EFFECTS OF MOBILE PHASE COMPOSITION ON THE SEPARATION OF CATECHOLAMINES BY LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

A. ISIMER
N. E. BASCI
A. BOZKURT
S. O. KAYAALP

SUMMARY: In this study, the effects of ion-pairing agents and other ingredients of mobile phase on the separation of nor adrenaline (NA), adrenaline (A) and dopamine (DA) were studied in reversed-phase high performance liquid chromatography with electrochemical detection. The effects of two ion-pairing agents, hexane-sulphonic acid sodium salt (HSA) and pentane-sulphonic acid sodium salt (PSA), have been compared with widely used octane-sulphonic acid sodium salt (OSA). In phosphate based mobile phase, increasing the concentration of OSA and HSA increased the capacity factors (K) of all catecholamines (CAs) in the standard mixture and higher concentration of HSA was required to reach the same values. PSA had minimal effect on the retention times at the same molar concentration range. In ammonium acetate based mobile phase, increasing the concentration of the ammonium acetate significantly reduced the K values of all CAs when HSA was used as an ion-pairing agent. Alteration of the ammonium acetate concentration in PSA containing mobile phase had minimal effect on K values. In contrast to the results obtained with phosphate buffer, increasing the pH of the ammonium acetate based mobile phase resulted in an increase in the K values of all compounds investigated. HSA was found to be a suitable ion-pairing agent for liquid chromatographic separation of CAs in either phosphate or ammonium acetate based mobile phases and the applicability of the method was demonstrated by analysis of CAs in different rat tissues after alumina extraction.

Key Words: Catecholamines, liquid chromatography, ion-pairing agents, electrochemical detection.

INTRODUCTION

Catecholamines (CAs) produce many important physiologic effects in the brain, the cardiovascular system and other organs. The concentration of the major CAs, nor adrenaline (NA), adrenaline (A) and dopamine (DA) in biological tissues is modified by physiological factors and influenced by pharmacological agents. It is therefore essential to be able to elucidate its variation in quantitative chemical tests and in turnover studies. Moreover, plasma and urinary CA levels and their measurements are of clinical importance as a diagnostic tool in clinical chemistry laboratories. Introduction of high performance liquid chromatography/electrochemical detection (HPLC-ED) has shown to offer high selectivity and sensivity for the analysis of CAs and related compounds in complex media (5, 7, 8). Most of the published HPLC-ED methods for determination of these compounds have employed reversed-phase technique and purification procedure via alumina or ion-exchange.

Retention of CAs on a reversed-phase column can be manipulated by (i) changing the pH of the mobile phase, (ii) adding a counter ion to the mobile phase to retain a charged molecule of interest, (iii) ion pair extraction or (iv) adding an organic modifier to the mobile phase. Since

From Department of Pharmacology, Gülhane Military Medical Academy, From Department of Pharmacology, Hacettepe University, Medical School, Ankara,Türkiye.

problems with various interferences can be solved in a number of ways, phosphate buffered mobile phases have been investigated in detail (4, 9). Furthermore, addition of an aliphatic sulfonate as a paired ion was necessary to enhance the separation of CAs and especially to achieve the resolution between adrenaline (A) and 3,4-dihydroxybenzylamine (DHBA), a useful internal standard (10). Octanesulfonic acid (OSA) was found to be the paired-ion of choice. One of the alternative mobile phase solutes is ammonium acetate. Recently, Bartlett (2) reported that ammonium acetate system was superior to conventional phosphate based systems for the separation of CAs. The present study was intended to investigate the effects of adding paired ions of varying carbon chain length such as, pentanesulfonic acid (PSA) or hexanesulfonic acid (HSA) and organic modifier, and of altering pH and buffer content of mobile phase on the separation of DOPA, NA, A, DA and DHBA in reversed phase HPLC-ED.

MATERIALS AND METHODS

Chemicals

HPLC-grade acetonitrile was purchased from Fisher Scientific (Pittsburgh, PA, USA). Pentanesulfonic acid sodium salt, hexanesulfonic acid sodium salt, octanesulfonic acid sodium salt, nor-adrenaline, adrenaline, dopamine and 3,4-dihydroxybenzylamine were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were analytical grade.

