Journal of Chromatography, 329 (1985) 147–152
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17 736

Note

Determination of catecholamines by high-performance liquid chromatography with catalytic photometric detection

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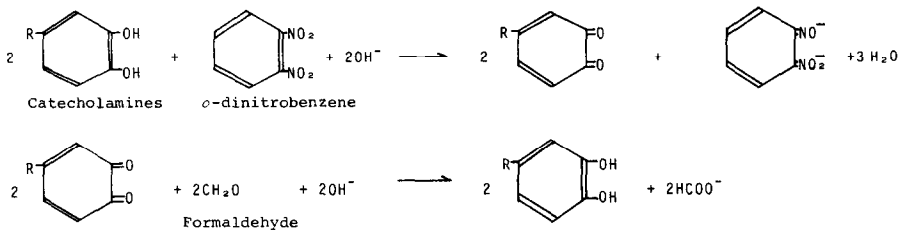
(Received March 19th, 1985)

Catecholamines play an important rôle in the function of the nervous system. Though present at low levels, these compounds can be extracted with acceptable recoveries and readily separated by high-performance liquid chromatography (HPLC) using ion-exchange[1,2] or reversed-phase[3–5] columns. HPLC has been coupled to highly sensitive detection systems in order to detect trace amounts of the compounds. Fluorimetric detection systems following post-column derivatization of the compounds are most often used[6–8]. Such detection methods are sensitive enough for various biological fluids, but the selectivity of the post-column reaction is not always sufficient.

Formaldehyde reduces *o*-dinitrobenzene to nitrophenylhydroxylamine dianion (λ_{max} = 560 nm) under alkaline conditions. Feigl and Neto[9] found that this redox reaction is accelerated by 1,2-diketones and some quinones, and can be employed in a spot test for compounds based on their catalytic effects. We have reported that the reaction is catalysed by compounds having a catechol group, particularly adrenaline, in alkaline dimethyl sulphoxide as solvent[10].

In this paper, the catalytic reaction is used as a photometric detector in a continuous-flow HPLC system for catecholamines. The compounds are separated by HPLC on an ODS column, and then measured photometrically, based on their catalytic effect on the reaction. This method permits the assay of authentic L-DOPA, dopamine, noradrenaline and adrenaline at the picomole level. The catalytic action of the catecholamines on the reduction of *o*-dinitrobenzene by formaldehyde is thought to occur as shown below.



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0021-9673/85/$03.30

EXPERIMENTAL

Materials

All reagents and solvents were analytical grade obtained from commercial sources. L-DOPA, dopamine (DA) hydrochloride, L-noradrenaline (NA) and L-adrenaline (AD) were purchased from Nakarai Chemicals (Kyoto, Japan). Standard solutions (10 m*M*) of the catecholamines were prepared in 0.1 *M* hydrochloric acid. Formaldehyde (FA), *o*-dinitrobenzene (DNB), sodium hydroxide and dimethyl sulphoxide (DMSO) were obtained from Wako Chemicals (Osaka, Japan). The reaction solution was prepared by dissolving 130 mg DNB in 250 ml of DMSO, followed by addition of 250 ml of FA (12 *M*). DMSO was purified by a refrigeration concentration method. The column (25 cm × 4.6 mm) packed with Zorbax ODS (5 μm, spherical particles) was purchased from DuPont (Wilmington, DE, U.S.A.).

Apparatus

A schematic diagram of the HPLC catalytic photometric system used is shown in Fig. 1. The catecholamine solutions were injected onto the reversed-phase HPLC analytical column using a Model KHP-U1-130 injector (Kyowa Seimitsu, Tokyo, Japan) with a 50-μl injection loop (B). The column temperature was ambient. The eluent, 0.1 *M* potassium dihydrogenphosphate–0.1 *M* hydrochloric acid (R_1), was pumped through the column (C) at a flow-rate of 0.5 ml/min using a Model BIP-1 pump (P_1) (Jasco, Tokyo, Japan). Solutions of 7 *M* sodium hydroxide (R_2) and 3 m*M* DNB + 6 *M* FA (R_3) were mixed with the column effluent, each at a flow-rate of 0.5 ml/min, in the four-way connector (D) attached to the exit of the column. The resulting mixture then flowed into a PTFE reaction coil (8 m × 0.5 mm I.D.) (E).

