CHROMSYMP. 2886

Determination of catecholamines by automated pre-column derivatization and reversed-phase column liquid chromatography with fluorescence detection

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ABSTRACT

A highly selective and sensitive method for fluorescence determination of catecholamines (CAs) after automated derivatization with 1,2-diphenylethylenediamine (DPE)/potassium ferricyanide-based reagent is described. The reaction is specific for catechol compounds and was shown to be very reliable for analysis of CAs (noradrenaline, adrenaline, dopamine) in plasma and urine. However, in spite of its high sensitivity the method has not yet achieved wide application, probably because of a rather complicated manual derivatization and reaction times of 40–60 min. The present method describes an optimized automated procedure utilizing the CMA/200 refrigerated microsampler. Usually, 10-μl samples cooled at 4°C were mixed with 13.5 μl of acetonitrile–ferricyanide reagent and then with 7.5 μl DPE–bicine as a second reagent; 29 μl were aspirated into the sampling loop and heated to 80°C. After 6.5 min reaction time, samples were injected onto a 100 × 4 mm column packed with Nucleosil C$_{18}$, 3 μm particle size. CAs (including internal standards α-methyl-noradrenaline and isoproterenol) were separated within 8 min using 0.05 M acetate buffer pH 7.0–40% acetonitrile–8% methanol at a flow-rate of 1 ml/min. The detection limits for CAs were 2–5 fmol, which is about 2–4 times better than electrochemical detection used under similar chromatographic conditions. Furthermore, fluorescence detection is more reliable for routine use in clinical laboratories because the detector is much simpler to maintain. The method could be used for automated analysis of CAs in plasma and urine extracts and for microdialysis perfusates.

INTRODUCTION

Since the first reports on the use of electrochemical detection for liquid chromatographic determinations of catecholamines (CAs) and their metabolites [1,2], this technique has become widely used and modified for analysis of monoamines in tissue and body fluids. The main advantages of electrochemical detection of catechol and indol compounds in HPLC are high sensitivity, relatively inexpensive apparatus and the possibility of automation.

On the other hand, the electrochemical detector reacts to all compounds that can be oxidized at a given potential of the working electrode. This increases the risk of interferences in the chromatogram and necessitates troublesome maintenance of the detector. Polishing the elec-

trode often means several hours of delay while waiting for the baseline signal to stabilize.

Recently, a fluorescence (FL) method for CA detection based on derivatization with 1,2-diphenylethylenediamine (DPE) was described [3–12]. The high fluorescence yield of this reaction allows detection of 2–10 fmol of CAs even on standard-bore (4 mm I.D.) columns. CAs can be derivatized either before injection onto the reversed-phase column [4–6,8–11] or after separation on the ion-exchange system [7]. Pre-column derivatization is preferable to the post-column approach, which requires extra pumps, coils or other reactors, causing dispersion of peaks and delay in the analysis. Furthermore, separation of precolumn-derivatized DPE–CAs on the reversed-phase system allows the use of gradient elutions [10,11] and reduces the problems associ-

ated with accumulation of interferences on the column. The most serious limitation of the pre-column method is the need for several manual pipetting steps and an incubation time of 45–60 min. The long reaction times, large final volumes of derivatized samples and the need for expensive fluorescence detectors are probably the main reasons why this method has not yet gained wider attention.

This paper reports a method for the automated pre-column derivatization of CAs with two reagent solutions by using an HPLC autosampler with a heated loop. The optimal parameters for reagent composition, their sequence, reaction time and temperature are discussed.

EXPERIMENTAL

Chemicals

The catecholamines noradrenaline (NA), adrenaline (A) and dopamine (DA) were obtained from Sigma (St. Louis, MO, USA). Isoproterenol (IP) and α-methyl-noradrenaline (α-MNA) were purchased from Research Biochemicals (Natick, MA, USA), potassium ferricyanide (PFC), acetonitrile, methanol and sodium acetate were from Merck (Darmstadt, Germany). Meso-DPE (R,S-form) was a kind gift from Dr. F. Boomsma (University Hospital, Rotterdam, Netherlands) or later obtained in either R,R-(+) or S,S-(−) form from Aldrich (Steinheim, Germany).

