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Evaluation of fused-core and monolithic versus porous silica-based C18 columns and porous graphitic carbon for ion-pairing liquid chromatography analysis of catecholamines and related compounds

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ABSTRACT

This paper evaluates the performances of reversed-phase (RPLC) and ion-pairing chromatography (IPLC) coupled with UV detection for the analysis of a set of 12 catecholamines and related compounds. Different chromatographic columns (porous C18-silica, perfluorinated C18-silica, porous graphitic carbon, monolithic and fused-core silica-based C18 columns) were tested using semi-long perfluorinated carboxylic acids as volatile ion-pairing reagents. Much more promising results were obtained by IPLC than by RPLC and important improvements in analytes peak symmetry and separation resolution were observed when using the "fast chromatography" columns (monolithic and fused-core C18) under IPLC conditions. For UV detection, a satisfactory separation of the 12 selected analytes was achieved in less than 20 min by using a fused-core particles column (Halo C18) and a mobile phase composed of a 1.25 mM nonafluoropentanoic acid aqueous solution and methanol under gradient elution mode. The chromatographic method developed can be directly coupled with electrospray ionization tandem mass spectrometry (ESI–MS/MS) in positive ionization mode and 10 solutes among those selected can be observed. The presence of the acidic ion-pairing reagent in the mobile phase makes this system incompatible with negative ionization mode and thus unable to detect the two acidic compounds that only responded in negative mode. In terms of MS detection, Monolithic C18 column proved to be the best one to reach the lowest detection limits (LODs) (from 0.5 ng mL^{-1} to 10 ng mL^{-1} depending on the neurotransmitter). The applicability of the optimized LC–MS/MS method to a "real world" sample was finally evaluated. The presence of the matrix leads to signal suppression for several solutes and thus to higher LODs.

1. Introduction

Catecholamines and indolamines play a significant role in the nervous system as central and peripheral neurotransmitters. The concentration level of these compounds in different biological fluids or tissues can offer important information about the state of health of the person. Among the biological amines there are three catecholamines known to occur in vivo: adrenaline (A) (epinephrine), noradrenaline (NA) (norepinephrine) and dopamine (DA) [1]. Their physiological precursors are tyrosine (Tyr) and 3,4-dihydroxy-phenylalanine (DOPA), while homovanillic acid (HVA), 3-methoxytyramine (3-MT) and 3,4-dihydroxy-phenylacetic acid (DOPAC) are some of their metabolites present in the organism. Serotonin (S) is an indolamine that is present in many tissues (blood platelets, lining of the digestive tract, brain). It is produced in the body from tryptophan (Trp) and metabolized into 5-hydroxyindole-3-acetic acid (5HIAA). These compounds are markers for the diagnosis and treatment of different diseases like: asthma, myocardial infarction, Parkinson's disease [2], pheochromocytoma [3] or neuroblastoma [4].

Concerning separation techniques for neurotransmitter analysis, liquid chromatography (LC) is widely used but capillary electrophoresis has been also reported [5–7]. Different LC systems have been described using UV [8] or fluorescence [9] detection however today, the most widespread technique for the investigation of these molecules in biological samples is ion-pairing chromatography (IPLC) associated with an electrochemical detection (ECD) [10–14]. Lately the mass spectrometry (MS) detection has been extensively used as this mode of detection has the advantage of providing additional structural information about the eluted compounds [15–22]. ECD is still the most sensitive detection mode with detection limits that can reach 0.01 ng L^{-1} [23] however, its specificity is limited to distinguishing only a family of compounds from other ones in relation to differences into their reduction or oxidation potential values and this is not sufficient when a new metabolite is formed. Thus, in view of a structural identification for

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any new metabolite detected in biological samples, the complementary information obtained by MS detection is very important. This explains the interest for a chromatographic method that would be compatible with both mass spectrometry and electrochemical detection.

Reversed-phase liquid chromatography (RPLC) either on octadecyl [24] or porous graphitic carbon (PGC) [20,25] columns and highly aqueous mobile phases using formic or acetic acid as additive have been used for catecholamine analysis. These polar compounds are often derivatized in order to obtain less polar compounds more easily retained on non-polar supports [26–29]. In order to effectively increase the retention of ionizable polar compounds, an alternative approach to RPLC is IPLC [30]. Regarding catecholamine separation, IPLC system using sodium octyl/dodecyl sulfonate or related compounds as ion-pairing agent is probably the most popular method [13,31–34] associated with ECD. Unfortunately, these ion-pairing reagents are not volatiles and then not compatibles with an MS detection. In order to overcome this inconvenient, volatile ion-pairing agents such as perfluorinated carboxylic acids [9,35–37] have been tested showing encouraging results. The use of hydrophilic interaction liquid chromatography (HILIC) has also been reported [38,39].

The aim of our project is to study the feasibility of replacing the actual IPLC–ECD method by one compatible with both ECD and MS detection for the analysis of catecholamines and related compounds either presenting a biological interest or can be physiologically or therapeutically present in biological samples. This includes a preliminary study of the performances of different RPLC and IPLC systems satisfying two major criteria: (i) volatile mobile phase with a minimum of 5% organic modifier for more favorable electrospray MS ionization conditions and (ii) baseline resolution between the target solutes to be compatible with an electrochemical detection. This preliminary study will be further followed by the coupling of the optimized chromatographic systems with MS detection and electrochemical detector, in order to offer a versatile chromatographic system. Comparison of different analytical instruments or chromatographic supports is very useful as it help scientists to choose among them, in relation to their analytical requirements (high sensitivity or more specificity).

