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Journal of Chromatography B, 801 (2004) 323–329
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Analysis of catecholamines and related substances using porous graphitic carbon as separation media in liquid chromatography–tandem mass spectrometry

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Received 26 May 2003; received in revised form 21 November 2003; accepted 24 November 2003

Abstract

Capillary porous graphitic carbon (PGC) columns have been utilized for separation of several catecholamines and related compounds (i.e. L-tyrosine, L-DOPA, 3-*O*-methyl-DOPA, dopamine, 3,4-dihydroxy-phenyl-acetic acid (DOPAC), homovanillic acid, noradrenaline, vanillomandelic acid and adrenaline) on-line with electrospray ionization tandem mass spectrometry (ESI–MS/MS). The use of a mobile phase without ion-pairing agents and with high content of organic modifier facilitated the coupling to the selective and sensitive mass spectrometric detection. Minimum detectable sample concentration (MDC sample) for noradrenaline, dopamine and L-tyrosine in a standard solution was estimated to 3, 10 and 30 nM, respectively (3 S/N corresponds to MDQ for L-tyrosine of approximately 8×10^{-14} mol). The developed strategy was applied for analysis of brain tissue, i.e. a substantia nigra (ns) sample.

Keywords: Catecholamines; Porous graphitic carbon; L-DOPA; Dopamine; Polar analytes

1. Introduction

Catecholamines are biogenic amines containing an amino function on a side chain together with the catechol function (i.e. hydroxyl groups at 3- and 4-positions on a benzene ring) and they play an important role in the nervous system for numerous organisms [1]. For instance, dopamine (DA) and L-DOPA are endogenous substances that have been reported to play a substantial role in, e.g. Parkinson's disease where the dopaminergic neurons in selective brain regions slowly degenerate and eventually die. When L-DOPA is administrated to the patient, an increase in the dopamine production in the surviving neurons takes place, thereby increasing the amount of dopamine available for release. To be able to study the pathophysiology resulting in Parkinson's disease and neurological disorders in general, it is essential to be able to analyze catecholamines and related substances in biological specimens.

Catecholamines and the related substances contain acidic and/or basic functions. In order to retain these small polar analytes, octadecyl silica materials (ODS, C_{18}) together with minute amounts of organic modifiers and/or ion-pairing agents as well as gradient elution are commonly used [2–10]. The mass spectrometric detector, which offers attractive selectivity and structural information (in contrast to, e.g. electrochemical detection (ECD)), is however not compatible with non-volatile mobile phase additives [11]. In addition, the use of gradient elution will alter the electrospray ionization conditions during the chromatographic run which may complicate quantification, thus the need for improved analytical methods.

The use of capillary liquid chromatography offers several benefits compared to conventional sized LC [12]. If the sample amount is limited, for example, the use of capillary liquid chromatography is beneficial in order to receive a sensitive separation and detection methodology. The coupling to mass spectrometric detection is also favored when capillary LC is used since there is no need for flow-splitting devices after the column. By using packed capillary porous graphitic carbon (PGC) [13–16] columns, it has earlier been shown by our group [17] that L-DOPA and four metabo-

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doi:10.1016/j.jchromb.2003.11.036

lites can be separated with an MS compatible mobile phase.

The aim of the present study was to further utilize the unique properties of packed capillary PGC columns to separate and analyze by MS/MS an even more complex mix of very closely related analytes (e.g. L-tyrosine, L-DOPA, 3-*O*-methyl-L-DOPA, dopamine, DOPAC, homovanillic acid (HVA), noradrenaline (NA), vanillomandelic acid (VMA) and adrenaline) that can appear together in endogenous samples. The developed strategy was subsequently applied to the analysis of a dissected nuclei (i.e. substantia nigra (sn)) isolated from porcine brain tissue.

2. Experimental

2.1. Chemicals

3,4-Dihydroxy-phenylalanine (L-DOPA), 3-*O*-methyl-L-DOPA (3-*O*-MD), dopamine, 3,4-dihydroxy-phenyl-acetic acid (DOPAC), noradrenaline (NA, arterenol) and homovanillic acid were obtained from Sigma Chemicals Co. (St. Louis, MO, USA). Adrenaline (A), DL-vanillomandelic acid and L-tyrosine (T) were purchased from Aldrich (Steinheim, Germany). The analytes are presented in Table 1. Methanol (LiChrosolv), acetonitrile (LiChrosolv), perchloric acid (pro analysis (pa)) and formic acid (analytical reagent grade) were purchased from Merck (Darmstadt, Germany) and ammonium formate (analytical reagent grade) from BDH Laboratory Supplies (Poole, UK).

