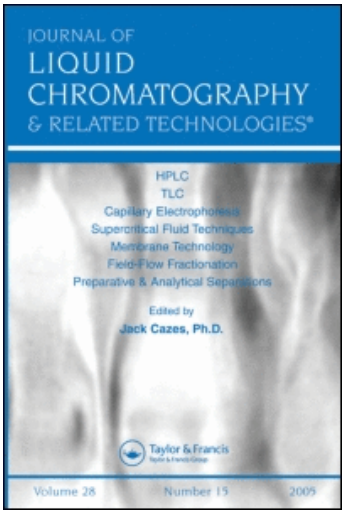

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Morier-teissier, E. , Drieu, K. and Rips, R.(1988) 'Determination of Polyamines by Pre-Column Derivatization and Electrochemical Detection', Journal of Liquid Chromatography & Related Technologies, 11: 8, 1627 — 1650
To link to this Article: DOI: 10.1080/01483918808076726
URL: http://dx.doi.org/10.1080/01483918808076726

JOURNAL OF LIQUID CHROMATOGRAPHY, 11(8), 1627-1650 (1988)

DETERMINATION OF POLYAMINES BY PRE-COLUMN DERIVATIZATION AND ELECTROCHEMICAL DETECTION

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ABSTRACT

Precolumn derivatization with o-phtalaldehyde (OPA) and thiol, combined with HPLC was used to measure the polyamines spermine, spermidine, putrescine and cadaverine. The derivatives were quantified by electrochemical detection instead of fluorescence; the optimum potential from the working electrodes was + 0.65 V. Best results were obtained with a derivatization mixture containing excess OPA and thiol, methanol and borate buffer, pH 9.5 . Spermine gave two derivative peaks; the broad minor peak seemed to be an intermediate which could be converted to the major derivative by increasing either the methanol content of the derivatization mixture or the reaction time. The best reaction time was 10 min ; the detector response was linear from 1 pmole to 10 nmoles and the sensitivity limit was from 200 fmoles for spermidine to 1.5 pmoles for spermine. The column retention times could be reduced by the addition of methanol or dimethylcyclohexylamine (DCA) to the eluent. Substitution of

H_3PO_4 by $HClO_4$ improved peak quality. The best elution of standards was obtained with $HClO_4$/DCA and 80 % methanol. A gradient from 60 to 90 % methanol gave improved separation when required for biological samples.

INTRODUCTION

Polyamines appear to be intimately involved in the processes of cell growth and maturation, especially in tissues which actively synthesise RNA and protein. For example, polyamine levels are very high in human and mouse foetal tissues, but decrease rapidly after birth (1, 2). Similarly, there is evidence that measurement of polyamines in pathological tissue specimens can be useful in the diagnosis of cancers and in the assessment of anticancer treatments (3, 4).

Polyamines may be of particular importance in the central nervous system. Although there is little direct evidence of their functional role, they are present in high concentrations (5). There is a high affinity uptake for spermine in the rat cerebral cortex and an active transport of spermine in the cerebral tissue (6). Newly synthesized polyamines may serve as intracellular messenger in Ca^{++} dependent release of neurotransmitters (7). Moreover a "neurotransmitter" or "neuromodulator" role for spermine has been discussed (6). Uptake of choline and of dopamine are inhibited by spermine and by spermidine (8). L-Dopa, precursor of dopamine and noradrenaline, cause significant elevations of brain putrescine levels in L-Dopa treated groups of rats (9).

Relations are even more evident between polyamines and γ -aminobutyric (GABA), a metabolic pathway from putrescine to GABA has been described since 1974 (10) aside from the

classical decarboxylation of glutamic acid. Some agents which modify the metabolism of GABA also modify the concentration of putrescine (11).

To study the concommitant variations of polyamines and GABA in response to neuropharmacological agents, we developed a method allowing their measurement in fragment of animal brains as we did for catecholamines (12) and for GABA (13).

