CAPSULES
notes and applications from Bioanalytical Systems, Inc.

Chromatography Conditions For Biogenic Amines In Brain

Purpose

The simplest approach to measuring biogenic amines and metabolites in tissue homogenates is to inject supernatant directly. This is often the only approach possible if compounds of different charge and polarity are to be determined. What works for catecholamines, for example, may exclude indoles.

The success of the assay then falls upon the chromatographic conditions, rather than the extraction steps before injection. This note describes appropriate chromatographic conditions for gross injections of tissue homogenates, without prior cleanup.

Proposed Methods

The compounds principally encountered in brain homogenate are listed in T1 according to their functional groups.

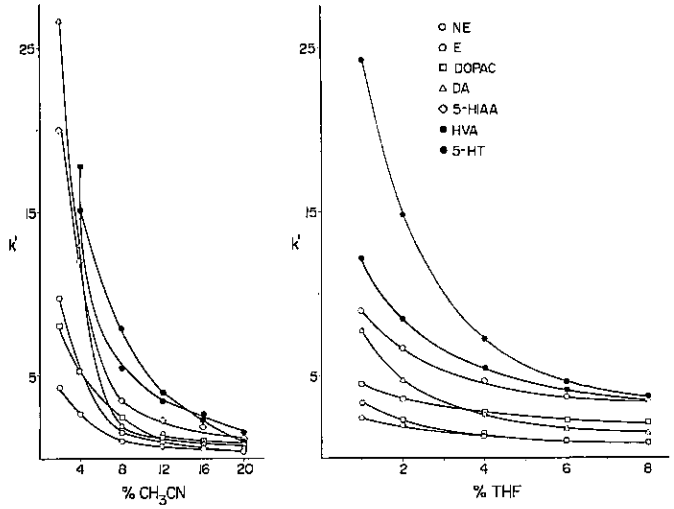


Figure 1. The effect of CH_3CN and THF on the capacity factors of seven neurochemicals.

Compounds may be separated on the basis of their hydrophobicity, charge, and size. Charge and hydrophobicity are principally involved in reverse phase ion pair chromatography. The variables of interest are the nature and concentration of the ion pairing agent, the pH, ionic strength, solvent strength, and temperature.

Ion pairing agent. The catecholamines are extremely hydrophilic. Retention on a hydrophobic C_{18} support is therefore negligible unless ion pairing agents are added. The use of sodium octylsulfate is common. This moderately hydrophobic detergent physically modifies the column's surface by providing numerous immobilized sites of negative charge. Since the catecholamines will be protonated at pH 8, an "ion exchange" mechanism is roughly in effect.

The surface concentration of ionic sites may be increased by using a higher octylsulfate concentration in the mobile phase, or by using a longer chain length detergent. Solvent strength reduces the surface concentration of ion exchange sites.

Retention of cations is inversely related to ionic strength.

pH. The ion pair separation of the catecholamines is little affected by pH, since pKa's are greater than 9. Elution of DOPAC and HVA is sensitive to pH, however, since their pKa's are 4.5 - 5.0. Retention is maximal at pH 2 (neutral charge on analyte) and minimal at pH 6 (charge is -1).

A pH of 3.0 retains DOPAC and HVA in positions between the catecholamines and indoles.

Solvent strength. A general trend is the decrease of retention with increasing solvent strength. THF (tetrahydrofuran) is a stronger eluent than CH_3CN, which, in turn, is a stronger eluent than methanol. Secondary equilibria permit unique selectivities to be obtained with solvents such as THF or isopropanol.

Temperature. Increasing temperature decreases retention but generally improves peak shape. EC

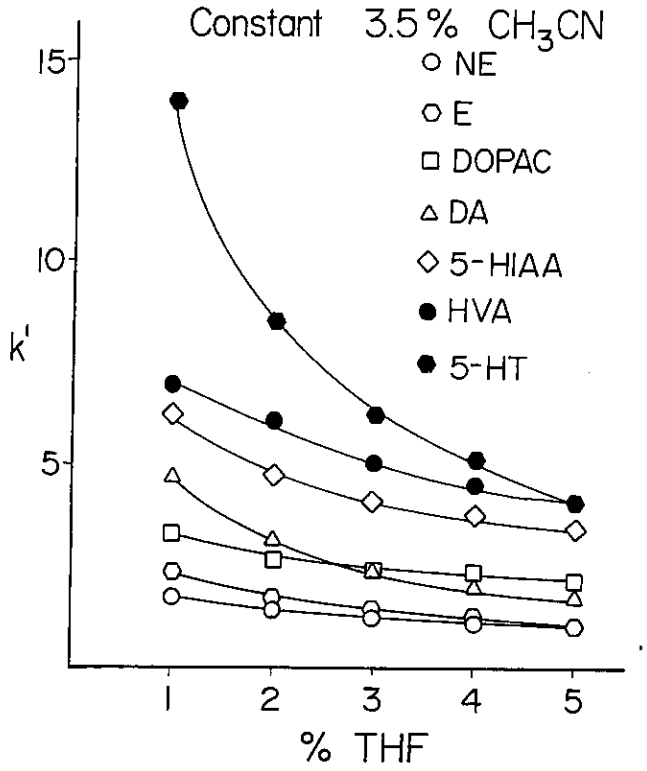


Figure 2. The effect of multiple solvents (THF and CH_3CN) on the capacity factors of the solutes.

detector noise degrades above 45°C, so try to work in the 30-40°C range.

Conditions

System: Original data was generated with a Bioanalytical Systems LC-304 liquid chromatograph. Current BAS products are the modular BAS 400 (isocratic) or BAS 200 (gradient) systems. Both are popular in the field for the tissue homogenate assay.

