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# DETERMINATION OF CATECHOLAMINES IN URINE (AND PLASMA) BY LIQUID CHROMATOGRAPHY AFTER ON-LINE SAMPLE PRE-TREATMENT ON SMALL ALUMINA OR DIHYDROXYBORYLSILICA COLUMNS

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## SUMMARY

The possibilities of the on-line pre-concentration of catecholamines on small columns packed with aluminium oxide (10 μm) or dihydroxyborylsilica (5 μm) prior to high-performance liquid chromatography (HPLC) were investigated. For both pre-concentration materials the influence of the pH of the mobile phase on the retention characteristics was studied for the catecholamines and some of their derivatives lacking the catechol and/or the amine function. The recovery of the catecholamines and the loading capacity on some pre-columns was then investigated. The compatibility of the pre-concentration system with several HPLC systems, viz., reversed-phase ion-pair, ion-exchange and reversed-phase ion-pair partition chromatography, was studied. Combined with amperometric or fluorimetric detection, the method was applied to the determination of free catecholamines in urine, using dihydroxybenzylamine or epinine as internal standards. An example of the determination of catecholamines in plasma containing 100 pg/ml of each catecholamine is also shown.

## INTRODUCTION

Liquid chromatography (LC) is well accepted as a method for the assay of catecholamines in body fluids such as plasma and urine. Generally, prior to LC a sample pre-treatment is necessary, and a first objective of this pre-treatment is

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clean-up of the sample. Depending on the solute concentration and the detection limits of the method, a second objective may be the pre-concentration of the catecholamines to improve their detectability. With post-column derivatization to their trihydroxyindoles followed by fluorimetric detection [1, 2] or electrochemical detection of the underivatized catecholamines [3–5], detection limits of 5–10 pg can be obtained. Applying native fluorescence detection, these low figures can only be obtained in miniaturized systems [6], whereas if wide-bore columns are used the detection limits are 100–500 pg [7]. This means that for urine, with concentrations of the free catecholamines of about 5–200 ng/ml [8, 9], pre-concentration is only necessary in non-miniaturized systems combined with native fluorescence detection. For plasma, with catecholamine concentrations of ca. 10–600 pg/ml [10–12], a pre-concentration step is always necessary.

Over the last twenty years, alumina extraction [13, 14] has been by far the most popular method for the pre-treatment of catecholamines from body fluids. Good results have also been reported using ion-exchange chromatography [15, 16]. Recently, some promising sample preparation methods have been described based on selective complexation at high pH between catecholamines and boric acid-derived materials, viz., boric acid gels [8, 17, 18] and diphenyl borate [19].

Until now, sample pre-treatment has mainly been carried out by off-line techniques, which usually are laborious and can give rise to a poor precision. On-line sample pre-treatment is more convenient to the operator, while generally the precision of the method is improved. In principle, on-line sample pre-treatment can be performed by both liquid–solid and liquid–liquid extraction. Kraak [20] described a method for on-line liquid–liquid extraction, using diphenyl borate, for the assay of catecholamines in urine. Goto and co-wørkers [4, 9, 21] adapted a micro-LC system for on-line sample preparation by liquid–solid chromatography, using small PTFE pre-columns filled with alumina. They assayed the catecholamines in urine and plasma with run times of about 1 h. Hansson et al. [22] investigated the characteristics of a laboratory-synthesized boronic acid–silica for the on-line pre-concentration of catechols and applied the system to the assay of 3,4-dihydroxyphenylacetic acid (DOPAC) in urine and brain tissue. Edlund and Westerlund [23] described a method for the direct determination of catecholamines in urine and plasma. This method incorporates two sample pre-treatment steps, viz., (i) on a boronic acid affinity column and (ii) on a reversed-phase ion-pair column. Kilts et al. [24] developed a method for the assay of catecholamines in plasma, combining off-line alumina sample pre-purification and on-line cation-exchange enrichment.

In this paper, we present the results of an investigation of the application of small columns packed with alumina (10 $\mu$m) or dihydroxyborylsilica (5 $\mu$m) to the on-line pre-treatment of catecholamines in urine and plasma samples prior to their determination by LC with electrochemical or fluorimetric detection.

## EXPERIMENTAL

### *Apparatus*

The apparatus was constructed from two SP 740 B pumps (Spectra-Physics, Santa Clara, CA, U.S.A.), a U6K injection system (Waters Assoc., Milford, MA,

U.S.A.) with a 2.5-ml sample loop and a Valco (Houston, TX, U.S.A.) six-way valve with the sample loop replaced by a pre-column. Stainless-steel pre-columns were laboratory made (65×4.6, 30×2.1 and 4×2.8 mm I.D.) or obtained commercially (10×2.0 and 6×2.0 mm I.D.) as a pre-concentration unit (Chrompack, Middelburg, The Netherlands). Detection was performed by means of an LS-4 spectrofluorimeter (Perkin-Elmer, Norwalk, CT, U.S.A.) with excitation and emission wavelengths set at 278 and 317 nm, respectively or an SFM 23 LC spectrofluorimeter (Kontron, Zürich, Switzerland) with excitation and emission wavelengths set at 280 and 310 nm, respectively, or by means of an amperometric detection system consisting of an E 230 potentiostat/amplifier (Bruker, Karlsruhe, F.R.G.) and a laboratory made wall-jet detector cell equipped with a glassy carbon working electrode, set at a potential of +0.65 V vs. the Ag/AgCl reference electrode. Peak areas were calculated with an Autolab Minigrator (Spectra-Physics) or a Chromatopac-E1A (Shimadzu, Kyoto, Japan).

*Packing procedures for the pre-columns*

The longer pre-columns (65×4.6 and 30×2.1 mm I.D.) were slurry packed with a DSHF-302 high-pressure air amplifier booster pump (Haskel, Burbank, CA, U.S.A.) by means of a technique described elsewhere [25] using ethanol–water (4:6, v/v) as the dispersing solvent and methanol as the displacing solvent. The 4×2.9 mm I.D. pre-column was packed by hand, using a microspatula. The pre-columns used in the Chrompack pre-concentration unit were packed by hand with a column packing device (Chrompack), especially constructed for this purpose, using a slurry of the material in methanol.