Apparatus

The liquid chromatographic pump was either a Spectra-Physics SP-8800 pump (San Jose, CA, USA) or a Pharmacia LKB 2249 gradient pump (Uppsala, Sweden). The mobile phase was degassed on-line by using a CMA/260 degasser (CMA/Microdialysis, Stockholm, Sweden). Automated derivations and injections were performed using a CMA/200 refrigerated microsampler. An LC-22C temperature controller (Bioanalytical Systems, W. Lafayette, IN, USA) was used to heat the sampling loop of the CMA/200. Derivatized CAs were detected either by a Smidadzu RF-535 spectrofluorometer (Kyoto, Japan) or later by a CMA/280 fluorescence detector (CMA/Microdialysis). The excitation

and emission wavelengths of the former were set to 350 nm and 480 nm, respectively. The CMA/280 detector operates at fixed wavelengths with excitation and emission maxima at 330–365 nm and 440–530 nm, respectively. Chromatograms were recorded and integrated using an SP-4290 integrator (Spectra-Physics). Separations were performed on a 100×4 mm cartridge column (H. Knauer, Berlin, Germany) packed with Nucleosil 120 C_{18}, 3 μm particle size, protected with a 5×4 mm guard column packed with the same material.

Derivatization and chromatography

The manual derivatizations were performed as described previously [8]. Briefly, 40 μl of CA standard sample (NA, A and DA at $1 \cdot 10^{-8}$ M each), stored in 0.01 M hydrochloric acid, were pipetted into a plastic Eppendorf tube, followed by 50 μl of acetonitrile, 10 μl of bicine buffer, 20 μl of DPE and 4 μl of PFC. Concentrations of these stock solutions were: 1.75 M bicine buffer, pH 7.05, adjusted with 10% NaOH; 0.1 M DPE in 0.1 M HCl; and 20 mM PFC in water. The mixture was incubated in a water bath shielded against light at 37°C for 45 min and then 50-μl volumes were injected onto the column.

For automated derivatizations, different combinations of DPE, PFC, acetonitrile and bicine were tested. Two pairs of reagents (R1, R2 and R3, R4) were prepared by mixing different volumes (shown in parentheses) of the stock solutions: R1, acetonitrile–PFC (25:2); R2, DPE–bicine (2:1); R3, bicine–PFC (5:2); and R4, acetonitrile–DPE (5:2). For derivatizations of CAs, a pair of reagents, R1 then R2, R2 then R1, R3 then R4 or R4 then R3, was placed into the autosampler. Normally, 10 μl of samples were mixed with 13.5 μl of R1 and 7.5 μl of R2, or alternatively 4 μl of R3 and 17.5 μl of R4. It is supposed that no reaction was activated at this stage since samples and reagents were stored at +4°C in the CMA/200 autosampler. After mixing in the vials, each sample was aspirated into the heated loop and allowed to remain there for a preset period of time. The optimal reaction time found was 6.5 min at 80°C.

The mobile phase for isocratic separations consisted of 0.05 M sodium acetate buffer, pH

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7.0, 40% acetonitrile and 8% methanol, as reported elsewhere [8]. The flow-rate was 1.0 ml/min.

RESULTS AND DISCUSSION

The optimal temperature of the reaction loop for DPE derivatization of NA, A and DA is shown in Fig. 1. As seen, for a fixed 5 min reaction time the maximal fluorescence yields (expressed as peak heights) for all CAs were achieved at 80°C. At this temperature, the optimal reaction time was found to be 6.5 min (Fig. 2). Here, the variation of reaction times from 5 to 8 min did not have such a profound effect on the FL yields as increasing the temperature. Thus it may be expected that higher temperature can shorten the reaction time significantly, while still maintaining the same sensitivity as for manual derivatization at room temperature or 37°C. Indeed, almost identical peak heights were measured for the CA standard sample derivatized automatically as compared with that derivatized manually: NA 92%, A 87%, DA 106%, and internal standards α-MNA 73%, IP 73% (expressed as a percentage of 80°C/6.5 min reaction vs. 37°C/45 min reaction).