Apparatus

The chromatographic system consisted of a Model 6000A solvent delivery system, a Model U6K injector, a Model 730 data module and a μBondapak C_{18} reversed-phase stainless-steel analytical column (10 μm particle size, 30 cm x 4.0 mm I.D.) protected with a guard column packed with Bondapak C_{18}/Corasil (Waters Assoc. Milford, Mass., USA). Catecholamines were detected using a Waters Model 460 electrochemical detector which was equipped with a glassy-carbon working electrode set at an electrode potential of + 0.6 V versus an Ag/AgCl reference electrode.

Standard solutions

Catecholamine (NA, A and DA) stock solutions were prepared separately at a concentration of 6 mM in 0.1 N HCl containing 5 mol/l sodium metabisulphite. All solutions were stored in dark at -20 ^{o}C and remained stable for at least one month. A working standard mixture of CAs was prepared daily in mobile phase to contain 0.6 μM of each and an injection volume of 20 μl was employed.

Mobile phase preparation

Aqueous solutions of 0.1 mol/l sodium dihydrogen phosphate and 0.34 mmol/l EDTA and varying concentrations of acetonitrile and an ion-pairing agent were made up in distilled water. The required pH value was adjusted with phosphoric acid. Another series of experiments were performed with ammonium acetate buffer system, in which mobile phase included 0.25 mol/l ammonium acetate, 0.34 mmol/l EDTA and varying concentrations of acetonitrile and an ion-pairing agent. The mobile was phase buffered with glacial acetic acid to required pH value. The mobile phases were filtered through a 0.45 μm Type HA filter (Millipore, Bedford, MA, USA). Mobile phase flow rate was maintained at 1.0 ml/min through the system. The capacity factor of CAs, K, is defined as follows:

$K = (t_R - t_o) / t_o$

where t_R is the retention time of CA and t_o is the retention time of void volume of the chromatographic system.

Application to biological samples

Rats were killed by decapitation and tissues were quickly removed. Weighed tissue samples were homogenized in ice cold 0.4 N perchloric acid containing 0.1% EDTA and 0.1% $Na_2S_2O_5$. Homogenates were centrifuged at 8000 g for 15 min. Supernatants were separated and stored -20^{o}C. Catecholamines were extracted from thawed supernatant using batch extraction with alumina at alkaline pH which was carried out as previously described (1). A 20 μl of alumina eluent in 0.1 mol/l perchloric acid was injected into the HPLC system.

RESULTS AND DISCUSSION

Effects of phosphate based mobile phase composition on the capacity factors of catecholamines:

Ionic strength of mobile phase is known to significantly affect chromatographic behavior of the CAs in ion-paired reserved-phase chromatography. Moyer and Jiang (10) reported that the retention time was not affected by varying concentrations of phosphate but the electrochemical detector responses of CAs were altered. The optimum responses were obtained with phosphate buffer at a concentration of 0.1 mol/l. Therefore, this concentration was used in all experiments done with phosphate based mobile phase.

As shown in Figure 1, increasing concentrations of OSA and HSA increased the K values of all components of the standard mixture. However, higher molar concentrations of HSA was required to reach the same values. In contrast, PSA had minimal effect on the retention times, at the same molar concentration range.

The effects of increasing pH on the capacity factors of CAs were shown in Figure 2. No significant change was observed in both HSA and PSA containing mobile phases. Bartlett (2) and Kontur, *et al.* (6) also showed that increasing the pH (3.5-5.0) of the phosphate buffered mobile phase did not affect the retention time of CAs.

Increasing the acetonitrile content of phosphate based mobile phase reduced the K values of CAs in a predictable way regardless of the type of ion-pairing agent used.