MATERIAL AND METHODS

1. Chromatographic System

The HPLC system included two Spectroflow 400 pumps (Kratos, Ramsey, NJ, USA) and a gradient control (Kratos) with a Spectroflow 480 manual injector (Kratos). The 250 x 4.6 mm Spherisorb Sup, RS (Prolabo, Paris, France) column contained a ODS 2 phase with 5 µm particle size. The BAS LC4 (West Lafayette, Ind, USA) electrochemical detector was fitted with a vitreous carbon working electrode and a Ag/AgCl reference electrode. The potential used was 0.65 V, the sensitivity was 5 to 50 nA and the output was 1 V.

2. Reagents

Ortho-phtalaldehyde (OPA) (Janssen, Beerse, Belgium)
Ethanethiol (ESH) (Merck-Schuchard, Darmstadt, Germany)
Mercaptoethanol (MSH) (Prolabo, Paris, France)
t-Butylthiol (Janssen, Beerse, Belgium)
Putrescine dihydrochloride (Put) (Sigma, St Louis, MO)

Cadaverine dihydrochloride (Cad) (Sigma, St Louis, MO)
Spermidine trihydrochloride (Spd) (Sigma, St Louis, MO))
Spermine tetrahydrochloride and spermine base (Spm) (Sigma)
1,6-Hexane diamine dihydrochloride (HDA) (Aldrich, Strasbourg, France)
N-N, dimethylcyclohexylamine (DCA) (Merck-Schuchard, Darmstadt, Germany)
Other compounds are pure grade (orthophosphoric acid, perchloric acid, methanol, ethanol, acetonitrile).

3 Derivatization Reagent

Three reagents were used :

- Skaaden and Greibrokk reagent.
- Reagent A : 10 mg OPA in 9 ml methanol, 50 µl of thiol (ESH, MSH or t-butylthiol), plus 1 ml of a 0.1 M potassium tetraborate buffer prepared by dissolving 0,78 g of boric acid in 100 ml water and bringing to pH 9.5 with 2N NaOH.
- Reagent B : 10 mg OPA in 1 ml methanol, 50 µl thiol, plus 9 ml of borate buffer.

All reagents were prepared fresh each week.

4. Standard

The polyamines stock solutions were 10^{-4} M, dissolved in water or 0.2 M $HClO_4$.

5. Derivatization Reaction

100 µl of stock polyamine solution in water and 100 µl of the OPA-thiol reagent A or B were mixed together and a 20 µl was

injected after 10 min (2 min for reagent B) with a potential of 0.65 V and an intensity of 50 nA.

100 µl of stock solution (or biological extracts) in 0.2 M $HClO_4$ and 100 µl of tetraborate buffer (0.1 M, pH = 9.5) were mixed with 100 µl of the OPA-thiol reagent A and treated as above.

6. Chromatography Eluent

a) Buffer composed of 0.2 M H_3PO_4 and 0.1 M DCA, pH of 3.0 Addition of 80 % MeOH gave an apparent pH of 4.0.

b) 0.1 M H_3PO_4 or 0.1 M $HClO_4$ mixed with 60, 80, 90 % MeOH and adjusted to pH 4 with 1 M NaOH.

c) 0.1 M H_3PO_4 or 0.1 M $HClO_4$ mixed with 60, 80 or 90 % MeOH and adjusted to pH 4 with DCA.

All buffers were filtered and degassed through 0.45 µm filters (Millipore, San Franscico, Ca, USA) before use.

7. Fluorescence Spectra

Spectra were recorded every 10 min between 2 and 60 min with a Jobin-Yvon JY 3D spectrofluorimeter, after that water or 10^{-4} M spermine in water was mixed (vol/vol) with reagent A or reagent B.

8. Biological sample preparation

Male mice (26-30 g, 6 weeks old) were sacrified by decapitation between 9 and 10 a.m. to avoid circadian changes

(14, 15) and the brains were quickly removed from the skull. Brains were washed in cold saline, blotted dry and dissected on ice (brain-stem, cerebellum, hypothalamus, hippocampus, corpus striatum and the remaining part). All samples were stored at -80°C until extraction. Samples were homogenized in a Potter Elvejem containing 500 µl of 0.2 M $HClO_4$ and centrifuged at 8 000 g and 4°C for 10 min. The protein content of the tissue pellets were measured by the Lowry method (16).