In this report, we present the separation of a set of 12 catecholamines and related compounds (A, NA DA, Tyr, DOPA, HVA, 3-MT, DOPAC, S, Trp, 5HIAA and DHBA) on different chromatographic columns: conventional C18-silica, perfluorinated (PFP)-silica, porous graphitic carbon (PGC), monolithic C18-silica and fused-core C18 columns. Then, the most outstanding chromatographic systems were coupled to MS/MS detection and compared in terms of detection limits. The applicability of LC–MS/MS method to "real world" samples was finally tested.

2. Experimental

2.1. Chemicals and reagents

HPLC-grade acetonitrile (MeCN) and methanol (MeOH) were purchased from J.T. Baker (Noisy-le-Sec, France) and perchloric acid from VWR Prolabo (Darmstadt, Germany). Ammonium acetate and ammonium formate, acetic acid, formic acid and trifluoroacetic acid (TFA) were purchased from Fluka (St.-Quentin-Fallavier, France). Heptafluorobutyric acid (HFBA), nonafluoropentanoic acid (NFPA) and pentadecafluorooctanoic (PDFOA) were purchased from Sigma–Aldrich (Saint-Quentin-Fallavier, France). Adrenaline (A), noradrenaline (NA), dopamine (DA), tyrosine (Tyr), 3,4-dihydroxy-phenylalanine (DOPA), homovanillic acid (HVA), 3-methoxytyramine (3-MT), 3,4-dihydroxyphenylacetic acid (DOPAC), serotonin (S), tryptophan (Trp), 5-hydroxyindole-3-acetic acid (5HIAA) were purchased from Sigma–Aldrich (Saint-Quentin-Fallavier, France). 3,4-dihydroxybenzylamine (DHBA) was purchased from Fluka (Saint-Quentin-Fallavier, France)

Deionised (18 MΩ) water, purified using an Elgastat UHQ II system (Elga, Antony, France) was used for preparation of analyte and mobile phase solution.

Marvin 4.1.11 software (ChemAxon, Budapest, Hungary) was used to calculate the analyte pK_a and log D values

2.2. Standards and solutions

Stock standard solutions of each catecholamine, indolamine, and metabolite prepared at a concentration of 1000 μg mL^{-1} were obtained by dissolving the adequate weighed amount of each compound with 0.2 M perchloric acid. The use of perchloric acid is dictated by the fact that the neurotransmitter analysis was inscribed in a larger study aiming at analyzing these molecules in brain extracts, which are prepared in perchloric acid. Thus it was found important to maintain identical conditions in the whole method development process. All stock solutions were stored at −80 °C. The injected solutions were obtained by diluting the corresponding stock standard solutions in the mobile phase in order to obtain a final analyte concentration about 5–10 μg mL^{-1}.

For the brain extract preparation, the sheep encephalon was dissected out of the skull and was separated in different regions that were weighed and then immersed in cold 0.2 mol L^{-1} perchloric acid at the ratio of 5 mL g^{-1} tissue. The brain tissue was homogenized by sonication or using a Potter apparatus. The tissue homogenate was centrifuged at 20,000 × g for 1 h at 4 °C. The supernatant was utilized as the brain extract and stored at −80 °C. Just before analysis, the brain extract was filtered through a 0.45 μm syringe filter (Millipore) and an aliquot (500 μL) of the filtrate was mixed in 500 μL of an aqueous solution of NFPA 1.25 mM. 20 μL of the so prepared sample were injected in the HPLC system.

2.3. Instrumentation

The chromatographic systems consisted of a Merck-Hitachi quaternary pump model Lachrom L-7100 (Darmstadt, Germany), a Rheodyne (Cotati, CA, USA) model 7725 injection valve fitted with a 20 μL loop, column oven Jet Stream 2 Plus and a 785A UV-visible HPLC Detector (Applied Biosystems, Courtaboeuf, France). The UV detection was carried out at 280 nm in order to obtain maximal absorbance for all the compounds. Physicochemical parameters of the different columns studied are reported in Table 1.

MS detection was realized with Perkin-Elmer Sciex (Forster City, CA, USA) API 300 or API 3000 mass spectrometers with triple-quadrupole and Turbo Ionspray as ion source. The mass spectrometers were operated in positive ionization mode. The optimized MS parameters were the following: ion spray voltage 5800 V, nebulizer gas was compressed air at a flow rate of 1.2 L min^{-1}, curtain gas was nitrogen at a flow rate of 0.9 L min^{-1}, source temperature 300 °C and focusing potential (FP) 100 V. The values for the declustering potential (DP), the entrance potential (EP) and the collision energy (CE) are different for each selected transition and they are presented in Table 2. For the LC–MS/MS coupling, a split was necessary at the mass spectrometer entry, this split was of 1/3 for the columns operating at a 1 mL min^{-1} flow rate (PGC, porous silica-based C18 and monolithic C18) and 1/5 for the fused-core column that had a 1.5 mL min^{-1} operating flow rate.

The chromatographic data handling was accomplished using EZChrom Server software (Merck, Darmstadt, Germany) for the UV detection and Analyst (Applied Biosystem MDS Sciex) for the MS detection.

Table 1
Physicochemical parameters of the columns used for this study.

