OPTIMIZATION OF NEUROTRANSMITTERS SEPARATION UNDER HILIC CONDITIONS

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Abstract: This paper presents the optimization of a hydrophilic interaction chromatographic method for the separation of eleven catecholamines, indolamines and their precursors and metabolites. The studied parameters are: the organic modifier nature and percentage, the salt nature and concentration, the mobile phase pH and the column temperature. The best results in terms of separation were obtained using a mobile phase composed of acetonitrile and ammonium formate (150 mM pH 3) 85:15 v:v.

Keywords: catecholamines, HILIC, neurotransmitters

1. INTRODUCTION

In the nervous system, neurotransmitters are the most common class of chemical messengers. Neurotransmitters can be divided into: small amino acids and biogenic amines [1]. Catecholamines (dopamine, norepinephrine and adrenaline) (Table 1) are neurotransmitters of the monoamine family which are synthesized from the same amino acid (tyrosine) and therefore have a similar structure consisting of an aromatic nucleus with two hydroxyl groups (the catechol nucleus) and an ethylamine as side chain [2].

Serotonin (S) belongs to the family of indole-type neurotransmitters. It is synthesized from tryptophan (TRP). S is the neurotransmitter for which has the largest number of receivers.

Catecholamines and indolamines, as well as their metabolites, are polar and non-volatile molecules. These are substances that are very sensitive to light, oxygen, temperature and pH. That is why they must be kept in an acid medium (pH less than 3), protected from light and at a low temperature. Miki and Sudo [3] carried out a study showing the effect of pH, storage time and temperature on the stability of catecholamines (DA, A and NA) in urine. They were able to observe that at a pH $\leq$ 7, the urine kept at less than 4 °C do not lose significant amounts of catecholamines for at least the first 7 days, while at pH 10 they degrade rapidly even at -20 °C. This study highlights the importance of temperature on the conservation of catecholamines.

In the human body, catecholamines and indolamines, as well as their metabolites, proved to be markers in the diagnosis of different diseases, like: Alzheimer's disease, Parkinson's disease, pheochromocytoma or neuroblastoma [4-7].

Because of the low physiologic concentrations of these compounds and the tendency of the catechol group to be easily oxidized, quantification of catecholamines presents numerous difficulties.

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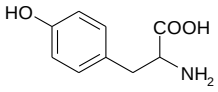
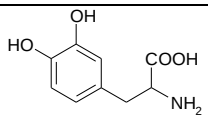
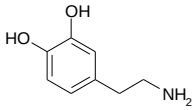
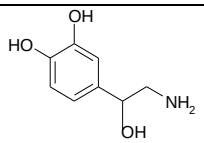
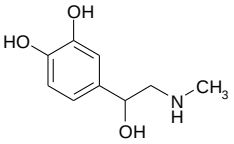
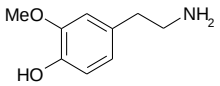
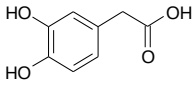
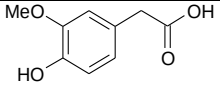
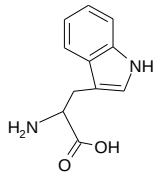
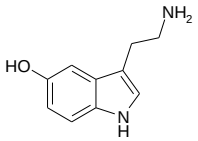
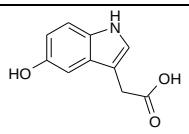
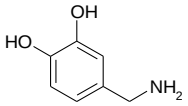
Although reversed phase chromatography (RPLC) is the most currently used method for the analysis of catecholamines and related compounds [8-13], recently hydrophilic interaction liquid chromatography (HILIC) began as an alternative for the separation of neurotransmitters [6, 14-16]. In HILIC, the stationary phases are polar and the mobile phases are composed of water and an important amount (60-98 %) of organic solvent, leading to the elution of the analytes in their increasing polarity order [17-20].

Table 1. Structure of the target compounds.

Name	Structure	Name	Structure
tyrosine (TYR)		dihydroxyphenylalanine (DOPA)	
dopamine (D)		noradrenaline (NA)	
adrenaline (A)		3-metoxytyramine (3-MT)	
dihydroxyphenylacetic acid (DOPAC)		homovanilic acid (HVA)	
tryptophan (TRP)		serotonin (S)	
5-hydroxyindolacetic acid (5-HIAA)		dihydroxylbenzylamine (DHBA)	

The aim of this study was to optimize a HILIC method for the separation of 11 catecholamines, indolamines, and their metabolites and precursors (dopamine, norepinephrine, adrenaline, serotonin, 5-hydroxyindolacetic acid, dihydroxyphenylacetic acid, homovanilic acid, 3-metoxytyramine, tryptophan, dihydroxyphenylalanine and tyrosine) that could be coupled to mass spectrometric (MS) detection for the quantification of the target compounds in biological matrixes. In order to be able to do that, a highly volatile mobile phase with a reasonable amount of salt is need to insure the lowest MS detection limits.