RESULTS AND DISCUSSION

1. Electrochemical Detection

Polyamines are not electrochemically active, but the derivatives formed by reaction with the reagent OPA-thiol should have low potentials similar to those of aminoacids; they have not been studied yet.

The best sensitivity was obtained by determining the optimal working potential by plotting intensity vs applied potential (Figure 1). The derivatives of spermidine (Spd), putrescine (Put), cadaverine (Cad) and two derivatives obtained for spermine (Spm A and Spm B) were studied. Each polyamine derivative is detected with a potential as low as 0.3 V, the curves show a sharp maximum at 0.65 V and decrease there after. Similar results were obtained by increasing or decreasing the potential, therefore these results are not due to passivation of electrodes. These variations are different from those of the aminoacids, ornithine, histidine, tryptophane, lysine, tyrosine, arginine and valine (17), glutamic acid and GABA (18). Each aminoacid derivative is detected at a potential as low as 0.3 or

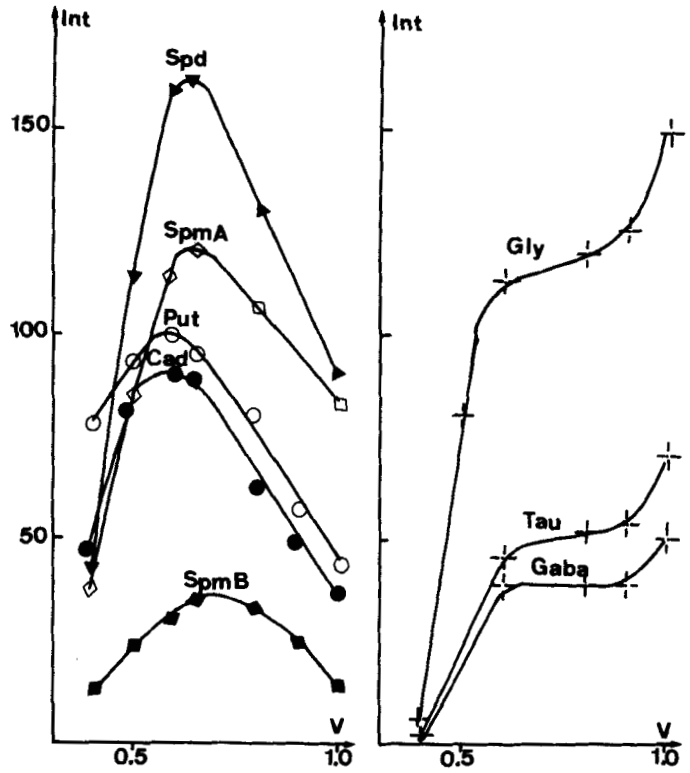

FIGURE 1. Variation of electrochemical activity of derivatives of polyamines or aminoacids and OPA-thiol with working electrode potential.

0.4 V, the response increases rapidly to give a plateau between 0.6 and 0.8 V before increasing again and the same variations were obtained for some of them with our electrochemical system. The optimal potential for polyamines was found to be + 0.65 V.

2. Derivatization of Polyamines

The factors modifying this reaction are :

- the nature and the concentration of the amine,
- the quantity of alcohol,

- the concentration of OPA,
- the nature and the concentration of the thiol,
- the concentration and pH of the borate buffer,
- the reaction time.

The parameters used with a fluorimetric detection by Skaaden and Greibrokk (19) were taken as a point of departure. Each parameter was then varied and its effects noted. All assays were performed using 10 nmole of each of the 4 polyamines.

Influence of Alcohol

Spermine gives two peaks with electrochemical detection system under the conditions indicated by Skaaden and Greibrokk (19) (Table 1, reagent SK): a main sharp peak A (RT = 12 min) and a second wide peak B (RT = 50 min). Spermidine itself have only one sharp peak (RT = 32 min). Peak A corresponds to the one found by Skaaden, the broad peak B is difficult to quantify.

TABLE 1

Proportions of Reagents in the Three Derivatization Mixtures Tested (nmoles)

Reagent	SK	B	A
Amine	2,5	10	10
OPA	750	750	750
ESH	10.000	10.000	10.000
Borate	9.000	9.000	1.000
Water	90 µl	90 µl	10 µl
Alcohol	10 µl	10 µl	90 µl

Preliminary assays show that addition of EtOH, or better, MeOH, to the derivatization medium or increasing the reaction time decreased peak B and increased peak A.