Stationary phase nature	Trade name	Manufacturer	L × Ø (mm)	Specific surface area ($m^2 g^{-1}$)	Carbon load (%)	Particle size (μm)	Endcapping
Octadecyl-bonded silica	Discovery HS C18	Sigma Aldrich	150 × 2.1	320	20	3	Yes
Octadecyl-bonded silica	Supelcosil ABZ + Plus	Sigma Aldrich	150 × 4.6	170	12	5	Yes
Pentafluorophenyl-propyl-bonded silica	Pursuit PFP	Varian	150 × 2.1	200	6.3	5	Yes
Octadecyl-bonded silica	Onyx	Phenomenex	100 × 4	300	18	Monolithic	Yes
Octadecyl-bonded silica	Halo C18	Advanced Materials Technology, Inc	50 × 4.6	150	–	1.7 (fused-core)	Yes
Porous graphitic carbon	Hypercarb	Thermo	100 × 4.6	120	100	5	–

3. Results and discussion

The 12 solutes selected for our study were divided into three groups: six biogenic amines (DA, NA, A, 3-MT, S and DHBA (as internal standard)), three amino acids (Tyr, DOPA, Trp) and three carboxylic acids (HVA, DOPAC, 5HIAA). Fig. 1 presents their chemical structures and their associated pK_a and log D (pH 3) values. To prevent degradation of analytes, the chromatographic separation was carried out under acidic conditions (pH ≤ 3) [40]. In a purely aqueous buffer at pH 3, the six biogenic amines are protonated, thus bearing a net positive charge. HVA, DOPAC and 5HIAA, containing carboxylic functions, with respective pK_a values of 3.9, 3.6 and 4.2, are only partially dissociated, thus bearing a partial negative charge. For the three amino acids, the amine functions are protonated whereas the carboxylic functions, with respective pK_a of 2.0, 1.6 and 2.5, are essentially deprotonated resulting in the presence of zwitterionic compounds, with a nominal net charge equal to zero.

3.1. Reversed-phase chromatography on conventional porous RP packing material

Three commercially available analytical columns were first selected for our study: (i) one among the new generation of porous reversed phase material based on high purity silica gel (Discovery HS C18) and recommended by its supplier for LC/MS applications without detectable bleed; (ii) one perfluorinated phase (Pursuit PFP) for alternative selectivity to traditional alkyl phases towards polar analytes [41–43] and (iii) a porous graphitic carbon (PGC) for its high ability to retain and separate polar and hydrophilic analytes [44–47]. Table 1 reported the physicochemical parameters of these columns.

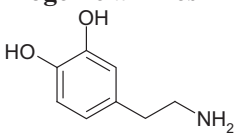
On the octadecyl bonded silica column we tested (Discovery HS C18) no satisfactory set of conditions for appropriate retention and separation of all the selected compounds could be found using a mixture of organic solvent and ammonium formate or acetate buffer pH 3 as volatile mobile phase. The insufficient retention for Tyr, DA, DOPA, NA, A et DHBA with mobile phases containing more than 5% organic solvent (MeOH or MeCN) lead us to give up the reversed-phase chromatography on C18 columns. The perfluorinated phases are an alternative for the C18 phases indeed, in addition to the dispersive interactions available on alkyl phases, the pentafluorophenyl (PFP) phases allow also dipole–dipole, π–π, charge transfer and ion-exchange interactions [41–43]. Better results in terms of catecholamine separation were obtained using this support type associated with a mobile phase composed of 10% MeOH and 90% of a 10 mM ammonium acetate solution pH 3 (Fig. 2a). Nevertheless this separation is not sufficient for an accurate electrochemical detection, considering the coelution of A, DHBA and DOPA.

The analysis on PGC of some of the compounds we are interested in, was previously reported under reversed-phase conditions [25,48,49]. Using similar conditions to those presented by Törnkvist et al. [25] (a mobile phase composed of 60% MeOH and 40% of a 5 mM aqueous solution of ammonium formate, pH 3), higher retentions were observed on PGC than on the other supports however, some coelutions are registered, like those of NA and A or DA and DHBA. Moreover a too high retention was observed for 5HIAA (retention time superior to 1 h). This fact is probably due to its partial negative charge in addition to its rather flat structure (indole ring) as previously described for this type of compounds on PGC [50,51]. When MeOH was replaced by the same amount (60%) of MeCN in mobile phase, an important decreasing in retention was observed for all the analytes resulting in a coelution of seven compounds (NA, A, DHBA, DA, Tyr, DOPA and 3-MT) near the void volume and a satisfactory retention for the other solutes except 5HIAA. Fig. 2b shows the analysis of the selected solutes by RPLC–UV on PGC column under gradient elution mode. The elution gradient is based on simultaneously increasing the percentage of MeCN and decreasing the salt concentration. A satisfactory separation of the first eluted analytes (NA, A, DHBA, DA, Tyr, DOPA 3-MT) was observed whereas S and Trp were poorly separated, DOPAC gave bad peak shape and width and, 5HIAA was too much retained. To sum up, it was not possible to achieve a reversed-phase chromatographic method for neurotransmitter analysis that would respond to our criteria thus, the addition of different ion-pairing reagents in the aqueous mobile phase was investigated once more for compatibility with MS requirements such as volatility.

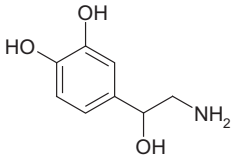
Table 2
Optimized values of the source and collision cell parameters for the catecholamine analysis on Sciex API 300 mass spectrometer.