2.2. Solution preparation

Stock solutions with concentrations between 2.5 and 14.3 mM of the analytes were prepared in 0.05 M hydrochloric acid, water or mobile phase. Stock solutions were stored in darkness at −80 °C. Infusion solutions were prepared from the stock solutions and diluted either with 60% methanol or mobile phase, i.e. methanol–ammonium formate buffer pH 2.9 and ionic strength 0.05 M (60:40, v/v) to a concentration between 10 and 100 μM.

2.3. Brain tissue

Porcine brains were obtained immediately after death at the local abattoir, and transported on ice to our laboratory. Substantia nigra was dissected out, homogenized in liquid nitrogen and frozen immediately at −80°C, until biochemical analysis. The analytes were extracted from the matrix by addition of 0.5 M formic acid. The sn (158 mg) was vortexed together with 565 μl 0.5 M formic acid and ultrasonicated for 2 min on ice. The extract was centrifuged for 30 min (25000 × *g*) in 4 °C, and the supernatant was divided to three vials (100, 100 and 300 μl) and stored at −80 °C until analysis.

2.4. Chromatographic and mass spectrometric instrumentation

The PGC columns (Hypercarb 5 μm, part no. 35005-000, batch PGC230R3, Hypersil, Runcorn, UK) were packed according to a procedure described elsewhere [17]. A liquid chromatographic system composed of a pump (Jasco PU-980), a manual six port injection valve (Valco Instruments, Houston, TX, USA) with a 2.5 μl external loop, a packed PGC capillary column and an ultra violet (UV) absorbance detector (Jasco UV 975) operating at 280 nm with a capillary flow cell (UZ-JA97 CAP; LC Packings, Amsterdam, The Netherlands) or a triple quadrupole mass spectrometer (API III+, PE-Sciex, Concord, Ont., Canada). During the MS experiments, the conductive PGC material was de-coupled from the high voltage used in the electrospray process. After the chromatographic column, an empty fused silica tubing (Polymicro Technologies) with internal diameter less than the packed column (e.g. 150 μm) was coupled by means of a teflon tubing. The untreated fused silica tubing was then coupled by a grounded union (Valco Instruments) to a fused silica capillary tubing (Polymicro Technologies) of internal diameter 50 μm connected to the mass spectrometer inlet, see Fig. 1.

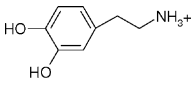
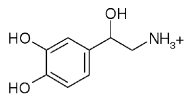
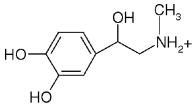
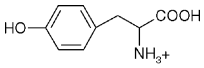
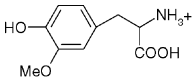
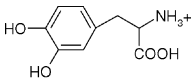
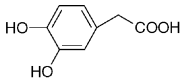
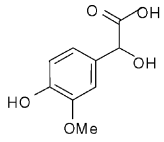
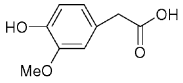
The mass spectrometer was operating with pneumatic assisted electrospray (IonSpray) and both positive- and negative-ionization modes were utilized as sample ionization techniques. In order to optimize the detection of the analytes, MS infusion experiments were performed with the different solutions of the analytes in mobile phase (i.e. methanol–ammonium formate buffer pH 2.9 and ionic strength 0.05 M, 60:40 v/v). The ion spray voltage (ISV) and orifice potential (OR) were varied. The chosen potentials were used during product ion scan infusion experiments with the individual components using different collision energies (10–20 eV). The recorded precursor ions and fragments are shown in Table 1. Multiple reaction monitoring (MRM) spectra for the analytes are shown in Fig. 2. The mass spectrometric parameters were then set to the following values: ion spray voltage: 4000–4500 V and −3500 V in positive and negative mode respectively and orifice lens (OR): 35 V and −35 V for positive and negative electrospray ionization, respectively. During the MS/MS experiments, a collision energy of 10 eV was used. The collision gas was argon with 99.9999% purity (6.0 AGA, Stockholm, Sweden).