Spermine measurement was optimized by maximizing the production of peak A and noting the changes in the other polyamine derivatives. This was done by substituting methanol for the ethanol of the original Skaaden and Greibrokk reagent and by adding increasing amounts of water or methanol to the derivatization reaction mixture. The reaction was allowed to proceed for 2 or for 10 minutes and the derivatives were chromatographed and quantified (figure 2).

Peak B was formed after 2 min of reaction with a low-methanol mixture and the amount increased with increasing methanol content. However, when reaction was for 10 min, increasing the methanol content decreased peak B and increased peak A. The addition of methanol also increased the production of the other polyamine derivatives; but the corresponding peaks produced after 10 min of reaction were equal to or slightly smaller than those formed after 2 min. Two derivatization mixtures were finally selected, A and B (table 1). Reagent A plus a 10 min reaction was used to obtain spermine peak A together with the three other polyamine derivatives; Reagent B and 2 min was used to obtain spermine peak B or aminoacid derivatives (figure 3).

The composition of the three derivatization mixtures are shown in table 1.

The rationale for these derivatization mixtures is partly based on the data of Simons and Johnson (20), who showed that isoindoles tended to be unstable in aqueous solution, and the results of Skaaden and Greibrokk (19) showing that the stability

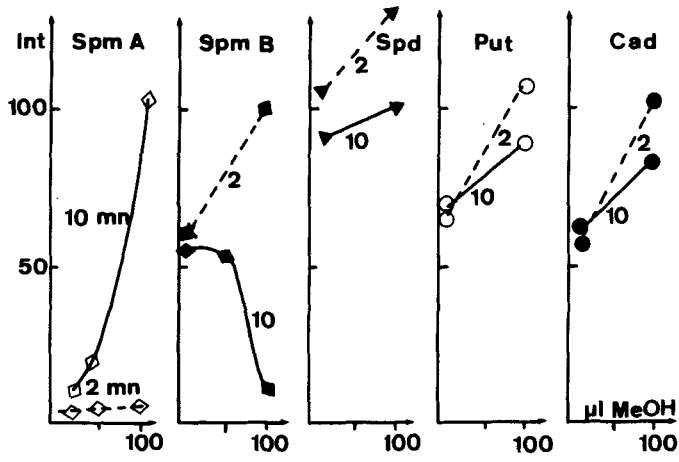

FIGURE 2. Effect of MeOH concentration on the formation of derivatives of polyamines and OPA-thiol. 100 µl of 10^{-4} M solution of polyamine, 100 µl of MeOH or water, 100 µl of Skaaden reagent.

of some OPA-thiol derivatives of polyamines could be increased by replacing 10 % ethanol with 50 % ethanol or propanol. However, the formation of two derivative peaks from spermine is probably a solvation problem. The reaction of this polyfunctional molecule can be incomplete under unfavorable condition, but the addition of methanol, a good solvent for both OPA and isoindole, may favour the formation of the end-product and/or its elution.

Influence of o-Phtalaldehyde and Thiol Concentrations

The OPA/amine ratio in the Skaaden reagent is 300 and the thiol/amine ratio is 4 000. These proportions give good results and assays with OPA and thiol quantities equal to 1/10, 1/2, 2 and 10 times the above give identical results for both the