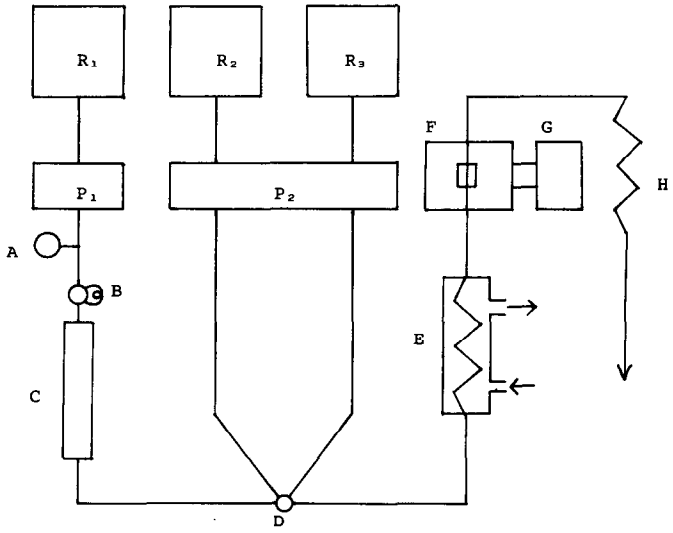

Fig. 1. Flow diagram of the HPLC catalytic photometric system. R_1 = Eluent (0.1 *M* potassium dihydrogen phosphate in 0.1 *M* hydrochloric acid); R_2 = reagent solution (7 *M* sodium hydroxide); R_3 = reagent solution (3 m*M* DNB + 6 *M* FA in DMSO); P_1 and P_2 = double plunger-type pumps; A = pressure gauge; B = loop injector; C = separation column; D = four-way connector; E = reaction coil; F = spectrophotometer equipped with flow cell; G = recorder; H = back-pressure coil.

The coil was maintained at 80°C in a water-bath controlled by a Model CTR-240 water circulator (Yamato Scientific, Tokyo, Japan). The absorbance at 560 nm was monitored with a JASCO Model UVIDEC-VI spectrophotometric detector equipped with an 8-μl cell (F), and recorded with a Model FBR-251A recorder (G) (Toa Denpa Kogyo, Tokyo, Japan). The outlet of the flow cell was connected to a back-pressure coil (10 m × 0.5 mm I.D.) (H).

RESULTS AND DISCUSSION

Catalytic reaction

Solvent. Various aqueous solvent systems were examined for their effect on the catalytic reaction. Although methanol and methyl cellosolve were adequate solvents, the most satisfactory was dimethylsulphoxide (DMSO). The results are shown in Table I. In all cases, solutions containing 50% organic solvent, 6 *M* FA, 3 m*M* DNB and water and 5 *M* sodium hydroxide were used. In DMSO, the catalytic activity was higher to those in other solvent systems. It was dependent on the composition of the solvent system (Fig. 2). An increase in the content of DMSO resulted in a proportional increase in catalytic activity. Since DMSO is very polar and has a strong tendency to solvate cations, it accelerates reactions in which anions play an important rôle. These results suggest the participation of anions in the catalytic reaction.

Sodium hydroxide concentration. The effect of sodium hydroxide on the reaction was examined in the concentration range 3–9 *M*. The absorbance increased exponentially with increasing sodium hydroxide concentration, and even 9 *M* sodium hydroxide was not sufficient to attain the maximum absorbance (Fig. 3). From the results, the absorbance increased linearly according to the power 2–4 of the sodium hydroxide concentration. The baseline at 9 *M* sodium hydroxide was unstable because of incomplete mixing in the reaction coil.

Formaldehyde concentration. The dependence of the absorbance on the formaldehyde concentration was studied over the range 2–6 *M*. The absorbance increased with increasing formaldehyde concentration, with maxima at 4.5 *M* for DOPA, DA and NA, but no maximum was attained with AD (Fig. 4).

Temperature. The effect of temperature was examined in the range 25–80°C. An increase in the reaction temperature resulted in an increase in absorbance, as

TABLE I

EFFECT OF SOLVENTS ON THE CATALYTIC REACTION

Conditions: 3 m*M* DNB + 6 *M* FA in 50% aqueous organic solvent; 5 *M* NaOH; reaction temperature 60°C; sample size 50 μl, containing 5 nmol of each catecholamine. Otherwise as described in the Experimental.

