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Determination of catecholamines by ion chromatography and electrochemical detection

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Abstract

An ion chromatographic method was developed for the fully automated routine clinical determination of urinary free catecholamines noradrenaline, adrenaline and dopamine with an internal standard (3,4-dihydroxybenzylamine). Simultaneous clean-up and separation of the analytes are achieved by an on-line purification step and a column-switching technique. The whole procedure requires 45 min. Both the prepurification microcolumn and the analytical column contain cation exchangers. Electrochemical detection was optimized with two working electrodes set at +500 mV for the cleaning step, and +700 mV for detection. Analytical recoveries for all three catecholamines were 90–95% and the detection limits (signal-to-noise ratio = 3) were 5.0, 10.0 and 10.0 pg for noradrenaline, adrenaline and dopamine, respectively.

1. Introduction

Catecholamines play an important role as neurotransmitters, having a marked influence on the vascular system and metabolic processes; disorders such as hypertension, neural crest tumours and Parkinsonism are indicated by excesses of these compounds [1]. Simple and selective methods are required for the routine determination of catecholamines in biological samples. Their determination is usually based on chromatographic separations using reversed-phase [2–5], cation-exchange [6] and supercritical fluid chromatography [7] coupled with electrochemical or fluorimetric detection. In most instances a preconcentration step is required owing to the lack of sensitivity and in order to purify samples before the analysis. Extraction procedures, either solid–liquid (namely alumina microcolumn [8], metal-loaded silica [4,9,10], immobilized boronates [11] or shielded hydrophobic phase columns [12]) or liquid–liquid (namely catecholamine–borate complexes [4,13]) have been developed in addition to a fully automated HPLC procedure [14], and the recoveries of analytes ranged between 63% and 100%. In cation-exchange chromatography [6] 3,4-dihydroxybenzylamine could not be used as an internal standard because it was co-eluted with adrenaline and more recently this method has been combined with a purification step based on an ion-exclusion procedure [15]. A highly sensitive determination of biogenic amines has been obtained by coulometric detection with multiple electrodes [16].

The aim of this work was to develop a routine method for the determination of catecholamines in urine samples based on cation-exchange sepa-

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ration with an on-line purification step and electrochemical detection.

2. Experimental

2.1. Apparatus and materials

The HPLC instrumentation was composed by a Gilson (Villiers-le-Bel, France) Model 305 and Model 307 double-pump system, a Model 401 diluter, a Model 232 autosampler equipped with a Rheodyne (Cotati, CA, USA) Model 7010 liquid chromatographic injector (100-μl sample loop) and a Model 7000 six-port switching valve. A Model Coulochem II electrochemical detector (ESA, Bedford, MA, USA) equipped with a Model 5011 dual coulometric–amperometric detector cell and a Hewlett-Packard (Palo Alto, CA, USA) Model 3393A reporter–integrator were used.

Ionpac CG-5 (13 μm) and Ionpac CG-10 (8.5 μm) polymeric cation-exchange microcolumns (Dionex, Sunnyvale, CA, USA) (50 × 4 mm I.D.) were used for sample prepurification and as a guard column, respectively. The analytical column was a Dionex Ionpac CS-10 (8.5 μm) (250 × 4 mm I.D.). Standard solutions were introduced into a 100-μl injection loop or loaded on to the Ionpac CG-5 for the prepurification procedure. After optimization (see below), the washing solution, for on-line sample purification, was water–methanol (90:10, v/v) containing 1.5 m*M* sodium formate at a flow-rate of 1.0 ml/min. The mobile phase was water–methanol (88:12, v/v) containing 120 m*M* sodium hydroxide and 260 m*M* formic acid and isocratic elutions were performed at a flow-rate of 0.9 ml/min. Prior to use, the eluent was filtered through a 0.2-μm membrane filter. Fig. 1 shows the on-line system and steps for the sample prepurification and chromatographic separation.

High-purity water obtained with a Milli-Q system (Millipore, Bedford, MA, USA) was used for preparing all solutions. Sodium hydroxide, formic acid, phosphoric acid and hydrochloric acid were purchased from Carlo Erba (Milan, Italy) and methanol from Riedel-de Haën (Schering, Selze, Germany). All reagents were of HPLC grade.

