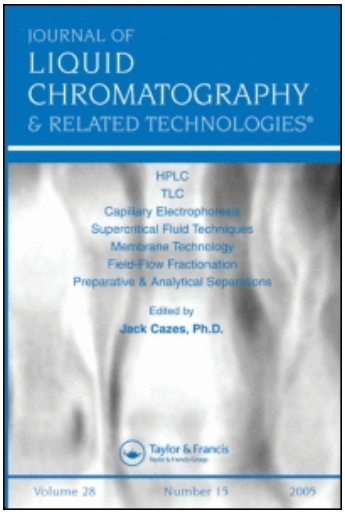


Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE QUANTIFICATION OF PUTRESCINE AND CADAVERINE AS THEIR ELECTROACTIVE DERIVATIVES: APPLICATION TO BIOLOGICAL SAMPLES

E. Bousquet[a]; R. Acquaviva[b]; A. Spadaro[a]; A. Vanella[b]
[a] Dipartimento di Scienze Farmaceutiche, Facoltà di Farmacia, Università di Catania, Catania, Italy [b] Istituto di Chimica Biologica, Facoltà di Farmacia, Università di Catania, Catania, Italy

Online publication date: 17 November 1999

To cite this Article Bousquet, E. , Acquaviva, R. , Spadaro, A. and Vanella, A.(1999) 'HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE QUANTIFICATION OF PUTRESCINE AND CADAVERINE AS THEIR ELECTROACTIVE DERIVATIVES: APPLICATION TO BIOLOGICAL SAMPLES', Journal of Liquid Chromatography & Related Technologies, 22: 19, 2957 — 2974
To link to this Article: DOI: 10.1081/JLC-100102071
URL: http://dx.doi.org/10.1081/JLC-100102071

J. LIQ. CHROM. & REL. TECHNOL., 22(19), 2957–2974 (1999)

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE QUANTIFICATION OF PUTRESCINE AND CADAVERINE AS THEIR ELECTROACTIVE DERIVATIVES: APPLICATION TO BIOLOGICAL SAMPLES

E. Bousquet,[1,*] R. Acquaviva,[2] A. Spadaro,[1] A. Vanella[2]

[1] Dipartimento di Scienze Farmaceutiche
Facoltà di Farmacia
Università di Catania
V.le A. Doria 6
95125 Catania, Italy

[2] Istituto di Chimica Biologica
Facoltà di Farmacia
Università di Catania
V.le A. Doria 6
95125 Catania, Italy

ABSTRACT

A high performance liquid chromatographic method coupled with an electrochemical detector for the separation and quantification of putrescine and cadaverine as their electroactive derivatives has been developed. Two different solvent column systems were used to determine putrescine, cadaverine, spermidine, spermine, and 1,3-diaminopropane (IS) in rat cerebral cortex. The lower detection limit was less than 80 fmol. The electrochemical response was linear in the range 80-240 ng/mL.

These chromatographic procedures are suitable for quantification of putrescine and cadaverine in animal tissues. Because of the simplicity, specificity, sensitivity, and ease of the operations, the method is applicable in both research and clinical laboratories.

INTRODUCTION

The polyamines (putrescine, cadaverine, spermidine, and spermine) are straight-chain aliphatic amines, present in significant amounts in all living cells. Polyamines (PA) are known to play a fundamental role in cell growth, proliferation and differentiation including cancer growth and tissue regeneration.[1,2] Recently, it has been suggested that N-methyl–D-aspartate-induced neurotoxicity might be mediated, in part, by increased PA levels.[1-5] Paschen et al.[3] reported that putrescine (PUT) levels increased markedly during cerebral post-ischemic reperfusion, whereas spermidine (SPD) and spermine (SPM) levels did not vary significantly, and that the degree of cell necrosis correlated with PUT levels.[3] The increased PUT levels are the result of the activation of ornithine decarboxylase ODC,[6] the enzyme that converts ornithine to PUT.

Several approaches for separating and measuring the PA in biological samples have been used. These included thin-layer chromatography,[7] gas-liquid chromatography,[8-10] radioimmunassay[8] and high performance liquid chromatography methods.[11-13] The HPLC methods with pre-column derivatization included reversed-phase chromatography of fluorescent compounds such as dansyl[14,15] or *o*-phthalaldehyde (OPA) derivatives.[16,17] Moreover cation-exchange chromatography with post column derivatization with UV-VIS[18-20] or fluorescence[21-24] detection has been developed. The most widely used method employs a cationic column with formation of the OPA-derivatives and measurement by fluorescence detection, or more recently, by electrochemical detection.[25] However, a significant problem with OPA derivatization is the instability of the derivatives,[26-28] requiring post-column derivatization to ensure reproducibility.

In this paper we describe an improved analytical procedure with pre-column derivatization, using 2,5-dihydroxybenzaldehyde (2,5-DHBA) for the quantification of PA through their electroactive derivatives (2,5-DHB-PA), allowing electrochemical detection (ECD). HPLC-ECD represents a very sensitive method, providing enhanced selectivity as a result of the limited number of substances which can undergo redox reactions under certain conditions.[29-31] For this purpose we established a novel pre-column derivatization of the PA, in particular PUT and cadaverine (CAD), with 2,5-DHBA to form electroactive derivatives measurable by HPLC-ECD using 1,3-diaminopropane (DAP) as an internal standard (IS).

We developed isocratic elution systems, which permit the separation of PA from the main endogenous products, which are usually present in rat cerebral cortex. In particular, the new method turned out to be efficient, rapid, reproducible, and sufficiently simple for the quantification of PUT and CAD in biological samples.