Catecholamine	*Absorbance*		
	DMSO	*Methyl cellosolve*	*Methanol*
DOPA	0.0970	0.0592	0.0325
DA	0.0336	0.0208	0.0144
NA	0.0832	0.0544	0.0384
AD	0.397	0.351	0.112

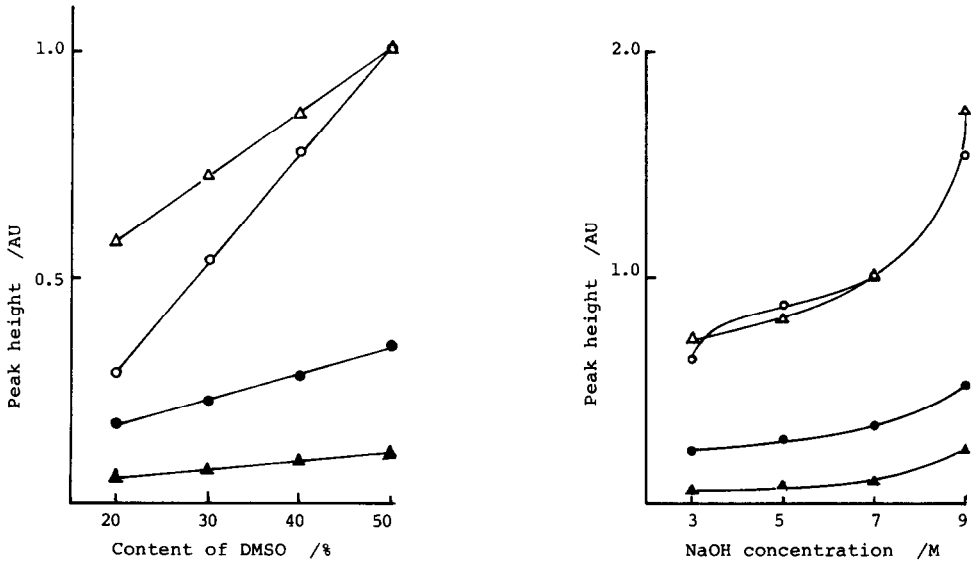

Fig. 2. Effect of DMSO on the catalytic reaction. Sample size: 50 μl containing 5 nmol of each catacholamine. Other conditions as described in the Experimental. △—△, DOPA; ▲—▲, DA; ●—●, NA; ○—○, AD.

Fig. 3. Effect of NaOH on the catalytic reaction. Details as in Fig. 2.

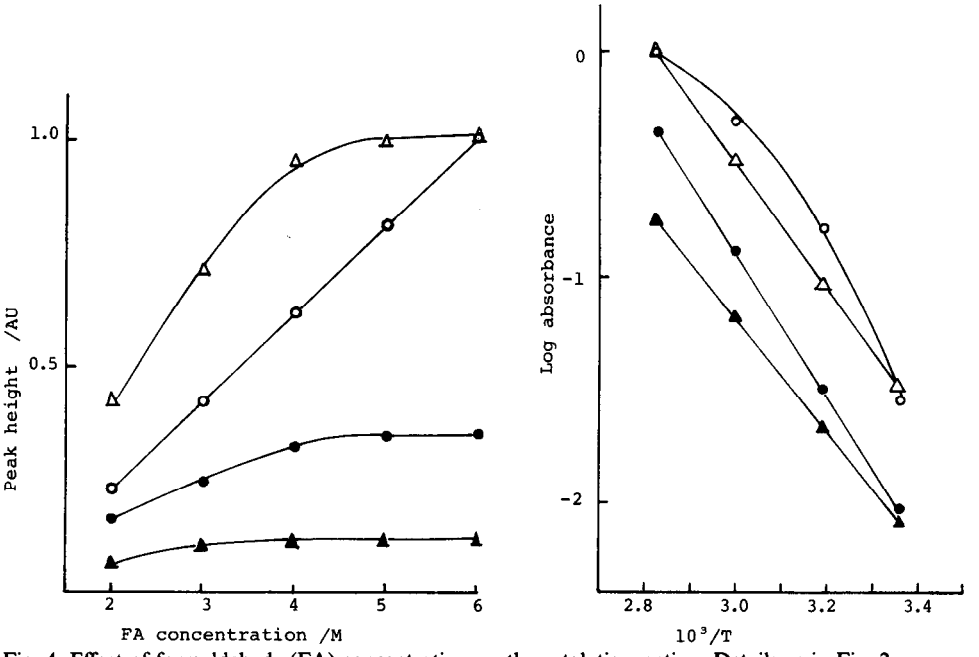

Fig. 4. Effect of formaldehyde (FA) concentration on the catalytic reaction. Details as in Fig. 2.