2. EXPERIMENTAL SETUP

The optimization of the chromatographic separation was carried out using an Agilent 1100 series (Waldbronn, Germany) system with: pump, auto sampler with 5 µL loop, column oven and UV-diode array detector (the set wave length was 280 nm). The column was kept at 20 °C for all the experiments with the exception of the study of the temperature influence were it was varied between 20 and 40 °C. Chemstation software version A.08.03 (Waters) was used for the chromatographic data handling. All the analysis were realized using bear silica column Silica Uptisphere Strategy 100A 5µm HIS, L x Φ = 250 x 2.0 mm, 5µm (Interchim, France).

All the chemicals and reagents were of analytical grade and were purchased as follows:

- A, DOPAC, DA, DOP0A, HVA, 5-HAIA, 3-MT, NA, S, TRP and TYR from Sigma-Aldrich (St-Quentin Fallavier, France);
- 3,4 dihydroxybenzylalanine (DHBA - internal standard), ammonium formate ($HCOONH_4$) and acetate (CH_3COONH_4), formic (HCOOH) and acetic acids (CH_3COOH) from Fluka (St.-Quentin-Fallavier, France);

- perchloric acid ($HClO_4$) from VWR Prolabo (Darmstadt, Germany);
- acetonitrile (ACN) and methanol (MeOH) from J.T. Baker (Noisy le Sec, France).

The analytes and mobile phase solution were prepared using purified water, produced by the Elgastat UHQ II system (Elga, Antony, France).

For each of the 12 compounds stock standard solutions of 1000 $\mu g \cdot mL^{-1}$ were prepared dissolving the appropriate weighted amounts in $HClO_4$ (0.2 $mol \cdot L^{-1}$), that were stored at -80 °C. The solutions injected in the chromatographic system were prepared by diluting the stock solutions mixtures of buffer and ACN in order to have an injection solvent as close as possible to the mobile phase. The injection solvent composition plays an important role in HILIC [21].

3. RESULTS AND DISCUSSION

The influence of different chromatographic parameters, such as: organic modifier nature and percentage, salt nature and concentration, column temperature and mobile phase pH, on separation of the target compounds was studied.

For this study we have selected dihydroxylbenzylamine (DHBA) as internal standard. Due to the high pH sensitivity of the catecholamines, the mobile phase pH was kept at 3. Under these conditions our analytes are bearing different charges as follows [15]:
- DA, NA, A, S, 3-MT and DHBA (biogenic amines) are protonated and thus, positively charged;
- DOPA, TYR and TRP (amino acids) have the carboxylic functions deprotonated and the amine functions protonated resulting in zwitterionic compounds that have the global charge zero;
- DOPAC, HVA and 5-HIAA (carboxylic acids) having the pKa values of 3.6, 3.9 and 4.2, respectively, are most probably partially dissociated and thus, partially negatively charged.

3.1. Influence of the organic modifier type and percentage content of in the mobile phase

As expected in the HILIC mode the increase of the organic modifier (ACN) percentage in the mobile phase lead to the augmentation of the retention factor (k) of most of the target compounds. For example, for TYR the retention factor doubled when the ACN percentage increased from 70 to 80 %, further increase to 90 % led to a 12 times increase of the k value. On the other hand, no significant modifications of the retention factor of the acidic compounds (DOPAC, HVA, 5-HIAA) (Figure 1).

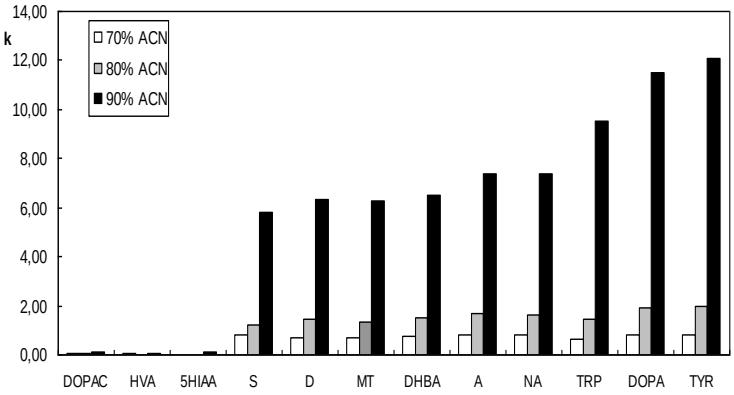


Fig. 1. Variations of the retention factors for analytes with the ACN percentage.

