



Jornal Brasileiro de Patologia e Medicina Laboratorial

ISSN: 1676-2444

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Sociedade Brasileira de Patologia Clínica/Medicina Laboratorial

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Jornal Brasileiro de Patologia e Medicina Laboratorial, vol. 46, núm. 6, diciembre, 2010, pp. 455-461

Sociedade Brasileira de Patologia Clínica/Medicina Laboratorial
Rio de Janeiro, Brasil

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Rat brain polyamines: an analytical method validation

Primeira submissão em 15/07/10
Última submissão em 13/08/10
Aceito para publicação em 13/08/10
Publicado em 20/12/10

Poliâminas no cérebro do rato: validação de método analítico

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| key words | abstract |
|----------------------|---|
| Validation | The validation of the analytical technique for the determination of polyamines in cerebral tissue using HPLC based on o-phthalaldehyde post-column derivatization is described. The polyamines were separated in a LiChrospher100 RP18 column. Elution gradient was formed with two mobile phases: A (sodium acetate 0.1 M + sodium octanesulphonate 0.01 M, pH = 4.5) and B (sodium acetate 0.2 M + sodium octanesulphonate 0.01 M)/acetonitrile (10:3), pH = 4.5) in a 1.2 ml/min flow rate. The derivative eluent was monitored by fluorescence (excitation, 345 nm; emission, 455 nm). Besides excellent linearity (putrescine, $r = 0.9816$; spermidine, $r = 0.9920$; spermine, $r = 0.9901$), the technique demonstrated intra and inter-day precision ($\leq 20\%$) as well as recovery (spermidine = 92.56%; spermine = 84.47%). Quantification limits were 0.22 pM for putrescine, 76.44 pM for spermidine and 51.44 pM for spermine. The method demonstrated to be robust, simple and highly reproducible for polyamine determination in tissues. |
| Analytical technique | |
| HPLC | |
| Polyamines | |

| resumo | unitermos |
|--|--|
| A validação técnica analítica para determinação de poliâminas em tecido cerebral utilizando cromatografia líquida de alta eficiência (HPLC) e derivação pós-coluna com o-ftalaldeído é descrita. A separação das poliâminas deu-se em coluna LiChrospher 100 RP18. O gradiente de eluição foi formado por duas fases móveis A (acetato de sódio 0,1M + octanossulfonato de sódio 0,01 M) e B (acetato de sódio 0,2 M + octanossulfonato de sódio 0,01 M)/acetonitrila (10:3), fluxo de 1,2 ml/min. O eluente foi monitorado por fluorescência (excitação, 345 nm; emissão, 455 nm). Além da excelente linearidade (putrescina, $r = 0,9816$; espermidina, $r = 0,9920$; espermina, $r = 0,9901$), a técnica demonstrou adequada precisão intra e interdia ($\leq 20\%$) e recuperação (espermidina = 92,56%; espermina = 84,47%). Os limites de quantificação foram 0,22 pM para putrescina, 76,44 pM para espermidina e 51,44 pM para espermina. O método demonstrou ser consistente, simples e altamente reprodutível para a determinação proposta. | Validação Técnica analítica HPLC Poliâminas |

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Introduction

The polyamines are low molecular weight aliphatic cations occurring in all living organisms⁽¹⁵⁾. The biogenic polyamines evaluated in this study are putrescine ($\text{H}_2\text{N}(\text{CH}_2)_4\text{NH}_2$), spermidine ($\text{H}_2\text{N}(\text{CH}_2)_3\text{NH}(\text{CH}_2)_4\text{NH}_2$) and spermine ($\text{H}_2\text{N}(\text{CH}_2)_3\text{NH}(\text{CH}_2)_4\text{NH}(\text{CH}_2)_3\text{NH}_2$). Putrescine is synthesized in mammals from L-ornithine in a catalyzed reaction by ornithine decarboxylase, an enzyme limiting from the polyamine synthesis. Putrescine and S-adenosylmethionine are substrates for the spermidine synthesis, which is the precursor of spermine.

