experiments the precision which can be obtained with both methods was critically examined with DHBA and E as internal standards. The degree of precision for each of the catecholamines was determined for the two work-up methods by carrying aqueous

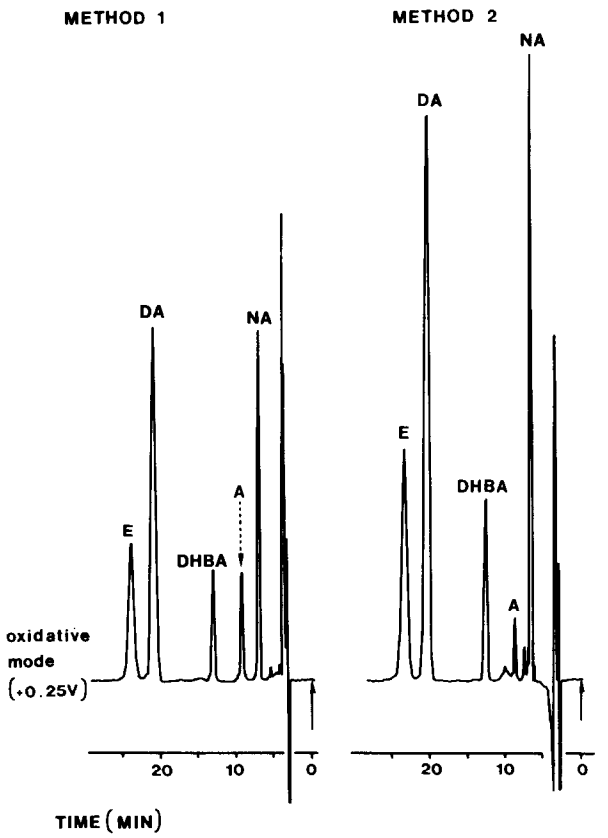

Figure 1
HPLC–ED chromatograms obtained in the oxidative mode for samples of a urine pool processed by means of the two sample work-up methods. Detection was carried out using a dual cell coulometric detector.

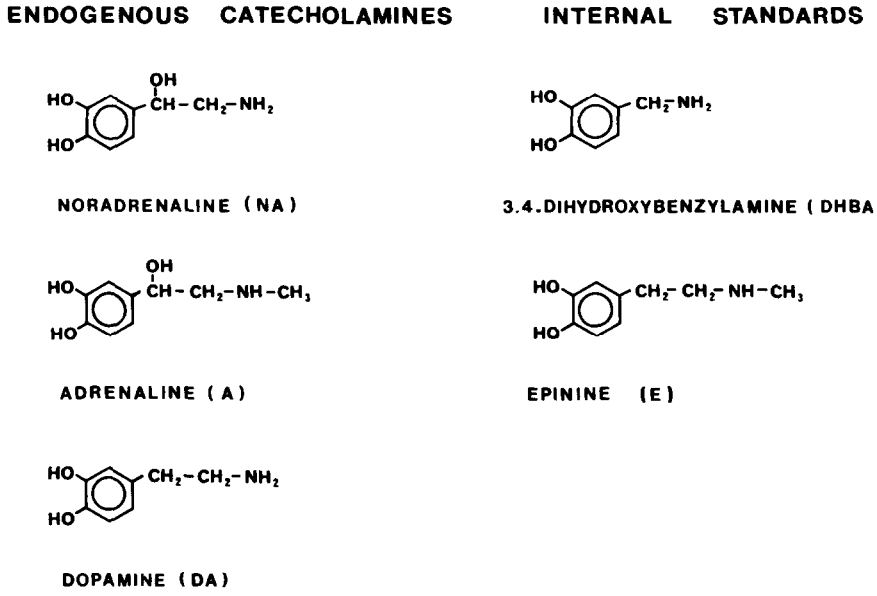

Figure 2
Chemical structures of endogenous catecholamines and structurally related internal standards.

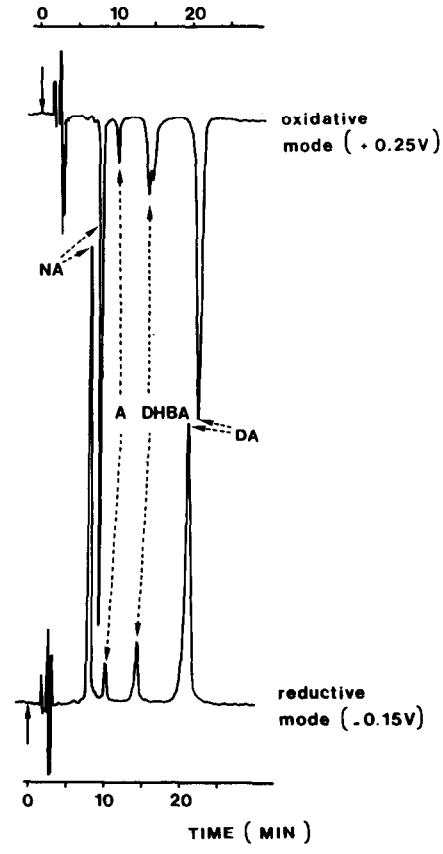

Figure 3
HPLC–ED chromatogram obtained for a urine sample of a patient with pheochromocytoma treated with labetolol. The sample was processed with method I. A metabolite of labetolol interferes with DHBA in the oxidative mode. Detection was carried out using a dual cell coulometric detector.