EXPERIMENTAL

Apparatus

The HPLC apparatus consisted of two Model 510 pumps, a Model 712 Wisp auto-injector, and an electrochemical detector (Model 5100A Coulochem, ESA, Bedford, MA, USA) which consisted of a control module and an analytical cell (Model 5010 Coulochem) containing two on-line porous graphite coulometric electrodes.

The analysis was performed in the oxidative mode. The ECD sensitivity range and response time were set at 100 nA and 10 s, respectively. Signals from the detectors were elaborated on an APC IV computer system (NEC, Boxborough, MA, USA) using Maxima 820 software (Waters Assoc., Milford, MA, USA).

Mass spectra were obtained on a Kratos MS 25 RF spectrometer. IR spectra were recorded on a Perkin-Elmer 1600 Fourier transformed spectrometer as KBr disks. The ^{1}H-NMR spectra were recorded at 300 MHz on a Bruker WP spectrometer in CD_3OD solutions with tetramethylsilane (TMS) as internal standard. Chemical shifts are expressed in ppm (δ).

Elemental analysis for C, H, N were obtained on a Carlo Erba 1106 analyzer (Milan, Italy) and agree with theoretical values to within ± 0.4%. UV absorption spectra were recorded on a Uvikon 860 (Kontron, Zurich, Switzerland) spectrometer in CH_3CN/MeOH (9:1 v/v). Analytical thin-layer chromatography (TLC) was performed on Merck 60 F_{254} silica gel plates.

Chemicals

2,5-dihydroxybenzaldehyde, 1,2-diamino-ethane (DAE) (IS), 1,3-diamino-propane, putrescine, cadaverine, spermidine, and spermine were obtained from Fluka (Buchs, Switzerland).

HPLC-grade methanol, acetonitrile, and water were purchased from Carlo Erba (Milan, Italy). Other chemicals used were of reagent grade or better. Wistar rats were obtained from Morini (Milan, Italy).

Table 1

Yield of Derivatization Reaction of PA, DAP (IS), and DAE (IS) with 2,5-DHBA

	Procedure A	Procedure B
2,5-DHB-DAE	80%	65%
2,5-DHB-DAP	85%	55%
2,5-DHB-PUT	86%	72%
2,5-DHB-CAD	88%	75%
2,5-DHB-SPD	65%	40%
2,5-DHB-SPM	62%	48%

Synthesis of Polyamine Standards, Electroactive Derivatives 2,5-DHB-PA (2,5-DHB-PUT, 2,5-DHB-CAD, 2,5-DHB-SPD and 2,5-DHB-SPM) and ISs (2,5-DHB-DAE and 2,5-DHB-DAP)

2,5-DHB-PA and ISs were obtained according to the following procedures.

Procedure A

A solution of a suitable polyamine (PUT, CAD, SPD, SPM) or ISs (DAE, DAP) 1.1 mmol in 10 mL of $CHCl_3$ was added slowly to 2,5-DHBA (2.2 mmol) in 5 mL of $CHCl_3$. The mixture was stirred at room temperature (21°C) for 1h, and 2,5-DHB-PA was separated as precipitate.

The reaction of 2,5-DHB-PUT, 2,5-DHB-CAD, 2,5-DHB-DAE, and 2,5-DHB-DAP was checked by TLC using hexane:AcOEt (3.5:6.5 v/v) as eluent, while for 2,5-DHB-SPD and 2,5-DHB-SPM we used NH_3:MeOH:AcOEt (0.5:1.5:8 v/v/v) as eluent. The precipitate was removed by filtration, washed with $CHCl_3$ and dried under vacuum. The yields, for each PA are reported in Table 1.

Procedure B

A solution of 2,5-DHBA (2.2 mmol) in 2 mL of absolute ethanol was added to glacial CH_3COOH (2.2 mmol). Subsequently, suitable polyamines (PUT, CAD, SPD, SPM) or ISs (DAE, DAP) 1.1 mmol were added. The mixture was stirred at 60°C for 2h and 2,5-DHB-PUT, 2,5-DHB-CAD, 2,5-DHB-DAE, and 2,5-DHB-DAP were checked by TLC using as eluent hexane:AcEt (3.5:6.5 v/v), while NH_3:MeOH:AcOEt (0.5:1.5:8 v/v/v) was used as eluent for 2,5-DHB-SPD and 2,5-DHB-SPM. The precipitate was removed by filtration, washed with absolute ethanol, and dried under vacuum. The yields, for each PA, are reported in Table 1.

2,5-DHB-DAE

Yellow crystal, UV: 345nm, ε ($M^{-1}cm^{-1}$) 7225; 256 nm, ε ($M^{-1}cm^{-1}$) 13800; I.R. (KBr, cm^{-1}) 3278 (OH), 1635 (C=N); ^{1}H-NMR (DMSO), δ 8.47 (s, 2H, CH=N); 6.79-6.62 (m, 6H, Ar); 3.87-3.35 (m, 4H, CH_2-N); MS (m/z): 300 (M^+), 245, 174, 136.

2,5-DHB-DAP

Red crystal, UV: 348nm, ε ($M^{-1}cm^{-1}$) 6825; 254nm, ε ($M^{-1}cm^{-1}$) 13878; I.R. (KBr) cm^{-1} 3279 (OH), 1638 (C=N); ^{1}H-NMR (CD_3OD), δ 8.37 (s, 2H, CH=N); 6.80-6.69 (m, 6H, Ar); 3.64-3.32 (m, 4H, CH_2-N); 1.83-1.72 (m, 2H, CH_2); MS (m/z): 314 (M^+), 265, 204, 182, 107, 83.