*Chemicals and reagents*

The compounds used as chromatographic reference substances are listed in Table I. All other chemicals were of analytical-reagent or reagent grade and were used without further purification. Water was purified with a Milli-Q purification system (Millipore, Bedford, MA, U.S.A.). The pre-concentration materials were 100 Polyol dihydroxyborylsilica, mean particle size 5 $\mu$m, irregular (Serva, Heidelberg, F.R.G.) and Alox D aluminium oxide, mean particle size 10 $\mu$m, irregular (Macherey & Nagel, Düren, F.R.G.).

*Chromatography*

The experimental set-up as used for on-line sample pre-treatment and LC analysis of the catecholamines is shown in Fig. 1. At time $t=0$ the sample is injected by means of valve $V_1$ (Waters U6K) and transported to the pre-column $C_1$ by means of pump $P_1$, which pumps mobile phase 1 (the "pre-concentration and clean-up mobile phase") at a flow-rate $w_1$. After a washing step, at time $t_I$ valve $V_2$ (Valco) is switched and the catecholamines are desorbed from $C_1$ and injected on to the "analytical column" $C_2$, where separation is effected; this is achieved with mobile phase 2 (the "desorption and separation mobile phase"), which is pumped by $P_2$ at a flow-rate $w_2$. At time $t_{II}$ valve $V_2$ is switched back in order to recondition $C_1$ for pre-treatment of the next sample. Using the configuration as depicted in Fig. 1, the catecholamines are desorbed from $C_1$ by means

TABLE I

STRUCTURES, CLASSIFICATION AND ABBREVIATIONS OF THE COMPOUNDS INVESTIGATED

| Group | Structure | Name | Abbreviation | $R_1$ | $R_2$ | $R_3$ | Origin |
|---|---|---|---|---|---|---|---|
| Catecholamines | (3,4-HO, HO)-phenyl-CH($R_1$)-CH($R_2$)-$NHR_3$ | Noradrenaline | NA | OH | H | H | Fluka, Buchs, Switzerland |
| | | Adrenaline | A | OH | H | $CH_3$ | Boehringer, Ingelheim, F.R.G. |
| | | Dopamine | DA | H | H | H | Aldrich, Beerse, Belgium |
| | | α-Methyldopamine | α-Me-DA | H | $CH_3$ | H | MSD, West Point, PA, U.S.A. |
| | | Epinine | EPI | H | H | $CH_3$ | Sigma, St. Louis, MO, U.S.A. |
| | (3,4-HO, HO)-phenyl-$CH_2$-$NH_2$ | 3,4-Dihydroxybenzylamine | DHBA | | | | Aldrich |
| Derivatives | | | | | | | |
| Class I | ($CH_3O$, HO)-phenyl-CH($R_1$)-$CH_2$-$NHR_2$ | Normetanephrine | NMN | OH | H | | Sigma |
| | | Metanephrine | MN | OH | $CH_3$ | | Sigma |
| | | 3-Methoxytyramine | 3-MT | H | H | | Aldrich |
| Class II | (HO, HO)-phenyl-CH($R_1$)-$R_2$ | 3,4-Dihydroxyphenylalanine | DOPA | H | $NH_2CHCOOH$ | | Fluka |
| | | α-Methyldopa | α Me-DOPA | H | $NH_2C(CH_3)COOH$ | | MSD |
| | | 3,4-Dihydroxyphenylacetic acid | DOPAC | H | COOH | | Fluka |
| | | 3,4-Dihydroxyphenylethylene glycol | DOPEG | OH | $CH_2OH$ | | Aldrich |
| | | 3,4-Dihydroxyphenylethanol | DOPET | H | $CH_2OH$ | | Labkemi, Gothenburg, Sweden |
| Class III | ($CH_3O$, HO)-phenyl-CH($R_1$)-$R_2$ | Homovanillic acid | HVA | H | COOH | | Fluka |
| | | 3-Methoxy-4-hydroxyphenyl-ethylene glycol | MOPEG | OH | $CH_2OH$ | | Aldrich |

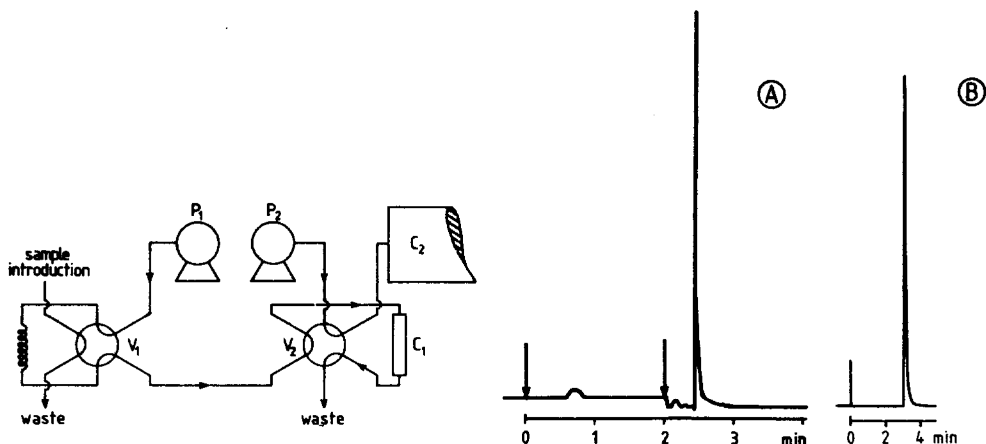


Fig. 1. Experimental set-up of the on-line pre-concentration system. $P_1$ and $P_2$=pumps; $V_1$ and $V_2$=valves; $C_1$=pre-column; $C_2$=separation column. See text for further explanation.