Analyte	$[M+H]^+$ m/z	Selected transition	CE (eV)	EP (V)	DP (V)
DHBA	140	140 < 123	12	10	20
DA	154	154 < 137	13	10	15
3-MT	168	168 < 151	12	2	10
		168 < 91	29		
NA	170	170 < 152	13	2	10
		170 < 135	25		
S	177	177 < 160	14	10	20
		160 < 115	30		
TYR	182	182 < 165	14	10	45
		182 < 136	18		
		182 < 123	22		
A	184	184 < 166	14	2	10
5HIAA	192	192 < 146	20	10	40
DOPA	198	198 < 181	13	10	50
		198 < 152	17		
		198 < 139	20		
TRP	205	205 < 188	14	10	10
		205 < 146	23		

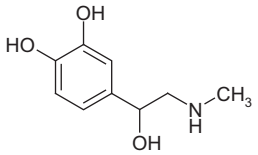
Biogenic amines



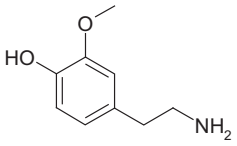
Dopamine (DA)
*log $D_{pH\ 3}$ -2.8
*$pK_{a\ NH2}$ 8.8
M = 153



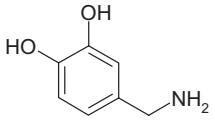
Noradrenaline (NA)
log $D_{pH\ 3}$ -4.0
$pK_{a\ NH2}$ 8.5
M = 169



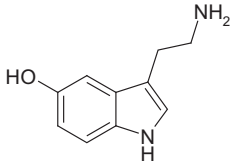
Adrenaline (A)
log $D_{pH\ 3}$ -3.5
$pK_{a\ NH2}$ 8.4
M = 183



3-Methoxytyramine (3-MT)
log $D_{pH\ 3}$ -2.5
$pK_{a\ NH2}$ 9.6
M = 167

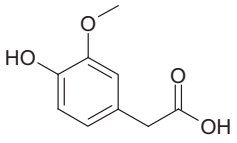


3,4-dihydroxybenzylamine (DHBA)
log $D_{pH\ 3}$ -3.3
$pK_{a\ NH2}$ 8.4
M = 139

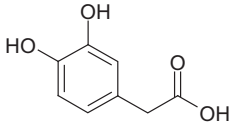


Serotonin (S)
log $D_{pH\ 3}$ -2.6
$pK_{a\ NH2}$ 10.0
M = 176

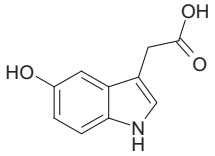
Acidic metabolites



Homovanillic acid (HVA)
log $D_{pH\ 3}$ 0.8
$pK_{a\ COOH}$ 3.9
M = 181

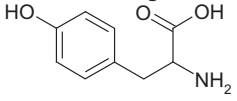


3,4-dihydroxyphenylacetic acid (DOPAC)
log $D_{pH\ 3}$ 0.5
$pK_{a\ COOH}$ 3.6
M = 167

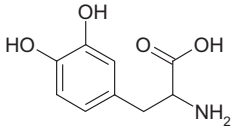


5 Hydroxyindole-3-acetic acid (5HIAA)
log $D_{pH\ 3}$ 0.6
$pK_{a\ COOH}$ 4.2
M = 191

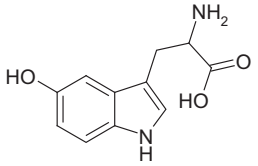
Amino acid precursors



Tyrosine (Tyr)
log $D_{pH\ 3}$ -2.0
$pK_{a\ COOH}$ 2.0
$pK_{a\ NH2}$ 9.1
M = 181



3,4-dihydroxyphenylalanine (DOPA)
log $D_{pH\ 3}$ -2.5
$pK_{a\ COOH}$ 1.6
$pK_{a\ NH2}$ 9.5
M = 197



Tryptophan (Trp)
log $D_{pH\ 3}$ -2.4
$pK_{a\ COOH}$ 2.5
$pK_{a\ NH2}$ 9.4
M = 204

Fig. 1. Structures of the studied neurotransmitters. *Marvin 4.1.11 software was used to calculate the pK_a and log D (pH 3).

3.2. Ion pairing chromatography on conventional porous RP packing material

Perfluorinated carboxylic acids and *n*-alkyl-amines are the two classes of volatile ion-pairing agents. As the majority of the selected analytes are stable under acidic conditions and present a protonable amino group we thought judicious to test the perfluorinated surfactants as ion-pairing reagents with PGC support and classical RP packing material (Supelcosil ABZ + Plus column).

3.2.1. Influence of the ion pairing agent nature

The nature (type and chain length of the hydrophobic group) of the ion pairing agent is considered to be an important parameter in the retention of charged solutes in IPLC then, different ion-pairing