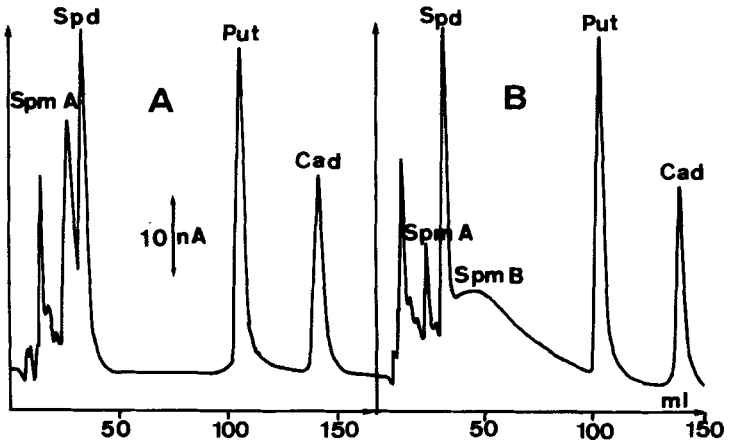

FIGURE 3. Isocratic separation of OPA-ethanethiol derivatives of polyamines with derivatization reagent A or B and phosphoric acid (0.2 M) and DCA (0.1 M) in 80 % methanol pH 4 on ODS-Hypersil (250 x 4.6 mm). 1 nmole of Spm, Spd, Put or Cad, sensitivity 100 nA.

polyamines and the blanks. The litterature values vary from 80 to 700 for OPA/amine and from 300 to 20 000 for thiol/amine. Cooper et al. (21), measuring amino acids, recommended 18/1 for OPA/amine and 2/1 for thiol/OPA. We retained the Skaaden OPA/amine ratio and the large thiol excess as this compound is very volatile. Mercaptoethanol derivatives were found to be less stable than ethanethiol derivatives, t-butylthiol derivatives were not much more stable, and gave high blanks and asymetrical peaks. Moreover the B peak of spermine took longer to be converted to the A peak. Thus, in this study we use ethanethiol.

Influence of pH

Borate buffers with pH values from 6 to 10 have been used to prepare the derivatization mixture. Spermine peak A exhibits

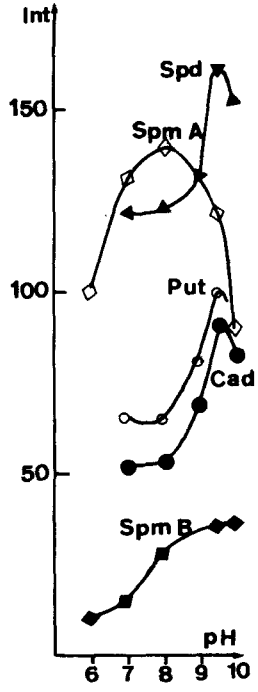

FIGURE 4. Effect of pH on the formation of derivatives of polyamines and OPA-thiol. 100 µl of a 10^{-4} M solution of polyamine, 100 µl of reagent A or B.

an ill defined maximum around pH 8, the three other polyamines (Figure 4) reacted maximally at pH 9.5, which corresponds to the pH proposed by Skaaden (9.4). Lowering the pH produced a marked decrease in the reaction due to protonation of the amine, making it inactive; at higher pH, the reaction yields are slightly lower, excepted for the spermine B derivative. Lysine, with two amino groups, has a maximum between pH 6 and 7, lower than that of other aminoacids (pH 8 -11) (16). Clearly, polyamines behave quite differently from aminoacids.

TABLE 2

pH	Height at 30 min / Height at 10 min %			
	Spm	Spd	Put	Cad
9.5	74	68	77	73
7	95	45	16	25

Isoindoles are less stable in low pH derivatization mixtures and give electrochemically inactive cyclic amides (20, 23). The percentage of degradation is higher at pH 7 than at pH 9.5 when the reaction time is increased from 10 to 30 min. This is particularly evident for putrescine and cadaverine (Table 2).

Kinetic of Degradation

Degradation was established using the conditions described in table 1 (figure 5). The B derivative of spermine is low and completely disappears in less that 10 min. The A derivative reaches its maximum only after 10 to 20 min and retains 74 % of its activity after 30 min. The spermidine peak is maximal at 5 min, those of putrescine and cadaverine are maximal at 2 min and remain at about 70 % after 30 min. None of these activities drop below 50 % after 1 hour.

These degradation kinetics are all for compounds in the presence of the derivatization medium. Degradation should be lower during elution as the compounds are no longer in contact with OPA and thiol (20) and are in a high-methanol medium.