Some of the mobile phases described contain small amounts of the highly volatile ether tetrahydrofuran (THF). For long- term retention time reproducibility, the mobile phase reservoir should be closed off with parafilm or a loose cap to retard evaporation. Detection was accomplished amperometrically at +800 mV vs. Ag/AgCl, on glassy carbon.

Flow Rate: 1.6 mL/min

Column: BAS Biophase ODS 5µ (P/N MF6017), 250 x 4.6 mm

Mobile Phase: The base mobile phase is a monochloroacetate (MCAA) buffer, pH 3.

Chloroacetate was chosen because it is a better buffer at pH 3 than either acetate or phosphate; it also is toxic to bacteria.

Base mobile phase: 0.15 M monochloroacetate with 0.86 mM sodium octylsulfate, pH 3. Dissolve 28.3 g monochloroacetic acid, 9.35 g NaOH, and 400 mg sodium octylsulfate in 2.0 L H_2O. Adjust pH to 3.0 with MCAA or NaOH as desired.

Typical mobile phase: 70 mL acetonitrile was diluted to 2.0 L with base mobile phase and filtered through a 0.2 µm nylon membrane. After vacuum filtration, the vacuum was broken; 36 mL THF was added and stirred magnetically. THF cannot be added before vacuum filtration because it is too volatile. Do not sparge the mobile phase.

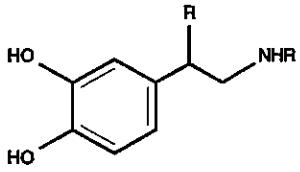
Results

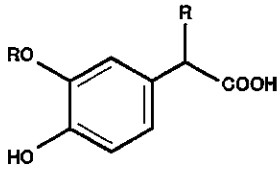
A systematic study of retention versus solvent concentration and type reveals that unique selectivity patterns can be obtained. As CH_3CN was varied, the elution order of DA and 5-HIAA and EPI and DOPAC interchanged once, while 5-HT and HVA exchanged twice. Satisfactory resolution within a desired k' of 10 was achieved at 8% and 12% (v/v) acetonitrile; however, the latter proved unacceptable for real samples. Resolution of the compounds in the required time span using only THF as the modifier was not achieved, either. (F1) NE and EPI coeluted when the concentration of THF was greater than 3%. At approximately 3.9% THF, the elution of DOPAC and DA inverted.

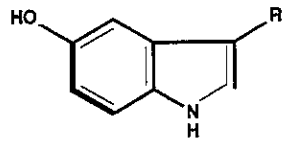
The concentration of CH_3CN was then fixed at 3.5% (v/v) while the THF content was varied to examine the elution of the neurotransmitters (F2). Satisfactory resolution of all compounds with k' less than 10 was achieved at 1.8% THF/3.5% CH_3CN. The selectivity of the two solvents used together was unique, not characteristic of either solvent alone. This effect with multiple solvents has also been described by Glajch et al(3). The THF drastically reduced the k' of 5-HT without appreciably affecting those of the earlier eluting solutes. A representative chromatogram is shown in F3. The separation was

Table 1.

Basic catechols	Acidic catechols and metabolites	Indoles
norepinephrine (NE)	3,4-dihydroxyphenyl-	5-hydroxytryptamine (5-HT)
epinephrine (EPI)	acetic acid (DOPAC)	5-hydroxytryptophan (5-HTP)
dopamine (DA)		
	homovanillic	5-hydroxyindole-3
	acid (HVA)	acetic acid (5-HIAA)







Primary Determinants of Retention

Ion pairing concentration	Solvent strength	Solvent strength
Ionic strength	pH	Ionic strength
pH		pH
Solvent strength		Ion pairing agent

sensitive to the concentration of THF. For example, increases from 14 to 20 minutes were observed with a 0.2% variation of THF concentration. Since THF is so volatile, the mobile phase should be kept in a tightly stoppered bottle to maintain reproducible retention times.

References

1. G.S. Mayer, R.E. Shoup, J. Chromatogr., 255(1983) 533- 544.

2. G.S. Mayer, R.E. Shoup, Anal. Chim. Acta, accepted.

3. J.L. Glajch, J.J. Kirkland, K.M. Squire and I.M. Minor, J. Chromatogr., 199(1980) 57.

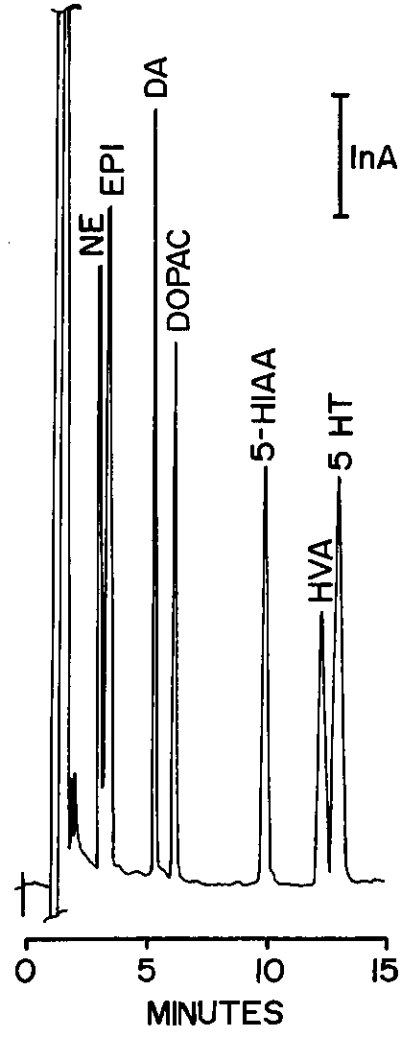


Figure 3. Chromatogram of a biogenic amine standard solution; 4 to 7 ng of each compound was injected.