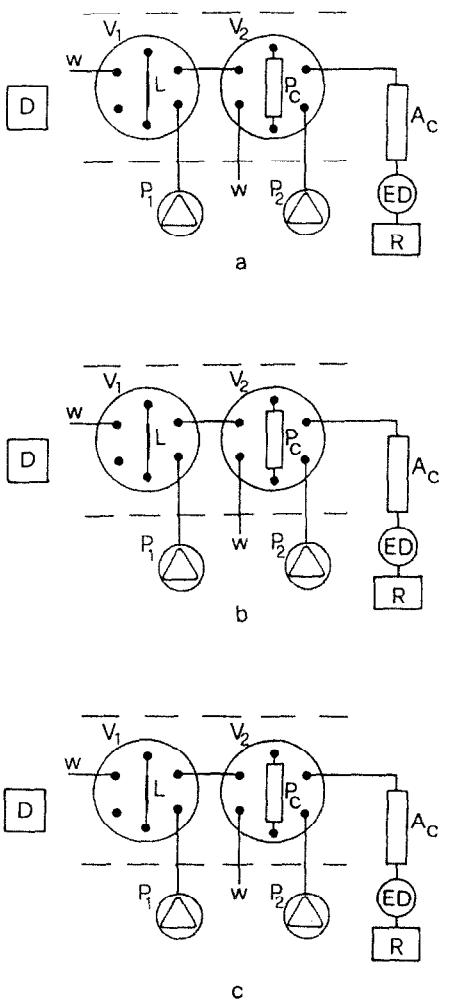


Fig. 1. Scheme and steps for sample purification and chromatographic elution. D = diluter–injector; L = loop (100 μl); P_1 and P_2 = pumps; V_1 and V_2 = six-port switching valves; P_c = column for sample purification (CG-5); A_c = analytical precolumn and column (CG-10, CS-10); ED = electrochemical detector; R = recorder. (a) V_1 and V_2 are switched, the sample is loaded into the loop (30 s), the purification column (CG-5) is washed and equilibrated, separation is run (20 min); (b) V_1 is switched, sample is transferred and purified into CG-5 (20 min), the analytical column is reconditioned; (c) V_2 is switched for 3 min and the sample is eluted into the analytical column.

Adrenaline (A), dopamine (DA), noradrenaline (NA) and 3,4-dihydroxybenzylamine (DHBA) were purchased from Sigma (St. Louis,

MO, USA). Stock standard solutions of A, DA and NA were prepared by dissolving 500 mg/l of each compound in 0.05 *M* H_3PO_4 and stored at −20°C. Working standard solutions (50 ng/ml A, 200 ng/ml DA and 50 ng/ml NA) were prepared daily by dilution with 0.05 *M* H_3PO_4 and finally diluted 1:1 (v/v) with 0.05 *M* H_3PO_4 solution containing 0.9 m*M* DHBA as an internal standard.

2.2. Urine samples

Urine samples (24 h collection) with 6 *M* HCl added (10 ml/l) were frozen at −20°C. Aliquots of urine were filtered through a Millex-GS 0.22-μm filter (Millipore, Saint-Quentin Yvelines, France), diluted 1:1 (v/v) with 0.9 m*M* DHBA and processed. Samples kept at 4°C or frozen at −20°C could be processed after 1 week and 6 months, respectively, without loss of analytes.

3. Results and discussion

3.1. Detection

A dual coulometric–amperometric detector cell (ESA Model 5011) was used for the detection of catecholamines. Different potentials were checked in order to optimize the sensitivity by total oxidation of the interfering compounds (first electrode) and to detect catecholamines (second electrode). Hydrodynamic voltammograms for the catecholamines (Fig. 2) show that their oxidation begins at 400 mV and a good sensitivity is reached at 700 mV. At lower values of the potential (200 mV) at the first electrode, with a constant value of 700 mV for the second electrode, all the interferents were not oxidized. The peak of DA was tailed and it became free from interferences at 400 mV or higher values. Low baseline noise and removal of interferents were obtained by working at potentials of 500 and 700 mV for the first and the second electrodes, respectively. Higher potentials at the second electrode showed an enhanced sensitivity, which is not required for this kind of sample.

