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Automated determination of polyamines by high-performance liquid chromatography with simple sample preparation

Hans M.H. van Eijk, Dennis R. Rooyakkers, Nicolaas E.P. Deutz*

Department of Surgery, Biomedical Center, University of Limburg, P.O. Box 616, NL-6200 MD Maastricht, Netherlands

Abstract

Recently, a new fully endcapped reversed-phase packing material, Inertsil, was introduced, especially suitable for the determination of basic compounds. We used this packing material to separate *o*-phthaldialdehyde (OPA) derivatives of amino acid derivatives completely from the OPA derivatives of spermine (SPM), spermidine (SPD), putrescine (PUT) and cadaverine (CAD). The obtained separation made the commonly used off-line extraction procedure redundant and thus an on-line sample clean-up was introduced. This enabled automation of the procedure resulting in a better reproducibility and a more efficient use of equipment. Furthermore, no studies are required to determine the extraction recovery.

The present method has a cycle time of 30 min. A linear response for each polyamine was found up to 250 pmol, with an R^2 ranging from 0.9981 (SPM) to 0.9998 (CAD). The limit of detection, calculated at a signal-to-noise ratio of 3, was 0.1 pmol, corresponding to a plasma concentration of 0.1 $\mu\text{mol/l}$. The coefficient of variation (C.V.) for the peak area was below 3% and for retention times below 0.5% ($n=15$).

In order to evaluate the applicability of the method, three different types of sample were chromatographed, e.g. urine (obtained from healthy human volunteers), pig plasma and sulfosalicylic acid homogenates of pig intestine biopsies. Tissue homogenates and urine-specimen could easily be quantitated, while plasma concentrations were just above the limit of detection, resulting in a plasma C.V. ranging from 4.8% (SPM) to 13.6% (SPD) and a tissue C.V. ranging from 2.1% (SPM) to 8.5% (CAD). The urinary C.V.s were not determined.

In conclusion, the present method provides an easy way to measure polyamine concentrations for most applications.

Keywords: Automation; Polyamines; Amino acids

1. Introduction

Polyamines are essential for normal growth and cellular differentiation. Several methods have been described for their determination [1–12], but most of these methods require an extraction procedure to remove interfering amino acid derivatives or reagent excess [1–3,5,6,8–10,12]. These features disable

automatization of the derivatization procedure and decrease reproducibility. Other methods employ ion-exchange chromatography and post-column derivatization, but they lack sensitivity [4,7,11].

Using reversed-phase chromatography, the complete separation of *o*-phthaldialdehyde (OPA)-polyamine derivatives from OPA-amino acid derivatives has not been accomplished before. The interaction of OPA-polyamine derivatives with residuing free silanol groups of most common reversed-phase packing materials, resulting in broad tailing peaks, prohibited this.

*Corresponding author.

Recently a new ultra-pure fully endcapped packing material, Inertsil, was introduced. This material has a high pH resistance, due to minimal silanol and trace metal interactions and is therefore ideal for the analysis of basic compounds. Using this new packing material, the complete separation of OPA-polyamine derivatives was obtained from the interfering OPA-amino acid derivatives of a physiological standard. Although in urine and plasma some interference of other unknown components still remains, the resulting separation is sufficient even in these complex samples. As a result, automatization of the derivatization procedure could be enabled, thereby decreasing the work load for analytical staff, increasing the reproducibility and resulting in a more effective use of equipment.

2. Experimental

2.1. Equipment

In the first set of experiments, a 250×3.2 mm I.D. Inertsil 5- μ m column (Phenomenex, Bester, Amstelveen, Netherlands) was used. The results of these experiments indicated that a shorter column with a larger I.D. would be more suitable. Therefore, the column was replaced later by a 200×4.6 mm I.D. column, equipped with a 10×4.6 mm I.D. pre-column. These other dimensions required delivery by another supplier (Applikon, Schiedam, Netherlands). The column was placed in a thermostatted Spark Mistral oven (Emmen, Netherlands) set at 10°C.