2,5-DHB-PUT

Red crystal; UV: 343nm, ε ($M^{-1}cm^{-1}$) 7583,3; 255nm, ε ($M^{-1}cm^{-1}$) 16083; I.R. (KBr cm^{-1}) 3200 (OH), 2845 (CH_2), 1600 (C=N); ^{1}H-NMR (CD_3OD), δ 8.35 (s, 2H, CH=N); 6.81-6.7 (m, 6H, Ar); 3.65-3.35 (m, 4H, CH_2-N); 1.82-1.80 (m, 4H, CH_2-CH_2); MS (m/z): 328 (M^+), 191, 137, 81, 69.

2,5-DHB-CAD

Red crystal; UV: 343nm, ε ($M^{-1}cm^{-1}$) 7139; 255nm, ε ($M^{-1}cm^{-1}$) 15085.5; I.R. (KBr cm^{-1}) 3280 (OH), 2940 (CH_2), 1649 (C=N); ^{1}H-NMR (CD_3OD), δ 8.32 (s, 2H, CH=N); 6.80-6.67 (m, 6H, Ar); 3.63-3.59 (m, 4H, CH_2-N); 1.80-1.71 (m, 4H, CH_2-CH_2); 1.53-1.47 (m, 2H, CH_2); MS (m/z): 341 (M^+), 323, 165, 137, 83.

2,5-DHB-SPD

Orange crystal; UV: 346nm, ε ($M^{-1}cm^{-1}$) 6512; 256nm, ε ($M^{-1}cm^{-1}$) 15243.9; I.R. (KBr cm^{-1}) 3285 (OH), 2845 (CH_2), 1620 (C=N); ^{1}H-NMR (CD_3OD), δ 8.32 (s, 2H, CH=N); 7.87 (s, 1H, NH); 6.80-6.68 (m, 6H, Ar); 2.73-2.68 (m, 4H, CH_2-N); 2.65-2.60 (m, 4H, CH_2-NH-CH_2); 1.89-1.84 (m, 2H, CH_2); 1.75-1.55 (m, 4H, CH_2-CH_2); MS (m/z): 385 (M^+), 343, 328, 201, 137, 110, 81.

2,5-DHB-SPM

Yellow crystal; UV: 347nm, ε ($M^{-1}cm^{-1}$) 5078; 230nm, ε ($M^{-1}cm^{-1}$) 17120.4; I.R. (KBr cm^{-1}) 3282 (OH), 2945 (CH_2), 1623 (C=N); ^{1}H-NMR (CD_3OD), δ 8.29 (s, 2H, CH=N); 6.21-6.45 (m, 6H, Ar); 3.68-3.63 (m, 4H, CH_2-N); 3.02-2.98 (m, 4H, CH_2-NH); 2.93-2.89 (m, 4H, CH_2-NH-CH_2); 2.07-2.00 (m, 4H, CH_2); 1.74-1.70 (m, 4H, CH_2-CH_2); MS (m/z): 442 (M^+), 425, 320, 265, 198, 137.

Standard Solutions

Standard solutions of 2,5-DHB-PA, 2,5-DHB-DAE and 2,5-DHB-DAP in the concentration range 80-240 ng/mL were prepared, diluting known amounts of acetonitrile stock solutions, (1μM/mL of the species), and analyzed by HPLC-ECD.

Optimization of the Derivatization Procedure

The derivatization was achieved by adding a solution of the individual PA and ISs in $CHCl_3$ to a solution of 2,5-DHBA in $CHCl_3$. The molar ratio between 2,5-DHBA and PA was fixed at 3:1. The reaction mixture was stirred at room temperature (21°C). After cooling an appropriate amount of the reaction mixture was diluted with acetonitrile and monitored by HPLC-ECD. To establish the optimum derivatization time, samples were taken at appropriate time intervals and immediately analyzed.

HPLC Analysis

Method A

Separations of 2,5-DHB-DAP, 2,5-DHB-PUT, 2,5-DHB-CAD were performed on a Hypersil C18 5μ (15 cm x 4.6 mm), with a direct connected guard column (Hypersil C18 5μ 4 mm x 10 mm) and eluted, isocratically with methanol:acetonitrile:potassium phosphate buffer 0.1 M at pH 7.5 with triethylamine (TEA) (25:25:50 v/v/v).

The mobile phase was filtered through GS-type filters (0.22 μm, Millipore, Bedford, MA, USA) and degassed on-line with a Model ERC-331 1 solvent degasser (Erma, Tokyo, Japan). Chromatography was performed at room temperature (21°C) with a flow-rate of 1.0 mL/min.

Method B

Separations of 2,5-DHB-SPD and 2,5-DHB-SPM were performed on a Hypersil CPS1 5μ (25 cm x 4.6 mm) and eluted isocratically, with methanol:TEA 1% solution at pH 7.45 with H_3PO_4 (85% p/v) (86:14 v/v).

The mobile phase was filtered through GS-type filters (0.22 μm, Millipore, Bedford, MA, USA) and degassed on-line with a Model ERC-331 1 solvent degasser (Erma, Tokyo, Japan). Chromatography was performed at room temperature (21°C) with a flow-rate of 1.0 mL/min.

Optimization of Electrochemical Detection

In order to optimize the detection of the electroactive derivatives, several parameters were examined: oxidation potential, hydrodynamic voltammogram, pH, and the ionic strength of the eluent.

Animals

Ninety male Wistar rats weighing 100-120 g were purchased from Morini (Milan, Italy). The rats were housed in a pathogen-free environment and fed a standard diet and allowed free access to water. The animals fasted over-night before surgery. During the experimental procedure for inducing cerebral ischemia the rectal body temperature was continuously monitored and maintained at 37.5°C throughout using a rectal probe.

