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Note

Simple approach for reversal of the epinephrine–norepinephrine elution order in ion-pair reversed-phase liquid chromatography

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Liquid chromatography with electrochemical detection is the most commonly used technique for catecholamine (CA) assay [1]. Nearly all separations are performed on reversed-phase (RP) silica with C_{18} or C_8 alkyl groups that enable the insertion into the sorbent structure of an anion surfactant, such as octyl sulphate (OS) or sodium dodecyl sulphate (SDS). These sulphates, dissolved in the mobile phase and coated onto the sorbent structure dynamically, act as ion-exchangers and result in increased retention of CAs trough ion-pair formation. Retention of the biogenic amines increases on addition of the negatively charged ion-pairing agent and becomes inversely proportional to the concentration of the positively charged counter-ion and the amount of organic modifier in the mobile phase.

In these separation systems CAs are eluted in the following order: norepinephrine (NE), then epinephrine (E), then dopamine (DA); dihydroxybenzylamine (DHBA), which is used mostly as an internal standard, is eluted between E and DA. This elution order may be disadvantageous when the concentration of late eluting compounds is low, as in case of free E and DA in plasma. The same is true for urinary E from an 8-h sleep period because there is a decrease to less than 10% of the total daily secretion and this fraction seems to be diagnostically important [2]. It is obvious that compounds with a low abundance should be eluted first so as to give as narrow peaks as possible. Although an ideal system, which would allow E and DA to be eluted prior to NE, has not yet been published, solutions for reversed elution order of E and NE have been described. Successful results were obtained by substituting RP silica for a weak cation-exchange stationary phase [3], by using a special surfactant N-methyloleoyl taurate with a C_{18} support [4,5] or by coating tri-*n*-butylphosphate on C_8-alkyl silica [6,7].

In addition, crown ethers, which are known to form complexes with cations, have been used [8]. It was found that 18-crown-6 ether forms a complex with

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primary amines but not with the secondary ones [9]. This resulted in longer retention times for primary amines when the crown ether was included in the mobile phase. We have now investigated the possibility of reversing the elution order of NE and E in a C_8 RP system, by including 18-crown-6 ether in the mobile phase. Moreover, dimethylformamide (DMF) was tested as a possible organic modifier instead of the usual acetonitrile or methanol.

EXPERIMENTAL

Chemicals

Octanesulphonic acid sodium salt, ethylenediaminetetraacetic acid disodium salt (EDTA) and 18-crown-6 ether were purchased from Fluka (Buchs, Switzerland), alumina (70–230 mesh, acidic grade I) was delivered by E. Merck (Darmstadt, F.R.G.). NE, E, DA, DHBA and isoprenaline (IP) were purchased from Sigma (St. Louis, MO, U.S.A.). Inorganic salts, DMF and other chemicals were obtained from Lachema (Brno, Czechoslovakia).

Procedure

Catecholamines were extracted from urine samples by using alumina extraction according the method of Maycock and Frayn [10], slightly modified by us. Briefly, 300-μl micropipette tips were filled with *ca.* 20 mg of alumina fixed by means of glass wool. A urine sample was mixed with an equal volume of 1 *M* Tris buffer (pH 8.6), and 200 μl of the mixture were applied to the column and percolated through by suction (reduced pressure created by peristaltic pump). After washing with 2 ml of water, CAs were eluted with 200 μl of 0.5 *M* acetic acid by means of a syringe An aliquot of the eluate was injected into the analytical system.

Chromatography

Separations were carried out on octylsilica column (250 mm × 4.6 mm I.D., 10 μm particle size) purchased from Serva (Heidelberg, F.R.G.). The HPLC system consisted of a Rheodyne 7125 injection valve with 200-μl sample loop, an FR-30 solvent-delivery pump (Knauer, Berlin, F.R.G.), a pulse dampener and an ED 641 VA electrochemical detector (Metrohm, Herisau, Switzerland). The detector was operated at +0.7 V *vs.* an Ag/AgCl reference electrode and glassy carbon working electrode at 5 nA full scale.