Among the various physiologic functions of the polyamines, we can categorize its performance as a second messenger, nutrient, metabolic regulator, growth factor, anti-oxidant, DNA, RNA and membrane stabilizer⁽¹⁷⁾.

The cells developed mechanisms in order to assure the rigorous regulation of the intracellular polyamine levels through biosynthesis processes, degradation and transportation inside the organism⁽¹²⁾. Insufficient polyamines levels result in a deficit growth and, in some cases, cellular death, including apoptosis. The uncontrolled elevation of the polyamines can lead to cellular transformation and tumor genesis⁽¹¹⁾.

The objective of the present study was to validate a modified version of the polyamine determination as described by Löser *et al.*⁽¹⁰⁾ using reverse phase ion-pair high performance liquid chromatography (HPLC), followed by o-phthalaldehyde post column derivatization and fluorescence detection.

Methods

Animals

In these experiments, male Wistar (*Rattus norvegicus*) rats from the animal colony of Ribeirão Preto Campus, University of São Paulo were used. The animals were maintained on a 12:12-h light/dark cycle (lights on at 6:00 am) with room temperature kept at 23-25°C, and with free access to water and food. The experiments were performed in compliance with the recommendations of the Brazilian Society of Neuroscience and Behavior (SBNeC), which is based on the US National Institutes of Health Guide for Care and Use of Laboratory Animals.

Apparatus and chromatographic conditions

In the quantitative determination of polyamines in cerebral tissue, a chromatograph from Shimadzu

Corporation – model LC-10AD was used, equipped with two Shimadzu LC-10AD high pressure pumps, a Shimadzu CBM-10A gradient mixture unit, a Shimadzu SIL-10A auto-sampler and a Shimadzu CTO-10A oven to keep the column at $37 \pm 1^\circ\text{C}$. A LiChrospher® 100 RP 18 column was used for the polyamine separation, containing octadecilsilane (C18), with 120×4.0 mm i.d. and $5\mu\text{m}$ particle diameter. The column gradient flow was kept at 1.2 ml/min. A low pressure pump from Milton Roy Company with a 0.45 ml/min flow was used to accomplish the o-phthalaldehyde (OPA) post-column derivatization. The column eluent and OPA derivatizing agent were mixed in a T connection and, after passing through a polypropylene coil (1 m x 0.5 mm i.d.), as described by Seiler *et al.*⁽¹³⁾, were maintained in a water bath at $54 \pm 1^\circ\text{C}$, controlled by a thermostat. After reaction, the eluent-reagent mixture went through a Shimadzu RF535 fluorescence detector, with excitation and emission wavelengths of 345 nm and 455 nm, respectively.

Reagents

The following polyamine standards were acquired: putrescine (1.4-diaminobutane) 99% Sigma D13208 with a molecular weight of 88.15 MW, spermidine (free base spermidine Sigma S2626 with a 145.25 MW), spermine (free base spermine Sigma S3256 with a 202.34 MW) and the internal standard (1,7-diaminoheptane Sigma 32990 with a 130.23 MW). The octanesulfonic sodium (1-octanesulfonic sodium acid Vetec Brasil with a 234.29 MW), the ophthalaldehyde (ophthaldialdeyde Acros with a 134.13 MW) and the Brij35 detergent (Acros with a 1198 MW) were categorized as a pure level to HPCL. The percloric acid at 70% (Cinética Química, Brazil), the acetonitrile (J. T. Baker), the PA sodium acetate (Merck), the potassium hydroxide, ethanol, 2-mercaptoethanol, PA acetic acid (Vetec Brasil) and the PA boric acid (Vetec Brasil) were analytical. MILLEX-GS filters with 13 mm diameter and $0.22\mu\text{m}$ pore from Millipore (JBR13GP05) were acquired. The water used was from the usage of the Milli-Q (Millipore) system. For the protein dosage, the Coomassie blue binding method⁽²⁾ was implemented, using the Dye Reagent Concentrate (BIO-RAD Protein Assay) and bovine albumin (PIERCE Protein assay reagents).