Induction of Cerebral Ischemia and Post Ischemic Reperfusion

Experiments were performed on male Wistar rats, which possess the Willis polygon, where the bilateral clamping of the common carotid arteries results in a partial rather than total cerebral ischemia. The rats were anaesthetized by an i.p. injection of ethyl urethane (1.2 g/Kg b.w.) and made ischemic by bilateral clamping of the common carotid arteries for 20 min followed by 5h of reperfusion.

Ischemia was evaluated by measuring lactate levels according to Noll's method.[32]

Sample Preparation and Extraction Procedure

The animals, submitted to 20 min partial cerebral ischemia followed by 5h reperfusion, were killed by decapitation and the cerebral cortex was rapidly removed in the cold room and homogenized in phosphate buffer (5mM, pH 7, 1:4 w/v). The homogenate was added to an appropriate amount of DAP (IS), deproteinized by perchloric acid (5% v/v final concentration) and centrifuged at 10,00 x g for 15 min.

After centrifugation, the supernatant was neutralized (NaOH, 7M), lyophilized and stored at –80°C until use. Lyophilized and neutralized samples were measured after pre-column derivatization with 2,5-DHBA. After 1h incubation at room temperature an aliquot of the reaction mixture was filtered through a 0.45 μm membrane and then 5 μL injected into HPLC. Polyamine levels were expressed as nmoli/mg prot.

Protein Assay

Protein content was evaluated according the method of Lowry et al.[33]

Recovery and Precision

The recovery efficiency was evaluated in control animals. The tissue homogenate was split into two aliquots. The first aliquot was further divided in three parts. Different quantities of PA (0.1, 1, and 10 nm/mg protein) were added to each of these parts. The second homogenate aliquot was used to determine the protein and the basal levels of the polyamines that were subtracted from the values found in the first aliquot. The precision of the recovery was determined by repeating the procedure 6 - 10 times and calculating the RSD.

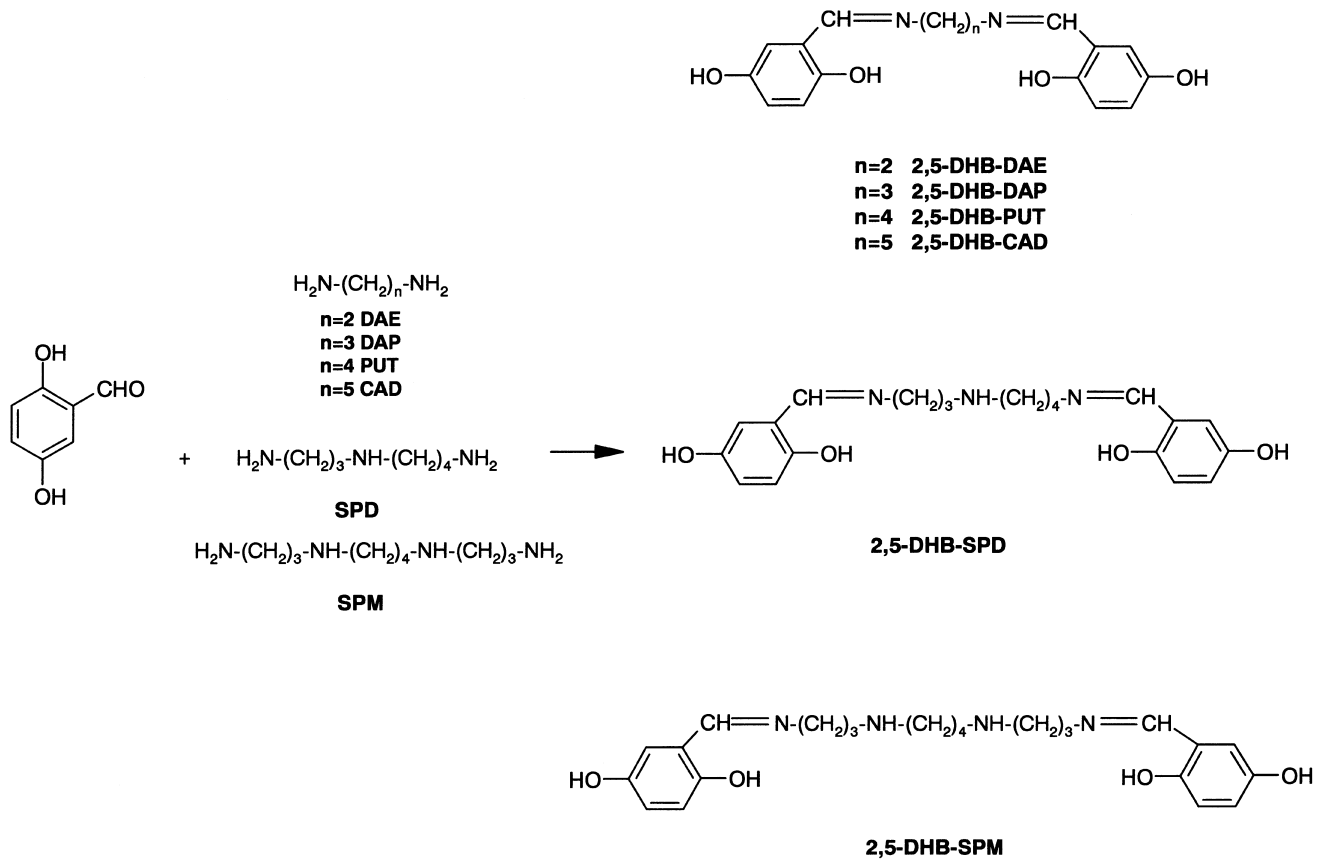
Statistical Analysis

Data are presented as means ± standard deviation. Two-side Student's t-test of differences between two sample means was used to assess the significance between groups. The significance level was set at $p < 0.05$.

