Journal of Chromatography, 286 (1984) 217–221
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 246

SIMULTANEOUS DETERMINATION OF SEROTONIN AND CATECHOLAMINES IN RAT BRAIN BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

COMPARISON OF THE MOST EFFICIENT TECHNIQUES

MIKLÓS PATTHY and RÓZSA GYENGE*
Institute for Drug Research, Szabadságharcosok útja 47-49, 1045 Budapest 4 (Hungary)

SUMMARY

Two methods have been developed for the concurrent measurement of biogenic amines in rat brain. After a simple extraction procedure, catecholamines and serotonin can efficiently be separated in a reasonably short time if a pellicular cation exchanger is used with appropriate buffers. The column capacity factor was studied as a function of sodium ion concentration in different buffers.

INTRODUCTION

Several micromethods for the determination of catecholamines, serotonin (biogenic amines) and their metabolites have been developed based on fluorescence[1–6], gas chromatography–mass spectrometry (GC–MS)[7–11] and radioenzymatic methods[12,13]. Liquid chromatography coupled with electrochemical detection (LC–EC) offers a degree of sensitivity and specificity which is probably unsurpassed by any other methodology. LC–EC methods have used reversed-phase[14], ion-pair reversed-phase[15] or ion-exchange liquid chromatography[16,17] for the separation of biogenic amines, their precursors and metabolites.

The sensitive and simultaneous determination of individual catecholamines and serotonin has always been difficult. With high-performance liquid chromatographic (HPLC) separations the main difficulty arises from the fact that the retention properties of noradrenaline (NA) and serotonin (5-HT) differ considerably. Previously published reversed-phase procedures using octadecylsilane or octylsilane columns have shown that NA is separated from the solvent front[18]. However, this separation is not consistent from column to column. On the other hand, such columns and solvent systems do not result in short retention times for 5-HT, $t_R > 60$ min. The addition of an ion-pairing agent, *e.g.*, octyl sulphonate, markedly increased the retention time of dopamine (DA) and especially that of 5-HT, but had little or no effect on the retention time of NA. Besides, most of the reversed-phase HPLC techniques require elaborate sample pre-purification prior to the analysis[19].

The present study was carried out in an attempt to show that ion-exchange

0021-9673/84/$03.00

LC–EC techniques using pellicular cation exchangers have distinct advantages over existing reversed-phase methods if biogenic amines without their metabolites are to be determined. These advantages include:

(a) minimum of sample pre-treatment due to the more selective separation methods

(b) longer lifetime for columns and detection cells (little or no organic solvent is needed in the eluent)

(c) remarkable sensitivity permitted by the very high yields of the sample preparation and by eluents that are highly compatible with electrochemical detection.

These advantages easily offset those expected from the somewhat higher plate numbers found in reversed-phase systems.

We have developed two methods for the concurrent measurement of biogenic amines in rat brain. One of them can determine adrenaline (A), DA and 5-HT with very low detection limits, the other is suitable for the measurement of NA, A, DA and 5-HT. Since 3,4-dihydroxybenzylamine was found to co-chromatograph with A in several systems, α-methyldopamine (α-MDA) proposed by Hallman *et al.*[20] was used as internal standard with both methods.

EXPERIMENTAL

Apparatus

The liquid chromatograph was constructed from individual components. A Varian 8500 high pressure pump was used and samples were injected by a home-modified, syringe-loaded Altex AX 201-56 (Tefzel) valve with a 100-μl loop operated in the partial loop mode. Glass columns (500 × 2 mm) dry packed with Zipax SCX (25 μm) or Vydac-CX (35 μm) pellicular cation exchangers were employed. The amperometric detector comprised a Bioanalytical Systems potentiostat LC-2A and a thin-layer type cell with carbon-paste working electrode. The electrode potential was +550 mV *vs.* an Ag/AgCl reference electrode. A Type OH-814/1 strip chart recorder (Radelkis, Budapest, Hungary) was used.

Chemicals

The water used in preparing solutions was deionized and then double glass-distilled. A citrate–acetate buffer was used as eluent. Before use, eluents were degassed *in vacuo*. Eluents: I, 8.6 g citric acid monohydrate, 10.2 g sodium acetate trihydrate, 1.6 ml glacial acetic acid and 3.6 g sodium hydroxide dissolved and diluted to 1 l in distilled water and adjusted to pH 5.3; II, 5.75 g citric acid monohydrate, 6.80 g sodium acetate trihydrate, 1.05 ml glacial acetic acid and 2.40 g sodium hydroxide dissolved and diluted to 1 l in distilled water and adjusted to pH 5.3; III, 95% eluent II and 5% methanol.