Previously, it was assumed that a high temperature could affect the reaction yields and reduce the stability of fluorescence derivatives [12], yet no systematic study has been made on the effects of temperature on reaction kinetics.
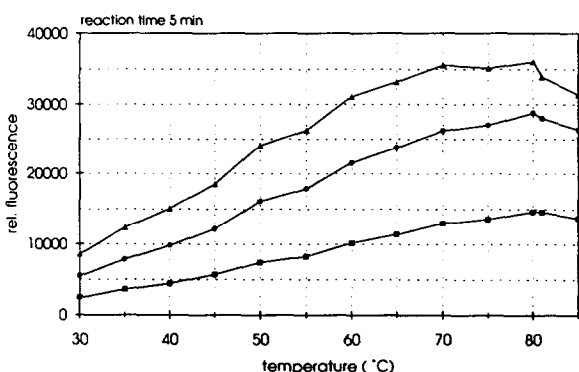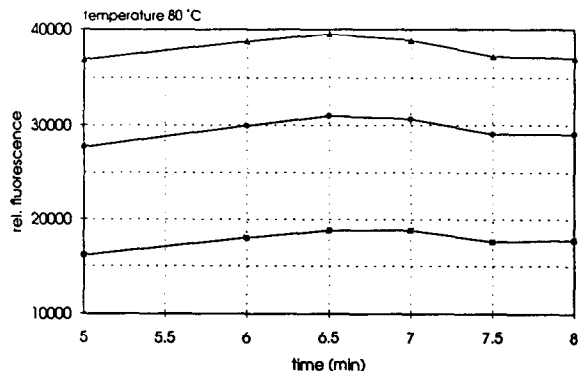

Fig. 2. Effect of reaction time on the fluorescence yields of DPE–catecholamine derivatives. $\blacktriangle$ = NA; $\bullet$ = A; $\blacksquare$ = DA.

More detailed studies were devoted to finding the optimal chemical conditions for the DPE reaction, such as pH, effect of accelerator (glycine, acetonitrile) and oxidant (PFC). Recently, the structure of the final DPE–adrenaline fluorophore was described [13]. It is postulated that, in the first step, CAs are oxidized to the corresponding adrenochromes by PFC. Also, coulometric oxidation is possible, which allows derivatization of hydroxymethylated CAs to their o-quinones [7]. Adrenochromes then react with DPE to form corresponding, highly fluorescent 2-aryl(4,5-dihydropyrrolo)[2,3-f]benzoxazoles [13].

Fig. 3A shows a typical chromatogram of 10 μl of a standard mixture of CAs, including internal standards α-MNA and IP, at concentrations of 0.1 pmol/10 μl each. Automated derivatization was performed at 80°C for 6 min with R1 and R2 as described in the Experimental section. The highest signal was observed for α-MNA; for CAs the largest peak was NA, whereas A and DA produced only 79% and 45% of the NA peak height. However, the extreme fluorescence intensity of DPE fluorophores allows detection to as low as 5 fmol of each catecholamine, as illustrated in Fig. 3B. It should be noted that electrochemical detection on a glassy carbon electrode can detect only 10–20 fmol of CAs under the same chromatographic conditions. A derivatized blank (water) sample is shown in Fig. 3C. No detectable peaks were observed in this sample, which suggests that the small peak
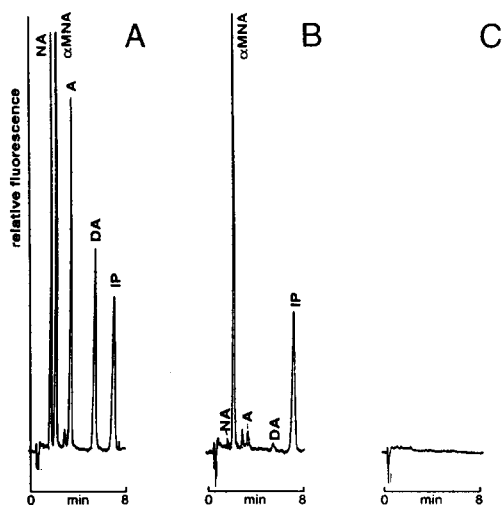

Fig. 1. Effect of reaction temperature on the fluorescence yields of DPE–catecholamine derivatives. $\blacktriangle$ = NA; $\bullet$ = A; $\blacksquare$ = DA.