RESULTS AND DISCUSSION

Stability and Separation of 2,5-DHB-PA

Electroactive derivatives were stable at pH values between 5.5 to 9.0. The typical acidic Shiff's bases reaction performed at pH lower than 5.5 (procedure B), is not efficient because the acidic pH promotes the degradation of the compounds. Whereas, when the derivatization of the PA was conducted in chloroform (Procedure A) an increased yield was observed (Table 1). In order to obtain an optimal chromatographic separation, different mobile phases and columns were evaluated. All attempts to obtain the contemporaneous isocratic elution of the PA in the same chromatographic run were unsuccessful (data not shown). DAP was used as internal standard because its electroactive derivative 2,5-DHB-DAP gave better chromatographic separation compared to 2,5-DHB-DAE. The chromatographic condition described in method A ensured an optimal separation of 2,5-DHB-PUT, 2,5-DHB-CAD, and 2,5-DHB-DAP. In this chromatographic condition 2,5-DHB-SPM and 2,5-DHB-SPD eluted after 30 min with a strong tailing and some degradation. We speculate that the lower stability of 2,5-DHB-SPD and 2,5-DHB-SPM is probably due to unreacted secondary amino groups[25] that are able to catalyze the hydrolysis of the iminic group. To improve the stability of these derivatives, therefore, we developed another chromatographic method for the separation of 2,5-DHB-SPD and 2,5-DHB-SPM (Method B). In this Method 2,5-DHB-SPD, 2,5-DHB-SPM, did not undergo substantial degradation and eluted within 15 min while 2,5-DHB-DAE,



Scheme 1. Derivatization reaction of PA, DAP (I.S.) and DAE (IS) with 2,5-DHBA to give the electroactive derivatives 2,5-DHB-PA, 2,5-DHB-DAP, 2,5-DHB-DAE.

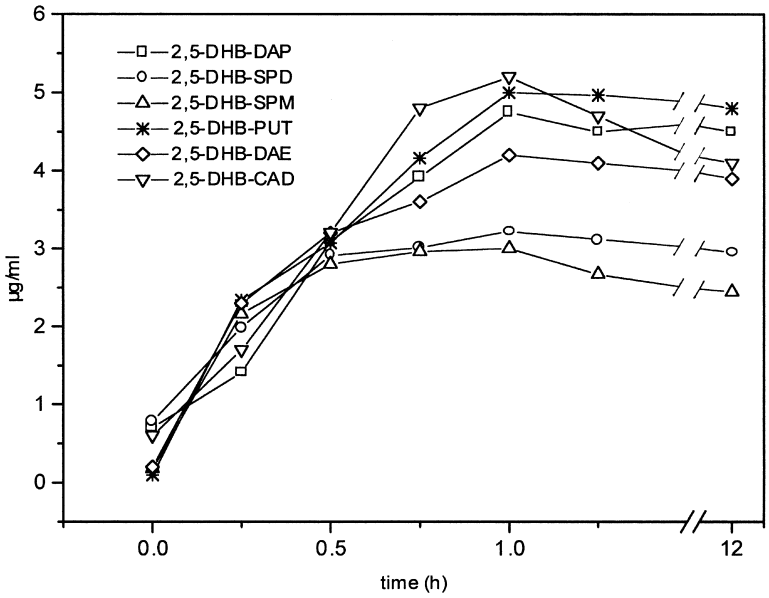


Figure 1. Curve of concentration of electroactive derivatives 2,5-DHB-PA, 2,5-DHB-DAP, and 2,5-DHB-DAE versus time.

2,5-DHB-DAP, 2,5-DHB-PUT, and 2,5-DHB-CAD eluted with a very small retention factor. The simultaneous elution of all the PA and IS would be possible under gradient conditions but this technique it is not ideal for ECD detection due to the strong baseline drift. On the other hand, some authors[3,34] have shown that PUT levels increased during post-ischemic reperfusion and that cellular necrosis is correlated to PUT and CAD levels. Therefore, we decided to focus our attention on PUT and CAD quantification, using method A for routine analysis and method B to check eventual variations of SPM and SPD concentration.

Optimization of the Derivatization Procedure

The scheme represents the derivatization reaction of PA, DAE, and DAP with 2,5-DHBA to give 2,5-DHB-PA, 2,5-DHB-DAE, and 2,5-DHB-DAP. The electroactive derivatives of the polyamine standards were obtained in one step (Procedure A). Experiments were performed to determine the optimum derivatization time to obtain maximum conversion of the polyamines. Figure 1 shows the trend of the derivatization reaction, which was complete after 60 min. The synthesized Schiff's bases showed stability in the reaction mixture up to 12h after the optimum.