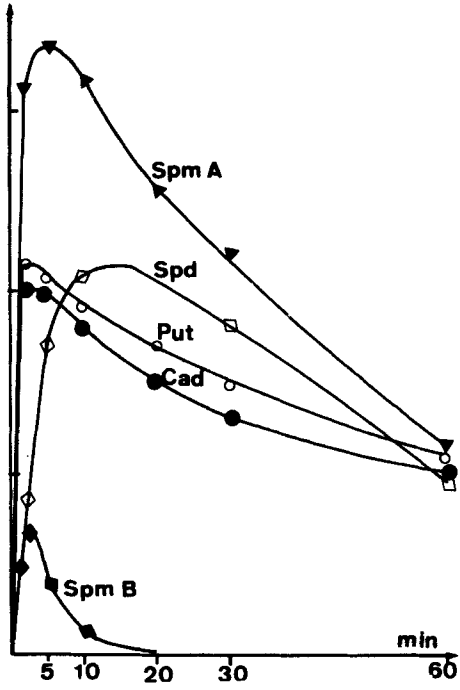

FIGURE 5. Stability of derivatives of polyamines in the derivatization reagent. 100 µl of 10^{-4} M solution of polyamines, 100 µl of reagent A or B.

The reaction time chosen was 10 min so as to obtain only the A derivative of spermine and to produce the best possible activity for each polyamine.

Use of Surfactants

While the addition of Brij increases the fluorescence of the isoindole of a basic aminoacid such as lysine (24), neither Brij or Tween 20 improved the electrochemical detection of polyamine derivatives.

Linearity and Limit of Sensitivity

A good linear relationship ($r = 0.99$) existed between polyamines concentration and the peak height over the range 1 pmole to 10 nmole when the reaction time was carefully controlled. Spermidine gave the best sensitivity, with a signal/noise of 2 for 200 fmoles. It was slightly lower for putrescine and cadaverine and 7 to 8 times lower for spermine.

The limit of sensitivity was 500 fmoles with OPA precolumn derivatization and fluorescence detection (19). It was 120 pmoles for spermidine and 12 pmoles for putrescine with OPA post-column derivatization (25). The detection limit with dansylated derivatives, was 500 fmoles with an ordinary fluorimeter and 25 fmoles with a particularly sensitive fluorometer (26).

Fluorescence Spectra of Spermine Derivatives

Two sets of spectra were recorded at a spermine concentration of 10^{-4} M after derivatization with both reagents A and B for 5, 10, 20, 30 and 60 min.

Both derivative A and derivative B gave fluorescence spectra with maxima at the same wavelenghts and with comparable intensities. Intensity decreased with time for both derivatives and it was not possible to show the transformation of one product into another one.

These spectra are similar to those described for the isoindole formed with propylamine and ethanethiol in ethanol (20).

3. Determination of the Chromatographic Conditions

Derivatives of polyamines with OPA-ethanethiol are very hydrophobic and are eluted on a C18 reverse-phase column in less than one hour if the mobile phase contains more than 80 % methanol. The following assays were performed with a fixed pH of 4.0 obtained with either DCA or NaOH.

Phosphoric Acid and DCA

Polyamine derivatives are eluted as asymetrical peaks with phosphoric acid and NaOH. Their retention times decreased, they become more symetrical and their heights increased as NaOH was progressively replaced by DCA. The two polyamines with secondary amino groups were the most sensitive to this effect. This mobile phase is the one proposed by Skaaden and Greibrokk (19).

Additives

Retention times were not modified by addition of the counter-ion heptane sulfonic acid (1 to 10 mM) or by the replacement of 20 % MeOH by 10 % THF or acetonitrile in the above mobile phase.

Effet of pH of the Mobile Phase

Changes in pH modify both retention times and shapes of the peaks. The retention times of the spermine B derivative decreased sharply with pH. The spermidine and spermine retention times slightly changes whereas those of putrescine and cadaverine derivatives were not affected. This is probably due

to protonation of the secondary amino groups or of an underivatized primary group.

An acidic mobile phase provides better resolution between spermine and spermidine and all peaks, especially the spermine B derivative, gave narrower peaks. At the same time, their stability decreased, the best overall pH was found to be 4.0 .

Phosphoric Acid

Resolution increased when perchloric acid was used in place of phosphoric acid. Addition of DCA still led to decreased retentions times, but the peak shapes were less sensitive to the presence of DCA. Addition of MeOH also led to decreased retention times, which were more marked for putrescine and cadaverine.