Fig. 2. Elution profiles of the catecholamines at low pH after pre-concentration at high pH. (A) On dihydroxyborylsilica. Column dimensions: 65×4.6 mm I.D. Mobile phase 1: 0.01 *M* phosphate, 0.01% (w/v) EDTA, pH 8.3; flow-rate ($w_1$), 1.5 ml/min. Mobile phase 2: 0.23 *M* perchlorate, pH 2.4; flow-rate ($w_2$), 1.25 ml/min. Injection of 1 ml of a mixture of A, NA and DA (180 ng/ml) in the pre-concentration mobile phase. $t_1$=2 min; elution from the pre-column by back-flushing. Detection: fluorimetric, LS-4. (B) On alumina. Column dimensions: 10×2.0 mm I.D. Mobile phase 1: 0.01 *M* Tris, pH 8.0; $w_1$=1.5 ml/min. Mobile phase 2: system II, see Experimental, pH 3.0; $w_2$=1.0 ml/min. Injection of 2 ml containing 50 ng/ml A, NA, DA and DHBA in 0.05 *M* EDTA, 0.01 *M* sodium metabisulphite, 0.01 *M* Tris, pH 8.0. $t_1$=3 min; elution by forward flushing. Detection: fluorimetric, SFM 23 LC.

of forward flushing. In some experiments back-flushing was applied. The configuration for back-flushing can be obtained by a simple reversal on $V_2$ of the connecting capillaries with $P_2$ and $C_2$.

The composition of the pre-concentration mobile phase is given under Results and Discussion. Separation of the catecholamines was achieved on three different chromatographic systems.

*System I: ion-exchange chromatography.* Zorbax 300 SCX, 7–8 μm mean particle size, spherical (DuPont, Wilmington, DE, U.S.A.), was used as the stationary phase in a 250×4.6 mm I.D. stainless-steel column; the mobile phase contained 0.01% (w/v) EDTA, 0.01% (w/v) sodium chloride and 0.05 *M* phosphate adjusted to pH 2.31 (90%, v/v) and methanol (10%, v/v).

*System II: reversed-phase ion-pair partition chromatography.* This phase system is a modification of systems described by Janssen et al. [26, 27]. The stationary phase was tri-*n*-butyl phosphate (Aldrich, Beerse, Belgium) coated on Nucleosil C8, 5 μm mean particle diameter, spherical (Macherey & Nagel). The mobile phase contained 0.25 *M* perchlorate and 0.002% (w/v) EDTA and was buffered at pH 2.5 or 3.0 with 0.05 *M* phosphate. The column (100×3.0 mm I.D.) and the eluent reservoir were thermostated at 294 K.

*System III: reversed-phase ion-pair chromatography.* This is a modification of a system described by Crombeen et al. [28]. Zorbax ODS, 7–8 μm mean particle

diameter (DuPont) was used as the stationary phase in a 250×4.6 mm I.D. stainless-steel column; the mobile phase contained 0.01% (w/v) sodium lauryl sulphate, 0.01% (w/v) sodium chloride, 0.01% (w/v) EDTA, buffered with 0.11 *M* phosphate to pH 3.5 (75%, v/v) and methanol (25%, v/v).

*Sample preparation*

After collection, the urine samples were acidified immediately to pH 2 with 85% phosphoric acid and after addition of the internal standard the samples were stored at 277 K. Prior to on-line sample pre-treatment the pH was adjusted to the appropriate value. The sample preparation procedure for plasma samples is given under Results and Discussion.

## RESULTS AND DISCUSSION

*Adsorption and desorption characteristics of catecholamines and some derivatives on alumina and dihydroxyborylsilica*

A first objective of this study was to obtain an insight into the retention behaviour of catecholamines and some derivatives (see Table I) on alumina (AL) and dihydroxyborylsilica (DHBS) as a function of pH. The class of catecholamines includes dihydroxybenzylamine (DHBA) and epinine (EPI), which are potential internal standards, and $\alpha$-methyldopamine ($\alpha$-Me-DA), a major metabolite of the antihypertensive drug $\alpha$-methyldopa ($\alpha$-Me-DOPA) [26, 27]. In the group of derivatives, a subdivision has been made based on the absence of the catechol and/or the amine function (see Table I). The retention of the compounds on the two pre-concentration materials was measured by injection of the standards on a 3-cm column, applying eluents of pH 2–8 and fluorimetric detection of the column effluent. The retention volumes are summarized in Table II. At low pH values (<5) neither the catecholamines nor their derivatives are retained. At pH 8 only the class III derivatives are still unretained. At this pH the derivatives possessing the catechol function (class II) or the amine function (class I) show a considerable retention, but only the catecholamines are retained very strongly. Note that the void volume of the column used in this experiment is smaller than 0.1 ml, which means that at pH 8 the capacity ratios for the catecholamines are larger than 2700 and 240 on AL and DHBS, respectively. This implies that on both materials pre-concentration of these compounds can be performed, even if short pre-columns are used. Pre-concentration of some derivatives, e.g., DOPA, $\alpha$-Me-DOPA, DOPAC, DOPEG and DOPET (see Table I for abbreviations), is possible but requires longer pre-columns while stringent limits are put on the injection and washing volumes. On the other hand, if these compounds could interfere in the assay of the catecholamines, they can easily be eluted from the pre-column in the washing step.