Effects of ammonium acetate based mobile phase composition on the capacity factors of catecholamines:

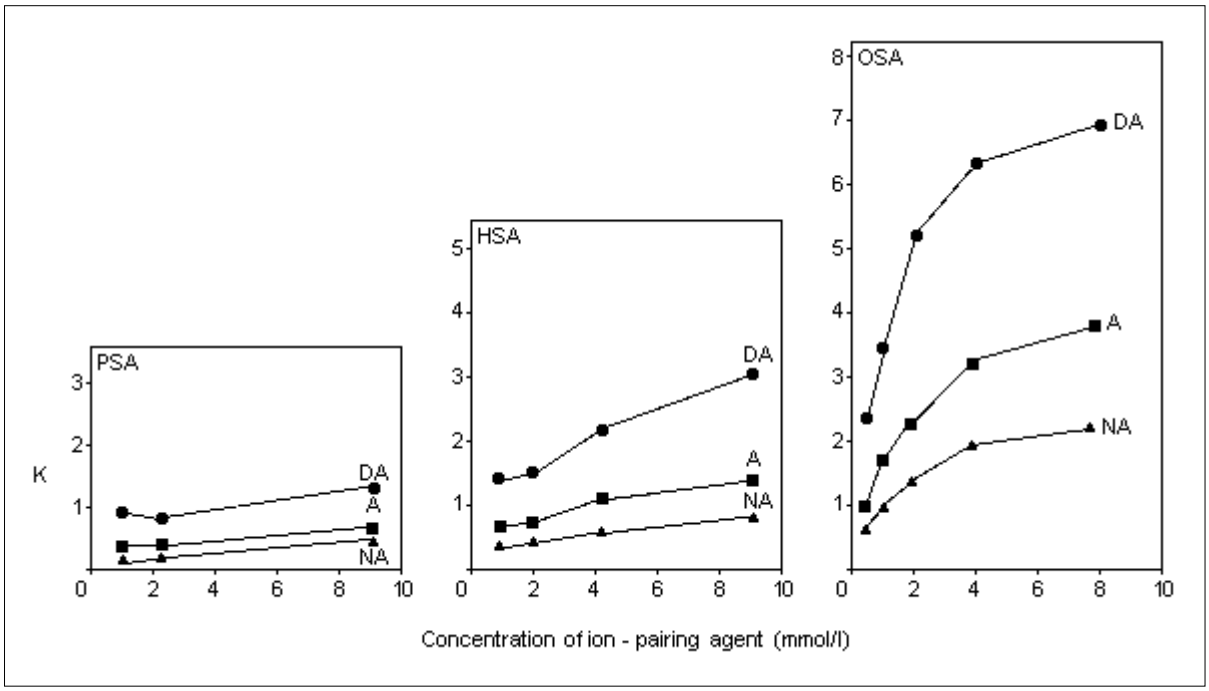

Figure 1: Effect of increasing the concentration of PSA, HSA and OSA in mobile phase upon the capacity factors (K) of catecholamines. Chromatography was performed on μBondapak C_{18} column with a mobile phase of 0.1 mol/l phosphate, 0.3 mmol/l EDTA, 3% (v/v) acetonitril, pH 3.5, flow rate 1.0 ml/min.