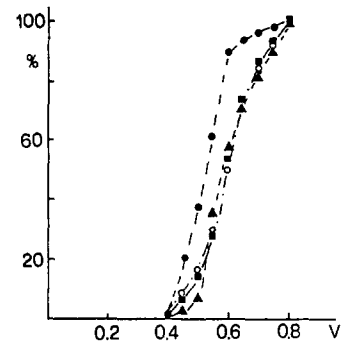


Fig. 2. Hydrodynamic voltammograms, applied voltage *vs.* detector response (as a percentage of maximum signal obtained). ■ = Noradrenaline; ○ = adrenaline; ● = dopamine; ▲ = 3,4-dihydroxybenzylamine.

3.2. Chromatographic conditions

In order to develop the separation procedure, the pH, ionic strength and organic modifier content in the eluent were optimized in the following way: 100 μl of standard solutions were injected and run without the prepurification column using water–methanol (90:10, v/v). The eluent was 260 m*M* formic acid with NaOH concentrations ranging from 72 to 144 m*M* and pH values between 3.03 and 3.55. The pH values of the aqueous solution were chosen according to the pK_a of the catecholamines (9.7, 10.3 and 9.9 for NA, A and DA, respectively [17]) so as to have the analytes in cationic form. In addition, low pH values prevented the oxidation of catecholamines that occurs in basic medium. Fig. 3

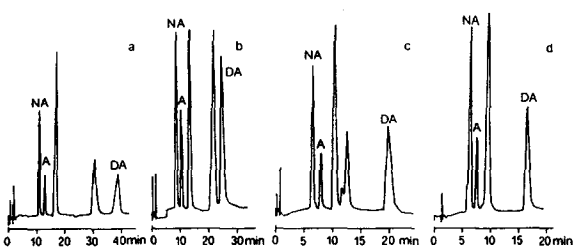
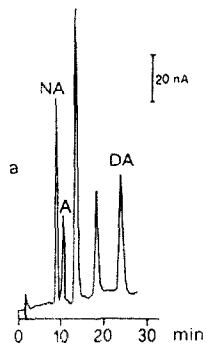


Fig. 3. Effect of sodium hydroxide concentration on retention times of noradrenaline (NA), adrenaline (A) and dopamine (DA). Chromatographic conditions: mobile phase, water–methanol (90:10, v/v) containing 260 m*M* formic acid and (a) 72 m*M* NaOH (pH 3.03), (b) 96 m*M* NaOH (pH 3.21), (c) 120 m*M* NaOH (pH 3.39) and (d) 144 m*M* NaOH (pH 3.55); flow-rate, 1.0 ml/min; injection volume, 100 μl.

shows that the retention times of the analytes decrease with increasing NaOH concentration, *i.e.*, Na^+ competes for cation sites, according to the ion-exchange mechanism. Experiments were also performed to evaluate the behaviour of the chromatogram as a function methanol concentration. Fig. 4 shows the chromatograms obtained for water–methanol mixtures containing 0, 10 and 20% methanol. The results indicate that the separation cannot be considered as a pure ion-exchange mechanism and a partition effect is also active. The best compromise between good resolution and acceptable retention times was obtained with water–methanol (90:10, v/v). For the analysis of urine samples a precolumn (CG-10) of the same kind as the separator was added to the system. This configuration caused an increase in the retention times of the analytes, and additional experiments showed that water–methanol (88:12, v/v) was the optimum composition of the eluent (Fig. 5).

3.3. Purification procedure

Owing to the complex matrix of urine, a clean-up step is required before the analysis. The loop was connected with a CG-5 cation-exchange microcolumn (see Section 2.1), which is characterized by a low hydrophobicity and the presence of some residual anion sites. This kind of column was selected in order to avoid strong retention of amines and oxidizable impurities present in urine that interfere in the determination of catecholamines. The following sequence was used: 100 μl (standard solution or sample with DHBA added as mentioned above) were loaded on to the loop, which was connected with the CG-5 column, where the sample was washed with water–methanol (90:10) containing 1.5 m*M* sodium formate at a flow-rate of 1 ml/min (actual pH 6.30). A detailed study was made to optimize the washing time in order to maximize the removal of interferences without decreasing the recovery of catecholamines. A washing time of 18 min was sufficient to remove the matrix and good reproducibility for the recovery of catecholamines



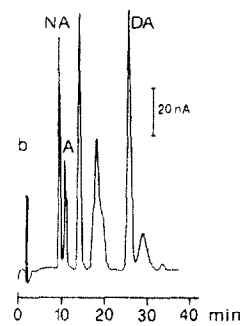


Fig. 5. Chromatograms obtained from: (a) standard (NA 50 ng/ml, A 25 ng/ml and DA 50 ng/ml); (b) urine sample (NA 34 ng/ml, A 21 ng/ml and DA 132 ng/ml). Chromatographic conditions: mobile phase, water–methanol (88:12, v/v) containing 120 m*M* NaOH and 260 m*M* HCOOH (pH 3.39); flow-rate 0.9 ml/min. Injection volume, 100 μl.