From the large difference in the retentions between the catecholamines and their deaminated derivatives, some conclusions can be drawn about the mechanism of interaction between these compounds and the pre-concentration materials. Apparently, the strong retention is the result of two different mechanisms, viz., the relatively strong and selective complexation between the catechol func-

TABLE II

RETENTION VOLUMES OF THE CATECHOLAMINES AND THE THREE CLASSES OF THEIR DERIVATIVES ON ALUMINA AND DIHYDROXYBORYLSILICA AS A FUNCTION OF pH

Column dimensions: 30×2.1 mm I.D. Mobile phase: 0.005 *M* phosphate with various pH values. Flow-rate: 1.3 ml/min. Injection of 1-2 μl of a solution of the compounds (50-5000 μg/ml) in 0.05 *M* phosphate buffer (pH 2.5). Detection: fluorimetric, SFM 23 LC.

| Compound | Retention volume (ml) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Alumina | | | | | Dihydroxyborylsilica | | | | | | |
| | pH 2.0 | 5.0 | 6.0 | 7.0 | 8.0 | pH 2.0 | 3.5 | 5.0 | 6.0 | 7.0 | 7.5 | 8.0 |
| A | 0.22 | 0.33 | 2.2 | 32 | 430 | 0.16 | 0.20 | 0.46 | 1.2 | 7.6 | 25 | 34 |
| NA | 0.20 | 0.35 | 2.6 | 40 | 700 | 0.16 | 0.20 | 0.39 | 1.0 | 5.2 | 16 | 24 |
| DA | 0.20 | 0.31 | 1.8 | 20 | 330 | 0.16 | 0.20 | 0.40 | 1.1 | 6.7 | 21 | 33 |
| α-Me-DA | 0.20 | 0.29 | 1.5 | 20 | 350 | 0.18 | 0.20 | 0.46 | 1.2 | 7.7 | 21 | 33 |
| EPI | 0.20 | 0.29 | 1.6 | 14 | 270 | 0.18 | 0.22 | 0.52 | 1.7 | 12 | 40 | 52 |
| DHBA | 0.20 | 0.31 | 2.9 | 45 | 520 | 0.16 | 0.20 | 0.44 | 1.3 | 9.8 | 32 | 43 |
| NMN | 0.20 | 0.25 | 0.43 | 0.64 | 0.79 | 0.18 | 0.17 | 0.23 | 0.54 | 0.76 | 0.82 | 0.92 |
| MN | 0.20 | 0.21 | 0.43 | 0.51 | 0.61 | 0.16 | 0.20 | 0.25 | 0.67 | 0.96 | 1.3 | 1.6 |
| 3-MT | 0.20 | 0.25 | 0.38 | 0.55 | 0.69 | 0.18 | 0.20 | 0.27 | 0.71 | 1.1 | 1.4 | 1.7 |
| DOPA | 0.20 | 0.21 | 0.22 | 0.73 | 12 | 0.18 | 0.20 | 0.23 | 0.43 | 0.53 | 1.1 | 1.5 |
| α-Me-DOPA | 0.20 | 0.23 | 0.27 | 0.51 | 10 | 0.18 | 0.20 | 0.23 | 0.40 | 0.51 | 0.89 | 1.3 |
| DOPAC | 0.20 | 0.21 | 0.20 | 0.20 | 0.26 | 0.30 | 0.46 | 0.79 | 1.7 | - | - | 2.3 |
| DOPEG | 0.20 | 0.21 | 0.25 | 0.35 | 3.0 | 0.22 | 0.22 | 0.29 | 0.55 | 0.75 | 3.1 | 3.5 |
| DOPET | 0.20 | 0.21 | 0.22 | 0.40 | 2.5 | 0.26 | 0.26 | 0.41 | 0.70 | 0.73 | 5.1 | 4.9 |
| HVA | 0.20 | 0.21 | 0.18 | 0.18 | 0.18 | 0.30 | 0.39 | 0.37 | 0.30 | 0.25 | - | 0.24 |
| MOPEG | 0.20 | 0.21 | 0.20 | 0.20 | 0.18 | 0.22 | 0.20 | 0.21 | 0.26 | 0.26 | 0.24 | 0.29 |

tion and the hydroxyl functions of the AL or DHBS [18, 22, 29] and the weaker ion–ion interaction between the positively charged amine function and negative charges on the AL and DHBS.

In pre-concentration processes, the "breakthrough volume" rather than the retention volume is the important parameter. At higher pH values (6–8), the catecholamines elute from the AL and DHBS column as very broad and tailing peaks with typical plate numbers on the 3-cm column of 5–20. This phenomenon, which has also been noted for a boronic acid–silica synthesized by Hansson et al. [22], is probably caused by the slow kinetics of the chemical equilibria involved in the retention of the catecholamines. As a consequence, the breakthrough volumes are about half those given in Table II.

The results presented in Table II give some guidelines for the selection of the conditions for on-line pre-concentration of the catecholamines. For pre-concentration a high retention is necessary whereas in the desorption step the retention should be negligibly small. For pre-concentration a pH of ca. 8 is the best choice.

Above this pH the risk of oxidation of the catecholamines increases considerably. Moreover, for DHBS an increase of the pH above 8 does not result in a further increase in the retention of the catecholamines and leads to a significant dissolution of the silica matrix, which frequently causes serious clogging of the pre-column. At pH $<5$ the retention of the catecholamines on both materials is negligible and therefore the mobile phase used for desorption of the catecholamines should have a pH $<5$.

In order to minimize the contribution of the pre-concentration process to peak broadening, it is very important to elute the catecholamines from the pre-column in a volume as small as possible. This implies that after valve switching the pH in the pre-column should change instantaneously from a basic to an acidic value. This is favoured by performing the pre-concentration step with a relatively low buffer concentration (0.005 or 0.010 *M* Tris or phosphate) and the desorption step with a more concentrated buffer of low pH. In Fig. 2, for both pre-concentration materials an example is shown of the elution profile of the catecholamines at low pH (2.4 for DHBS and 3.0 for AL) after pre-concentration at basic pH. The step gradient causes the catecholamines to be eluted from the pre-column in a small volume, which indicates that at pH $\leqslant 3$ the catecholamines are rapidly desorbed from the pre-concentration materials.