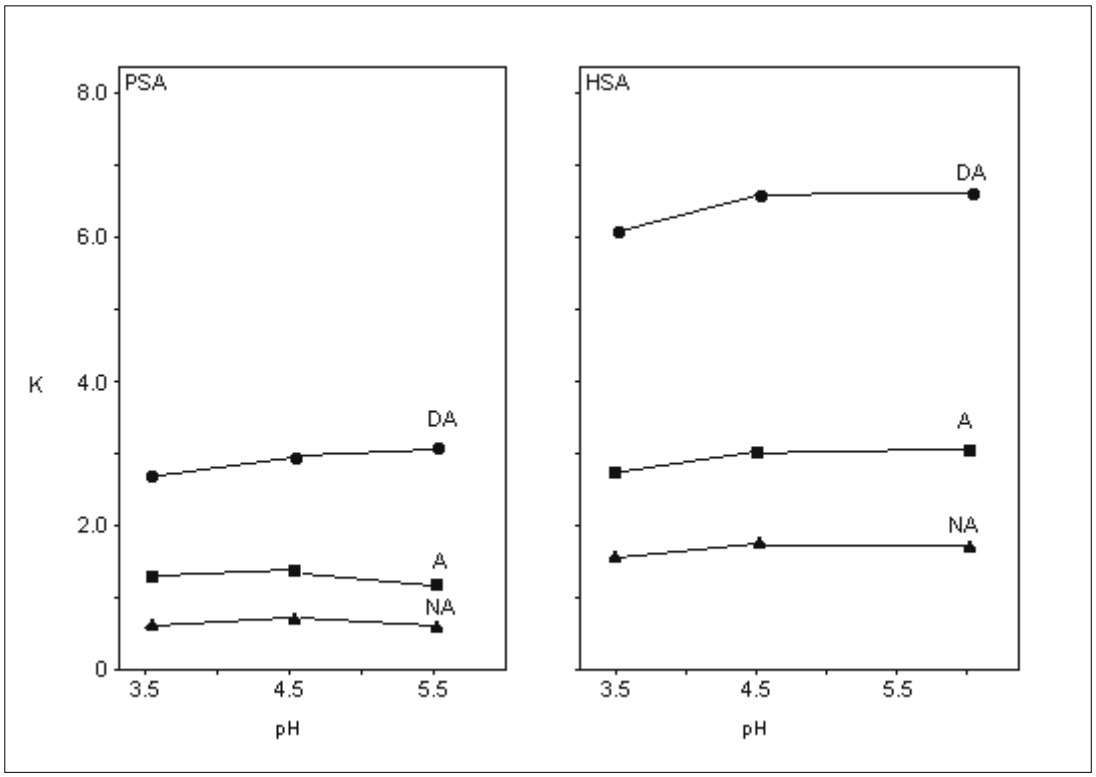

Figure 2: Effect of increasing pH of phosphate based mobile phase on the capacity factors (K) of catecholamines. Mobile phase consisted of either 1.2 mmol/l PSA or 8.5 mmol/l HSA; other conditions were as described in Figure 1.