Solutions

- Buffer A: 0.1 M sodium acetate and 0.01 M sodium octanesulfonate solution: obtained by the addition of sodium acetate (16.41 g) and sodium

octanesulfonate (4.69 g) to 2,000 ml Milli-Q water, having the pH adjusted to 4.5 by the addition of acetic acid.

- Buffer B: 0.2 M sodium acetate and 0.01 M sodium octanesulfonate solution was obtained by the addition of sodium acetate (32.81 g) and sodium octanesulfonate (4.69 g) to 2,000 ml Milli-Q water, having the pH adjusted to 4.5 by the addition of acetic acid. To an aliquot of this solution we add acetonitrile in a 10:3 (v/v) proportion in order to create Buffer B. Both buffers (A and B), were filtered, after agitation, through a cellulose (or nylon) organic phase filter with 0.45 µm pore and 47 mm diameter.
- Boric acid solution: obtained after addition of boric acid (12.34 g) and potassium hydroxide at 25% (25 ml) to 1,000 ml Milli-Q water, having the pH adjusted to 10.4 with addition of potassium hydroxide at 50%
- OPA-methanol solution: obtained after adding OPA (400 mg) to methanol (5 ml).
- OPA-2-mercaptoethanol derivatizing reagent solution: obtained after adding the boric acid solution (227.3 ml), 30% BRIJ35 (6.82 ml), 2-mercaptoethanol (4.55 ml) and the OPA-methanol solution (11.36 ml). The derivatizing reagent must be kept in a dark flask protected from light and pressurized under constant low helium flow.
- 0.2 M perchloric acid solution (PCA): obtained after addition of 70% PCA (4.31 ml) (MW = 100.46, density = 1.664 g/ml) to 250 ml distilled water.
- Internal standard solution: obtained after adding 1.7 diaminoheptane (0.039 mg) to buffer A (10 ml), which gives a 300 nmol/ml concentration.
- Polyamines Standards Mixture (PSM) for the standards curves and calibration runs, being made of:
 - ✓ 13,822 nM internal standard 1.7 diaminoheptane solutions (160 µl): obtained after adding 1.7 diaminoheptane (0.09 mg) to buffer A (50 ml).
 - ✓ 846.3 nM 1.4 diaminobutane (putrescine) solution (40 µl): obtained after adding 1.4 diaminobutane (0.000373 mg) to buffer A (5 ml).
 - ✓ 63.7 µM N-(3-aminopropyl)-1.4 diaminobutane (spermidine) solution (30 µl): obtained after

adding spermidine (0.23131 mg) to buffer A (25 ml).

- ✓ 32,122 nM N,N'-Bis(3-aminopropyl)-1.4 diaminobutane (spermine) solution (8 µl): obtained after adding spermine (0.03250 mg) to buffer A (5 ml).
- ✓ Buffer A (5,000 µl).

The final PSM content concentrations are: putrescine (6.77 nM), internal standard (44.22 nM), spermidine (76.44 nM) and spermine (51.40 nM).

Elution gradient

The elution gradient described in **Table 1** was used in the HPLC analysis. The column flux was 1.2 ml/min, with a 17 minutes elution run time, followed by a 8 minutes equilibration time, summing up to 25 minutes total analysis time.

Table 1 Buffers elution gradients used

| | Elution time (min.) | Buffer A | Buffer B | Buffer A | Buffer B |
|-------------------------|---------------------|----------|----------|----------|----------|
| | | % | % | flow | flow |
| | | | | ml/min | ml/min |
| | 0 | 50 | 50 | 0.6 | 0.6 |
| | 10 | 25 | 75 | 0.3 | 0.9 |
| | 12 | 0 | 100 | 0 | 1.2 |
| | 16 | 0 | 100 | 0 | 1.2 |
| | 17 | 50 | 50 | 0.6 | 0.6 |
| Equilibrium time (min.) | 8 | | | | |
| Total time (min.) | 25 | | | | |

Sample extraction procedure

The extraction procedure was conducted according to Löser *et al.*⁽¹⁰⁾. After weighting, the samples were frozen to -70°C, being reported that under this condition, we can wait up to six months for further analysis⁽⁶⁾.