Table 1
Regression characteristics of calibration curves obtained for NA, A and DA using DHBA and E as internal standard with different work-up methods

Compound measured: NA
Concentration aqueous standards: 18.9, 37.8, 56.7, 75.6, 94.5 and 13.4 ng ml^{-1}

Internal standard	DHBA					E				
Regression parameters	a	b	s^2	r	s/b	a	b	s^2	r	s/b
Method										
Method I	10.9400	1.6668	108.2487	0.9878	6.2	3.5333	1.5125	8.6462	0.9988	1.9
Aluminum adsorption	11.4733	2.2050	106.1292	0.9931	4.7	5.6867	0.9279	6.7610	0.9975	2.8
Ion pair extraction	3.5267	1.2364	24.8228	0.9948	4.0	0.2533	0.7321	1.5210	0.9991	1.7
Method II	2.4133	1.8703	2.4918	0.9998	0.8	0.8467	1.4228	0.2268	0.9999	0.3

Compound measured: A
Concentration aqueous standards: 6.1, 12.1, 18.2, 24.2, 30.3 and 36.4 ng ml^{-1}

Internal standard	DHBA					E				
Regression parameters	a	b	s^2	r	s/b	a	b	s^2	r	s/b
Method										
Method I	3.5046	1.1058	4.6192	0.9884	1.9	1.6474	1.0080	0.2869	0.9991	0.5
Alumina adsorption	1.5408	1.2821	2.5143	0.9953	1.2	0.7903	0.5393	0.0973	0.9990	0.6
Ion pair extraction	2.0147	0.6780	4.8852	0.9685	3.2	0.8497	0.3999	0.5097	0.9902	1.8
Method II	2.1047	1.3753	0.1971	0.9997	0.3	1.3926	1.0443	0.0524	0.9999	0.2

Compound measured: DA
Concentration aqueous standards: 38.2, 76.4, 114.5, 152.7, 190.9 and 229.1 ng ml^{-1}

Internal standard	DHBA					E				
Regression parameters	a	b	s^2	r	s/b	a	b	s^2	r	s/b
Method										
Method I	not calculated ($r < 0.9000$)					not calculated ($r < 0.9000$)				
Aluminum adsorption	−4.4834	7.8663	144.0116	0.9968	1.5	−0.9241	0.7902	0.9079	0.9999	1.2
Ion pair extraction	5.0709	1.2538	91.5346	0.9955	7.6	−0.7040	0.7425	6.1310	0.9991	3.3
Method II	2.1567	0.7413	1.1340	0.9998	1.4	0.8590	0.5639	0.2122	0.9999	0.8

standards through the complete or partial procedures and by evaluating the linearity and the regression characteristics of the calibration curves. The chemical structures of the catecholamines, NA, A and DA and of the internal standards, DHBA and E, are shown in Fig. 2. For some routine clinical measurements, it may be advantageous to avoid the use of DHBA as internal standard, e.g. in cases of urine of patients treated with labetolol, where a metabolite of labetolol was found to co-elute with DHBA (Fig. 3).

Table 1 summarizes the regression parameters for the calibration curves obtained with aqueous standards for the different catecholamines, using DHBA and E as internal standards. Method I resulted in linear calibration curves for NA and A, but not for DA with either internal standard. In an effort to find out why irreproducible results were obtained for DA, the adsorption onto aluminum oxide and ion pair extraction steps were tested independently. However, in these experiments, linear calibration curves were obtained for DA, so that an explanation for the peculiar behaviour of DA during the complete processing with method I is not obvious. As can be seen from the data in Table 1, method II resulted in linear calibration curves for NA, A and DA. The values obtained for the regression parameter s/b (in ng ml^{-1}), which may be regarded as a measure of precision, indicate that reproducibility is consistently higher with method II than with method I.

The reproducibility of both methods was also evaluated by analysing six samples of a pooled urine sample (Table 2). These results demonstrate that method II enabled the determination of urinary catecholamines at physiological concentrations with a precision

Table 2
Reproducibility of methods I and II using DHBA and E as internal standard for NA, A and DA. Six samples of a urine pool were analysed and the concentrations were estimated by using a calibration curve and applying reversed least-squares linear regression

Compound	NA			
Method	I		II	
Internal standard	DHBA	E	DHBA	E
Concentration ± S.E.* (ng ml^{-1})	130.6 ± 27.9	84.6 ± 3.1	91.4 ± 5.7	91.8 ± 3.2
RSD*	21.4%	3.7%	6.2%	3.5%
Compound	A			
Method	I		II	
Internal standard	DHBA	E	DHBA	E
Concentration ± S.E.*	28.65 ± 6.21	21.82 ± 7.89	11.45 ± 0.75	11.67 ± 0.32
RSD*	21.7%	36.2%	6.6%	2.7%
Compound	DA			
Method	I		II	
Internal standard	DHBA	E	DHBA	E
Concentration ± S.E.*	not calculated		208.8 ± 8.9	209.6 ± 3.0
RSD*			4.3%	1.4%

* Abbreviations: S.E. = standard error; RSD = relative standard deviation.

that is markedly superior to that obtained with method I. Acceptable relative standard deviations (RSD) ranging between 1 and 7% were obtained with method II with DHBA or E as internal standards. The data also indicate that the highest precision was achieved using E as internal standard. On the basis of these results, method II has been selected for routine measurement of urinary NA, A and DA in the authors' laboratory. The reproducibility obtained with method II consisting of cation-exchange followed by adsorption onto aluminum oxide is comparable to results reported by Moyer *et al.* for work-up by adsorption onto aluminum oxide followed by adsorption onto a boric acid gel [9].

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