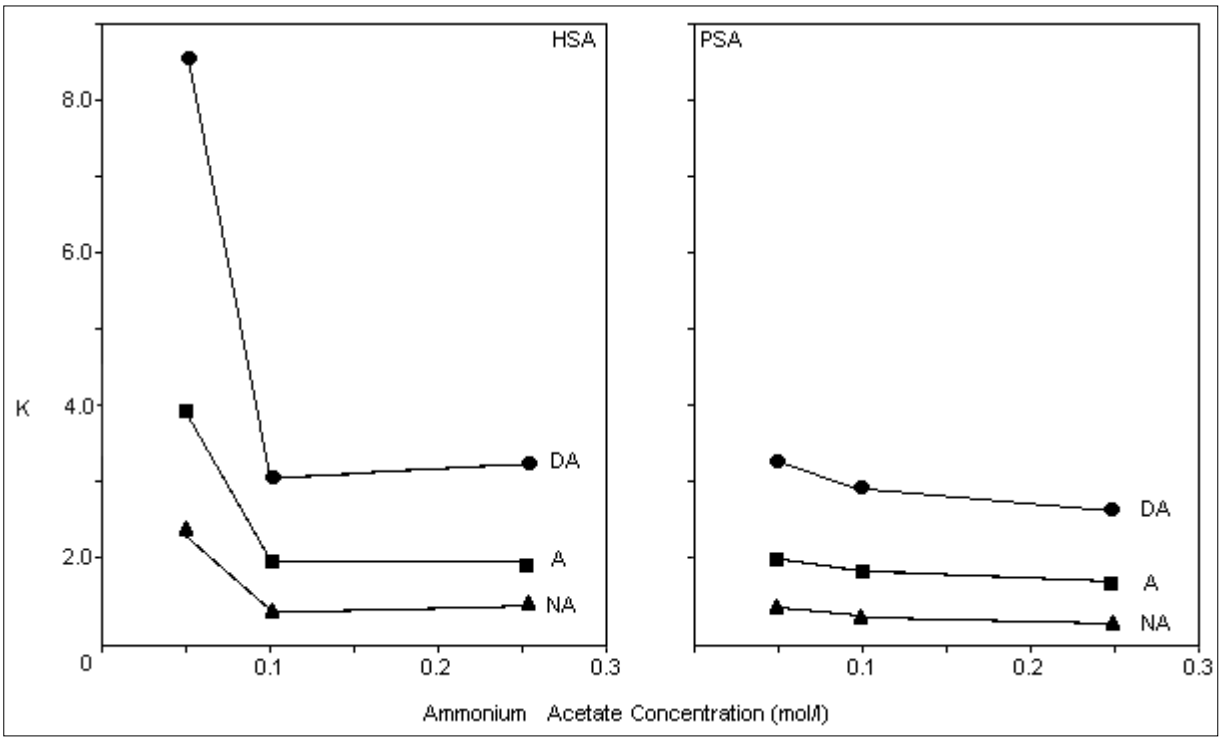

Figure 3: Effect of ammonium acetate concentration of mobile phase on the capacity factors (K) of catecholamines. Mobile phase consist ed or either 8.5 mmol/l HSA or 2.3 mmol/l PSA. A μBondapak C_{18} column used with 1% (v/v) acetonitrile, 0.3 mmol/l EDTA and pH 4.5 in a flow rate of 1.0 ml/min.

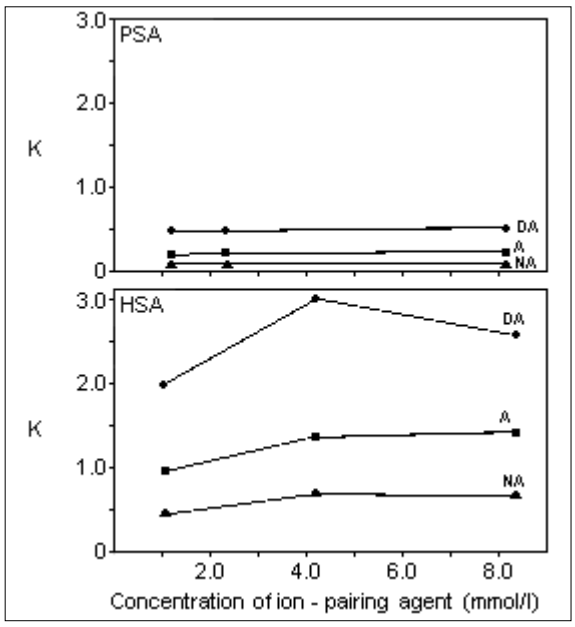

Figure 4: Effect of increasing the concentration of PSA and HSA in mobile phase on the capacity factors (K) of catecholamines. Chromatography was performed on a μBondapak C_{18} column with a mobile phase of 0.25 mol/l ammonium acetate, 3% (v/v) acetonitril and pH 4.5 in a flow rate 1.0 ml/min.

Increasing the molar concentration of ammonium acetate significantly reduced the K values of all CAs when HSA was used as an ion-pairing agent (Figure 3a). In contrast to this observation, alteration of the ammonium acetate concentration in PSA containing mobile phase had minimal effect on K values (Figure 3b).

The effects of increasing concentrations of ion-pairing agents on the separation of CAs were shown in Figure 4. Results obtained with HSA and PSA in ammonium acetate based mobile phase resembled those seen in phosphate based mobile phase.

Increasing the acetonitrile content of mobile phase reduced the K values of CAs in a predictable manner regardless of the type of ion-pairing agent used, which was the same in the phosphate based mobile phase.