In the analysis day, after defrost the sample at ambient temperature, a 0.9% saline solution was added to the eppendorf containing the sample in a 1:20 (weight [g]/volume [ml]) proportion followed by a homogenization with a sonicator (Ultrasonic Processor Model GE 130PB) for 4 times of 5 seconds. After that, 250 µl of the homogenate sample was removed and separated in another eppendorf, while the remaining sample was frozen to -70°C for

posterior protein quantification. The internal standard (1.7 diaminoheptane, 300 nM/ml) solution (50 µl) and 70% 0.2 M PCA (700 µl) were added to the homogenate. The mixture was then centrifuged at 3200 g for 5 minutes and the supernatant was filtered by a Millex (0.22 µm) filter and 20 µL were injected into the HPLC.

The PSM, routinely used in calibrations to obtain the analytical curve, which used to be done before the sample analysis, contained, in the 20 µl injected into the HPLC, 27.08 picomoles of putrescine, 176.92 picomoles of the internal standard (1.7-diaminoheptane), 305.76 picomoles of spermidine and 205.6 picomoles of spermine.

Technical analysis validation

The technical analysis validation, confirming its applicability to the intended purpose, was accomplished based on the following parameters: linearity, detection and quantification limits, precision and recovery, following the FDA⁽¹⁾ and ICH⁽⁸⁾ recommendations.

Results and discussions

Chromatographic methodology

In **Figure**, the chromatogram shows the retention times of putrescine (about 6 minutes), internal standard (11 minutes), spermidine (14 minutes) and spermine (16 minutes) during the sample analysis from the cerebral tissue of the rat.

Linearity

The linearity of the polyamines responses was examined to ensure that the procedure can provide quantitative

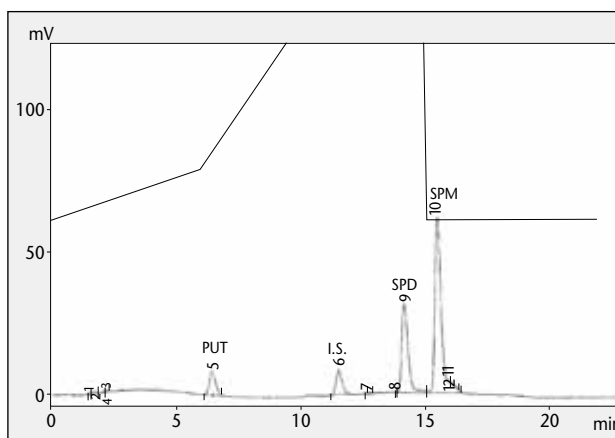


Figure – Chromatogram of the cerebral tissue of the rats showing the retention peaks for putrescine (5), internal standard (6), spermidine (9) and spermine (10).

analysis. Therefore, analytical curves were built starting from the solution obtained from the addition of the PSM (200 µl) to Buffer A (200 µl) with triplicate analysis of 10, 20, 30, 40 and 50 µl injected solution. The linear regression analysis for the relation between the injected concentration and the detector response for putrescine (PUT), spermidine (SPD) and spermine (SPM) as well as correlation coefficients and linearity equations are shown below in **Table 2**.

Precision

The precision, defined as the agreement pattern between the results of individual tests when the procedure is applied to multiple aliquots of a homogenized sample, was accomplished through the evaluation of the repeatability, or intra-assay or intra-day, precision, and the intermediate, or inter-assay or inter-day, precision⁽¹⁴⁾. Three male Wistar rat brains were submitted to the same extraction procedure described above, each brain supplying a homogenized solution. Fifteen samples from each homogenized solution were analyzed in quintuplicate ($n = 5$) for intra-day assay and triplicate ($n = 15$) for inter-day assay.