The replacement of ACN with MeOH lead to the decrease of the retention coefficients of all the compounds and thus to their insufficient separation (data not shown). Under these conditions for the following experiments ACN was used as organic modifier.

3.2. Influence of the salt nature and concentration on the retention factor

For the influence of the salt concentration on the retention of the analyses, $HCOOH_4$ concentration in the aqueous phase was varied between 20 and 150 mM. That led to an overall concentration of alt in the mobile

phase that was comprised between 3 and 22.5 mM. The retention increased only for the two amino acids, TYR and DOPA, for the rest of the compounds the salt concentration did not have a significant influence (Figure 2).

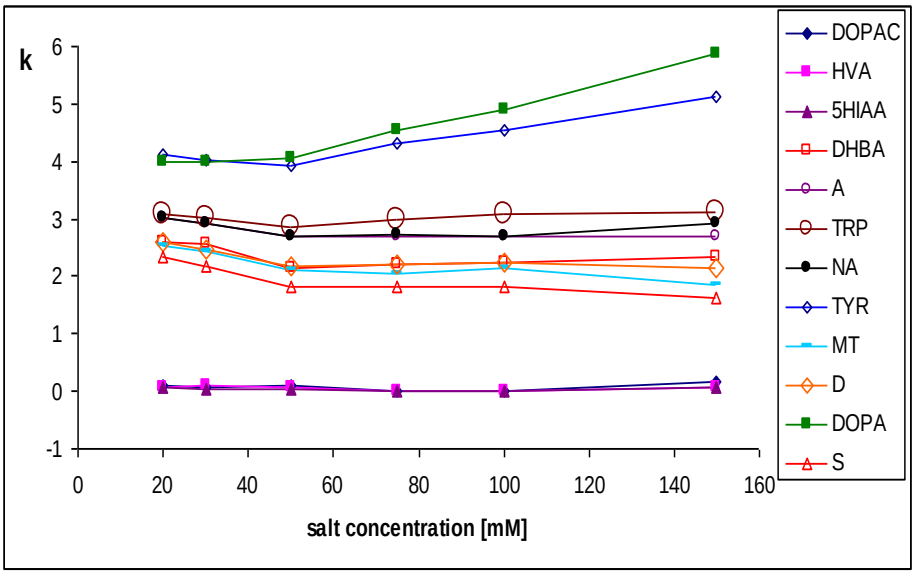


Fig. 2. Influence of the salt concentration on the retention factor.

In order to keep our mobile phase compatible with a MS detection the added salt needs to be volatile, under this conditions there are a limited number of salts that could be used to replace $HCOONH_4$ [15]. Thus, the replacement of ammonium formate by ammonium acetate in the mobile phase didn't lead to better separation of the target compounds.

3.3. The influence of pH on the retention factor

In HILIC electrostatic interactions between the stationary phase and the charged or chargeable analytes can play a role in their retention [15]. The increase of the mobile phase pH leads to the deprotonation of the hydroxyl groups of the silica stationary phase that could offer new interactions possibilities for the analytes. More over the global charge of some analytes can also change and thus the exploration of the mobile phase pH influence on the retention seems appropriate. However, the pH influence, in the tested range (3-7), on retention factor for neutral and basic compounds is insignificant (data not shown). That is probably due to the fact that in the studied pH domain the global charge of these compounds does not undergo important variation. On the other hand, for the acid compounds (DOPAC, HVA, 5-HIAA) the charge differences are more important. One would expect that the acids, bearing a negative charge, would be less retained due to electrostatic repulsions with the negatively charged silica stationary phase. To our surprise, the retention factor for acid compounds increased 5 to 10 times with the pH increase (Figure 3). Never the less, the overall separation didn`t undergo significant improvement and taking into account the fact that the higher the pH the less stable the compounds are, we have decided to keep pH 3 for the used mobile phase.