The mobile phase contained 50 m*M* ammonium acetate, 100 mg/l OS and EDTA (pH 5.4); DMFA (0.5 vol.-%) was added as described in Results. The flow-rate was 1.0 ml/min.

RESULTS AND DISCUSSION

The advantage of octylsilica RP material compared with octadecylsilica is its

lower affinity for the biogenic amines, so that admixture of a larger amount of an organic modifier (acetonitrile, methanol) is unnecessary. It may even be possible to omit the organic solvent at all and the total time of analysis will still be reasonable (Fig. 1A). In such a system the biogenic amines can be analysed within

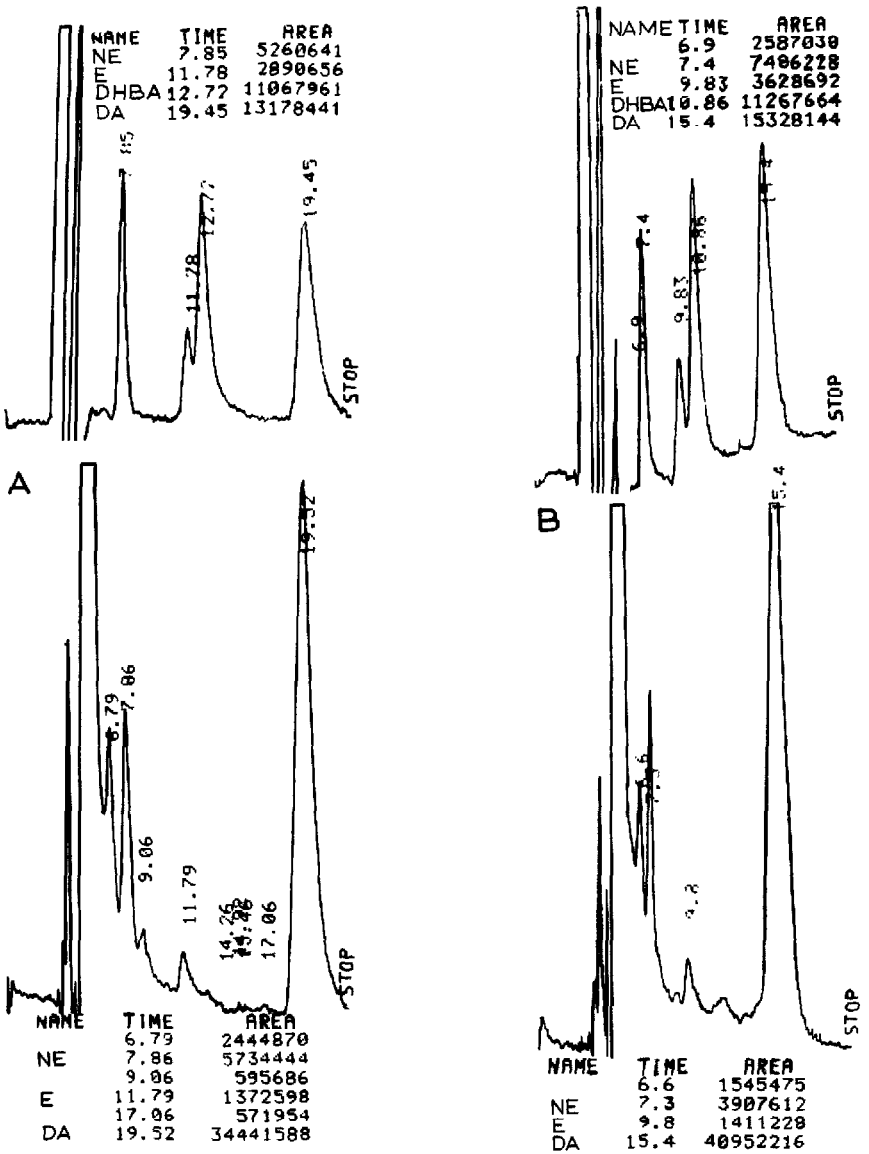

Fig 1 HPLC analysis of CAs on an RP-8 column using ammonium acetate (pH 5.4) mobile phase as described in Experimental. (A) Mobile phase without DMF, (B) with DMF (0.5 vol -%) as organic modifier. Upper traces, CA standard mixture (20, 10, 40 and 60 pmol of NE, E, DHBA and DA), lower traces, CAs isolated from urine after alumina column clean-up (DHBA not added to the urine sample).