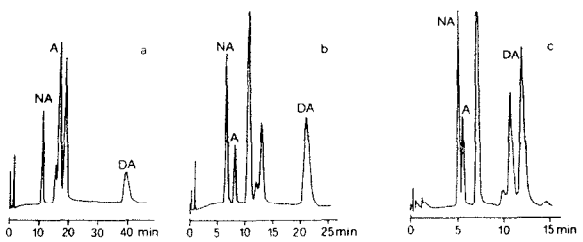


Fig. 4. Effect of methanol concentration on noradrenaline (NA), adrenaline (A) and dopamine (DA) retention times. Chromatographic conditions: mobile phase, water–methanol: (a) (100:0, v/v), (b) (90:10, v/v), (c) (80:20, v/v) containing 96 m*M* NaOH and 260 m*M* HCOOH (pH 3.21). Injection volume, 100 μl.

resulted up to 23 min. A washing time of 20 min was selected.

3.4. Recovery

The recovery was determined by comparing the peak areas obtained by direct injection of the standard solutions with those obtained by following the column-switching purification procedure with the same amount of catecholamines (n = 10). Volumes of 100 μl (50, 25, 100 and 100 ng/ml for NA, A, DA and DHBA, respectively) were injected and purified as indicated above. The recoveries found were NA 96%, A 90%, DA 94% and DHBA 95% with R.S.D.s of 2.6, 3.8, 2.9 and 2.0%, respectively. Urine samples analysed as such or spiked gave the same recoveries. During the optimization and validation of the method, 1000 samples were processed without a decrease in the column efficiency.

3.5. Linearity, detection limits and precision

Under the optimized chromatographic conditions, linear relationships between catecholamine (CAT) concentration and peak-area ratio (CAT/DHBA) (concentrations 0.20–1600 ng/ml NA, 0.20–800 ng/ml A and 0.78–3200 ng/ml DA) gave correlation coefficients of 0.9999, 0.9999 and 0.9998 for NA, A and DA, respectively. The detection limits were 5.0 pg (NA), 10.0 pg (A) and 10.0 pg (DA) at a signal-to-noise ratio of 3 (Table 1).

Table 2
Reproducibility for the whole procedure in the analysis of pooled urine (n = 10)

Catecholamines	Concentration (ng/ml)	S.D. (ng/ml)	R.S.D. (%)
Noradrenaline	23.65	0.45	1.89
Adrenaline	12.67	0.29	2.30
Dopamine	345.54	9.57	2.77

The reproducibility was evaluated by performing ten replicate analyses of an urine sample. Table 2 summarizes the results obtained.

3.6. Time optimization

The whole procedure of purification, separation and determination was improved by evaluating also the time required to transfer all the sample from clean-up column to the analytical column and the time to wash the prepurification column. Experiments with standard solutions and samples showed that after 3 min catecholamines are totally removed from the prepurification column and 20 min are required to wash it with the same solution as used to purify samples. Taking into account the time required for sample clean-up (20 min), by coupling the autosampler for sample injection, analysis and prepurification simultaneously performed required about 45 min.

Table 1
Linear response range and detection limit for catecholamines (n = 14)

Catecholamine	Linear range (ng/ml)	Correlation coefficient	Regression equation[a]	Detection limit (pg)
Noradrenaline	0.20–1600	0.9999	$y = 0.0019 + 0.0544x$	5.0
Adrenaline	0.20–800	0.9999	$y = 0.0178 + 0.0328x$	10.0
Dopamine	0.78–3200	0.9998	$y = 0.3797 + 0.0288x$	10.0

[a] y = Peak area ratio (CAT/DHBA); x = catecholamine concentration (ng/ml).

4. Conclusions

The ion chromatographic procedure developed for the simultaneous purification and determination of noradrenaline, adrenaline and dopamine gave the possibility of routine application to the analysis of urine samples with high sensitivity, good precision and a short time of analysis.

5. Acknowledgements

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6. References

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