In the precision and recovery studies there were no results regarding putrescine, which concentration in the used samples was below the detection limit. The intra and inter day coefficients obtained in the method precision are summarized in **Tables 3** and **4** and are according to what was expected ($\leq 20\%$)⁽¹⁾.

Recovery

For the polyamines recovery analysis, a whole rat brain was used. Fifteen samples of an homogein sample

Table 2 Linear regression analysis of the relation between concentration and peak area of putrescine (PUT), spermidine (SPD) and spermine (SPM)

| Substance | Corr. coef. (r) | Slope (a)±SD | Intercept-X | Intercept-Y (b) |
|-----------|-----------------|---------------|-------------|-----------------|
| PUT | 0.981631 | 16530 ± 858.8 | 1.233 | -20380 ± 18670 |
| SPD | 0.992018 | 3093 ± 105.2 | -5.579 | 17260 ± 25830 |
| SPM | 0.990051 | 2865 ± 108.9 | 8.071 | -23130 ± 17970 |

Table 3 Intra-day precision in quintuplicate showing mean (picomoles), standard deviation (SD), standard mean error (SME) and coefficient of variation (CV) of the measures

| Intra-day precision | | | | | | | | |
|---------------------|-------|-------|-------|----------|------|--------|--------|--------|
| SPD | | | | SPM | | | | |
| Days | 1 | 2 | 3 | | Days | 1 | 2 | 3 |
| Mean | 287.8 | 291.5 | 236.7 | ANIMAL 1 | Mean | 126.2 | 115.7 | 83.19 |
| SD | 3.234 | 11.45 | 5.38 | | SD | 24.14 | 3.884 | 6.073 |
| SME | 1.867 | 6.612 | 2.477 | | SME | 13.94 | 2.746 | 3.037 |
| CV | 1.12% | 3.93% | 2.34% | | CV | 19.13% | 3.36% | 7.3% |
| Days | 1 | 2 | 3 | | Days | 1 | 2 | 3 |
| Mean | 229.2 | 268.2 | 254.6 | ANIMAL 2 | Mean | 84.21 | 117.8 | 104.1 |
| SD | 5.739 | 22.53 | 15.02 | | SD | 4.759 | 10.41 | 11.54 |
| SME | 2.869 | 10.08 | 6.718 | | SME | 2.128 | 4.655 | 5.161 |
| CV | 2.5% | 8.4% | 5.9% | | CV | 5.65% | 8.84% | 11.08% |
| Days | 1 | 2 | 3 | | Days | 1 | 2 | 3 |
| Mean | 277.4 | 243.4 | 218.3 | ANIMAL 3 | Mean | 69.96 | 163.5 | 39.01 |
| SD | 25.44 | 8.075 | 2.814 | | SD | 11.57 | 20.09 | 0.891 |
| SME | 11.38 | 4.038 | 1.625 | | SME | 5.176 | 11.6 | 0.4455 |
| CV | 9.17% | 3.32% | 1.29% | | CV | 16.54% | 12.29% | 2.28% |

Table 4 Precision calculation of 3 days ($n = 15$) showing the average (picomoles), the standard deviation (SD), the standard mean error (SME) and the coefficient of variation (CV) of the measures

| Intra-day precision | | | |
|---------------------|------|--------|--------|
| | | SPD | SPM |
| ANIMAL 1 | Mean | 267.4 | 101.7 |
| | SD | 29.4 | 19.73 |
| | SME | 8.16 | 6.24 |
| | CV | 10.99% | 19.4% |
| ANIMAL 2 | Mean | 249.1 | 102 |
| | SD | 24.52 | 16.71 |
| | SME | 6.33 | 4.31 |
| | CV | 9.84% | 16.37% |
| ANIMAL 3 | Mean | 252.60 | 73.44 |
| | SD | 36.89 | 10.47 |
| | SME | 9.53 | 3.7 |
| | CV | 14.6% | 14.26% |

obtained from the extraction procedure described above were analyzed in triplicate, with and without addition of known quantities of polyamine standards: 92.56% and

84.47% were the recovery percentage for spermidine and spermine, respectively.