ca. 22 min, and extraneous peaks in the urine sample do not disturb the analysis. Among widely used organic modifiers, such as acetonitrile or methanol, we missed one that proved to be very effective in reduction of the retention time,

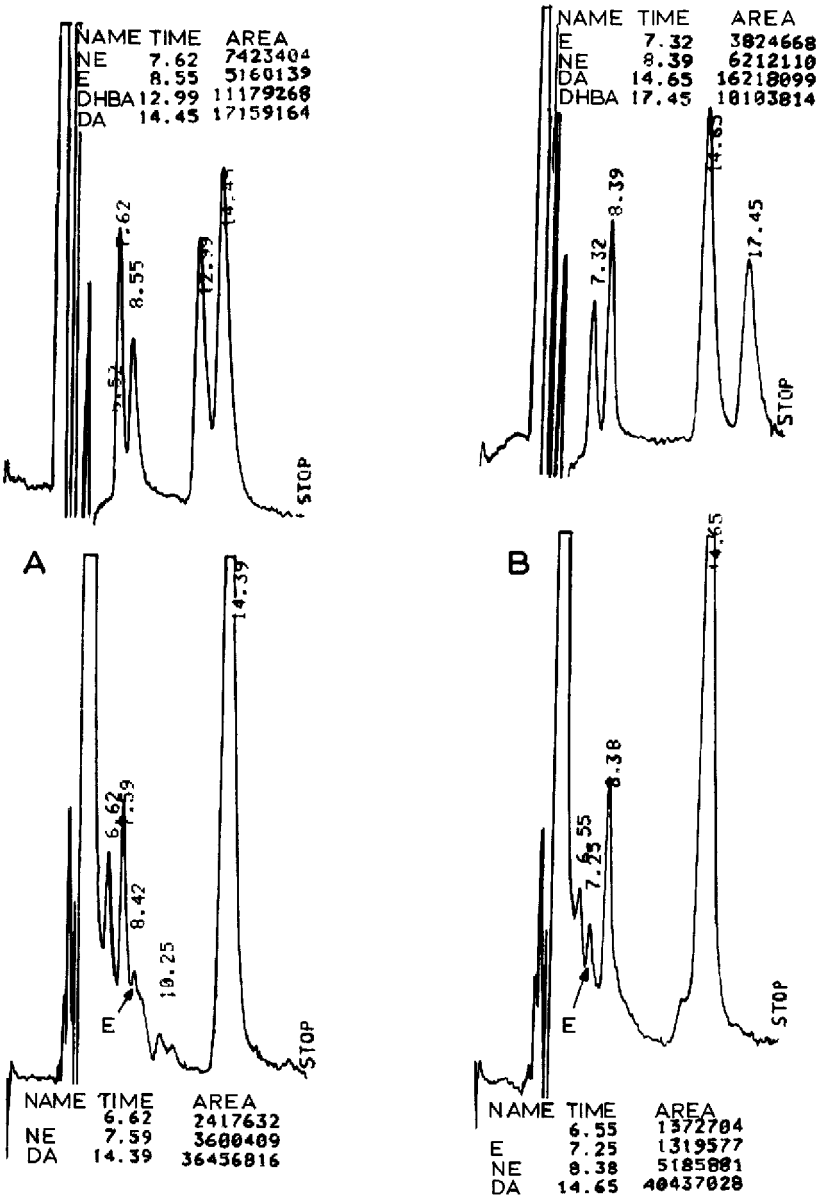

Fig. 2 HPLC analysis of CAs on an RP-8 column using ammonium acetate mobile phase with DMF (0.5 vol -%) as organic modifier, and with the addition of 18-crown-6 ether. (A) 0.15 m*M* (40 mg/l); (B) 0 65 m*M* (180 mg/l). Upper and lower traces as in Fig. 1

namely DMF. Added to the aqueous phase in a proportion of only 0.5%, it caused a substantial decrease in retention times of E, DHBA and DA. Furthermore, the quantitative response from DA was improved by *ca.* 15–20% (Fig. 1A and B). Thus, there was good reason to add DMF to the mobile phase, as not only the elution but also the resolution and peak shape improved.

