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Note

Simple and fast analysis of adrenaline and noradrenaline in plasma on microbore high-performance liquid chromatography columns using fluorimetric detection

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The concentration of catecholamines in body fluids can provide valuable information about certain metabolic disorders in man associated with hypertension [1], psychiatric syndromes [2] and adrenal or neuronal tumours [3]. Therefore there is a need for a fast and sensitive method to analyse these compounds in body fluids, preferably in plasma.

Among available techniques such as fluorimetry [4], gas chromatography [5, 6] and radioimmunoassay [7], high-performance liquid chromatography (HPLC) combined with electrochemical [8, 9] or fluorimetric detection [10] has shown to be eminently suited for this analytical problem. However, in our hands, with HPLC until now reliable results could be only obtained with samples containing relatively large catecholamine concentrations as found in urine or tissues. With plasma samples, in which the common catecholamine concentrations are very low and moreover the available sample quantity is limited, a large scattering in the results is found.

The analysis in plasma has to be performed close to the detection limit (i.e. small signal-to-noise ratio). This is a typical case of limitation in sample amount and in combination with a concentration sensitive detection for which small-bore columns offer improved detectability:

$$c_i^{max} = \frac{Q_i}{\sqrt{2\pi}\,\epsilon_m\, A_s\,(1 + k'_i)\,\sqrt{LH_i}} \qquad (1)$$

where c_i^{max} = the maximal concentration (the peak height) of a solute at the end of the column,

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Q_i = amount of solute injected (injection volume × concentration of the solute in the sample),
ϵ_m = mobile phase porosity,
A_s = cross-sectional area of the column,
L = length of the column,
H_i = theoretical plate height.

According to eqn. 1, the detectability will be improved, at constant injected amount of solute, by decreasing the cross-sectional area of the column, the length of the column and the theoretical plate height (the capacity ratio k' has usually already been optimized). This points to short small-bore columns filled with small particles, providing the contribution of the external peak broadening caused by the injection and detection system can be kept small.

In the present study we report the results of an investigation into the applicability of 200 × 1.2 mm I.D. columns for the analysis of catecholamines in 0.5—1 ml of plasma, using on-column concentration and fluorimetric detection. Fluorimetric detection was chosen instead of electrochemical detection, which is quite suitable to combine with microbore columns, because of its greater selectivity which simplifies the sample pretreatment.

EXPERIMENTAL

Apparatus

The liquid chromatograph consisted of a high-pressure pump (Eldex Type B 100-5-2, Menlo Park, CA, U.S.A.), a flow-through manometer acting as a pulse dampener, a high-pressure sampling valve (Rheodyne 7125, Berkeley, CA, U.S.A.), and a fluorimeter (Perkin Elmer, LS-4, U.S.A.) equipped with a 3-μl cell. The wavelengths were set at 278 nm (excitation) and 317 nm (emission). The columns were of 316 stainless steel with dimensions of 200 × 1.2 mm I.D. The inner surface of the tubes was extensively polished before packing [11]. The columns were directly fitted into the injection valve in order to minimize extra peak broadening.

Materials

All solvents were of analytical grade and used without any pretreatment. For the liquid—liquid extraction of the catecholamines (see ref. 9) the quality of *n*-octanol was found to be of crucial importance in order to obtain high recoveries and clean extracts. In this respect, excellent results were obtained with *n*-octanol p.a. from Riedel-De Hean (F.R.G.).

The reversed-phase support was Hypersil ODS 5 μm (Shandon, U.K.).

Procedures

Chromatography. The microbore columns were packed by a slurry technique [11], at constant flow-rate (1.2 ml/min) and a maximum pressure of 600 bars. The slurry liquid consisted of tetrachloromethane + 5% (v/v) methanol and the displacer liquid was pure methanol. The columns were washed with 10 ml of methanol and then equilibrated with the mobile phase. The mobile phase consisted of water—methanol (3:1, v/v) containing 0.15 *M* acetic acid buffer (pH 4.7) 0.04—0.06% (w/v) sodium dodecyl sulphate, 0.01% (w/v) EDTA and 0.01% (w/v) sodium chloride.

Extraction. For the isolation of adrenaline and noradrenaline from plasma, use was made of a selective liquid—liquid extraction system [9]: 0.5—1 ml of plasma was mixed with 0.5 ml of a 2 *M* NH_4OH—NH_4Cl buffer of pH 8 containing 0.2% (w/v) diphenylborate—ethanolamine (DPBEA) and 0.5% (w/v) EDTA. To this mixture 2.5 ml of the extraction solvent, consisting of *n*-heptane containing 1% (w/v) *n*-octanol and 0.25% (w/v) tetraoctylammonium bromide, were added, intensively mixed for 2 min and centrifuged for 5 min at 1200 *g*. Then 2 ml of the organic phase were transferred into a conically shaped glass tube, and 1 ml of *n*-octanol and 0.125 ml of 0.08 *M* acetic acid added. After intensively mixing for 2 min and centrifugation for 5 min at 1200 *g*, 5—100 µl of the aqueous layer were injected into the HLPC system.