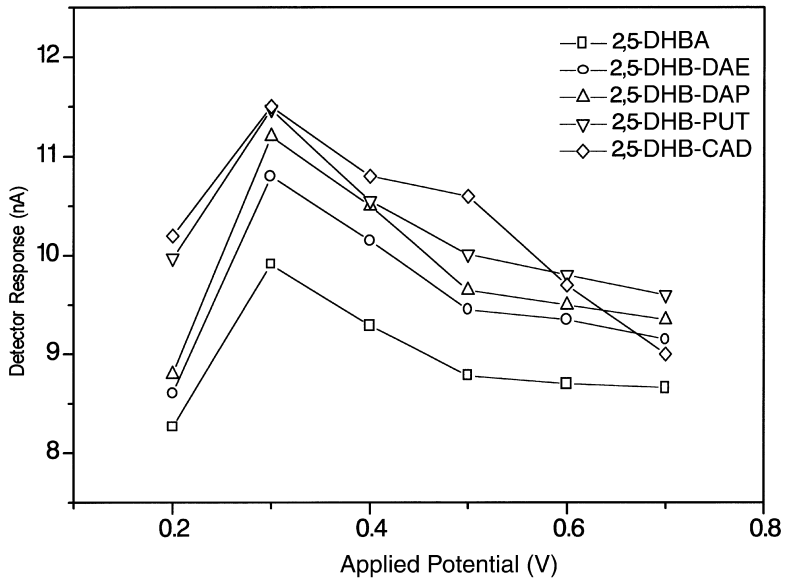


Figure 2. Hydrodynamic voltammograms of the 2,5-DHB-PUT, 2,5-DHB-CAD, 2,5-DHB-DAP, and 2,5-DHB-DAE with Method A.

Optimization of Separation and Detection

Several parameters were examined in order to optimize the electrochemical detection of the electroactive compounds. Under the chromatographic conditions described above (Method A), the derivatives responded at ECD oxidation potentials higher than + 0.2 V. Enhanced signals were obtained as the working potential was increased from + 0.2 V to + 0.7 V. With additional applied potential, no further increases in iminic peak height occurred and a rise in the background current was observed. Electroactive properties of the compounds 2,5-DHB-PUT, 2,5-DHB-CAD, 2,5-DHB-DAP, and 2,5-DHB-DAE were examined using their hydrodynamic voltammograms (Fig. 2). Figure 2 indicates that the best potential is + 0.3 V because, for higher potentials, the response detected decreases progressively for all electroactive derivatives.

The ECD performance separation of 2,5-DHB-PUT, 2,5-DHB-CAD, and 2,5-DHB-DAP was markedly influenced by the ionic strength and by the pH of the mobile phase. With increasing concentrations of the potassium phosphate buffer (from 0.05M to 0.1M), an increase in the response was observed. No significant improvement in detector response was achieved by further increase

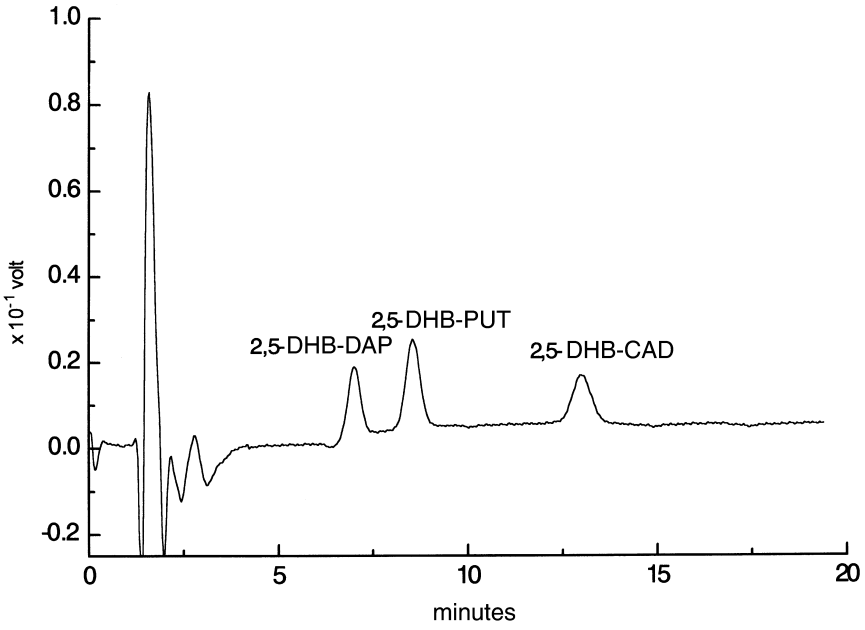


Figure 3. Typical HPLC-ECD chromatogram of 5 μL standard mixture of electroactive derivatives: 2,5-DHB-PUT (9.0 min), 2,5-DHB-CAD (13.5 min), and 2,5-DHB-DAP (7.5 min).

in the potassium phosphate molarity, which was consequently fixed at 0.1 M and pH 7.5. In fact, we observed that the peak areas decrease at pH values outside the 5.5 - 9.0 range. The chromatographic separation of 2,5-DHB-SPD and 2,5-DHB-SPM (Method B) was examined using their hydrodynamic voltammograms. The best potential is + 0.3 V because for higher potentials the response decreased progressively for all electroactive derivatives. Also, here we obtained the same trend in terms of pH observed in Method A, and therefore we used a mobile phase consisting of the methanol:TEA 1% solution pH 7.45 with H_3PO_4 (86:14 v/v).

Chromatography A

Figure 3 shows a representative HPLC chromatogram with ECD detection of the standards. Chromatographic separations were carried out under isocratic reversed-phase conditions on a Hypersil C18 column. The mobile phase consisted of the ternary mixture methanol:acetonitrile:potassium phosphate 0.1 M at pH 7.5 with TEA (25:25:50 v/v/v), at a flow-rate of 1 mL/min with an injection volume of 5 μL. The analysis was completed within 15 min and the

retention times were 7.5 min, 9.0 min, and 13.5 min for 2,5-DHB-DAP, 2,5-DHB-PUT, and 2,5-DHB-CAD, respectively. The chromatographic system gave a good separation of these electroactive derivatives.

This method is applicable to rat cerebral cortex and it does not present interference with endogenous substances containing amino groups.