3. Results and discussion

3.1. The choice of PGC

PGC is known to have special chromatographic properties for separation of polar analytes. The stationary phase has proved advantageous for the separation of L-DOPA, dopamine, DOPAC, HVA and 3-*O*-MD [17] and now also for VMA, L-tyrosine, noradrenaline and adrenaline. In Fig. 3,

Table 1
Catecholamines and related substances. Recorded precursor ions and fragments during the MRM (multiple reaction monitoring) experiments are given

Analyte	Recorded precursor ion	Recorded fragments (*m*/*z*)	Retention order
Dopamine (DA)	 m/z $[M+H]^+$ =154.2	137.1, 119.1	2
Noradrenaline (NA)	 m/z $[M+H]^+$ =170.2	152.1, 135.1	1 (co-eluting with A)
Adrenaline (A)	 m/z $[M+H]^+$=184.2	166.1, 135.1	1 (co-eluting with NA)
L-tyrosine (T)	 m/z $[M+H]^+$=182.2	165.1, 136.1	3
3-*O*-Methyl-L-DOPA (3-OMD)	 m/z $[M+H]^+$=212.2	195.1, 166.1	7
L-DOPA	 m/z $[M+H]^+$=198.3	181.0, 152.1	5
DOPAC	 m/z [DOPAC+formate]=213.0	167.0, 123.0	6
Vanillomandelic acid (VMA)	 m/z [VMA+ammonium]=216.2	199.2, 181.1	4
Homovanillic acid (HVA)	 m/z [HVA+formate]=227.0	181.1, 137.2	8

the qualities of PGC are demonstrated and additional catecholamines and related substances are analyzed in a single run using an isocratic mobile phase with high content of organic modifier without non-volatile additives.

3.2. Mass spectrometric detection and the importance of pH

In mass spectrometric detection, usually positive electrospray ionization is used for the detection of basic compounds whereas negative electrospray ionization is generally to prefer for acidic analytes since the compounds may then be detected as the protonated or deprotonated molecule respectively. Although pH in the sprayed droplets may differ from the pH in the mobile phase [18], it is important to choose an optimal pH, not only for the separation, but also for the detection of the analytes [11]. In the present study, both basic and acidic compounds were included, see Table 1, thus the decision of ionization mode was not that straight forward. The low pH (pH 2.9) used during the separation step results in the presence of negatively charged, positively charged and un-charged analytes. Although it is possible to switch between positive and negative ionization during MS analysis,

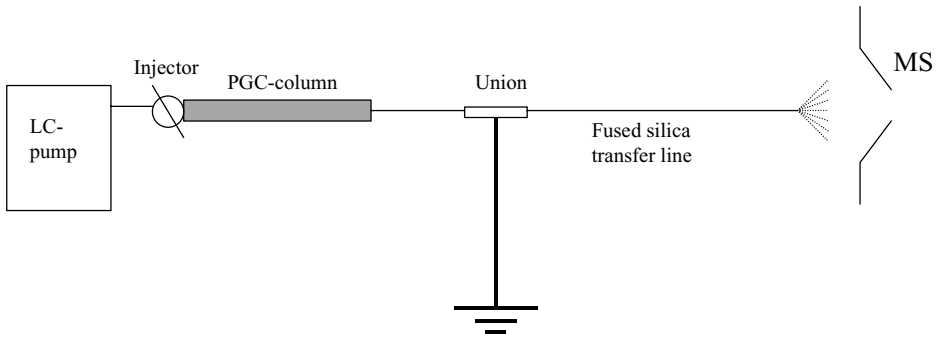


Fig. 1. The system set-up for a PGC column coupled to MS/MS detection. The conductive chromatographic PGC column is decoupled from the high voltage supply by grounding the stainless-steel union between the PGC capillary column and mass spectrometric inlet.

it is always more convenient to stay at one polarity mode. However, since the compounds separated in the present study both contain acidic and/or basic functions, they may not be detected in the same ionization mode.

The detection of noradrenaline, adrenaline and dopamine was performed using positive electrospray ionization, see Table 1 and Fig. 2a–c. The analytes are protonated at pH 2.9 and the protonated molecules were chosen as precursor ions whereas fragments resulting from loss of water and/or ammonia was used as product ions for noradrenaline and dopamine. The dominant fragment for adrenaline resulted from loss of water.

L-Tyrosine, 3-*O*-MD and L-DOPA were also recorded using positive electrospray ionization (Fig. 2d–f). It should, however, be noted that the low pH used during the separation is not optimal for the MS detection of these compounds. The amine functions are protonated whereas the carboxylic functions are not fully protonated (pK_a for L-DOPA is 2.32) resulting in the presence of un-charged analyte. Still, it was possible to record the protonated molecules and fragments resulting from loss of either ammonia or formic acid were chosen as product ions.