It must be noted that the pH of the NH_4OH—NH_4Cl buffer should not exceed a value of 8, as at higher pH the methylated catecholamines, metanephrine and normetanephrine, present in plasma can be partly converted into adrenaline and noradrenaline and thus give rise to misleading results.

RESULTS AND DISCUSSION

Chromatography

The efficiency of the microbore columns was tested with a chromatographic set-up with negligible external peak broadening [11] using methanol—water (8:2, v/v) as the mobile phase and toluene ($k' = 2$) as the solute. Under these experimental conditions the plate number varied between 15,000 and 17,000, at a linear velocity of 1 mm/sec. This corresponds to a reduced plate height of about 2.5. When installing the microbore column in the chromatographic set-up for the catecholamines, the observed plate number dropped to about 8000. This loss can be attributed to the significantly greater external peak broadening

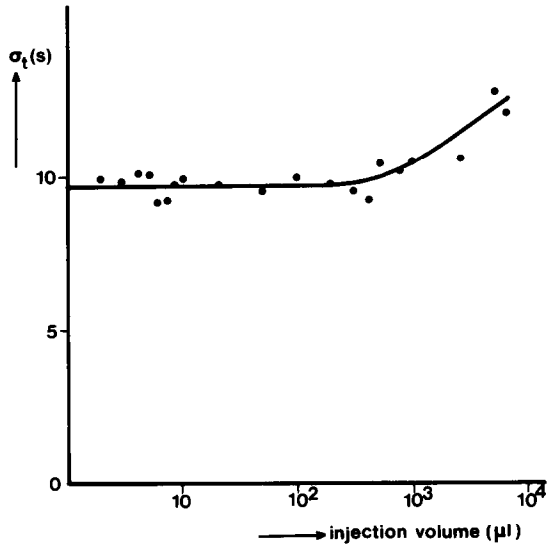

Fig. 1. The standard deviation of the elution peak of noradrenaline (σ_t) as a function of the injection volume of an aqueous test solution on a microbore column. For conditions, see experimental section.

caused by the injection and detection system and by the type of phase system and solutes used. Despite this loss in efficiency, the microbore columns still show an efficiency comparable to a 150 × 4.6 mm I.D. column. Thus the profit of less dilution in the detection of catecholamines can be still exploited, providing the same sample quantity can be injected as on the usual sized columns. Unfortunately the sample volume that can be injected on the microbore columns without losing efficiency is very small (< 1 μl) when the sample is dissolved in the mobile phase. Under these conditions the application of microbore columns is less favourable. However, it is possible to inject much larger sample volumes, without influencing the efficiency, when the sample can be concentrated at the top of the column. This occurs when the sample is dissolved in a non-eluting liquid [12]. On reversed-phase columns, water behaves as a non-eluting liquid and thus is very suitable as a sample liquid.

In order to test water as non-eluting sample liquid and to determine the sample volume which can be injected under these conditions, the standard deviation of noradrenaline was measured as function of the injection volume of the aqueous sample. The results of these measurements are shown in Fig. 1. The standard deviation was chosen for monitoring the efficiency rather than the theoretical plate height because of the uncertainty in measuring the retention time when injecting samples in non-eluting liquids. From Fig. 1 it can be seen that the standard deviation of the peak is virtually constant up to an injection volume of 1 ml and then slowly increases at larger injection volumes. These results show that, when injecting the catecholamines in an aqueous

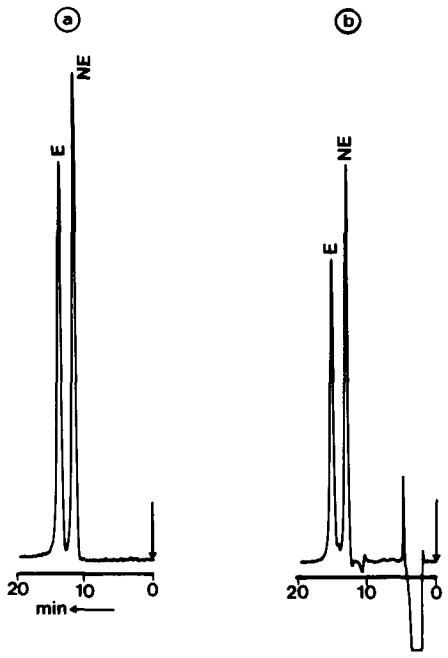

Fig. 2. Chromatograms of a test mixture of adrenaline (E) and noradrenaline (NE) on a microbore column: (a) 0.5-μl injection of the catecholamines dissolved in the mobile phase; (b) 100-μl injection of the catecholamines dissolved in water. For conditions, see experimental section. Sodium dodecyl sulphate concentration = 0.04% (w/v).

solution, similar injection volumes can be applied as on normal sized columns. This is demonstrated in Fig. 2, which shows chromatograms of a 0.5-μl injection of a solution of catecholamines in the mobile phase and of a 100-μl injection of an aqueous solution of the catecholamines.