Chromatography B

Chromatographic separations of 2,5-DHB-SPD and 2,5-DHB-SPM were carried out with isocratic elution on a Hypersil cyano CPS1 column. The mobile phase consisted of the mixture methanol: TEA 1% solution pH 7.45 with H_3PO_4 (86:14 v/v), at flow-rate of 1 mL/min with an injection volume of 5 μL. The analysis was completed within 15 min and the retention times were 6.6 min, and 10.8 min for 2,5-DHB-SPD, and 2,5-DHB-SPM, respectively.

Linearity and Detection Limit

The linearity of response was examined for each 2,5-DHB-PA, analyzing 5 solutions in the range 80-240 ng/mL. The coefficients of linear regression of the standard curves were consistently greater than 0.99.

Detection limit was determined using progressively lower concentrations of electroactive derivatives for a signal/noise ratio of 5:1 (n=5) with an injected volume of 5 μL. The lower limit of detection was less than 80 fmol for each PA.

Recovery and Precision

The accuracy of the assay was determined by repetitive analysis of control tissue homogenates spiked with PA standards at 0.1, 1.0, and 10.0 nm/mg of protein. An aliquot of homogenate was used to assess the basal level of the different PA and the protein amount. The accuracy of the assay was evaluated by comparing the measured concentration, subtracted from the corresponding value of basal polyamine, to its known value. The repeatability of the method was evaluated by replicating analyses and is expressed as RSD. The results obtained are reported in Table 2.

Polyamines Levels in Rat Cerebral Cortex

We confirmed that bilateral clamping of the carotid arteries induced cerebral ischemia, as demonstrated by a significant increase in lactate levels in ischemic animals when compared to sham-operated controls.

Table 2

Recovery and Precision in the Analysis of PA

Compound	Known Conc. nmol/mg Prot.	Accuracy Mean ± SD[a]	RSD (%)[a]
PUT[b]	0.1	97.5 ± 3.2	3.85
	1	98.2 ± 2.8	2.98
	10	99.8 ± 1.6	2.20
CAD[b]	0.1	96.8 ± 4.1	4.10
	1	97.3 ± 3.1	3.85
	10	98.6 ± 1.5	2.98
SPD[c]	0.1	89.6 ± 4.2	4.11
	1	90.2 ± 3.1	3.84
	10	92.1 ± 1.9	2.98
SPM[c]	0.1	90.1 ± 3.2	3.65
	1	92.3 ± 2.8	3.51
	10	94.6 ± 1.7	3.05

[a] n = 5. [b] = Method A. [c] = Method B.

The homogenate, from rat cerebral cortex, was subjected to derivatization with 2,5-DHBA and HPLC analysis using the proposed ECD system. The high sensitivity achieved by ECD monitoring allowed an accurate quantification of the PUT levels in cerebral cortex of post-ischemic reperfused rats.

Figure 4 shows a typical chromatographic separation by HPLC-ECD of 2,5-DHB-PUT and 2,5-DHB-DAP from cerebral cortex of post-ischemic reperfused rats.

In the cerebral cortex of rats subjected to ischemia, and following reperfusion, we noted a significant increase of PUT levels 0.86 ± 0.25 nmol/mg prot. ($p < 0.05$) compared to control levels 0.15 ± 0.019 nmol/mg prot. ($p < 0.05$), while SPD and SPM showed no significant variation ($p > 0.05$) when compared to controls 6.35 ± 0.71 and 7.09 ± 0.42 nmol/mg prot., respectively. In this biological condition, no CAD was found.

The significant increase of PUT levels in the rat cerebral cortex during post-ischemic reperfusion confirms that ODC activity and PUT levels may contribute to ischemia-related delayed neuronal death. The lack of CAD is correlated to the absence of necrosis.

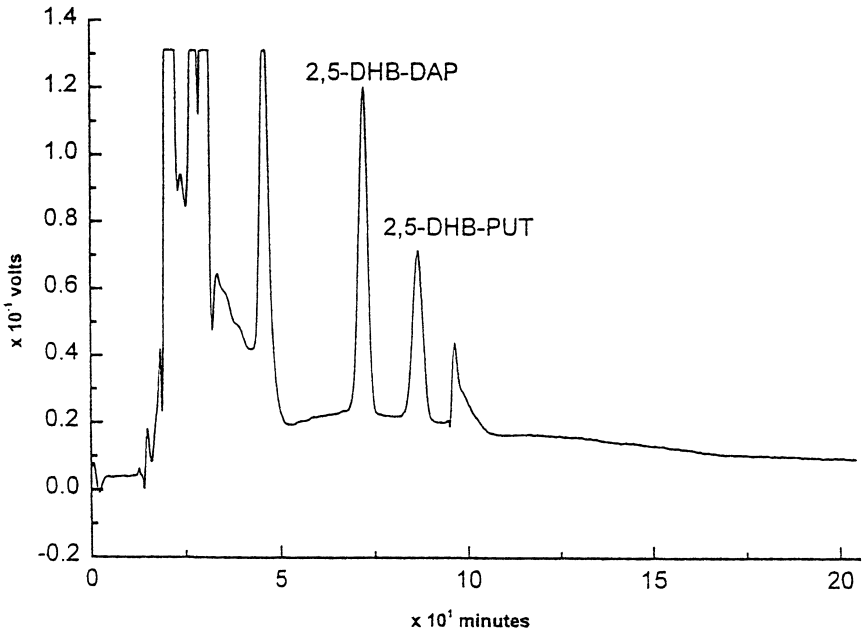


Figure 4. Representative chromatogram of 2,5-DHB-PUT (9.0 min) and 2,5-DHB-DAP (7.5) in cerebral cortex of post-ischemic reperfused rats.