Methanol was purchased from Merck, dopamine hydrochloride, noradrenaline hydrochloride and adrenaline from Chrompack (The Netherlands) and α-methyldopamine hydrobromide from Merck Sharp & Dohme (Rahway, NJ, U.S.A.). All other chemicals were of analytical reagent grade. Catecholamine stock solutions were made up in 0.1 *M* trichloroacetic acid containing glutathione (20–40 μl/ml) as antioxidant. Dilute solutions were prepared daily.

RESULTS AND DISCUSSION

The main parameter studied in our chromatographic system was the column capacity factor, k', which is a measure of the degree of retention of a solute compared to that of a non-sorbed compound:

$$k' = \frac{t_R - t_0}{t_R}$$

Since a pH value of 5,3 was found optimal for both the selectivity of the cation-exchange separations and the amperometric oxidation of the biogenic amines, k' values were determined at constant pH, as a function of the sodium ion concentration in neat aqueous citrate–acetate buffers as well as in citrate–acetate buffers containing

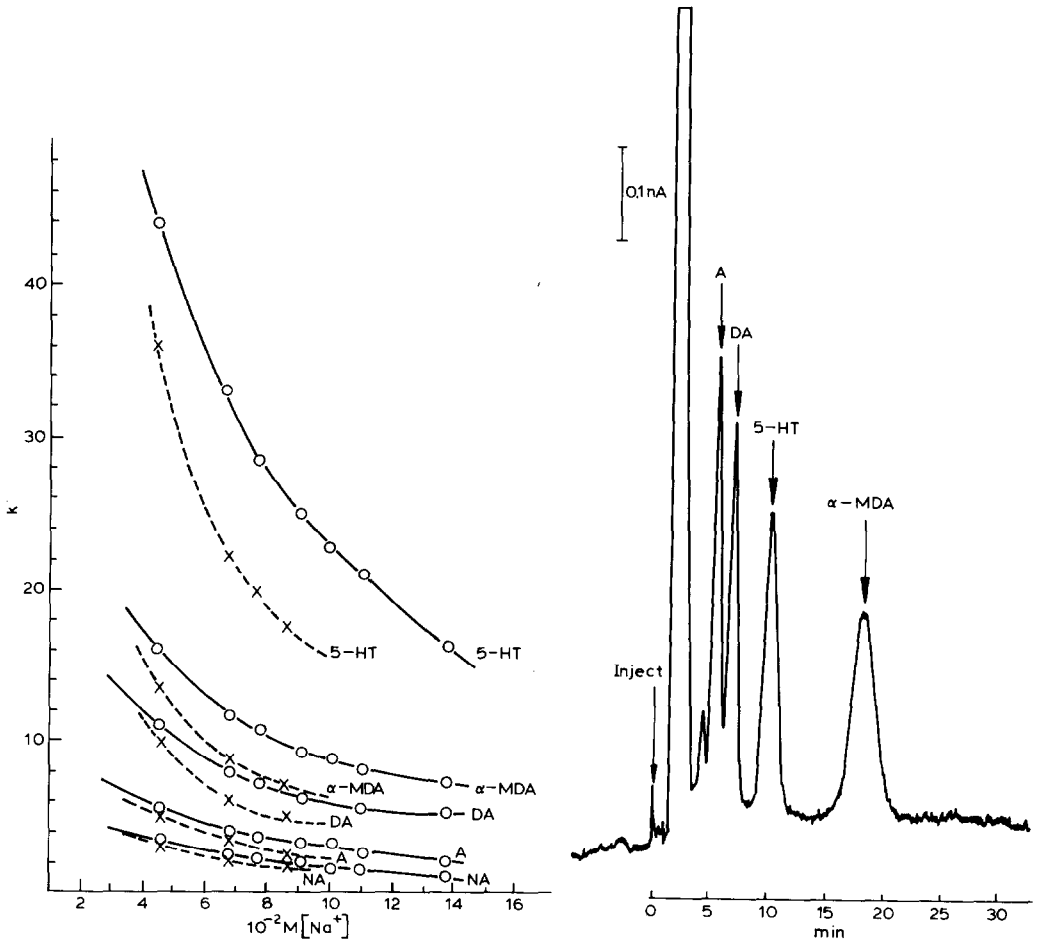

Fig. 1. Effect of sodium ion concentration on capacity ratio, k', in neat aqueous citrate–acetate buffers (○) and citrate–acetate buffers containing 5% methanol (×).