Throughout this work forward flushing was applied for the small pre-columns ($4\times2.8$, $10\times2.0$ and $6\times2.0$ mm I.D.) and back-flushing was applied for the longer pre-columns ($65\times4.6$ and $30\times2.1$ mm I.D.) for desorption of the catecholamines (see Fig. 2 and below). For the small pre-columns backward and forward flushing yielded the same results in terms of efficiency; for the longer pre-columns some extra band broadening was observed in the forward flushing mode owing to dispersion in the pre-column during the desorption step.

### *On-line coupling with HPLC*

For the on-line pre-concentration system as described here, in principle three different liquid phases are needed: one for pre-concentration and clean-up of the sample, a second for desorption of the catecholamines from the pre-column and a third for their separation. Obviously, if the same mobile phase can be used for desorption and separation of the catecholamines, the experimental set-up is simplified considerably. Applying this approach, we investigated the compatibility of some chromatographic systems frequently used for the assay of catecholamines [5, 26–28] with the on-line pre-concentration system. Fig. 3A shows an example of the separation of the catecholamines by means of ion-exchange chromatography after on-line pre-concentration of 1 ml of the standards on a DHBS pre-column. Compared with direct injection of the same amount (50 ng) of each compound in a small volume (see Fig. 3B), there is no significant extra band broadening and peak heights decrease by about 10%.

Fig. 4 shows some characteristics of the combination of reversed-phase ion-pair partition chromatography for the separation step and AL for the pre-concentration step. Fig. 4A shows the separation of the catecholamines after direct injection of 6 $\mu$l of the standards on the separation column (mass injected 1 ng per compound). If the same amount is introduced by means of the pre-concen-

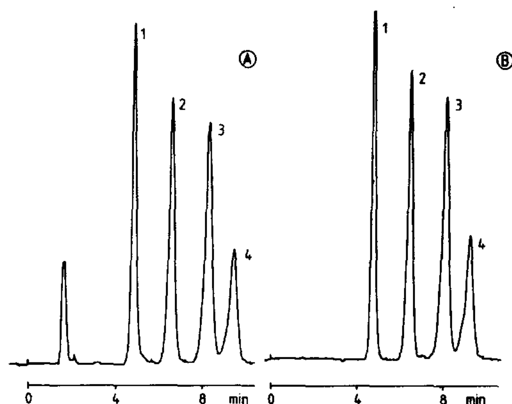


Fig. 3. Comparison between separation of the catecholamines after on-line pre-concentration on dihydroxyborylsilica (A) and direct separation of the catecholamines (B). Separation: system I, see Experimental. Detection: fluorimetric, LS-4. (A) Injection via the pre-column (30×2.1 mm I.D.) of 1 ml of a mixture (5 ng/ml per component) of (1) NA, (2) DHBA, (3) A and (4) DA in mobile phase 1 [0.01 *M* phosphate, 0.01% (w/v) EDTA, pH 8.1]; $w_1 = 1$ ml/min. Mobile phase 2: system I, see Experimental; $w_2 = 1.3$ ml/min. $t_I = 2$ min (indicated in the figure as $t = 0$ min); $t_{II} = 5$ min; elution from the pre-column by back-flushing. (B) Direct injection on the analytical column of 50 $\mu$l of a mixture containing 100 ng/ml of each component. Mobile phase: system I, see Experimental; flow-rate 1.3 ml/min.

tration column the results are as shown in Fig. 4B (volume injected 6 $\mu$l) and Fig. 4C (volume injected 2 ml). For the three biogenic catecholamines the peak heights and chromatographic efficiencies are similar if 6 $\mu$l are introduced directly or via the pre-column. If the catecholamines are introduced via the pre-column in 2 ml (the only situation in which real pre-concentration occurs) there is some extra band broadening and the peak heights decrease by about 20%. For DHBA, introduction of 6 $\mu$l via the pre-column causes a decrease in peak height of about 25% (see Fig. 4A and B), which is caused by a recovery lower than 100% (see below). If DHBA is pre-concentrated on-line from an injection volume of 2 ml (see Fig. 4C) a further decrease in peak height of about 20% is observed, which is the same behaviour as for the biogenic catecholamines.

The third chromatographic system which was tested in combination with the on-line pre-concentration system was a reversed-phase ion-pair system (see Experimental, System III) with a mobile phase pH of 3.5 (0.11 *M* phosphate). Although this phase system produces efficient separations [28], serious peak broadening occurs when combined with the on-line pre-concentration system. For this reason this phase system was not further investigated.

*Quantitative aspects*

The linearity of the method was investigated in the range from 400 pg to 640 ng injected per component by means of on-line pre-concentration of 2-ml portions containing equal amounts of A, NA, DA and EPI. In this experiment a small AL pre-column (6×2.0 mm I.D.) was used for pre-concentration and separation was performed with reversed-phase ion-pair partition chromatography (condi-