CONCLUSIONS

The derivatization of PA with 2,5-DHBA yields stable sensitive electroactive derivatives, which are detectable by HPLC-ECD. The sensitivity obtained here was higher than that of methods reported in literature. The applied potential permits the selective oxidation of iminic derivatives without interference from compounds present in the complex biological matrix given the limited number of substances which can undergo redox reactions under these conditions. The chromatographic procedures are suitable for the separation of putrescine and cadaverine from animal tissues. Because of the simplicity and ease of operation, this method is applicable in both research and clinical laboratories.

ACKNOWLEDGMENT

This work was supported by the Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST), Rome, Italy.

REFERENCES

1. R. J. Dempsey, M. W. Roy, D. E. Cowen, D. J. Combs, Neurol. Res., **10**, 141-144 (1987).

2. W. Pashen, G. Rohn, C. O. Meese, B. Djuricic, R. Schmid-Kastner, Brain Res., **343**, 9-16 (1988).

3. W. Pashen, R. Schmid-Kastner, J. Hallmayer, B. Djuricic, Neurochem. Pathol., **9**, 1-20 (1988).

4. R. Ransom, N. Stec, J. Neurochem., **51**, 830-836 (1988).

5. C. Carter. **The Neuropharmacology of Polyamines**, Academic Press Ltd., London, 1984.

6. P. Kleihues, K. A. Hossmann, A. E. Pegg, K. Kobayoshi, V. Zimermann, Brain Res., **95**, 61-73 (1975).

7. N. Seiler, B. Knodgen, J. Chromatogr., **164**, 155-168 (1979).

8. F. Bartos, D. Bartos, D. P. Grettie, R. A. Campbell, L. J. Marton, R. G. Smith, G. D. Daves Jr., Biochem. Biopys. Res. Commun., **75**, 915-919 (1977).

9. M. D. Denton, H. S. Glazer, D. C. Zellner, F. G. Smith, Clin. Chem., **19**, 904-907 (1973).

10. A. G. Giumanini, G. Chiavari, F. L. Scarponi, Anal. Chem., **48**, 484-489 (1976).

11. G. Taibi, M. R. Schiavo, J. Chromatogr. B. Applic., **614**, 153-158 (1993).

12. M. Marcè, D. S. Brown, T. Capell, X. Figueras, A. F. Tibucio, J. Chromatogr. B. Applic., **66**, 329-335 (1995).

13. S. Asotra, P. V. Mladenov, R. D. Burke, J. Chromatogr. **408**, 227-233 (1987).

14. N. E. Newton, K. Ohno, M. M. Abdel-Monem, J. Chromatogr., **124**, 277-285 (1976).

15. N. Seiler, B. Knodgen, F. Eisenbeiss, J. Chromatogr., **145**, 29-39 (1978).

16. J. E. Folk, M. H. Park, S. I. Chung, J. Schrode, E. P. Lester, H. L. Cooper, J. Biol. Chem., **255**, 3695-3700 (1980).

17. B. Kneip, M. Raymondjean, D. Bogdanovsky, L. Bacner, G. Shapira, J. Chromatogr. **162**, 547-559 (1979).

18. L. J. Marton, D. H. Russell, C. C. Levy, Clin. Chem., **19**, 923-926 (1973).

19. H. Tabor, C. W. Tabor, F. Irreverre, Anal. Biochem., **55**, 457-467 (1973).

20. C. W. Gehrke, K. C. Kuo, R. L. Ellis, T. P. Waalkes, J. Chromatogr., **143**, 345-361 (1977).

21. L. J.Marton, P. L. Y. Lee, Clin. Chem., **21**, 1721-1724 (1975).

22. G. G. Shaw, I. H. S. Al-Deen, P. M. Elworthy, J. Chromatogr. Sci., **18**, 166-170 (1980).

23. J. R. Shipe Jr. , J. Savory, Ann. Clin. Lab. Sci., **10**, 128-136 (1980).

24. F. Perini, J. B. Sadow, C. V. Hixson, Anal. Biochem., **94**, 431-439 (1979).

25. P. G. Zambonin, A. Guerrieri, T. Rotunno, F. Palmisano, Anal. Chim. Acta, **251**, 101-107 (1991).

26. K. Mopper, D. Delmos, Anal. Chem., **56**, 2557-2560 (1984).

27. D. L. Hogan, K. L. Kraemer, J. I. Isenberg, Anal. Biochem., **127**, 17-24 (1982).

28. D. C. Turnell, J. D. H. Cooper, Clin. Chem., **28**, 527-531 (1982).

29. S. Tirendi, O. Prezzavento, F. Tateo, E. Bousquet, J. Chromatogr., **18**, 1933-1945 (1995).

30. A. M. Krstulovic, H. Colin, G. A. Guiochon, "Electrochemical Detectors for Liquid Chromatography," in **Advances in Chromatography**, J. C. Giddings, E. Gruska, J. Cazes, P. R. Brown, eds, Marcel Dekler, Inc., New York, 1984, vol. 24, pp 301-332.

31. E. Bousquet, V. Cavrini, R. Gatti, A. Spadaro, J. Chromatogr., **21**, 2873-2866 (1998).

32. F. Noll, **Methods of Enzymatic Analysis**, Vol. 6., H. U. Bergmeyer, ed., Verlag Chemie, Weinheim & Basel.

33. O. H. Lowry, N. J. Rosebrogh, A. L. Farr, R. G. Randal, J. Biol. Chem. **193**, 265-275 (1951).

34. C. Di Giacomo, V. Sorrenti, R. Acquaviva, A. Campisi, G. Vanella, J. R. Perez-Polo, A. Vanella, Neurochem. Res., **22**, 1145-1150 (1997).

Received January 25, 1999
Accepted April 26, 1999
Manuscript 4960

Request Permission or Order Reprints Instantly!