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ORIGINAL RESEARCH PAPER

OPTIMISATION OF A HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY METHOD FOR CATECHOLAMINES AND RELATED MOLECULES ANALYSIS

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Abstract: A simple and specific method for the analysis of 11 compounds (catecholamines, their precursors and their metabolites) has been developed using hydrophilic interaction chromatography. Adrenaline, noradrenalin, dopamine, serotonin, 3,4-dihydroxy-phenylalanine, 3-methoxytyramine, tryptophan, homovanillic acid, tyrosine, 3,4-dihydroxy-phenylacetic acid, 5-hydroxyindole-3-acetic acid and 3,4-dihydroxybenzylalanine (as internal standard) were separated on a TSK gel amide 80 column. The influence of parameters such as organic modifier type and content, salt nature and concentration, *p*H as well as column temperature on the selectivity were investigated. The optimized mobile phase consisted of a 20 mM ammonium acetate aqueous solution buffered at *p*H 3 and acetonitrile (20:80 v/v) mixture.

Keywords: *catecholamine, HILIC optimization, indolamine, neurotransmitters, TSK gel Amide 80*

INTRODUCTION

Catecholamines are known as neurotransmitters. Three of them occur in vivo: adrenaline (A) (epinephrine), dopamine (DA) and noradrenaline (NA) (norepinephrine) [1, 2], they are produced in the body from tyrosine (Tyr) and 3,4-dihydroxy-phenylalanine (DOPA). Their most important metabolites are: homovanillic acid (HVA), 3-methoxytyramine (3-MT) and 3,4-dihydroxy-phenylacetic acid (DOPAC). One of the most important body indolamine is serotonin. It is implicated in the body functioning as it influences sleep, mood, depression or anxiety. Tryptophan (Trp) and 5-hydroxyindole-3-acetic acid (5HIAA) are serotonin's precursor and main metabolite, respectively. Catecholamines and indolamines, along with their metabolites can play the role of markers in the diagnosis and/or treatment of numerous diseases, like: myocardial infarction, asthma, Parkinson's disease, pheochromocytoma or neuroblastoma [3, 4]. However, the detection of neurotransmitters remains an analytical and technical challenge due to their low concentrations in biological samples and the tendency of their catechol group to be easily oxidized.

Different high performance liquid chromatography systems have been used for catecholamine analysis, most often using electrochemical detection due to its high sensitivity and selectivity [5 – 12], but UV [13] and fluorescence [14] detection have also been reported. Lately mass spectrometric detection has been extensively used because of the relatively high sensitivity and complementary structural information [15 – 21]. Reversed phase chromatography (RPLC) using octadecyl [21 – 23] or porous graphitic carbon [15, 21, 24] columns was often used for the analysis of catecholamine with mobile phases whit high water content. Ion-pair reversed-phase liquid chromatography is one of the most popular techniques used for catecholamines analysis because of the polar and ionizable nature of these compounds. Satisfactory separations have been obtained with either non volatile [8 – 10, 25 – 28] or volatile [21, 29, 30] ion-pairing reagents. The literature also presents some capillary electrophoresis methods [31 – 33].

Lately, hydrophilic interaction liquid chromatography (HILIC) appears to be an interesting alternative to RP chromatography for applications involving polar compounds such as neurotransmitters [11, 12, 34]. In HILIC, polar stationary phases are typically associated with highly organic mobile phases containing between 2 and 40 % water. Under these chromatographic conditions, the analytes are eluted in an increasing polarity order, as in normal phase chromatography [35 – 38]. The mechanism of HILIC separation is rather complicated as hydrophilic interactions are seconded by electrostatic and hydrogen bond interactions. These interactions can vary substantially with the variation of additives, such as acids or salts, in the mobile phase.

The aim of our study was to develop a new separation method based on hydrophilic interaction chromatography to determine eleven catecholamines, precursors and metabolites (A, NA, DA, S, Tyr, Trp, DOPA, HVA, 3-MT, DOPAC, 5HIAA) (Figure 1). The optimized separation method should be compatible with mass spectrometric detection as it is supposed to be fatherly used for these compounds determination in different biological matrices. Thus the separation method needs to provide:

i) satisfactory retention by excluding these analytes from the void volume where ion suppression is maximal given that most of the matrix compounds are eluted in this area;
ii) maintaining selectivity among all the compounds of interest and other interfering solutes of the matrices when tandem MS detection is not specific enough.