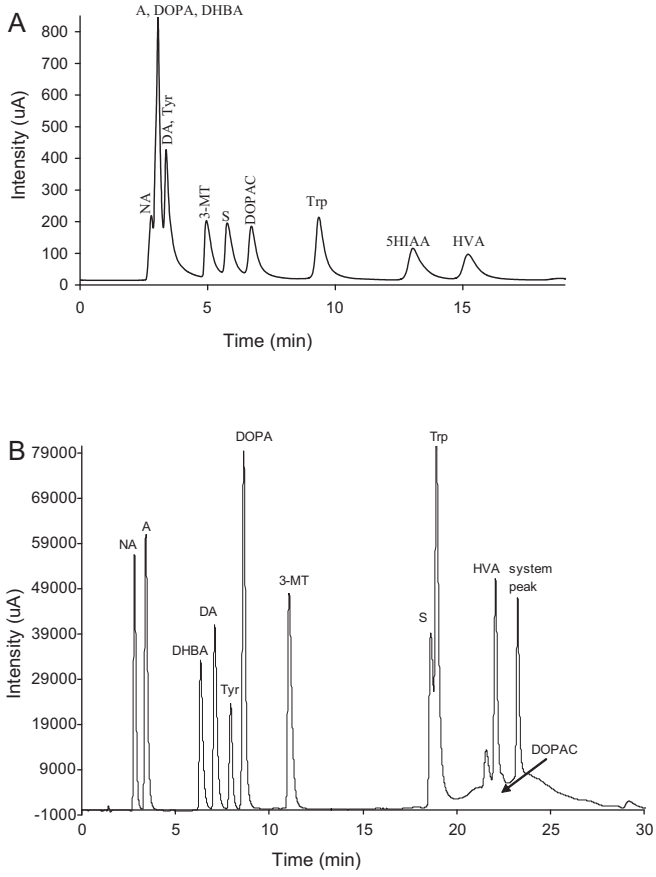


Fig. 2. RPLC–UV analysis of catecholamines on porous RP packing material. (a) Column: Pursuit PFP (150 mm × 2.1 mm I.D., 5 μm); isocratic mobile phase: MeOH/CH_3COONH_4 10 mM, pH 2.9, (10/90 v/v); flow rate: 0.2 mL min^{-1}; detection UV at 280 nm. (b) Column: PGC Hypercarb (100 mm × 4.6 mm I.D., 5 μm); gradient elution: solvent A: MeCN/$HCOONH_4$ 50 mM pH 3 (10/90 v/v), solvent B: MeCN/$HCOONH_4$ 100 mM pH 3 (70/30 v/v). Gradient: from 0 to 30% B in 15 min, then from 30 to 100% B in 5 min and finally 100% B in further 5 min; flow rate: 1 mL min^{-1}; detection UV at 280 nm.

agents were tested: trifluoroacetic acid (TFA), heptafluorobutyric acid (HFBA), nonafluoropentanoic acid (NFPA) and pentadecafluorooctanoic (PDFOA). On both tested supports (silica-based C18 and PGC), the following phenomena were observed: (i) in accordance with an ion pairing mechanism [30,37], the retention is increased with the increase of the perfluorinated chain for the six biogenic amines bearing a net positive charge at pH 3 and for the three amino acids for which the amine functions are protonated; (ii) the retention is decreased with the increase of the perfluorinated chain for the three acidic metabolites (DOPAC, HVA and 5HIAA) bearing a partial negative charge at pH 3. As these compounds cannot form any ion-pair with the perfluorinated carboxylic acids, their retention can only be ensured by reversed-phase mechanism. Moreover, it was well established [37] that adsorbed quantities of surfactant on PGC or C18 support increase with surfactant concentration as well as with the increase of the surfactant alkylchain length leading to a decrease in the number of free C18 ligands on the support surface that could ensure retention for acidic analytes. Therefore this may explain the decrease in retention for acidic compounds when using long-chain ion-pairing agents in the mobile phase. As a compromise for our further studies, NFPA was selected as ion-pairing agent. Moreover the use of surfactant with a short side chain in mobile phase involves that the chromatographic system has a faster equilibration time [37,51].

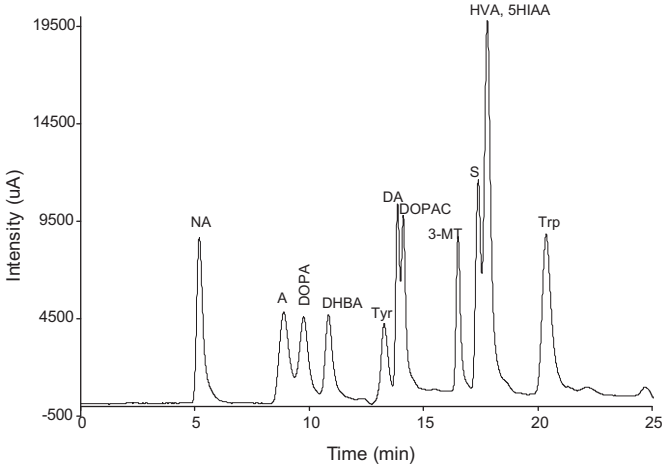


Fig. 3. IPLC–UV analysis of catecholamines using porous silica-based C18 support. Column: Supelcosil ABZ + Plus (150 mm × 4.6 mm I.D., 5 μm); gradient elution: solvent A: 1.25 mM NFPA in water, solvent B: MeOH. Gradient: 0% B for 5 min; from 0 to 10% B in 5 min, then from 10 to 20% B in 0.1 min, and finally 20% B in further 15 min; detection UV at 280 nm.

3.2.2. *Influence of the organic modifier nature and percentage*

As expected, under isocratic conditions, the elution strength of MeCN is higher than MeOH. When MeOH is used rather than MeCN, differences in selectivity were observed on PGC support. To reduce analysis time, it seems more promising to use MeCN as organic modifier on the PGC support whereas the lowest retention offered by the C18 support leads to select MeOH as organic modifier on this support type in order to ensure sufficient analyte retention. In order to analyze all the 12 selected analytes, it was necessary to investigate gradient elution conditions. Unfortunately, even under optimized gradient conditions, baseline separation of the 12 analytes could not be achieved on the silica-based C18 column: HVA and 5HIAA are coeluted and their peak is poorly separated from that of S as well as DA and DOPAC are poorly separated as shown in Fig. 3. Insufficient separation was also observed on the PGC support with a coelution for S, Trp and 5HIAA (data not shown). Thus, even under IPLC conditions, neither of the tested supports (PGC and Supelcosil ABZ + Plus) offers us the possibility of a dual electrochemical and MS detection. In order to improve the separation efficiency, finally we tested columns designed for fast chromatography, as these supports are considered also to provide higher column efficiency.