Our HPLC system consisted of a Model 2248 pump, a Model 2252 controller, a Model 2256 solvent conditioner, a Model 2248-201 low-pressure binary mixing panel (all from Pharmacia, Woerden, Netherlands). The automated pre-column derivatization was executed using a WISP 715 sample processor (Waters, Etten-Leur, Netherlands), equipped with a cooled sample storage compartment and a 48-position sample tray. Fluorescence was monitored using a Jasco Model 820FP fluorescence detector (B&L systems, Zoetermeer, Netherlands) equipped with a xenon lamp and a 12- μ l flow-cell. Measurements were made at an excitation wavelength of 330 nm and an emission wavelength of 445 nm. Data

were collected on-line by a Model 900 interface (Perkin Elmer/Nelson, Gouda, Netherlands) and processed by a Tandon Model MCS 486/50 personal computer (Amsterdam, Netherlands) running Model 2700 (Turbochrom: version 3.2) software (Perkin Elmer/Nelson) under Microsoft Windows, version 3.1.

2.2. Reagents and solvents

All solutions were prepared with ultra-pure water, generated by a Super-Q system (Millipore, Etten-Leur, Netherlands). All chemicals used were of analytical grade (Pierce, Oud Beijerland, Netherlands), solvents of chromatographic grade (Janssen Chimica, Amsterdam, Netherlands).

The *o*-phthalaldehyde (OPA) reagent was prepared by dissolving 15 mg OPA in 0.25 ml of methanol, adding 2.25 ml of potassium borate buffer (Fluoraldehyde reagent solution: 1.0 mol/l, pH 10.4: Pierce, Brunschwig, Amsterdam, Netherlands) and 15 μ l of 3-mercaptopropionic acid (3-MPA). This reagent was placed in a 4.0-ml WISP vial with a self-sealing silicone rubber, Teflon-coated stopper (Waters).

Solvent A was a 125 mmol/l sodium citrate buffer, pH 6.50, containing 50 ml of tetrahydrofuran (THF) per litre. Solvent B consisted of the citrate buffer, acetonitrile and THF (45:40:15, v/v/v).

Polyamine standards were prepared by dissolving pure chemicals (Sigma, Amsterdam, Netherlands) in water to a final concentration of 250 μ mol/l each. These standards were divided into 1-ml portions and stored at -80°C.

2.3. Sample preparation

Plasma

Heparinized pig blood samples were obtained from pre-implanted catheters and collected on ice, followed by immediate centrifugation (5 min at 4°C). The obtained plasma was deproteinized with solid 5-sulfosalicylic acid (SSA) (4 mg/100 μ l) plasma, frozen immediately in liquid nitrogen and stored at -80°C. Before analysis, samples were thawed at 4°C, vortex-mixed vigorously and centrifuged at

11 000 g in a Hereaus Model Biofuge for 10 min at 4°C. From the clear supernatant 40 µl was mixed with 80 µl water and 80 µl Fluoraldehyde reagent buffer (end concentrations: 0.5 mol/l potassium borate, pH 10.4) and stored in the cooled (7°C) sample compartment of the autosampler until analysis.

Tissue

Pig jejunal mucosa samples were obtained by biopsy and immediately frozen in liquid nitrogen and stored at –80°C. Before analysis, they were transferred to a preweighed 1.5-ml eppendorf vial, containing 0.1 g of glass beads (1.0-mm diameter) and 100 µl of an ice-cold 5% sulfosalicylic acid (SSA) solution. The vials were weighed again, whereafter the tissue was homogenized in a mini-bead beater (Cole Parmer/Applikon, Schiedam, Netherlands), set at high speed for 1 min. Next, the vials were centrifuged and processed as described before.

Urine

After an overnight fast, urine was collected from healthy male human volunteers. Aliquots were centrifuged 5 min at 4°C and processed in the same way as plasma.

2.4. Automated pre-column derivatization and gradient elution

This automated pre-column derivatization procedure has been extensively tested for amino acid analysis [13,14]. In short, employing the WISP 715 'auto-standards' method with the OPA-reagent placed at position 1 of the tray, the automated pre-column derivatization was executed during the first 4.5 min of the run. First, 5 µl of the OPA reagent is injected at zero flow, thus the reagent remains in the sample loop. One minute later (the minimum delay time of the sampler) 5 µl of a sample is injected also into the loop. In the next 2.5 min the pump is activated and the flow is slowly increased to 0.1 ml/min, thus allowing sample and reagent to mix and react in the sample loop and the supplying capillaries of the column, after which the gradient elution starts. In the first 10 min of the gradient all

Table 1
Gradient conditions

Time (min)	Flow-rate (ml/min)	% A	% B
0	0	65	35
2.5	0.020	65	35
2.7	0.040	65	35
3.5	0.100	65	35
4.5	0.600	65	35
4.6	1.200	65	35
10.0	1.200	45	55
13.0	1.200	43	57
18.0	1.200	35	65
21.0	1.200	0	100
22.0	1.200	0	100
24.0	1.200	65	35
30.0	0.600	65	35
31.0	0.000	65	35

amino acid adducts elute. In the next 10 min polyamine derivatives elute, after which regeneration and equilibration of the column takes place. Gradient profile is described in Table 1.