The catecholamines are isolated from 0.5—1 ml of plasma by liquid—liquid extraction and then back-extracted into 125 μl of aqueous phase. From Fig. 1 it can be seen that, if necessary, the whole 125-μl extract (corresponding to 0.5—1 ml of plasma) can be injected onto the microbore column without influencing the performance.

Analysis of plasma samples

The developed method was applied to plasma samples of essentially healthy persons and of patients. Fig. 3a shows a chromatogram of a 50-μl injection of the extract from plasma of a healthy person. The catecholamine levels were found to be 120 pg/ml adrenaline and 200 pg/ml noradrenaline. Fig. 3b shows a chromatogram of a 20-μl injection of the plasma extract of a patient suffering from hypoglycaemia. The catecholamine levels are significantly higher (2.1 ng/ml adrenaline and 2.3 ng/ml noradrenaline) which simplifies the analysis. Fig. 3a and b shows that owing to the selective liquid—liquid extraction system and the selective measurement via native fluorescence a very clean chromato-

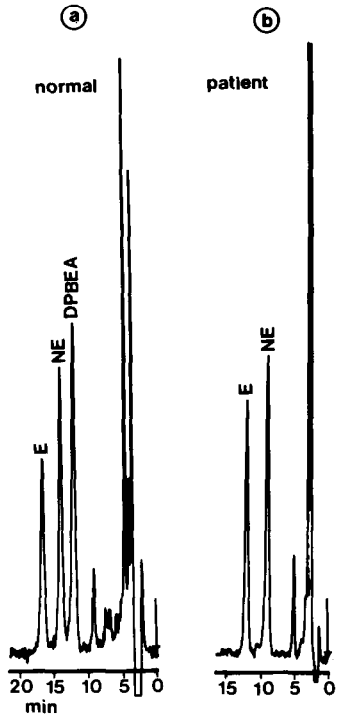

Fig. 3. Chromatograms of extracted plasma samples on the microbore HPLC system: (a) 50-μl injection of an extract obtained from 1 ml plasma of a healthy person; (b) 20-μl injection of an extract obtained from 1 ml plasma of a patient suffering from hypoglycaemia. For conditions, see experimental section. Sodium-dodecyl sulphate concentration = 0.06% (w/v). Peaks: E = adrenaline; NE = noradrenaline; DPBEA = diphenylborate—ethanolamine.

gram is obtained, which allows detection of adrenaline and noradrenaline at levels of about 10 pg/ml of plasma. The relatively large peak eluting in front of noradrenaline in Fig. 3a is diphenyl borate used in the extraction procedure. This peak appears only at very sensitive detector setting and shifts under the noradrenaline peak when the sodium dodecyl sulphate concentration in the mobile phase is $< 0.06\%$ (w/v).

Quantitative aspects

The precision and linearity of the chromatographic method were determined by injecting 50 μl of aqueous solutions of adrenaline and noradrenaline (20—16,000 pg/ml) and measuring the peak area or peak height. The calibration curves were linear over the investigated concentration range, with regression coefficients of 0.9998. The precision of the chromatographic method was determined from replicate analyses of a test mixture of the catecholamines. The relative standard deviation was found to be $\pm$ 0.5% (n = 25) at 500 pg injected amount. This is comparable to the results commonly obtained on normal sized columns.

The detection limit, defined as three times the standard deviation of the noise, was determined to be 5 pg for both catecholamines.

The recovery and reproducibility of the extraction were determined with aqueous test samples, containing 1 ng/ml of both adrenaline and noradrenaline. For both solutes the recovery was found to be 98.5% $\pm$ 0.5 (n = 6), in agreement with earlier reports [9]. The reproducibility of the whole analytical method was determined from replicate extractions of a pooled plasma sample and analysis of the extract by microbore HPLC. For adrenaline a mean value of 430 $\pm$ 8 pg/ml (n = 9) and for noradrenaline 706 $\pm$ 10 pg/ml (n = 9) was found.

CONCLUSIONS

Microbore columns, combined with on-column sample concentration and fluorimetric detection, are eminently suited for the analysis of adrenaline and noradrenaline in 0.5—1 ml of plasma down to levels of 10 pg/ml. The precision and reproducibility of microbore columns are comparable to those obtained on normal sized columns. In our experience microbore columns show a better column stability than normal sized columns. More than 800 plasma extracts could be analysed without any column deterioration.

Analysis of adrenaline and noradrenaline and possibly dopamine at the fg/ml level on microbore columns and fluorimetric detection looks feasible by applying a laser excitation source. This is now the subject of investigation in our laboratory.

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