Fig. 5. Effect of temperature on the catalytic reaction. Details as in Fig. 2.

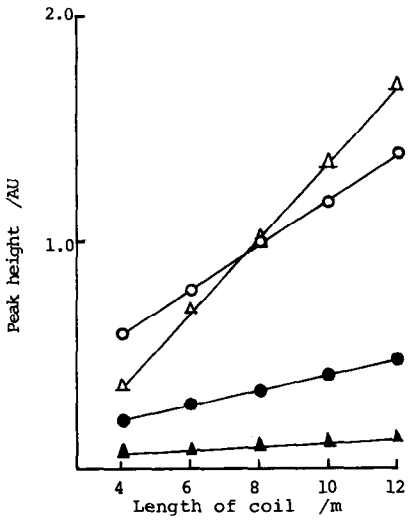

Fig. 6. Effect of the length of the reaction coil on the catalytic reaction. Details as in Fig. 2.

expected. Fig. 5 illustrates the plots of logarithmic absorbance *vs.* $1/T$. From the slopes of the straight lines, apparent activation energies for DOPA, DA and NA were 59, 48 and 44 kJ/mol, respectively. The plot for AD was convex, suggesting that the catalytic reaction comprises two steps having different rates, namely the oxidation of the catalyst and its regeneration process as shown before in the scheme.

Reaction time. The changes in absorbance as a function of the length of the reaction coil (0.5 mm I.D.) at a flow-rate of 1.5 ml/min (0.5 ml/min (DMSO + 0.5 ml/min sodium hydroxide + 0.5 ml/min eluent) are shown in Fig. 6. The absorbance increased linearly with increasing residence time, but the resolution decreased. For example, doubling the length of the reaction coil (18 m) resulted in a 50% increase in the peak area of AD but a 25% decrease in the resolution, due to overlapping with the peak of NA (Fig. 7).

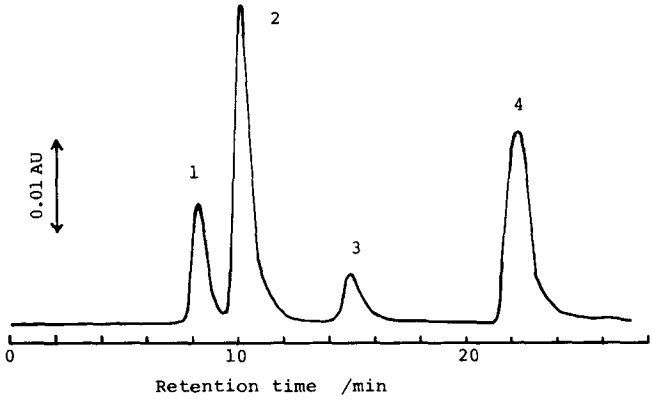

Fig. 7. Chromatogram of a synthetic mixture of catecholamines. Sample size: 50 μl containing 500 pmol of each catecholamine. Other conditions as described in the Experimental. Peaks: 1 = noradrenaline; 2 = adrenaline; 3 = dopamine; 4 = DOPA.

o-Dinitrobenzene concentration. The reaction was found to be independent of the concentration of DNB over the range 2–8 m*M*.

Separation

The separation of DOPA, DA, NA and AD was studied by reversed-phase HPLC with the catalytic photometric detection system. Fig. 7 shows a typical chromatogram obtained under optimum conditions as described in the Experimental. The lower limits of determination were 5 pmol for DOPA, 50 pmol for DA, 20 pmol for NA and 5 pmol for AD (injected amount). The relative standard deviations were 6.45 and 6.25% for 5.0 pmol each of DOPA and AD, respectively. A linear relationship was found between the absorbance (peak area) and amounts up to 20 nmol for DOPA, 150 nmol for DA, 60 nmol for NA and 20 nmol for AD.

CONCLUSIONS

The present method is the first HPLC method involving the separation of catecholamines and post-column reaction based on their catalytic effect. The HPLC system described enables the photometric determination of catecholamines at the picomole level. The system is especially suitable for the analysis of biological fluids in which the amount of AD is small relative to that of NA, because of its high sensitivity to AD.

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