3. Results

Pilot experiments had revealed that the last-eluting amino acid of a physiological standard elutes before the first polyamine OPA derivative, if the solvent pH is set above pH 6. To study the influence of the solvent buffer molarity under these circumstances, a citric acid buffer system, set to pH 6.4 for maximum buffer capacity, was used in an isocratic solvent system with 35% acetonitrile on a 250×3.2 mm I.D. Inertsil 5-µm ODS 2 column (Phenomenex). Within the range of 25 to 200 mmol/l, the best separation was found at 125 mmol/l.

Next, the influence of the solvent pH on the separation was studied in the range between 6 and 8, using a standard mixture containing all major physiological amino acids (Pierce) and the polyamines spermine (SPM), spermidine (SPD), putrescine (PUT) and cadaverine (CAD). At pH 6, the separation between the first-eluting polyamine (SPM) and the last-eluting amino acid, lysine (LYS), was not complete. Above pH 7, an unknown component (probably an OPA degradation product) coeluted

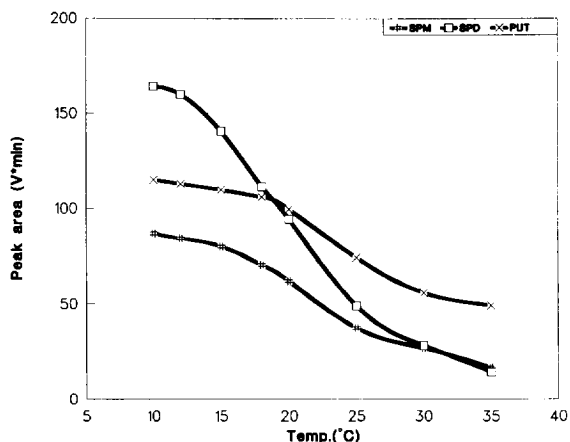


Fig. 1. Influence of the temperature on the on-column degradation of OPA-polyamine derivatives.

with PUT. Therefore, pH 6.5 was considered optimal.

Although the separation was now optimal, the fluorescence response of especially the SPD derivative was low and variable, compared to amino acid derivatives. As it is known that OPA-polyamine

derivatives degrade rapidly after their formation [1,3], the influence of the temperature on the on-column degradation rate was studied between 10°C and 35°C. With a decreasing column temperature, the fluorescence response increased, reaching a maximum at 10°C (Fig. 1). At this temperature, the column back-pressure was too high (30 MPa). For routine measurements a decrease in flow-rate or column length was thus required. As the obtained baseline separation in the standard allowed a decrease in column length, from this point the 200×4.6 mm column (Bischoff, Applikon, Schiedam, Netherlands) was applied for all further experiments. The decrease in back-pressure allowed application of a 10×4.6 mm pre-column, filled with the same packing material (resulting back-pressure 25 MPa), thus ensuring a longer column life time. For this column, an optimal gradient profile was developed (Table 1), resulting in an adequate separation (Fig. 2A) from all amino acids present in a physiological standard.

The linearity of the method was checked by injecting 5- μ l aliquots of polyamine standards with increasing concentrations. A linear response was