Fig. 3. (A) A typical chromatogram of 10 μl of CA standards at concentrations of 0.1 pmol/10 μl each. Internal standards α-methyl-noradrenaline (α-MNA) and isoproterenol (IP) were also included. (B) The limits of detection. A 10-μl aliquot of standard mixture contained $5 \cdot 10^{-10}$ M (5 fmol) NA, A and DA each and $1 \cdot 10^{-8}$ M internal standard. (C) A blank (10 μl water) sample was derivatized as described above.

eluted at 2.9 min, shown in Fig. 3A and B, is an impurity present in the internal standard (α-MNA).

The internal standard (α-MNA or IP) should be used for plasma or urine samples, which must undergo preseparation on solid phase (alumina or ion-exchange) cartridges or by liquid–liquid extraction [8]. In these cases the recoveries of extracted CAs can vary from 60 to 100%. For analysis of microdialysis samples, no preseparation steps are normally needed, and thus internal standards can be omitted. However, it is advisable to correct for any possible variations in derivatization yields and the precision of sample pipetting by adding the internal standard to one of the reagents. The best signal for internal standards without any interferences or multiple peaks was observed when α-MNA and IP were added to R2 (see Fig. 3B).

Another important factor affecting the final number and shape of peaks is the reagent composition and the sequence of mixing for each pair of reagents. The chromatograms in Fig. 4A–D illustrate the effect of the composition of
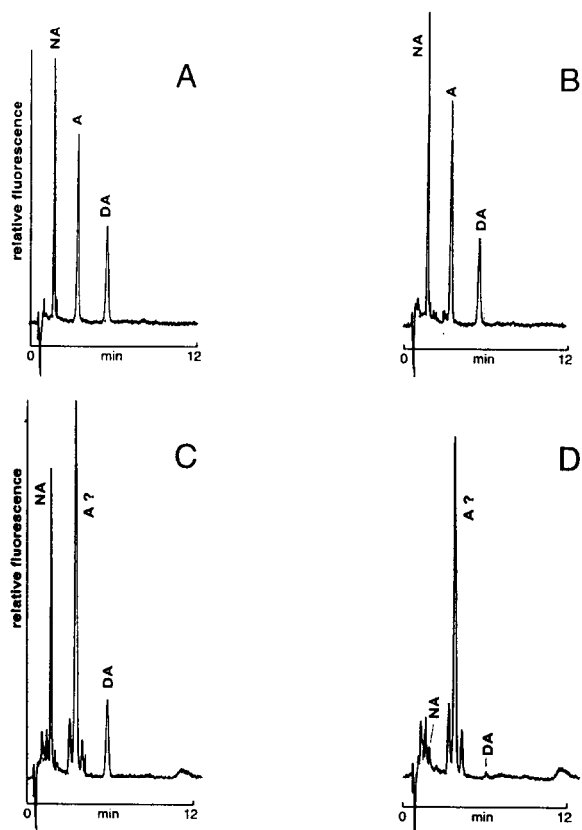

Fig. 4. Effects of reagent composition and mixing sequences on reaction yields of DPE-derivatized catecholamines. A 20-μl aliquot of $1 \cdot 10^{-8}$ M CA standards was derivatized with two pairs of reagents. For chromatograms B and D, the mixing order of reagents was reversed. Standards and reagents were kept at +4°C; the derivatization was performed at 65°C for 5 min: (A) 27 μl of acetonitrile–PFC + 15 μl of bicine–DPE, the optimal derivatization; (B) 15 μl of bicine–DPE + 27 μl of acetonitrile–PFC; (C) 7 μl of bicine–PFC + 35 μl of acetonitrile–DPE; (D) 35 μl of acetonitrile–DPE + 7 μl of bicine–PFC.

each reagent and the mixing order of the reagents on derivatization yields. The best results were achieved with R1 and R2, mixed in this sequence with a $1 \cdot 10^{-8}$ M CA standard (Fig. 4A). When the derivatization order was reversed, i.e. R2 was pipetted first followed by R1, a similar chromatogram was obtained, but with several small unidentified peaks. These data suggest that no significant chemical reaction occurs during pipetting of the reagents, since samples and reagents are stored at +4°C. The reaction starts when the mixture is aspirated into

the heated sampling loop. R1 and R2 are stable and give reproducible signals for at least 24 h. It is recommended that fresh reagents are prepared daily in order to prevent any source of impurities or carry-over from the samples.