Method modification for putrescine detection

The largest HPLC injection volume, from 20 μ L to 30 μ L, elevating from 1.5 times the solute mass, did not successfully result in detection of putrescine in the samples. After that, in an attempt to detect putrescine, we concentrated the samples in a way that the injected mass quintuplicated. Therefore, the original saline dilution proposed by Löser *et al.*⁽¹⁰⁾ was reduced from 1:20 to 1:10 and the PCA volume from the original proportion of 2.8:1 (homogenate volume /PCA volume) to 1.2:1. The additional internal standard volume remained in the same original 1/5 proportion of the homogenate volume. Consequently, for a homogenate volume of 250 μ L, for instance, we added 50 μ L of the internal standard, a procedure we maintained, and 300 μ L of PCA. Finally, to elevate 5 times the injected mass, we injected 30 μ L into the HPLC. These alterations in the method succeeded in the putrescine detection.

Detection and quantification limits

The detection limits for each polyamine were calculated considering a 2:1 signal/noise level. Following this criteria,

the detection limits obtained resulted in 0.11 picomoles for putrescine, 27.56 picomoles for spermidine and 20.69 picomoles for spermine.

For the quantification limit, the lowest analyte concentration that provides an answer which could be accurately quantified resulted in 0.22 picomoles for putrescine, 76.44 picomoles for spermidine and 51.40 picomoles for spermine, considering a 10:1 signal/noise relation.

Conclusion

The highly sensitive and reproducible chromatographic method developed by Löser *et al.*⁽¹⁰⁾, used in this study as well as in others^(9, 16, 18), proved to be adequate to the proposed polyamine analysis.

Under the chromatographic extraction conditions of the tissue polyamines used according to the reference description, putrescine was not detected for its concentration being below the detection methods reproduced by us. The measures for sample concentration elevated 5 times the final mass injected into the HPLC and putrescine became detectable and quantifiable in the samples. The different properties of the stationary phase of the columns used in this study (LiChrospher®) and by Löser *et al.* (NovaPak®), such as particle and pore size, surface area, carbon load, greater silanol groups exposition, with consecutive greater hydrophobicity and polarity, can identify and explain the difference in efficiency for basic compounds between the columns⁽³⁾. Gennaro *et al.*⁽⁵⁾, in a study about different

chromatographic C18 columns adequacy for the ionic interaction transference method in the HPLC, comparing the ruggedness in the transference of methods between five different commercial products, with silica 5 µm stationary phases (RP-18), concluded that the critical parameter to be considered in column change between methods is the mobile phase pH, which must be strictly controlled for performance maintenance. With the LiChrospher® column, the minimum pH deviations up to ± 2.5% in the mobile phase are tolerated⁽⁵⁾, which we considered in the present study. Löser *et al.*⁽⁹⁾ reported the usage of a 1:10 final dilution for sample concentration.

The polyamines concentration in tissues can be expressed in nanomoles per tissue gram and the normalization in milligrams of protein shows similar results, also noted by Deignan *et al.*⁽⁴⁾. The spermidine/spermine ratio has been used as a more reliable measure for the spermidine formation during the polyamine inter-conversion cycle⁽¹³⁾. Hixson *et al.*⁽⁷⁾ reported that the ratio spermidine/spermine is the less susceptible indicator to measure mistakes because it does not depend on a second measure for normalization, like the weight or protein content of the samples.

Acknowledgements

The authors express gratefulness to Prof. Dr. Pierina Sueli Bonato, from the Physics and Chemistry Department of the Farmaceutical Sciences Faculty of Ribeirão Preto (USP), for her valuable orientation on the polyamines chromatographic method validation.

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