Fig. 2. Separation of catecholamines (each 250 pg) A, DA and 5-HT using α-MDA as internal standard. Column: Zipax SCX (25 μm), 500 × 2 mm I.D. Eluent: citrate-acetate buffer (eluent I). Flow-rate: 0.6 ml/min. Chart speed: 10 cm/h. Temperature: ambient.

1 or 5% methanol. Fig. 1 shows the dependence of k' on sodium ion concentration in neat aqueous buffers and in those containing a small amount of methanol. The column was packed with Vydac-CX, a pellicular cation exchanger.

When the ionic strength of the buffer is below 4×10^{-2} M, methanol causes a dramatic decrease in sensitivity due to the substantial increase in the uncompensated resistance in the cell. At ionic strengths higher than 0.15 M the resolution becomes unsatisfactory and in the presence of methanol the resolution deteriorates further. Glutathione is a more effective antioxidant and stabilizing agent than sodium bisulphite and has a very sharp front peak which does not disturb the separation of NA.

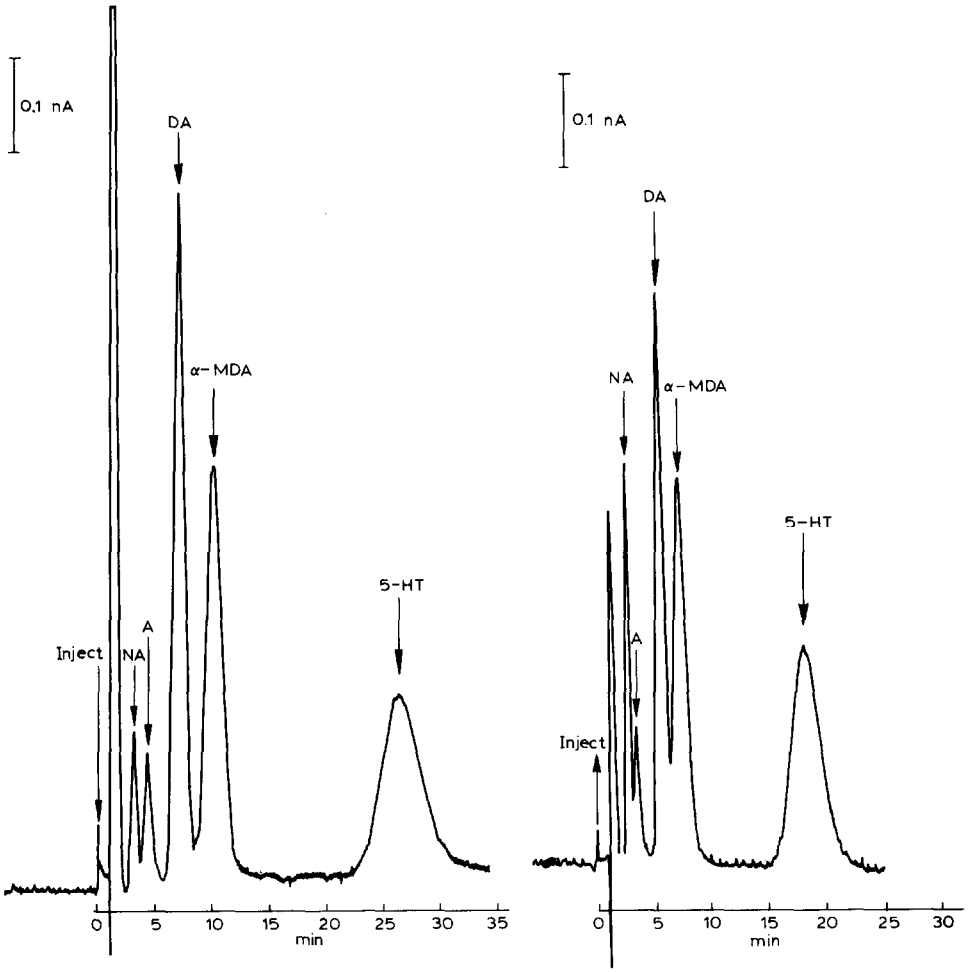

Fig. 3. Separation of NA, A, DA and 5-HT (80, 100, 400 and 200–250 pg, respectively) using α-MDA (200–250 pg) as internal standard. Column: Vydac-CX (35 μm), 500 × 2 mm I.D. Eluent: citrate–acetate buffer (eluent II). Other details as in Fig. 2.

Fig. 4. Separation of NA (250 pg), A (80 pg), DA (350 pg) and 5-HT (250 pg) using α-MDA (250 pg) as internal standard, as in Fig. 3 except that the eluent (III) contained 5% methanol.