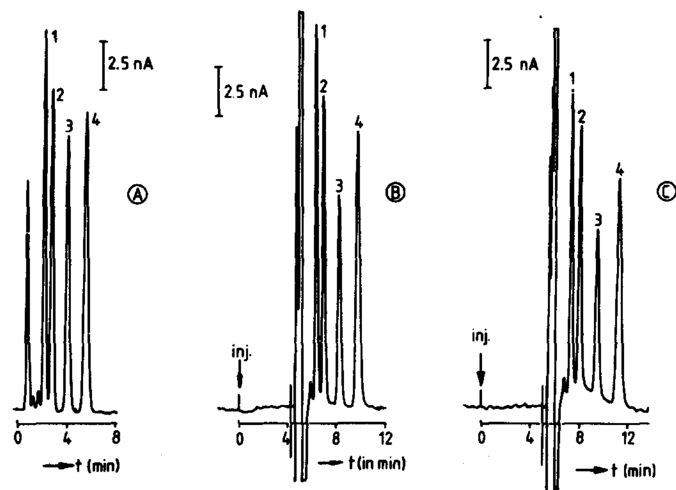


Fig. 4. Comparison between direct separation of the catecholamines (A) and separation after on-line pre-concentration on alumina (B and C). Separation: system II, see Experimental. Detection: electrochemical. (A) Direct injection on the analytical column of 6 $\mu l$ of a mixture (166 ng/ml per component) of (1) A, (2) NA, (3) DHBA and (4) DA in the mobile phase (system II, see Experimental, pH=2.5); flow-rate, 1.5 ml/min. (B) Injection via the pre-column (6×2.0 mm I.D.) of 6 $\mu l$ of the same mixture as in A. Mobile phase 1: 0.005 *M* Tris, pH 8.0; $w_1 = 1.6$ ml/min. Mobile phase 2 and $w_2$ as in A. $t_I = 4.5$ min; $t_{II} = 13$ min; elution from the pre-column by forward flushing. (C) Injection via the pre-column of 2 ml of a mixture (0.5 ng/ml per component) of the four catecholamines in 0.01 *M* Tris, 0.05 *M* EDTA, 0.01 *M* sodium metabisulphite, pH 8.0. $t_I = 5$ min; $t_{II} = 14$ min. Other conditions as in B.

tions as in Fig. 4C). For all four compounds plots of peak height or peak area versus amount injected are linear (with correlation coefficients of 0.9998 or better and intercepts which do not differ significantly from zero) up to a total amount injected of 1.28 $\mu g$ (i.e., 320 ng of each catecholamine). Only when a mixture containing 640 ng per component is pre-concentrated, both peak heights and peak areas are smaller than expected. Because the response of the electrochemical detector is still linear in this region, it can be concluded that the capacity of the pre-column is exceeded at a total amount injected of 2.56 $\mu g$. Thus, using electrochemical detection, the linear dynamic range of the method amounts to about 5 decades. However, this can be expanded further by using a larger pre-column.

The recovery of the catecholamines, including DHBA and EPI, after on-line pre-concentration was determined for both pre-concentration materials. The results are summarized in Table III. On DHBS, the recoveries generally approach 100%. However, for the smallest amount injected (5.5 ng) the deviation from 100% is higher, probably because the detection limits are approached and thus the accuracy and precision of the peak area measurements decrease. On AL recovery (determined at the 1-ng level) of A, NA and DA is ca. 100% but for EPI and DHBA the recovery is lower, viz., ca. 90 and 76%, respectively.

At the levels at which the recoveries were determined (1–58 ng injected per component; see Table III), the reproducibility for the on-line pre-concentration

TABLE III

RECOVERIES OF THE CATECHOLAMINES AFTER ON-LINE PRE-CONCENTRATION ON DIHYDROXYBORYLSILICA AND ALUMINA

For dihydroxyborylsilica the recoveries were calculated by comparison of the peak areas obtained by (a) pre-concentration of 1-ml portions [in 0.01 $M$ phosphate, 0.01% (w/v) EDTA, pH 8.1] and (b) direct injection of 116-$\mu$l portions (in the mobile phase of system I) containing the same amount of the catecholamines. Number of injections: 2–4. Separation: system I. Conditions of pre-concentration and separation: see Fig. 3A. Detection: fluorimetric, LS-4. For alumina the recoveries were calculated by comparison of peak areas obtained by (a) pre-concentration of 1-ml portions (0.05 $M$ EDTA, 0.01 $M$ sodium metabisulphite, 0.01 $M$ Tris, pH 8.0) and (b) direct injection of 6-$\mu$l portions (in the mobile phase of system II, pH 2.5), both containing 1 ng of the catecholamines. Number of injections: 5. Separation: system II. Conditions of pre-concentration and separation: see Fig. 4B and C, except for $w_1 = 1.0$ ml/min; $t_I = 4$ min; $t_{II} = 10$ min.

| Pre-concentration material | Amount injected (ng) | Recovery (%) | | | | |
|---|---|---|---|---|---|---|
| | | NA | A | DA | DHBA | EPI |
| DHBS | 58 | 98.5 | 97.4 | 100.5 | 98.3 | - |
| | 27 | 102.2 | 98.5 | 93.6 | 99.0 | |
| | 5.5 | 98.3 | 89.2 | 106.8 | 89.3 | - |
| AL | 1.0 | 98.0 | 99.4 | 99.3 | 76.2 | 90.0 |

mode is of the same order as that for the direct injection mode, with relative standard deviations of 1–6% ($n=2$–$5$).

## APPLICATIONS

### *Urine*

Fig. 5 shows a chromatogram obtained after on-line sample pre-treatment of 0.5 ml of urine (after centrifugation and pH adjustment) on a DHBS pre-column, applying ion-exchange chromatography with fluorescence detection. A good, clean

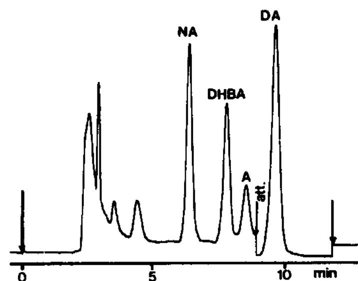


Fig. 5. Determination of the catecholamines in urine by means of on-line sample pre-treatment on dihydroxyborylsilica. Separation: system I, see Experimental. Injection of 0.5 ml of urine after addition of 100 $\mu$l of a solution of 100 ng/ml of DHBA, centrifugation and adjustment of the pH to 8.1. Conditions as in Fig. 3A. Urine concentrations of the catecholamines: A, 8 ng/ml; NA, 36 ng/ml; DA, 340 ng/ml.