DOPAC, VMA and HVA, contain carboxylic functions (pK_a values for DOPAC, VMA and HVA: 4.3, 2.3 and 4.4, respectively) and lack amine functions. At pH 2.9, DOPAC and HVA are predominately un-charged, whereas VMA is dominantly negatively charged. The choice of positive or negative electrospray ionization is then not obvious. In order to detect these analytes in positive electrospray ionization, it may be necessary to detect the sodium or ammonium adducts. In negative electrospray ionization it can be suitable to add a make-up flow after the column in order to, e.g. increase the pH. The analytes will then be deprotonated and increased sensitivity in negative ESI might appear. It may also be possible to add more mobile phase modifier or another modifier in a sheath flow after the column in order to change, e.g. the surface tension of the eluent and thereby decrease the detection limits [19]. In the present study, it was found that VMA was suited for both positive and negative electrospray ionization, whereas HVA and DOPAC gave (2–10 times) higher intensities for the fragments obtained in negative electrospray ionization mode. DOPAC (Fig. 2g), eluting close to, e.g. L-DOPA, was chosen not to be analyzed due to the inconvenience of switching back and forth between positive and negative electrospray ionization. VMA, however, was successfully detected in positive mode, see Fig. 4. The substantial difference in obtained intensity for the fragments for VMA compared to HVA and DOPAC in positive-ionization mode, may be due to a more stable fragment from VMA (Fig. 2h). Negative electrospray ionization was, hence, only utilized for the detection of HVA (Fig. 2i).

From Fig. 4, MDC sample was calculated for noradrenaline, dopamine and L-tyrosine to be 3, 10 and 30 nM, respectively (3 S/N for L-tyrosine corresponds to MDQ of approximately 8×10^{-14} mol). These values are in the same region as reported by others for similar compounds [20–23]. Detection limits of 30×10^{-15} mol have, on the other hand, been reported for biogenic amines analyzed with conventional sized LC coupled to electrochemical detection. The use of ECD in combination with miniaturized chromatographic columns is also very favorable since the principle of ECD is based on surface reactions, which can be miniaturized without sacrificing a significant amount of sensitivity. An inherent drawback of the ECD detection technique is, nevertheless, the risk of passivating the electrode surface (since the method is based on surface reactions). Moreover, MS detection is the detection of choice if structural evaluation of, e.g. metabolites is needed.

3.3. Analysis of endogenous levels of catecholamines in brain tissue

The developed strategy for separation of catecholamines and related substances using the PGC material as separation media in LC–MS/MS showed promising results also for analysis of biological samples. In Fig. 5, the supernatant of an acid treated and centrifuged substantia nigra sample (2.5 μl) was directly injected onto the PGC column. The analytes noradrenaline, L-tyrosine and dopamine were clearly detected in MRM mode, and the concentration of L-tyrosine in the injected sample has been estimated to approximately 14 μM corresponding to a concentration