Sample preparation

Before the measurements, all of the volumetric flasks, glass and polyethylene tubes, vessels, syringes, etc., have to be checked by HPLC for possible contaminants.

TABLE I

THE SENSITIVITY RANGES OF THE MOST FREQUENTLY USED METHODS

Abbreviations: RP = reversed phase; IE = ion exchange; F = fluorescence; NF = native fluorescence; DF = derivative fluorescence; OPA = *o*-phthalaldehyde; THI = trihydroxyindole; REA = radioenzymatic assay; no = not measurable with the mentioned method.

Method	*Sensitivity (pg)*				
	A	*NA*	*DA*	*5-HT*	*Histamine*
RP-HPLC-EC	20–1000	20–1000	20–1000	20–1000	
IE-HPLC-EC	10–100	10–100	10–100	10–100	
HPLC-F, NF	30–1000	30–1000	30–1000	20–200	
HPLC-F, DF, OPA	no	100–300	100–300	100–300	20–100
HPLC-F, DF, THI	10–100	10–100	no	no	
GC-MS	50–500	50–500	50–500	50–500	
REA	2–20	2–20	2–20	no	

Weighed whole rat brain tissue samples (1–3 mg) were placed in polyethylene tubes. After adding 200 μl of oxygen-free (deaerated by N_2) 0.2 *M* trichloroacetic acid containing 30–40 μg/ml glutathione, the samples were frozen and homogenized. The centrifuged brain extract was directly injected (40 μl) into the chromatographic system by using α-MDA as internal standard. The whole brain homogenate contained 800 pg DA per mg wet brain tissue and 500 pg 5-HT per mg wet brain tissue.

Figs. 2–4 show the systems used for the separation of biogenic amines. Table I summarizes the sensitivity ranges of the most frequently used methods.

REFERENCES

1 Y. Dalmaz and L. Peyrin, *J. Chromatogr.*, 145 (1978) 11–27.
2 K.-I. Okamoto, Y. Ishida and K. Asai, *J. Chromatogr.*, 167 (1978) 205–217.
3 C. A. Marsden, *J. Neurochem.*, 36 (1981) 1621–1626.
4 H. Svendsen and T. Greibrokk, *J. Chromatogr.*, 213 (1981) 429–437.
5 D. E. Mais, P. D. Lahr and T. R. Bosin, *J. Chromatogr.*, 225 (1981) 27–35.
6 S. Hori, K. Ohtani, S. Ohtani, K. Kayanuma and T. Ito, *J. Chromatogr.*, 231 (1982) 161–165.
7 J. C. Lhuguenot and B. F. Maume, *J. Chromatogr. Sci.*, 12 (1974) 411–418.
8 E. Gelpi, E. Peralta and J. Segura, *J. Chromatogr. Sci.*, 12 (1974) 701–709.
9 F. Artigas and E. Gelpi, *Anal. Biochem.*, 92 (1979) 233–242.
10 F. Artigas, E. Martinez and E. Gelpi, *J. Chromatogr. Sci.*, 20 (1982) 75–82.
11 J. J. Warsh, A. Chiu, P. P. Li and D. D. Godse, *J. Chromatogr.*, 183 (1980) 483–486.
12 P. Hjemdahl, M. Daleskog and T. Kahan, *Life Sci.*, 25 (1979) 131–138.
13 J. J. M. L. Hoffmann, J. J. Willemsen, T. Thien and T. J. Benraad, *Clin. Chim. Acta*, 125 (1982) 319–327.
14 J. Molnár and C. Horváth, *Clin. Chem.*, 22 (1976) 1497.
15 T. P. Moyer and N.-S. Jiang, *J. Chromatogr.*, 153 (1978) 365–372.
16 P. T. Kissinger, C. J. Refshauge, R. Dreiling and R. N. Adams, *Anal. Lett.*, 6 (1973) 465–477.
17 C. J. Refshauge, P. T. Kissinger, R. Dreiling, L. Blank, R. Freeman and R. Adams, *Life Sci.*, 14 (1974) 311–322.
18 L. J. Felice, J. D. Felice and P. T. Kissinger, *J. Neurochem.*, 31 (1978) 1461.
19 J. J. Warsh, A. Chiu and D. Godse, *J. Chromatogr.*, 228 (1982) 131–141.
20 H. Hallman, L.-O. Farnebo, B. Hamberger and G. Jonsson, *Life Sci.*, 23 (1978) 1049–1052.