3.3. *Ion pairing chromatography on fast chromatography support*

The use of columns with particle diameter below 2 μm has become a preferred approach in order to increase the speed, resolution or efficiency in HPLC. However, the use of these small particles often requires an ultra-performant HPLC systems able to withstand pressures that can exceed 1000 bar [53]. Nevertheless if we want to keep the conventional HPLC equipment generally designed to withstand up to 400 bar, fused-core particles columns can be a solution. Another solution to overcome pressure limitation in HPLC is represented by monolithic columns [54]. These two types of columns can operate at higher mobile phase flow rates than conventional totally porous silica columns (with porous particles of 3 or 5 μm) without loss of peak efficiency or overrate pressure increase.

3.3.1. *Ion pairing chromatography using monolithic silica-based C18 column*

The monolithic columns have different structure compared to conventional particular silica. While the typically used columns

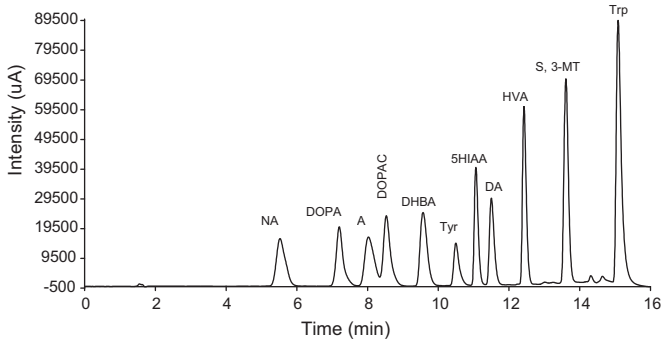


Fig. 4. IPLC–UV analysis of catecholamines using a monolithic column. Column: Onyx (100 mm × 4 mm I.D.); mobile phase: solvent A: 1.25 mM NFPA in water, solvent B: MeOH. Gradient starts at 5% B for 5 min, from 5 to 20% B in 3 min, then from 20 to 40% B in 4 min and finally 40% B in further 5 min; flow rate: 1 mL min^{-1}; detection UV at 280 nm.

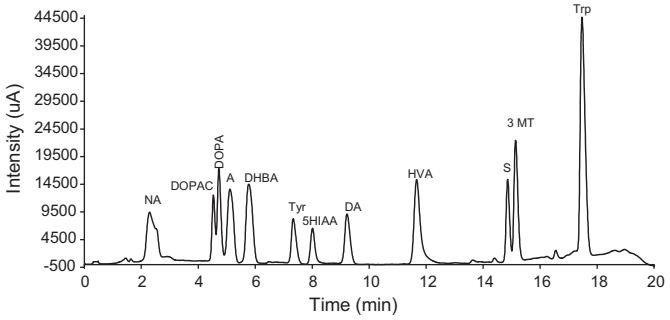


Fig. 5. IPLC–UV analysis of catecholamines using a fused-core column. Column: Halo C18 (100 mm × 4.6 mm I.D.); mobile phase: solvent A: 1.25 mM NFPA in water, solvent B: MeOH. Gradient: from 0 to 7% B in 10 min, then from 7 to 30% B in 8 min and finally 30% B in further 5 min; flow rate: 1.5 mL min^{-1}; detection UV at 280 nm.

are filled with spherical silica particles, monolithic columns are not formed by particles. They are made by sol–gel technology, which facilitates the formation of highly porous material, containing macropores (typically 2 μm) and mesopores (about 12 nm) in its structure. Such an LC column consists of a single rod of silica-based material with these two kinds of pores. The large pores are responsible for a low flow resistance and therefore allow the application of high eluent flow rates, while the small pores ensure sufficient specific surface area for separation efficiency [55,56].

From the best chromatographic conditions obtained on the 5 μm particles C18 column under IPLC conditions, we have first evaluated the isocratic analysis of the 12 solutes on the monolithic column (Onyx, 100 mm × 4 mm I.D.) with a mobile phase composed of a 1.25 mM aqueous solution of NFPA, pH 2.9 and MeOH (90/10 v/v). The specific surface area of the monolithic column (300 m^2 g^{-1}), almost twice larger than the conventional porous silica column (170 m^2 g^{-1}) implies a significant increase in the catecholamine retention under the same conditions of mobile phase and flow rate and this is observed in spite of a shorter length for monolithic column (see Table 1). With a 1 mL min^{-1} flow rate, the total analysis time of the catecholamine mixture is 55 min on the monolithic C18 column compared to only 18 min on the conventional totally porous silica column (Supelcosil ABZ + Plus).

In order to shorten the analysis duration on monolithic C18 support, two approaches were investigated. The first one was to increase the flow rate from 1 mL min^{-1} to 2 mL min^{-1}. Unfortunately, although the peak efficiency is not really affected, a separation without baseline resolution for the first eluted compounds (NA, DOPA, A, DOPAC, DHBA and Tyr) was obtained. The second approach was to evaluate gradient elution conditions at a flow rate of 1 mL min^{-1}. Fig. 4 shows the best separation obtained under optimized gradient conditions. A total analysis time of 16 min, symmetrical peaks, an elution far from the void volume for the first compounds and only one coelution (S and 3-MT) could be achieved under these conditions. Nevertheless, the electrochemical detection remains problematic because of the S/3MT coelution.