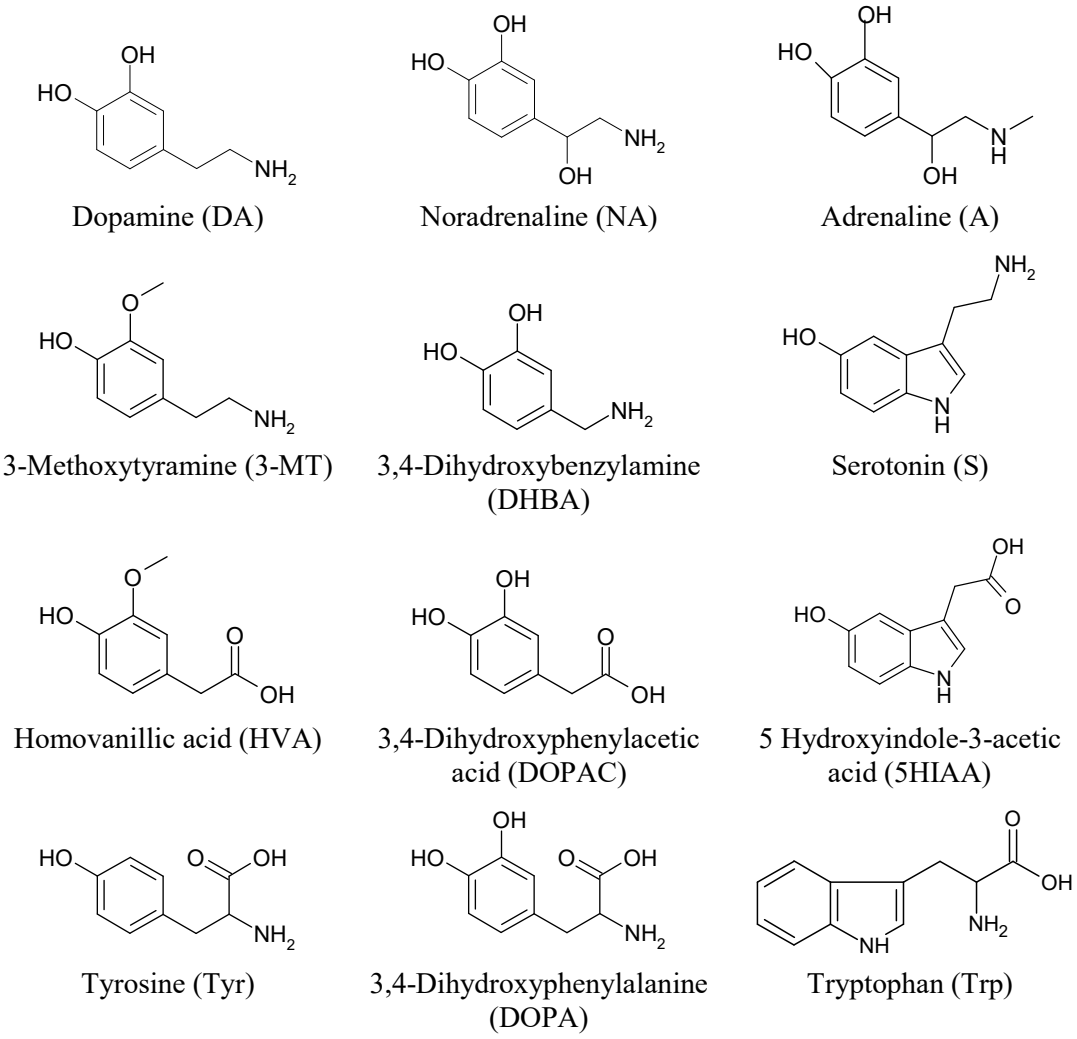

Figure 1. *Structures of the neurotransmitters and related compounds studied*

MATERIALS AND METHODS

Adrenaline, 3,4-dihydroxy-phenylalanine, dopamine, 3,4-dihydroxy-phenylacetic acid, homovanillic acid, 5-hydroxyindole-3-acetic acid, 3-methoxytyramine, noradrenalin, serotonin, tryptophan and tyrosine were obtained from Sigma-Aldrich (St-Quentin-Fallavier, France). The internal standard, 3,4 dihydroxybenzylalanine (DHBA), ammonium acetate and formate, acetic and formic acids were purchased from Fluka (St.-Quentin-Fallavier, France). The perchloric acid is produced by VWR Prolabo (Darmstadt, Germany). Acetonitrile (MeCN), acetone and methanol (MeOH) were HPLC-grade and were purchased from J.T. Baker (Noisy le Sec, France).