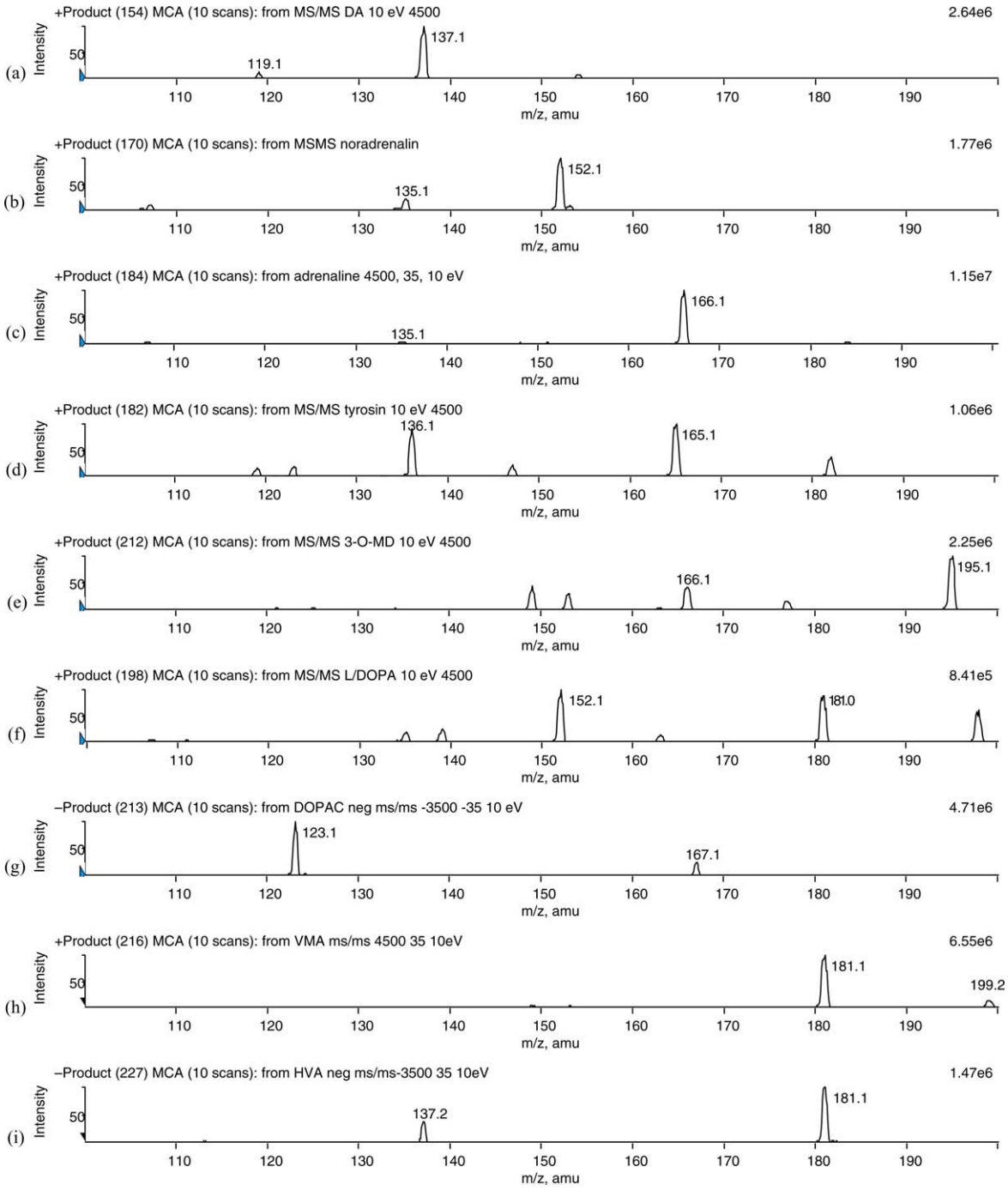


Fig. 2. Product ion scan spectra for the analytes: dopamine (a), noradrenaline (b), adrenaline (c), tyrosine (d), 3-*O*-methyl-L-DOPA (e), L-DOPA (f), DOPAC (g), VMA (h), and HVA (i). Experimental settings: see Section 2.4.

of 5×10^{-11} mol/mg substantia nigra. The concentrations of noradrenaline and dopamine were found to be approximately 7 times lower. During the analysis also a broad peak originating from *m*/*z* 154, i.e. the same *m*/*z* as for dopamine (Table 1), was seen after around 11 min. The peak profile may be explained by a saturation of the detector as the settings employed for the detector electronics caused the saturation level to be reached around 300,000–400,000 counts. Several sample pre-treatment techniques (including protein precipitation with perchloric acid together with or without addition of sodium EDTA and sodium sulfite) were tested but the broad peak eluted irrespectively of pre-treatment. The identity of this peak thus needs to be further investigated. It should, however, be noted that the broad peak does not co-elute with the other compounds of interest. The eluting broad peak, as well as other components in the mobile phase, e.g. impurities as oxidizing agents [24] or phtalates, may however disturb the long-term stability of the station-

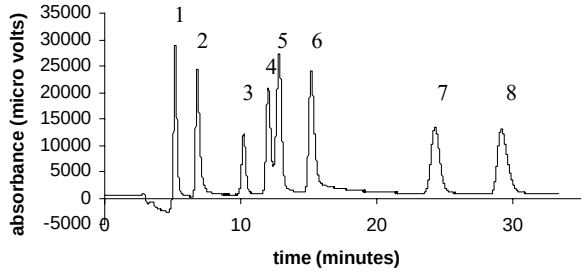


Fig. 3. Separation of L-DOPA and related substances (in a standard solution) on a porous graphitic carbon column using a volatile mobile phase. Elution order: noradrenaline (1), dopamine (2), L-tyrosine (3), vanillomandelic acid (4), L-DOPA (5), DOPAC (6), 3-*O*-methyl-L-DOPA (7), and homovanillic acid (8). Column: Packed PGC capillary column with an internal diameter of 200 μm and length of 15 cm. Mobile phase: methanol–ammonium formate buffer pH 2.9 (ionic strength: 0.05 M, 60:40, v/v). Flow rate: 2 μl/min. Injection volume: 2.5 μl. Injected concentration: 10 μM of each analyte. Detection: UV detection at 280 nm.