3.3.2. Ion pairing chromatography using fused-core C18 column

Fused-core particles are produced by "fusing" a porous silica layer onto a solid silica particle. Thus, the ability of this column to generate efficient separation comes not only from its small particle size (2.7 μm), but also from its 0.5 μm porous shell fused to a solid core particle. The fused-core silica materials providing the shorter diffusion mass transfer path for solutes are less affected in resolving power by increasing in mobile phase velocity than the sub-2 μm porous silica packings resulting in faster separations and higher sample throughput [52–56].

The column we used was a Halo C18 (50 mm × 4.6 mm I.D., 2.7 μm). Its specific surface area (150 m^2 g^{-1}) was comparable to that of a column packed with totally porous particles whereas its length is three times shorter (5 cm for the fused-core column and 15 cm for the conventional column). Under the same isocratic conditions (eluent: 1.25 mM NFPA in water/MeOH (95:5)) and the same flow rate (1 mL min^{-1}), higher retention and larger retention range were observed on the Halo C18 column than on a conventional porous silica-based C18 (Supelcosil ABZ + Plus). Thus, under these conditions the first eluted compound (NA) has a retention time of 3 min and the retention time of the last eluted one (Trp) is greater than 70 min (data not shown).

To reduce the analysis time, we increased the flow rate of the mobile phase from 1 mL min^{-1} to 1.5 mL min^{-1} and used mobile phase under gradient conditions. For the first time, a satisfactory separation of the 12 selected compounds was obtained in about 20 min (Fig. 5). Even if all the resolutions are not systematically higher than 1.5, this should not prevent the compound identification and quantification by mass spectrometry as well as by electrochemical detection.

3.4. Analysis of catecholamines by IPLC–MS/MS

MS detection has the advantage of providing structural information about the eluted compounds. Moreover, coelutions of compounds can be resolved in the event of different m/z ratios. Thus, the four optimized ion-pairing systems developed in our study on the 4 column types (5 μm particles C18, PGC, monolithic and fused-core) are compatible with mass spectrometry detection as no isobaric solutes are coeluted on none of the 4 columns. The chromatographic method developed can be directly coupled with electrospray ionization tandem mass spectrometry (ESI–MS/MS) in positive ionization mode and 10 solutes among the 12 selected can be observed. Unfortunately the presence in the mobile phase, of the negative ion-pairing reagent, makes this system incompatible with negative ionization mode and thus unable to detect the two acidic compounds (HVA and DOPAC) that only responded in negative mode.

The selective reaction monitoring (SRM) mode was used to monitor the parent and product ions for the tandem MS analysis of catecholamines and Table 2 sums up the MS/MS transitions selected for each solute.

3.4.1. MS detection limits

Table 3 summarizes the detection limits obtained with Applied-Biosystem/Sciex API 300 for the 10 solutes in IPLC–MS/MS using the four chromatographic systems under optimized conditions. The detection limits were calculated as the analyte concentration that gives a signal-to-noise ratio equal to 3, and they were determined

Table 3
Limits of detection (LOD) (ng mL^{-1}) obtained in IPLC–MS/MS (positive ionization mode) for each catecholamine.

Column	PGC	HALO C18		Supelcosil ABZ +		ONYX C18		
MS	API 300	API 300	API 3000	API 300	API 3000	API 300	API 3000	
Analyte	LOD in mobile phase	LOD in mobile phase		LOD in mobile phase		LOD in mobile phase	LOD in mobile phase	LOD in matrix
DHBA	800.0	50.0	25.0	10.0	2.5	2.5	2.0	5.0
DA	500.0	1000.0	100.0	10.0	5.0	2.5	1.0	1.0
3-MT	5.0	20.0	2.5	5.0	3.0	2.5	0.5	1.0
NA	1000.0	100.	50.0	5.0	10.0	5.0	10.0	50.0
S	5.0	>1000.0	100.0	5.0	10.0	2.0	1.0	1.0
TYR	40.0	10.0	5.0	10.0	2.5	4.0	3.0	-
A	1000.0	75.0	50.0	1.0	5.0	3.0	2.5	5.0
5HIAA	500.0	>1000.0	300.0	100.0	50.0	10.0	5.0	7.0
DOPA	100.0	250.0	50.0	10.0	25.0	5.0	10.0	10.0
TRP	5.0	*	*	10.0	5.0	*	*	-

* For the two chromatographic systems: Onyx C18 and Halo C18, gradient conditions led to a system peak observed at the retention time of Trp and following its specific selected transition consequently, the LOD determination cannot be obtained for Trp onto these two systems.
- Tyr and Trp are present in the matrix then their LOD cannot be estimated.