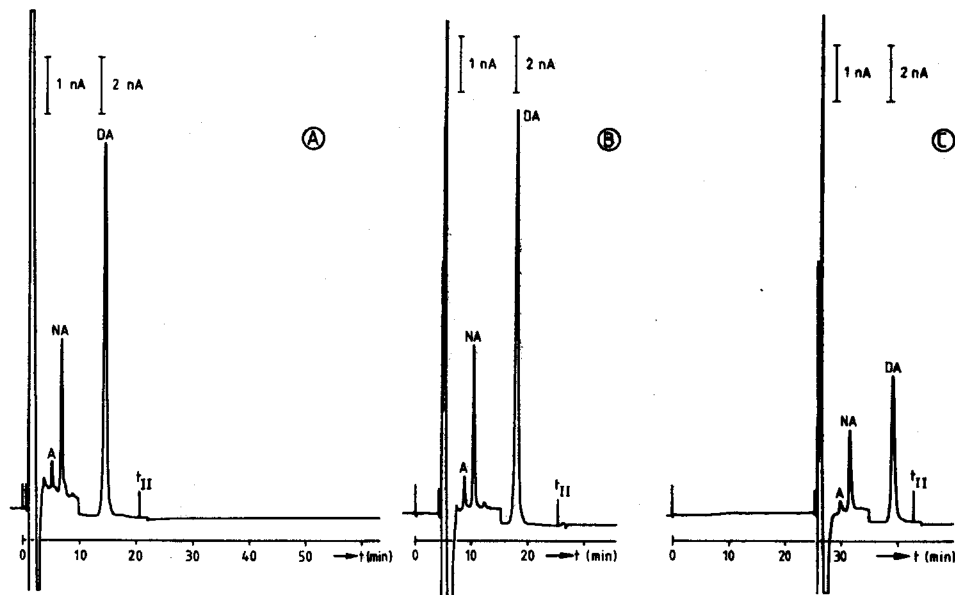


Fig. 6. Effect of variation of the washing volume on the clean-up of a urine sample on alumina. Injection of 30 $\mu$l of a urine sample after adjustment of the pH to 6.0. Dimensions of the pre-column: 6×2.0 mm I.D. $t_I$= (A) 0.5 min, (B) 4 min or (C) 25 min; for $t_{II}$, see figures. Other conditions as in Fig. 4B and C except for $w_2$=0.60 ml/min.

chromatogram is obtained and the native fluorescence of catecholamines is strong enough for the analysis of common concentration levels of these substances in urine. The concentrations of the catecholamines (see Fig. 5) are in good agreement with those reported [8, 9, 23].

When electrochemical detection is applied, a small volume of urine is sufficient for quantitation of the catecholamines. This is demonstrated in Fig. 6B, which shows a chromatogram obtained after on-line sample pre-treatment of 30 $\mu$l of urine on AL. Prior to injection the only pre-treatment of the urine is adjustment of its pH to 6. No internal standard was added to the urine but DHBA or EPI can be used for this purpose (see Fig. 6B). The concentrations of the catecholamines in this urine sample, as determined by means of the standard addition method (see below), were 6, 29 and 180 ng/ml for A, NA and DA, respectively. Again, these values are in good agreement with those reported by others (see above).

Fig. 6 also shows the effectiveness of the clean-up function of the pre-column in relation to the duration of the washing step; even if the washing volume is very small, i.e., 0.8 ml (see Fig. 6A), only a few minor interfering peaks are present, clearly demonstrating the excellent selectivity of the AL extraction step. DOPAC, if also retained on the pre-column, would seriously lengthen the run time because (i) its retention time on the analytical column is about 45 min, (ii) the urine concentrations are relatively high (about 1 $\mu$g/ml [22]) and (iii) its electrochemical detectability is good. However, DOPAC is not pre-concentrated on AL (see Table II) and, because DA is the last compound detected (see Fig. 6A), the

total analysis time, including a reconditioning step of 8 min, is about 20 min (reconditioning can be started after the elution of NA). If the washing volume is very large, e.g., 40 ml (see Fig. 6C), after the interfering compounds the catecholamines are also eluted from the pre-column. However, a significant percentage is still adsorbed on the AL, as can be predicted from Table II.

Under the conditions indicated in Fig. 6B the linearity of the method was investigated by standard additions of the catecholamines to urine. The calibration graphs (peak heights versus amounts added to urine) are linear up to at least ten times the normal urine concentrations of the catecholamines with correlation coefficients better than 0.9992 (maximum concentrations spiked in urine: A, 50 ng/ml; NA, 200 ng/ml, DA, 2000 ng/ml; each sample was injected in triplicate). This indicates that the capacity of the pre-column is not exceeded, which is not surprising; as described in the previous section, the loading capacity of the pre-column is larger than 1.28 $\mu$g whereas in the urine spiked with ten times the normal concentrations the total amount in 30 $\mu$l is only about 75 ng.

The recovery of the catecholamines was determined at ten times the normal concentrations in urine by subtraction of the peak area after and before standard addition ($n=4$) and comparison of this value with the peak area obtained by direct injection ($n=4$) onto the analytical column of the same amount in 30 $\mu$l. The recoveries were found to be 102, 103 and 99% for A, NA and DA, respectively. Relative standard deviations for subsequent 30-$\mu$l injections ($n=4$) are the same for peak-height and peak-area measurements and are about 7% for A and 3% for NA and DA at normal concentrations and about 2% for all three catecholamines at ten times the normal levels.

With regard to the possibilities of automating the method, it is advantageous if the pH of the injected urine can be kept as low as 6. At this pH the catecholamines are remarkably stable; if protected from light, even at room temperature no noticeable degradation is observed after several days. In contrast, at urine pH values of about 8, the catecholamines are stable for only a few hours, even in the presence of an antioxidant and cooled on ice. Thus, if before on-line pre-concentration the pH of the urine has to be adjusted to about 8, which is necessary when larger volumes have to be injected to improve the detectability, in an automated system special equipment is required to adjust the pH of the urine samples just before or during their injection.