The best separation pattern coupled with a good sensitivity for the four polyamine derivatives and for the internal standard 1,6-diaminohexane were obtained with 0.1 M perchloric acid in 80 % MeOH adjusted to pH 4 with DCA (Figure 6). The concentration of $HClO_4$ could, however, not be reduced since this resulted in peak tailing.

4. Estimation of Polyamines in Brain Samples

Extraction

In tissues polyamines are not conjugated and therefore hydrolysis is not necessary. Deproteinization and extraction are effected in 0.2 M $HClO_4$ (27). In the same time compounds soluble in acid aqueous solution are also extracted.

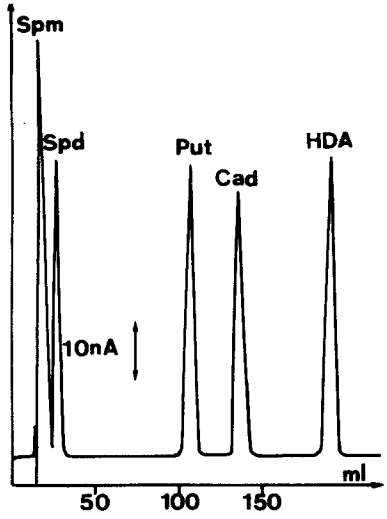

FIGURE 6. Isocratic elution of polyamine derivatives with derivatization reagent A and 0,1 M $HClO_4$/DCA in 80 % MeOH pH 4. 1 nmole of Spm, Spd, Put, Cad or MDA, sensitivity 100 nA.

Prederivatization clean-up

It is suggested for the concentration of polyamines from tissues and for the improvement of subsequent chromatographic separations. To verify that no peaks are hindered behind peaks attributed to polyamines, we prepurified some samples by passing them through silica gel cartridge (27).

Preparation of samples

Before reaction with OPA-thiol, a known amount of 1,6-diaminohexane, 2HCl, is added to the tissue extracts as an internal standard and perchloric extracts are adjusted to pH 7 by addition of a borate buffer. For quantitation, external

TABLE 3

	Spm	Spd	Put
Cerebellum	3.00 ± 0.15	6.08 ± 0.29	0.14 ± 0.07
Hypothalamus	1.37 ± 0.04	6.89 ± 0.20	0.23 ± 0.007
Hippocampus	2.22 ± 0.08	4.11 ± 0.15	nd
Medulla	0.61 ± 0.02	12.84 ± 0.41	0.16 ± 0.05
Corpus striatum	nd	2.34 ± 0.09	nd

standard mixtures are run at regular intervals in similar amounts. Others brain samples are spiked with polyamines and aminoacids in order to ascertain the non-interference of aminoacids with peaks attributed to polyamines.

Elution

Brain samples contain large amounts of aminoacids compounds which elute early in the chromatogram.; since OPA-thiol reacts with primary amines, this reponse cannot be eliminated. The best eluent is one containing perchloric acid instead of phosphoric acid and no DCA as retention times of spermine decreases with DCA. Spermine is easier to measure if eluted after GABA the most important and the last eluting aminoacid. With this mobile phase there is a sufficient long time between the peaks of GABA and the beginning of the first polyamine peak.

Polyamines together with aminoacids can be measured in the same run if a gradient of MeOH is used. This gradient elution is compatible with electrochemical detection at the

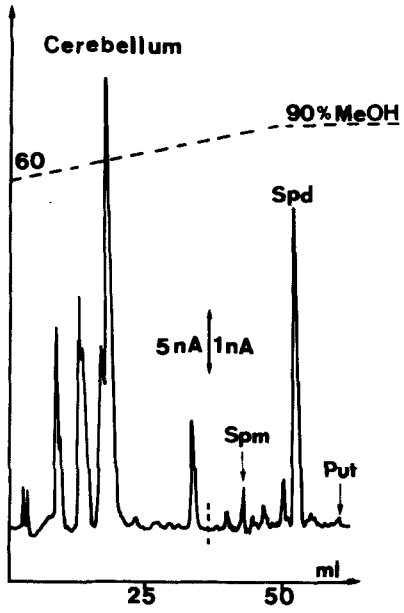

FIGURE 7 (a-e). Gradient elution of polyamine derivatives in tissue extracts with 0.1 M $HClO_4$/NaOH in 40 to 90 % MeOH in 50 min, pH = 4.

modest potential of 0.65 V and at a moderate sensitivity. In the case of this complete separation the elution program lasts 50 min. Figure 7 (a-e) illustrates this separation and Table 3 gives results in some brain areas in nmole/mg.