The purified water used for preparation of analytes and mobile phase solution, was obtained using an Elgastat UHQ II system (Elga, Antony, France).
The chromatographic system used to optimize the chromatographic separation consisted of an Agilent 1100 series (Waldbronn, Germany) system with: pump, auto sampler with 5 µL loop, column oven and DAD detector. The UV detection was carried out at 280 nm. The chromatographic data handling was accomplished using Chemstation software version A.08.03 (Waters). The separation was achieved using an amide bounded silica column, TSK gel Amide 80 (250 x 2 mm i.d.) (Tosoh Bioscience, USA).
Stock standard solutions were prepared at a concentration of 1000 $\mu g \cdot mL^{-1}$ and obtained by dissolving the adequate weighted amount of each compound in 0.2 $mol \cdot L^{-1}$ perchloric acid. All stock solutions were stored at -80 °C.
For optimization of the chromatographic method, the studied solutions were obtained by diluting the corresponding stock standard solutions in buffer/organic modifier mixture in order to have an injection solvent as close as possible to the mobile phase and a final analyte concentration of 5 or 10 $mg \cdot L^{-1}$.

RESULTS AND DISCUSSION

We divided the selected compounds (precursors, metabolites and catecholamines) into three groups:

i) biogenic amines: A, DA, NA, 3-MT, S;
ii) amino acids: Tyr, DOPA, Trp;
iii) carboxylic acids HVA, DOPAC, 5HIAA.

All solutes are highly polar and ionized depending on the mobile phase *p*H value. One of the difficulties of this work comes from the heterogeneity in the ionization state of the test compounds in a given sample. It is only under highly basic *p*H conditions that all these compounds are negatively charged. However, in this *p*H range, the catecholamines are unstable in solution due to their rapid oxidization. To prevent degradation of analyte, this work was carried out under acidic *p*H conditions (*p*H 3). Consequently, the six biogenic amines (DA, NA, A, S, 3-MT) and the selected internal standard (DHBA) are protonated, thus bearing a net positive charge; HVA, DOPAC and 5HIAA containing carboxylic functions, with respective *p*Ka of 3.9, 3.6 and 4.2, are only partially dissociated, thus bear a partial negative charge. For the three amino acids (Tyr, Trp and DOPA), the amine functions are protonated whereas the carboxylic functions, with respective *p*Ka of 2.0, 2.5 and 1.6, are essentially deprotonated resulting in the presence of zwitterionic compounds, with a global net charge equal to zero [37]. However, in the HILIC mobile phase, a large proportion of the composition is an organic solvent and it is a well-known fact that *p*H and *p*Ka values are affected by the presence of organic solvents. As a result, the ionization state of the above solutes might be somewhat different from what we expect, based on purely aqueous solutions.

Evaluation of stationary phase

Various hydrophilic columns can be employed in the HILIC mode, the most popular phases being underivatized silica stationary phases and polar derivatized silica (cyano, amide, aminopropyl, diol or zwitterionic phases) [39]. For aromatic carboxylic acids,

the amino-silica column can generate high retention and only low efficiencies [36]. These specific retention effects have been explained by ion-exchange interactions between analytes possessing acidic functionalities and the amino stationary phase that reinforces hydrophilic partition interaction. HILIC silica column, in contrast, can be negatively charged under the mobile phase conditions due to silanol deprotonation making it possible for the positively charged solutes (biogenic amines) to interact with the silica column through ion exchange resulting in stronger retention. At the same time, however, very weak retention was observed for negatively charged solutes due to repulsive electrostatic interactions with negatively charged silanols on the silica surface. Although commonly referred to as a polar phase, the absence of hydrogen bond donor capabilities causes a low HILIC retention on cyanopropyl silica [40, 41]. The TSK gel Amide 80 support consists of highly polar, yet intrinsically noncharged, functional groups (carbamoyl). The amide group is less reactive than the amine and lacks its basicity. Retention on this column type is thus less sensitive to eluent *p*H [39] and higher than that obtained on cyano silica due to possible hydrogen bonding interaction. Different HILIC applications on such a support have been reported: peptides [38], amino acids [42], oligosaccharides [43], and other polar compounds from natural products [44]. For all these reasons, the TSK gel Amide 80 was therefore employed for further optimization studies in order to separate catecholamines, precursors and metabolites under acidic conditions (*p*H 3).