R3 and R4 gave quite different chromatograms (Fig. 4C and D). When mixing the standard sample with R3 followed by R4, a large A peak was measured, compared with almost unchanged NA and DA peaks. At the same time, as many as five "degradation" peaks were eluted in the vicinity of the NA and A peaks. Reversing the derivatization order to R4 then R3 led to almost complete disappearance of NA and DA peaks, while there remained a large peak corresponding to the retention time of A. This peak, although not so high, also appears in a blank (water) sample derivatized by the same procedure. In both cases, several by-product peaks were eluted at the beginning of the chromatogram. It was concluded that the R3 + R4 pair is less 'safe' in terms of peak purity than R1 + R2. Because of many interfering peaks it is not possible to use internal standards such as α-MNA.

The main difference between R3,R4 and R1,R2 pairs is most probably the chemical stability of the mixed components themselves, rather than alternation of the reaction steps in the derivatization formula.

The chemical environment in which catecholamine samples are stored also plays a significant role in the derivatization efficiency, as documented in Fig. 5A and B. In both cases derivatizations were performed with R1 + R2 at 80°C/6.5 min as described in the Experimental section. Derivatization of 10 μl of a standard sample of CAs at concentrations of $5 \cdot 10^{-8}$ M each, which was stored in 0.3 mM glutathione, yielded dramatically suppressed peak heights for all compounds, except for A, with which some unknown products co-eluted (Fig. 5A). Glutathione is often used as an antioxidant during preparation of plasma samples for CA measurements. Obviously, it inhibits the effect of PFC in oxidizing CAs to their corresponding o-quinones.

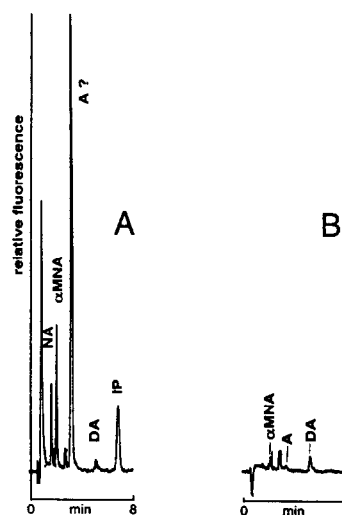Similarly, low pH can block the reaction, as shown for the CA standard stored in 0.1 M

Fig. 5. Effect of antioxidants and acids present in the CA extracts on derivatization yields of NA, A, DA and internal standards α-MNA and IP. A 10-μl aliquot of standard containing $5 \cdot 10^{-8}$ M catecholamines and internal standards was stored in: (A) 0.3 mM glutathione solution or (B) 0.1 M HCl. Derivatizations were performed as described in Fig. 4A.

hydrochloric acid (Fig. 5B). Lowering the concentration of HCl in CA standards to 0.01 M produced normal peak heights, as seen in Fig. 3A. The effect of the pH of the bicine buffer on the fluorescence intensity of CA derivatives has been detailed previously [8]. The highest FL yields are achieved at pH 6.5–7.0. This means that samples stored in strong acids should be neutralized before derivatization, or the pH of the DPE–bicine reagent should be increased to shift the final pH of the reaction mixture to the optimal range.

CONCLUSION

The present method for fluorescence detection of derivatized CAs possesses several advantages over electrochemical detection. Probably the main difference is easier maintenance and superior reliability of the FL method for routine use. High sensitivity and the possibility of using gradient elutions or microbore columns make the technique very suitable for automated analysis of CAs in plasma and urine extracts and for moni-

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toring the neurotransmitters NA and DA in microdialysis samples.

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