The maximum volume that can be injected depends on the dimensions of the pre-column, the pH of the urine sample and the material used for the extraction step. If more than 30 $\mu$l of urine, adjusted to pH 6, are injected on the $6\times2.0$ mm I.D. pre-column filled with AL, the catecholamines are not quantitatively recovered. If the $10\times2.0$ mm I.D. pre-column, again filled with AL, is applied, ca. 60 $\mu$l of urine (pH 6) can be injected, thus improving the detectability of the catecholamines by a factor of 2. However, using the $10\times2.0$ mm I.D. pre-column, the negative peak in the chromatogram (see Fig. 6) is extended and consequently the quantitation of A becomes difficult. It is worth mentioning that the temporary change in the mobile phase after $t_{II}$ leads to a relatively large disturbance of the baseline signal of the electrochemical detector, whereas it is hardly noticeable with the fluorimetric detector. After the volume of the pre-column, the potential

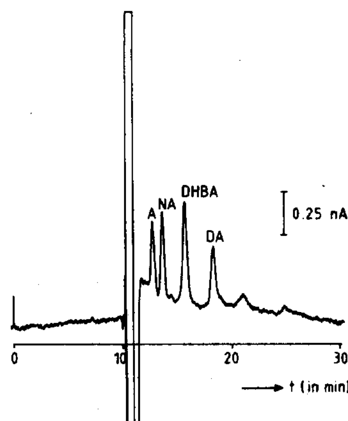


Fig. 7. Determination of the catecholamines in a spiked plasma sample by means of on-line sample pre-treatment on alumina. Separation: system II, see Experimental. Injection of 2 ml of a plasma extract, obtained from plasma containing 100 pg/ml of the four catecholamines (see text) after protein precipitation (20 parts of plasma with 1 part of 70–72% perchloric acid; vortex mixing, 2 min), centrifugation (1000 *g*, 10 min) and adjustment of the pH to 8.2 (by mixing of 10 parts of the cooled supernatant with 1 part of a solution containing 5 *M* sodium hydroxide, 1 *M* Tris, 0.5 *M* EDTA and 0.2 *M* sodium metabisulphite). Conditions as in Fig. 4B and C except for dimensions of the pre-column: 4×2.8 mm I.D.; $w_1$=0.8 ml/min; $w_2$=1.0 ml/min; $t_{I}$=10 min; $t_{II}$=10.5 min.

applied to the glassy carbon working electrode is an important parameter in determining the performance of the electrochemical detector; if this potential is set too high, the above-mentioned disturbance increases and some interfering peaks appear in the chromatogram. In order to maintain a high and stable response of the catecholamines at a potential of +0.65 V vs. the Ag/AgCl reference electrode, an electrochemical pre-treatment of the working electrode was applied periodically (about once every month). The pre-treatment consisted of a sequence of about 30 scans from 0 to +1.5 V and back to 0 V with a scan-rate of 20 mV/s and an interval of 30 s at 0 V between each scan [30].

Using the procedure as described in Fig. 6B, several hundred 30-µl aliquots of urine could be injected before deterioration of the pre-column material was observed, indicated by a decrease in the recovery of the catecholamines. After repacking of the pre-column with new material, which takes only a few minutes, the new pre-column can be used directly.

*Plasma*

Preliminary results indicated that the on-line sample pre-treatment method is also suitable for the determination of catecholamines in plasma (see Fig. 7). A blank plasma (obtained by oxidation of the catecholamines at pH 10) was spiked with 100 pg/ml of the four compounds, which are concentrations of the same order as those present in plasma [10–12]. After protein precipitation and pH adjustment, 2 ml were injected on to a small AL pre-column. A clean chromatogram is obtained (see Fig. 7), from which all four catecholamines can be readily quantified.

Investigations are in progress to apply the method to the assay of plasma cate-

cholamines after further automation of the sample pre-treatment. Because the catecholamines are stable only at acidic pH, the perchloric acid protein precipitation and centrifugation (see the legend to Fig. 7) are still performed off-line and as rapidly as possible. Adjustment of the pH to 8.2 forms the first step in the automated method after autosampling of the perchloric acid extract and prior to on-line pre-concentration on alumina.

## CONCLUSIONS

On-line pre-concentration of catecholamines can be performed on both alumina and dihydroxyborylsilica, although on the former material the retention of catecholamines is larger. Compounds that possess only a catechol or an amine function are retained to a certain extent at basic pH, but this retention is negligible compared with that of the catecholamines. This means that only a few compounds, viz., epinine and dihydroxybenzylamine, which can be used as internal standards, and $\alpha$-methyldopamine are pre-concentrated to the same extent as adrenaline, noradrenaline and dopamine.

Pre-concentration is best achieved at pH values of about 8, using a mobile phase with a low buffer concentration (0.005–0.010 $M$ phosphate or Tris). The catecholamines can be desorbed and eluted from the pre-column in a small volume by using a mobile phase with a low pH (2–3) and a higher buffer concentration (0.05–0.10 $M$). If the on-line pre-concentration is combined with a separation step by means of ion-exchange or reversed-phase ion-pair partition chromatography, the same mobile phase can be used for desorption and separation of the catecholamines and the contribution of the pre-concentration step to band broadening is relatively small.

The calibration graphs obtained with the on-line pre-concentration system are linear up to at least 1.28 $\mu$g injected on to the pre-column, the upper limit being mainly determined by the capacity of the pre-concentration material and thus by the dimensions of the pre-column. The recoveries are 90–100% for adrenaline, noradrenaline and dopamine on both pre-concentration materials, for epinine on alumina and for dihydroxybenzylamine on dihydroxyborylsilica. On alumina the recovery of dihydroxybenzylamine is lower, i.e. 76%.

The method is very suitable for the determination of catecholamines in urine. Using electrochemical detection, the catecholamines can be quantified in 30 $\mu$l adjusted to pH 6. When fluorimetric detection is applied, a larger volume, adjusted to a higher pH (e.g., 8.1) is injected. In both variants, but especially the former, the method can easily be automated.

Preliminary results indicate that on-line sample pre-treatment can also be used for the determination of catecholamines in plasma. Investigations are in progress on the further automation of this method and its application to the assay of plasma catecholamines at endogenous concentrations.

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