The effect of organic modifier content and nature

In HILIC mode, the mobile phase usually contains between 2 % and 40 % water, for the elution of compounds and it is necessary to include a salt in mobile phase to achieve acceptable reproducibility for chromatographic separations of charged species. First, the effect of organic modifier content (60 - 90 %) in the mobile phase was investigated. As expected in HILIC mode, catecholamine retention time increases with increasing organic modifier percentage in the mobile phase. A good compromise for retention was obtained using an eluent containing at least 80 % of organic modifier.

The eluting strength of three different organic modifiers (acetone, acetonitrile and methanol) in combination with 20 % of 20 mM ammonium acetate solution buffered at *p*H 3, was then compared and the results are reported in Figure 2. As expected methanol (Figure 2a) is the strongest elution solvent and consequently no sufficient retention was observed under these chromatographic conditions. On the other hand, acetonitrile (Figure 2c) was the weakest elution solvent, thus offering the best selectivity. Only one co-elution between A and DHBA (internal standard) was noticed when this solvent is used. Acetone (Figure 2b) has intermediate elution strength between MeOH and MeCN, but generated insufficient selectivity. These results are in good agreement with those presented by Li and Huang [45] concerning the HILIC separation of an antibiotic and its impurities for which MeCN appears less eluting than THF, isopropanol and methanol.

In order to separate A and DHBA, a ternary mobile phase was tested (Figure 2d). Half of the water content in mobile phase was replaced by MeOH to decrease the mobile phase elution strength. However, no improvement in selectivity was observed with this mobile phase (MeCN/MeOH/40 mM CH_3COONH_4 *p*H 3, (80:10:10 v/v/v)) as DHBA and A are still not separated. Moreover, the total analysis time was doubled and the

peak shapes were deteriorated (Figure 2d). Finally, no matter what organic modifier or combination of organic modifiers was used the elution order was not affected.

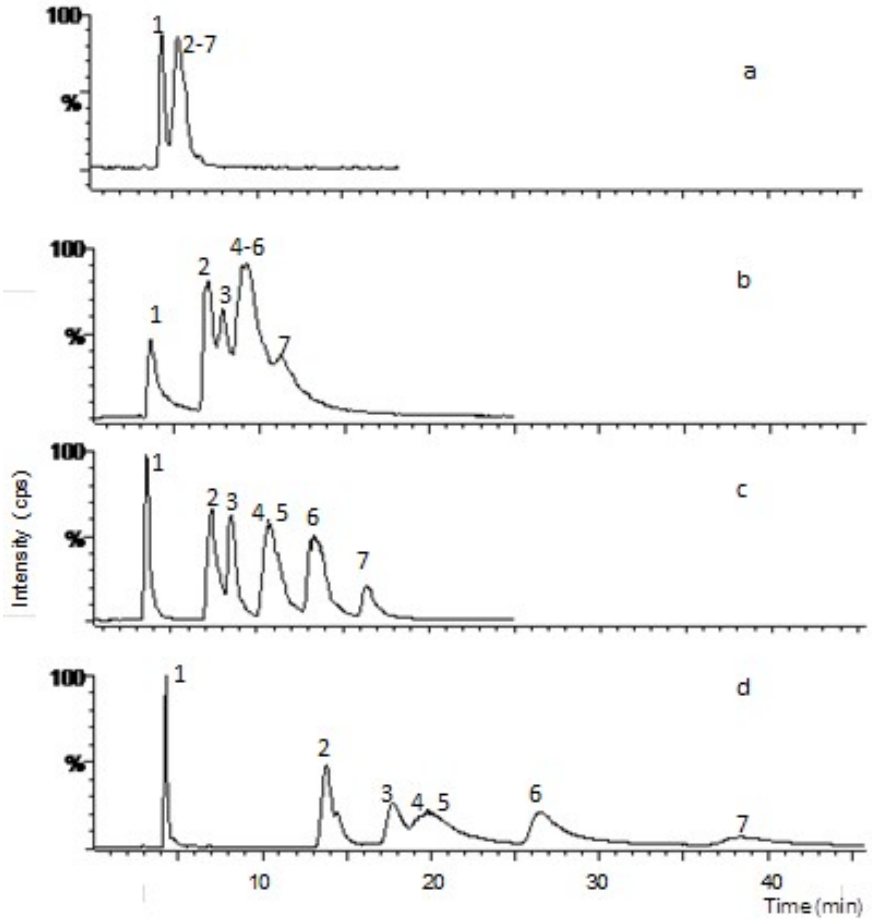

Figure 2. *Effect of organic modifier nature on separation*

Column: TSK gel amide 80 (250 x 2 mm I.D.). Column temperature: 20 °C.
Flow-rate: 0.2 mL·min^{-1}. Detection UV at 280 nm
Mobile phase:

a. Methanol / ammonium acetate (20 mM, *p*H 3) (80:20, v/v).
b. Acetone / CH_3COONH_4 (20 mM, *p*H 3) (80:20, v/v).
c. MeCN / CH_3COONH_4 (20 mM, *p*H 3) (80:20, v/v).
d. MeCN / MeOH / CH_3COONH_4 (40 mM, *p*H 3) (80:10:10, v/v/v).

Peaks: (1) 5HIAA, (2) S, (3) Trp, (4) A, (5) DHBA, (6) NA, (7) DOPA

The effect of salt nature and concentration

When ionic compounds are studied, the presence of salt in the mobile phase is necessary to ensure analyte elution and good peak symmetry. Choice of the type of salt is limited to the volatile salts that can be further used when coupling the chromatographic system to the mass spectrometric detection. That is why generally only ammonium acetate and ammonium formate are tested.

For a given salt concentration, when ammonium acetate is replaced by ammonium formate same analysis duration was obtained and elution order was similar, meaning that the two salts have the same elution strength for catecholamines (data not shown). Subsequent analyses were carried out using ammonium acetate.

Typical HILIC behavior involves an increase in retention when the salt concentration in the mobile phase is increased [46], although a decrease has been reported [36] if electrostatic interactions (as secondary retention mechanism) occur between the analytes and the stationary phase. In our case, the effect of the salt concentration depends on the nature of the compound. The retention of HVA, DOPAC, 5HIAA, Trp, Tyr and DOPA is not affected by changes in the salt concentration (Figure 3). On the other hand, the retention of the amino compounds (especially: DA, NA, A and DHBA) tends to decrease with the increase in salt concentration in the mobile phase. The existence of the two types of variations leads to different selectivity at the different salt concentrations. For example, an inversion of the elution order for the two pairs: NA, Tyr and DA, Trp, was observed when the concentration increases from 15 to 40 mM (NA, Tyr) or 15 to 50 mM (DA, Trp) (Figure 3).