Addition of 18-crown-6 ether to the mobile phase leads to complex formation with primary amines [9] and, consequently, to their longer retention on the column. We have found that the crown ether acts competitively with the ion-pair agent (OS) and displaces it, to a certain extent, from the silica matrix. This should result in decreased retention of the CAs, but in practice this is true for E only as the secondary amines are not complexed by the crown ether. The retention of the primary amines is, then, a result of an equilibrium formed between crown ether and OS in the sorbent matrix. The shift to longer retention due to complexation is compensated by a decrease in the number of OS binding sites, so that the retention of primary amines is almost unaltered, as shown in Figs. 1B and 2A. In contrast the retention of DHBA is much more influenced by the addition of crown ether than by the decrease in OS binding sites, so that the shift to longer retention is considerable. The total influence of crown ether on the retention

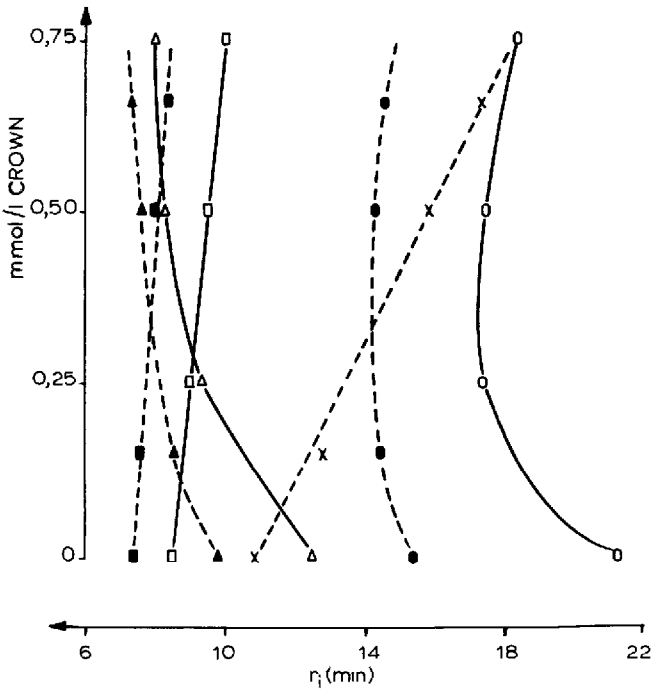

Fig 3 Influence of 18-crown-6 ether concentration in the mobile phase on the retention behaviour of CAs Full line, mobile phase without DMF; dashed line, mobile phase with 0.5 vol-% DMF. (■ and □) Norepinephrine, (▲ and △) epinephrine; (● and ○) dopamine; (×) DHBA

behaviour of CAs, including DHBA, is given in Fig. 3. Addition of 180 mg/l crown ether to the mobile phase with 100 mg/l OS proved to be effective enough to reverse the elution order of NE and E so that analysis of urinary CAs after alumina clean-up was successful (Fig. 2B).

The opposite behaviour to that of DHBA was observed with IP after the addition of the crown ether. IP is a secondary amine with a retention twice as long as that of DA when there is no crown ether in the mobile phase. However, it elutes before DA after the addition of an appropriate amount of crown ether (180 mg/l, Fig. 2B).

Finally, it should be noted that the affinity of crown ethers for the RP silica is greater than that of either OS or SDS. Saturation of the silica sorbent with the ion-pair agent requires overnight treatment, but equilibration with the crown ether-containing phase can be achieved within *ca.* 2 h. When the RP silica is treated with the crown ether first, the incorporation of ion-pair agent into the solvent matrix fails. However, the ether can easily be washed out with methanol or acetonitrile (10 column volumes).

CONCLUSION

The use of 18-crown-6 ether is one of the simplest ways to change the elution order of NE and E with a high degree of flexibility. In such systems IP can replace DHBA as the internal standard.

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