CONCLUSION

A combination of HPLC on a reversed-phase column, prederivatization with OPA-ethanethiol and electrochemical detection could be used for the direct assay of polyamines Spm, Spd, Cad and Put in deproteinated extracts of mouse brain.

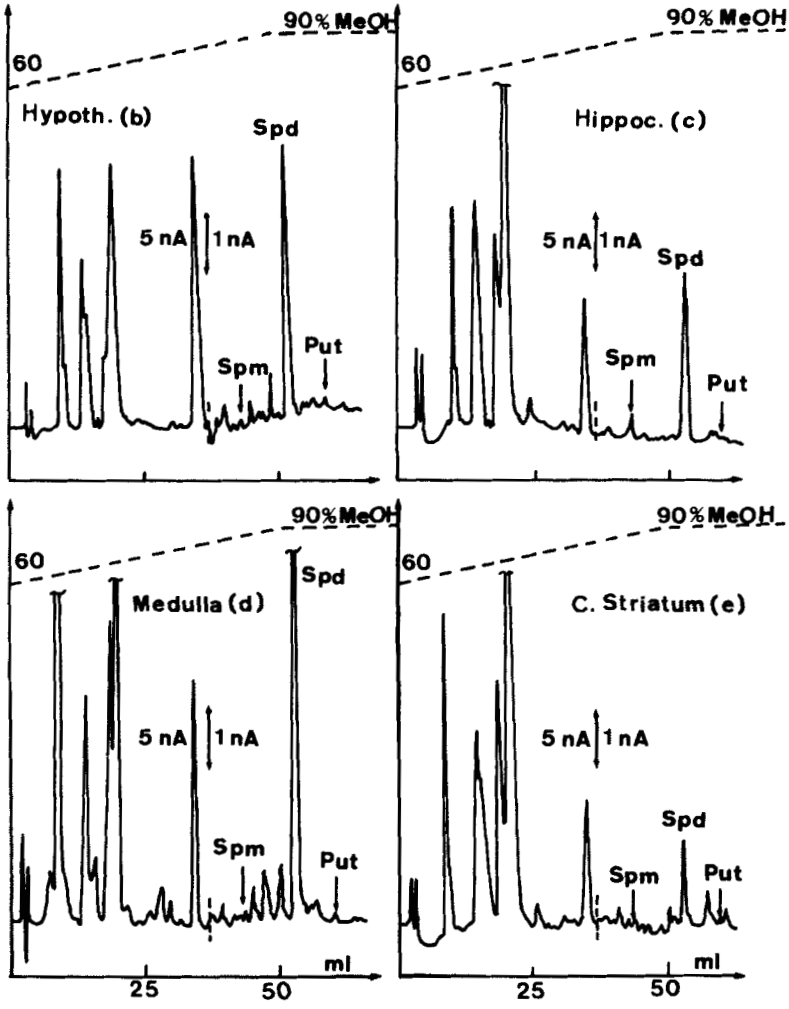

FIGURE 7 (continued)

This study shows that two derivatives of spermine are formed, one probably being an intermediate. The formation of only one derivative is obtained by increasing the quantity of methanol. The lowest detection limit for spermine was obtained using a derivatization medium containing a high quantity of MeOH with a reaction time longer than usual and a mobile phase

containing $HClO_4$ instead of H_3PO_4. With electrochemical detection, the background is better than with fluorescence and does not deteriorate as the reagents age.

The optimized system is rapid, simple, sensitive and flexible as the gradient elution may be adjusted to take care of the interfering amino compounds contained in the samples. This system can be automated to provide a convenient assay for polyamines in, for example, studies on the variations in polyamine levels in response to neuropharmacological agents.

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