In contrast to the results obtained with phosphate buffer, increasing the pH of ammonium acetate based mobile phase resulted in an increase in the K values of all compounds investigated (Figure 5). Results obtained with phosphate based mobile phase suggested that CAs would be separated independently of pH changes. However, the effects of pH were markedly different when ammonium acetate based mobile phase was used. One explanation of this difference caused by the mobile phase is that ammonium ion could function as a secondary ion-pairing agent interacting with stationary phase and with

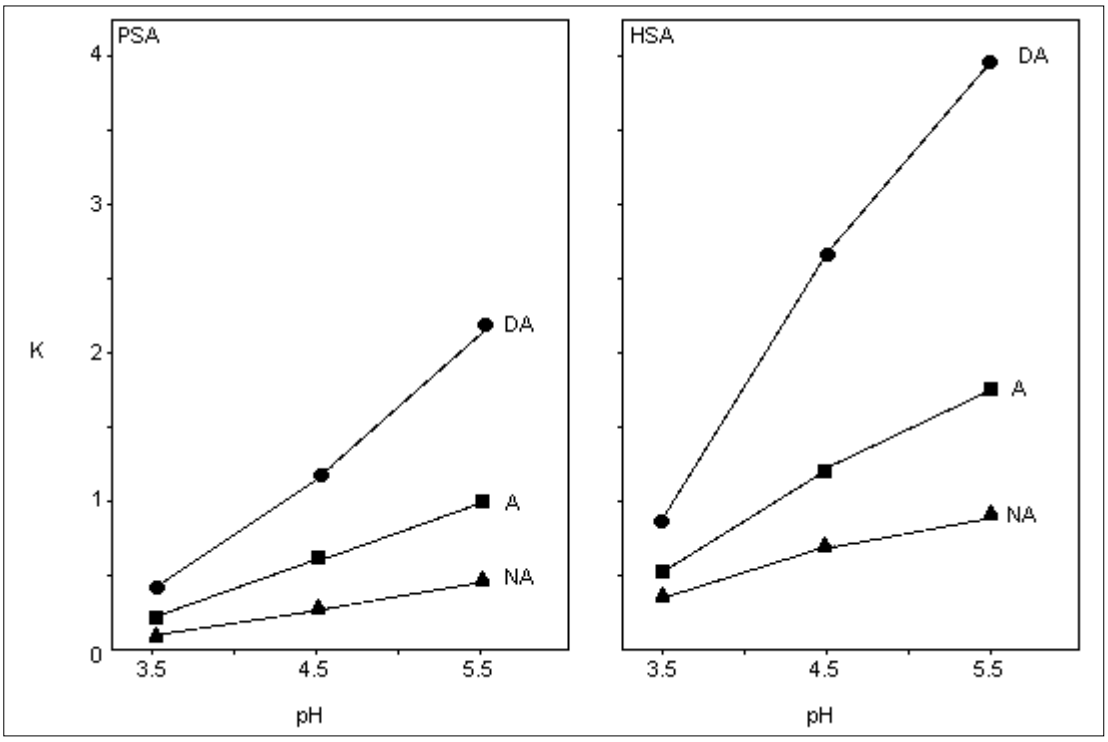

Figure 5: Effect of ammonium acetate mobile phase pH on the capacity factors (K) of catecholamines. Mobile phase consisted of either 2.3 mmol/l PSA or 8.5 mmol/l HSA. Other conditions were as described in Figure 4.

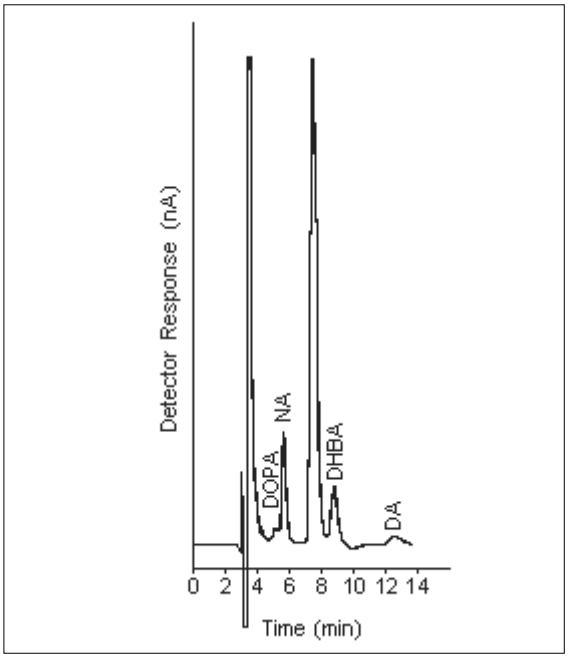

Figure 6: Chromatogram of adrenal gland supernatant. Chromatography was performed on a μBondapak C_{18} column with a mobile phase of 0.1 mol/l phoshphate, 3% (v/v) acetonitrile, 8.5 mmol/l HSA and pH 3.5 in a flow rate of 1.0 ml/min.