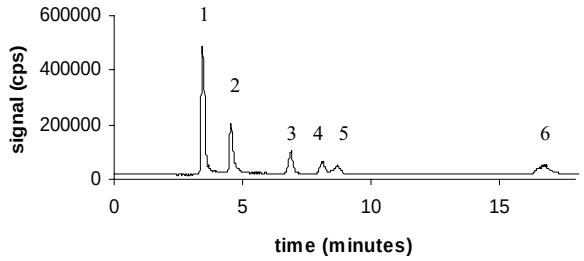


Fig. 4. Separation of L-DOPA and related substances on a porous graphitic carbon column using a volatile mobile phase and positive electrospray ionization mode. Elution order: noradrenaline + adrenaline (1), dopamine (2), L-tyrosine (3), vanillomandelic acid (4), L-DOPA (5), and 3-*O*-methyl-L-DOPA (6). Column: packed PGC capillary column with an internal diameter of 200 μm and length of 15 cm. Mobile phase: methanol–ammonium formate buffer pH 2.9 (ionic strength 0.05 M) (60:40, v/v). Flow rate: 3 μl/min. Injection volume: 2.5 μl. Injected concentration: 3 μM of each analyte. Detection: MS/MS detection (positive electrospray ionization mode) as described in Section 2 and Table 1.

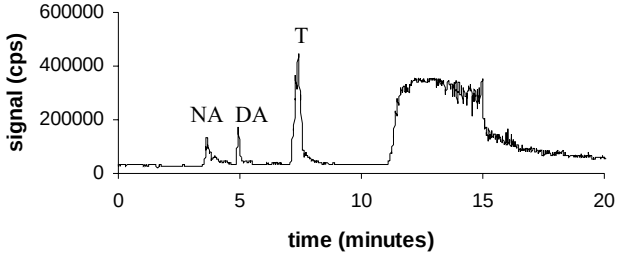


Fig. 5. Analysis of a substantia nigra sample using capillary liquid chromatography-mass spectrometry. Noradrenaline (NA), dopamine (DA) and L-tyrosine (T) are detected. Column: Packed PGC capillary column with an internal diameter of 200 μm and length of 15 cm. Mobile phase: methanol–ammonium formate buffer pH 2.9 (ionic strength 0.05 M, 60:40, v/v). Flow rate: 3 μl/min. Injection volume: 2.5 μl. Detection: MS/MS detection (positive electrospray ionization) as described in Section 2 and Table 1.

ary phase, something that needs further investigation. The benefit of using selective mass spectrometric detection is however clear, since co-eluting compounds originating from different *m*/*z* values still may be detected individually. The porcine brain sample was analyzed as an example of a complex biological sample and the possible use of PGC in combination with MS/MS for this type of analysis. It was hence not the scope of this paper to thoroughly quantify the detected analytes.

4. Conclusions

The present paper has demonstrated a strategy for successful separation and detection of catecholamines and some of the related substances in biological matrix, i.e. in less than one mg of mammalian brain tissue. The use of the unique properties of PGC made it possible to use a mobile phase of relatively high content of organic modifier without addition of ion-pairing agents. Furthermore, the elimination of non-volatile mobile phase additives facilitated the coupling to mass spectrometry. A previously published paper with PGC as stationary phase for separation of L-DOPA and related substances presented a separation of five compounds using a coupled column system with UV detection. In the present work, eight compounds can nicely be separated within 30 min using a more simplified chromatographic system with PGC as stationary phase. We also show that these similar compounds that are differently charged at low pH can be nicely separated and detected by use of PGC as stationary phase coupled to mass spectrometric detection. MS detection offers useful structural information and increased selectivity compared to the general UV detection. Thereby, an application was possible where a porcine brain sample was analyzed using this straightforward method. This was not possible by using only UV detection.

Acknowledgements

The PGC material was kindly donated by ThermoHypersil (UK). Financial support from Pharmacia, the Swedish Society for Medical Research and the Swedish Research Council (Grant 13123, 621-2002-5261, 621-2002-6821 and K5104-706/2001) are also acknowledged. Jonas Bergquist has a senior research position at the Swedish Research Council.

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