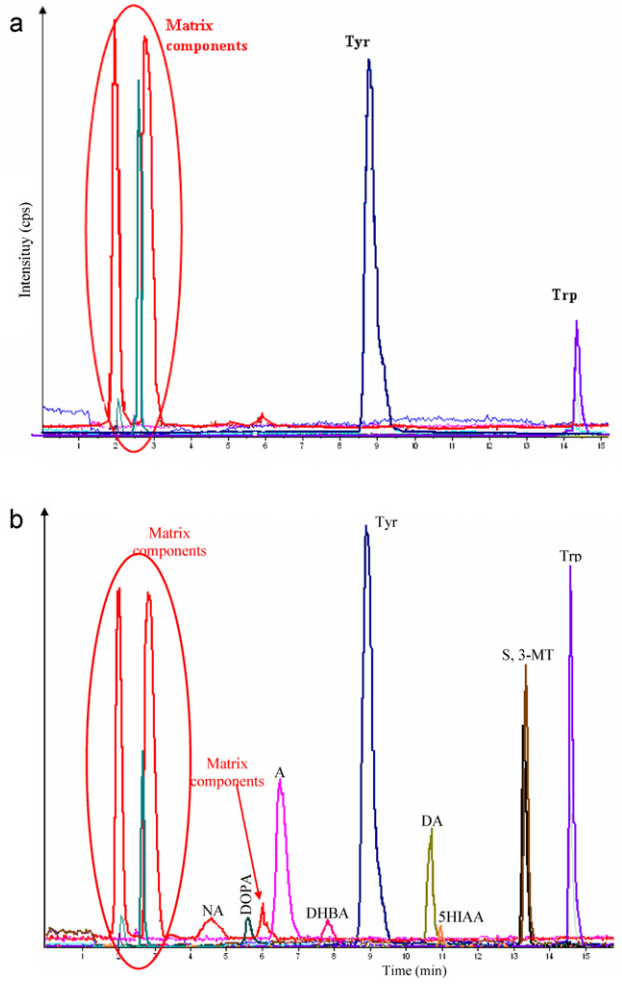


Fig. 6. IPLC–ESI–MS/MS (extracted ion current (XIC)) analysis of a sheep brain sample using a monolithic silica-based C18 support. (a) Brain extract. (b) Brain extract spiked with 100 ng mL^{-1} of each catecholamine.

by consecutive injection of the 10 analytes mixture with decreasing concentration values in both the mobile phase and a brain extract.

As we can notice in Table 3, in terms of MS detection, Monolithic C18 column proved to be the best support to reach the lowest detection limits (LODs) (from 0.5 ng mL^{-1} to 10 ng mL^{-1} depending on the neurotransmitter) and the highest detection limits were obtained for the separation realized on the PGC column. These LOD values evaluated in our study are in good accordance with reports concerning native amino acid analysis under similar chromatographic conditions [57,58] or catecholamine analysis [25]. Surprisingly, despite rather high peak efficiency, the fused-core particles column (Halo C18) lead to poorer detection limits than the other two systems using conventional porous particles C18 column (Supelcosil ABZ + Plus) and monolithic column (Onyx C18). This could be explained by the fact that with this support, the initial mobile phase composition was totally aqueous, involving a bad ESI spray stability at the analysis beginning and consequently, conditions not favorable to a sensitive detection. As expected, for most of solutes, LODs are better for Sciex API 3000 than for API 300 (Table 3) because of its more advanced technology.

Moreover, higher LOD values were found when the standards are prepared in matrix (sheep brain sample) in comparison with mobile phase. These results demonstrated that some interferences with other matrix components occurred during the MS detection step.

3.4.2. Analysis of a brain extract

As previously mentioned, the aim of this work was to optimize a chromatographic method that could be used for further qualitative and quantitative analysis of biological samples. Fig. 6 depicts an IPLC–MS/MS analysis of a sheep brain sample using the monolithic system as it offers both sufficient separation for MS detection and the best LODs. The sheep brain sample was analyzed as an example of a complex biological sample. The analytes Tyr and Trp were clearly detected in SRM mode (Fig. 6a) whereas NA, DOPA, A, DA, 5HIAA, S and 3-MT in contrast, cannot be detected in this sample. In Fig. 6b, the analysis of the same sheep brain sample spiked with 100 ng mL^{-1} of each catecholamine underscored the matrix interference that is mainly responsible for NA peak shape deterioration and for the high ionization suppression effect for 5 solutes (NA, A, DHBA, 5HIAA and 3-MT) as shown in Table 3. The standard addition method was applied in order to estimate the Tyr and Trp concentrations in the sheep brain sample and their concentration values have been estimated to 10.0 μg g^{-1} for Tyr and 0.6 μg g^{-1} for Trp (corresponding to a concentration of 5×10^{-11} mol mg^{-1} for Tyr and 3×10^{-12} mol mg^{-1} for Trp). These contents were in the same magnitude order to those evaluated previously in a porcine brain sample [25].

4. Conclusions

This paper summarizes a step-by-step optimization of an ion-pairing chromatographic method for the analysis of catecholamines and related compounds. Different supports were tested under ion-pairing conditions and we could see that the use of columns

dedicated to fast chromatography provided the most promising systems. The separation method optimized on a fused-core C18 column (Halo C18) allows a separation of the 12 catecholamines without coelution. The chromatographic method developed can be directly coupled with electrospray ionization tandem mass spectrometry (ESI–MS/MS) in positive ionization mode and 10 solutes among the 12 selected can be observed. The most sensitive system (lowest MS detection limits) proved to be the one using the monolithic column (Onyx C18) (from 0.5 ng mL^{-1} to 10 ng mL^{-1} depending on the neurotransmitter). The applicability of the optimized LC–MS/MS method to a "real world" sample was finally evaluated and a sheep brain sample was analyzed as an example of a complex biological sample. A determination of its content in Tyr and Trp was possible by the standard addition method.

The future of this work will be to couple the optimized systems with the electrochemical detector in order to improve the detection limits. For a sensitive electrochemical detection a post-column introduction of an additional salt solution will be investigated in order to increase mobile phase conductivity.

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