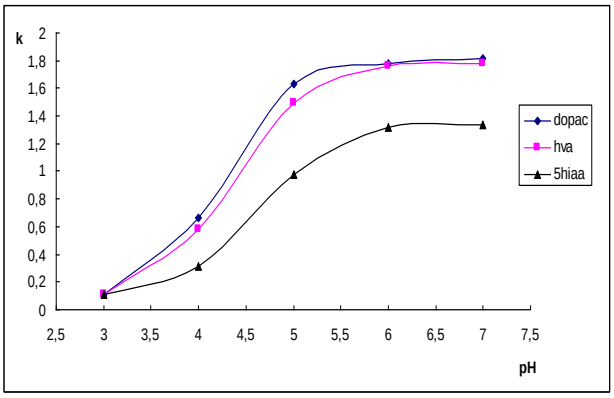


Fig. 3. pH influence on the retention factor for the acid compounds.

3.4. Influence of the temperature on retention factor

From the literature we could observe that the temperature effect was function of the analytes nature and of the interaction between the analytes and the stationary phase [22]. In this case, the variation of the column temperature between 20 and 40 °C doesn't have significant influence on the retention factors of the analytes (Figure 4). The temperature increase doesn`t lead to the separation improvement as retention decrease was observed for all solutes.

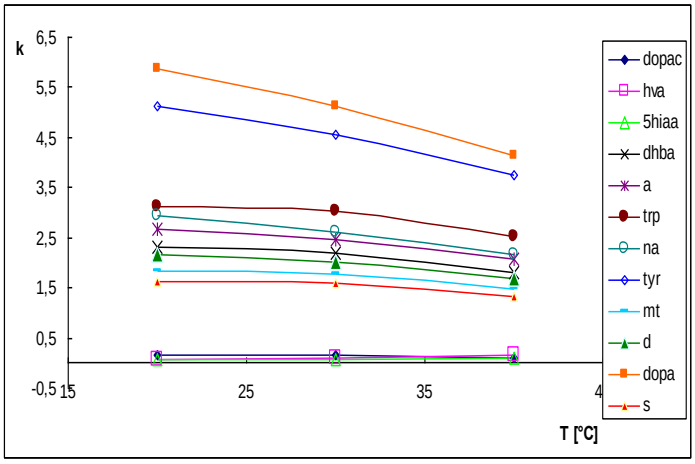


Fig. 4. pH influence on the retention factor.

4. CONCLUSIONS

The best analytes separation was obtained using a mobile phase composed of ACN and $HCOONH_4$ 85:15 v/v, the aqueous part of the mobile phase has the pH equal to 3 (buffered by the addition of formic acid) and the salt concentration of 150 mM. The column temperature is 20 °C. Under these optimized conditions good separation between the 12 analytes was obtained (Figure 5). The first eluded compounds are far from the void volume allowing their quantification in complex matrices. The analysis time is reasonable (less than 25 minutes) and the peak shapes are correct (all peaks are symmetrical).

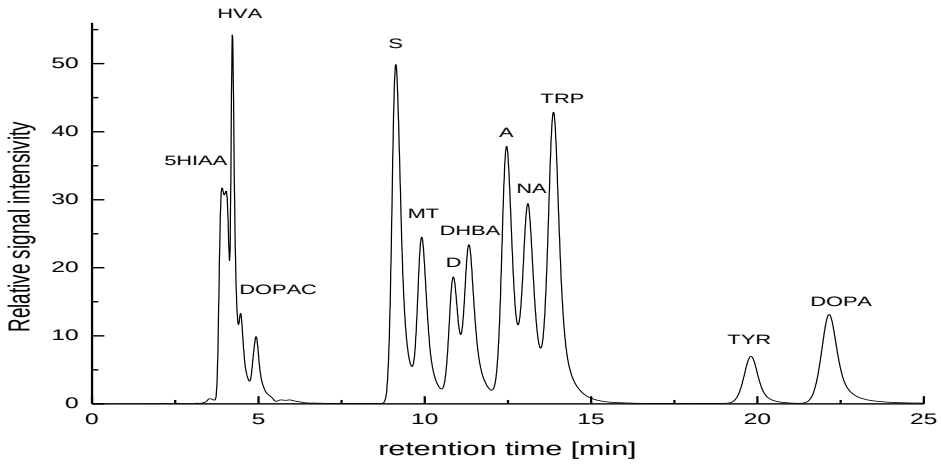


Fig. 5. Separation profile of 12 analytes under the optimized chromatographic conditions.

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