amines. This event is dependent on the pH which determines the degree of ionization of ammonium ion. Bartlett (2) also showed that in ammonium acetate based mobile phase, increasing the pH from 4.0 to 5.5 augmented the capacity factors of NA, A and DA.

Application to biological samples

Alumina extracts of rat adrenal glands were assayed for NA, A and DA. Chromatography was performed upon μBondapak C_{18} column (10 μm particle size, 30 cm x 4.0 mm I.D.) with a mobile phase consisting of 0.1 mol/l phosphate buffer, 8.5 mmol/l HSA, 0.34 mmol/l EDTA, pH 3.5 with a flow rate of 1.0 ml/min and a detection voltage of 0.6 volt. As shown in Figure 6, three CAs and internal standard (DHBA) were readily detected and values obtained agreed with results reported in the literature (3).

CONCLUSION

These observations suggest that HSA is a suitable ion pairing agent to achieve a baseline separation of CAs in both phosphate and ammonium acetate based mobile phases. However, the capacity factors of three CAs calculated at the same HSA concentration (8.5 mmol/l) and the K values for all CAs obtained in phosphate buffer were found to be about 120% higher than those obtained in ammonium acetate based mobile phase.

REFERENCES

1. Anton AH, Sayre DF : A Study of the Factors Affecting the Aluminium Oxide-Trihydroxyindole Procedure for the Analysis of Catecholamines. J Pharmacol Exp Ther, 138:360-374, 1962.

2. Bartlett WA : A Comparison and Clinical Applications of Ammonium Acetate and Phosphate Based Mobile Phases for the Separation of Catecholamines on Reversed Phase Columns. J Liq Chromatogr, 8:719-751, 1985.

3. Coulter CL, McMillen C, Browne CA : The Catecholamine Content of the Perinatal Rat Adrenal Gland. Gen Pharmacol, 19:825-830, 1988.

4. Horvath C, Melander W, Molnar I, Molnar P : Enhancement of Retention by Ion-Pair Formation in Liquid Chromatography with Non-polar Stationary Phases. Anal Chem, 49:2295-2305, 1977.

5. Kagedal B, Goldstein DS : Catecholamine and Their Metabolites. J Chromatogr, 429:177-233, 1988.

6. Kontur P, Dawson R, Monjan A : Manipulation of the Mobile Phase Parameters for the HPLC Separation of Endogenous Monoamines in Rat Brain Tissue. J Neurosci Meth, 11:5-18, 1984.

7. Krstulovic AM : Investigations of Catecholamine Metabolism Using High Performance Liquid Chromatography. Analytical Methodology and Clinical Application. J Chromatogr, 229:1-15, 1982.

8. Mefford IN : Application of High Performance Liquid Chromatographic-Electrochemical Detection to Neurochemical Analysis: Measurement of Catecholamines, Serotonin and Metabolites in Rat Brain. J Neurosci Meth, 3:207-215, 1981.

9. Molnar I, Horvath C : Reversed-Phase Chromatography of Polar Biological Substances : Separation of Catechol Compounds by High-Performance Liquid Chromatography. Clin Chem, 22:1497-1502, 1976.

10. Moyer TP, Jiang N : Optimized Isocratic Conditions for Analysis of Catecholamines by High-Performance Reversed-Phase Paired-Ion Chromatography with Amperometric Detection. J Chromatogr, 153:365-372, 1978.

Correspondence:
Askin Isimer
Gülhane Military Medical Academy,
Department of Pharmacology,
Etlik, 06018 Ankara, TÜRKIYE.