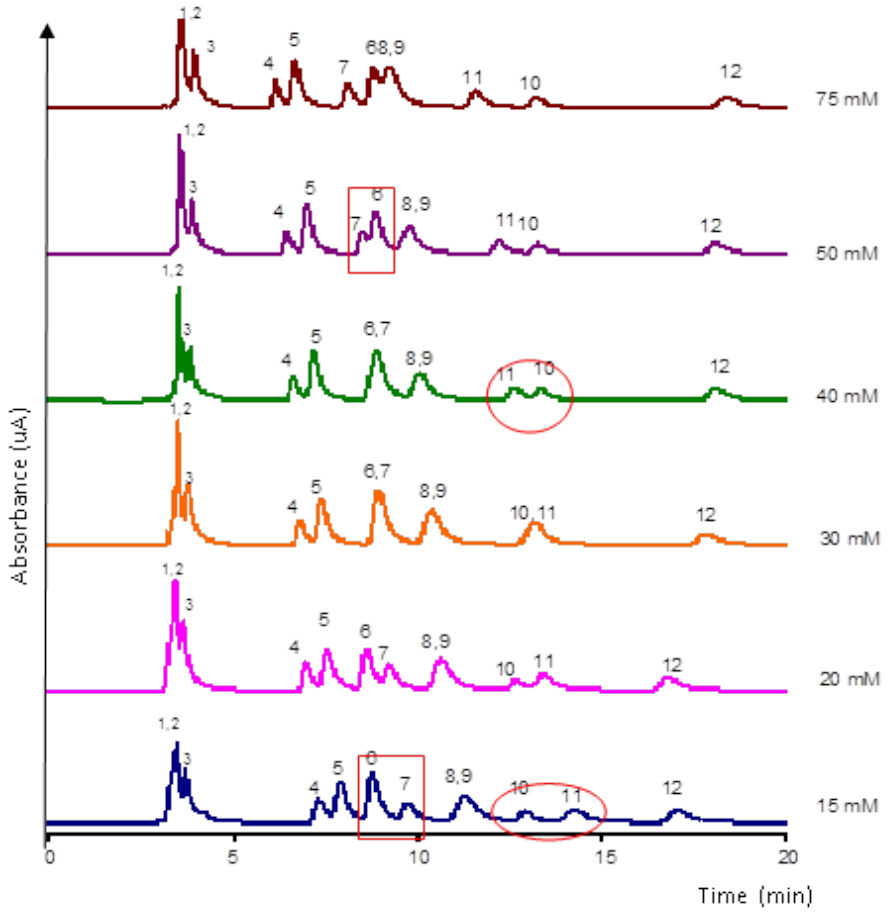

Figure 3. *Effect of salt concentration on selectivity*
Column: TSK gel amide 80 (250 x 2 mm I.D.). Column temperature: 20 °C.
Flow-rate: 0.2 mL·min^{-1}. Detection UV at 280 nm
Mobile phase: MeCN / CH_3COONH_4 (20mM, *p*H 3) (80:20, v/v).
Peaks: (1) HVA; (2) 5HIAA; (3) DOPAC; (4) 3-MT; (5) S; (6) Trp; (7) DA; (8) A; (9) DHBA; (10) Tyr; (11) NA; (12) DOPA

Our study confirms the presence of electrostatic interactions between the positively charged analytes and the TSK gel Amide column as previously reported by Alpert [35] for the analysis of basic amino acids on this column and a mobile phase composed of MeCN and triethylamine phosphate (TEAP) *p*H 4. These possible electrostatic

interactions imply the presence of a negative charge on the support surface, caused either by deprotonated residual silanols or by electron delocalization on the bounded amide group involving high electron density zones. Both hypotheses are plausible. First Quiming *et al.* [46] attributed to deprotonated silanols the electrostatic repulsions between their analytes (methyl uric acids) on their "neutral" diol bound silica column. Second, the comparison by Guo and Gaiki [36] of the TSK gel Amide column with ZIC HILIC and bare silica for the analysis of salicylic acid and its homologues, revealed, for the TSK column, a behavior closer to that of the bounded column, meaning that most likely the TSK gel column negative charge is generated by its bounded group rather than by its silica backbone.
For catecholamine analysis on TSK gel column, the presence of salt in mobile phase is necessary but better separations were obtained with lower salt concentrations (15 - 20 mM) than higher (30 - 75 mM) as reported in Figure 3. For further studies, a mobile phase containing 20 mM of ammonium acetate was retained rather than 15 mM due to higher peak efficiency.

Temperature effect

The retention of six solutes (A, NA, 3-MT, DOPAC, 5HIAA, Trp) was not modified when the column temperature was increased from 10 °C to 50 °C whereas for the other six (Tyr, DOPA, DA, DHBA, HVA and S) slight retention variations were observed (data not shown). For DA, DHBA, HVA and S, retention increased with temperature, whereas the opposite effect was registered for Tyr and DOPA. Finally, as no significant separation improvement was observed with an increase in temperature, for further investigations the column was thermostated at 20 °C.
After optimization, a mobile phase composed of MeCN/CH_3COONH_4 20 mM *p*H 3 (80:20, v/v) and a column temperature of 20 °C was found to provide the best results in selectivity and in total analysis time (Figure 3). Under these conditions, the separation was performed in less than 20 minutes and only two co-elutions are registered: peaks 1 and 2 (HVA, 5HIAA) and peaks 8 and 9 (A, DHBA (internal standard)). This new method developed on a TSK gel amide column, unlike that proposed by Gu *et al.* [40] on a cyano column, offers satisfactory retention by excluding these analytes from the void volume and has the advantage of achieving, under HILIC conditions, a baseline resolution between first A, NA, DA and second Tyr and DOPA. Moreover, HVA and DOPAC can be retained and analyzed simultaneously with the other solutes under HILIC conditions on amide column whereas this was not possible on cyano column. On the latter support type, they are retained only under RPLC conditions [40].

CONCLUSIONS

In this paper we have developed a HILIC method for the analysis of eleven catecholamines, indolamines and their precursors and metabolites. The most important parameters that influence the chromatographic separation have been investigated. The optimum chromatographic separation is achieved in the HILIC mode on a TSK gel Amide 80 column using a mobile phase composed of 80 % of MeCN and 20 % of CH_3COONH_4 20 mM at *p*H 3. This type of mobile phase is compatible with mass

spectrometric detection which enables us to fatherly use the optimized method for brain extract sample analyses.

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