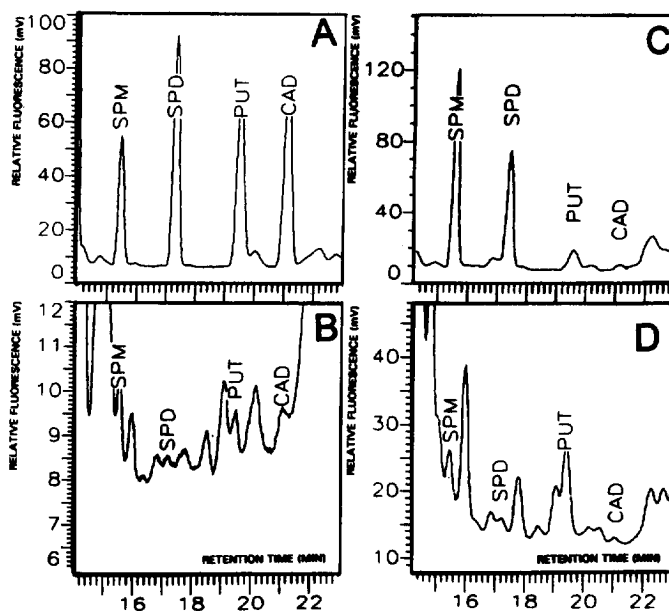


Fig. 2. Separation of polyamines in different sample matrices. Chromatographic conditions are discussed in the text. (A) Separation of a polyamine standard showing no interference by the presence of a physiological amino acid standard (25 pmol injected into each component). (B) Pig plasma (1 μ l pure plasma injected). (C) Pig intestine biopsy homogenate (sample amount equivalent to 0.5 mg wet weight). (D) Human urine (1 μ l pure urine injected).

Table 2
Reproducibility of the method

	Standard (<i>n</i> = 15, $\mu\text{mol/l}$)		Pig mucosa homogenate (<i>n</i> = 10, $\mu\text{mol/kg}$ wet weight)		Pig plasma (<i>n</i> = 10, $\mu\text{mol/l}$)		Human serum [2] (<i>n</i> = 30, $\mu\text{mol/l}$)	
	Mean \pm S.D.	C.V. (%)	Mean \pm S.D.	C.V. (%)	Mean \pm S.D.	C.V. (%)	Mean \pm S.D.	C.V. (%)
SPM	125 \pm 3.5	2.8	2140 \pm 45	2.1	0.56 \pm 0.03	4.8	0.18 \pm 0.05	28.6
SPD	125 \pm 3.3	2.6	1210 \pm 43	3.6	0.15 \pm 0.02	13.6	0.29 \pm 0.06	21.0
PUT	125 \pm 2.6	2.1	360 \pm 18	5.2	0.34 \pm 0.04	11.8	0.49 \pm 0.13	26.6
CAD	125 \pm 4.7	3.7	40 \pm 5	8.5	0.26 \pm 0.03	12.1	0.05 \pm 0.02	38.5

found up to 250 pmol per injection, with a R^2 ranging from 0.9981 (SPM) to 0.9998 (CAD). The limit of detection, calculated at a signal-to-noise ratio of 3, was 0.1 pmol. Considering a five-fold dilution factor for plasma (see Section 2.3) and an injection volume of 5 μl , this limit corresponds to a plasma concentration of 0.1 $\mu\text{mol/l}$.

The applicability of the method was evaluated by chromatographing three different types of sample, pig plasma and homogenates of pig intestine biopsies (both obtained from conscious unrestrained pigs recovering from a partial enterectomy or SHAM operation) and human urine (obtained from healthy human volunteers) (Fig. 2B, C, D). To exclude inter-experiment variations all samples were pooled from ten specimen each. An adequate separation and sufficient sensitivity was found to measure polyamine concentrations in all three matrices (Fig. 2).

The coefficient of variation (C.V.) was determined by injecting standards (*n* = 15) and pooled samples (*n* = 10) (Table 2). The variation in retention time was below 0.5% (not shown). For standards, the peak-area C.V. ranged from 2.1 to 3.7%, in tissue, it ranged from 2.1 to 8.5% and in plasma, the C.V. ranged from 4.8 to 13.6%. For urine, the C.V. was not determined.

4. Discussion

The liquid chromatographic separation of basic compounds and thus of polyamines has always been difficult. This was mainly due to their interaction with residuing silanol groups of the packing material, resulting in broad tailing peaks. To solve this problem, special solvent systems have been used, containing high salt concentrations and/or amines and

low pH values [1,9,11]. These systems, however, do not allow the on-line separation of OPA polyamine derivatives from OPA amino acids. Therefore, it has become common practice to extract the interfering amino acid derivatives before injecting the sample [1–4]. Not only does this make the method more laborious, it also introduces two problems.

The recovery of the extraction procedure has to be established and monitored constantly by the addition of an internal standard to the samples.

The use of the OPA label in this application introduces another problem. It is known that OPA-polyamine derivatives degrade quickly after their formation [1,3]. Stabilization of the formed derivatives was only partially successful, resulting in recoveries ranging from 31% to 93% after a 30 min delay time, as compared to immediate injection [3]. Direct injection after derivatization was impossible to automate as the reaction had to be preceded by the extraction procedure. Thus, the coefficients of variations resulting from the whole process were relatively large (plasma C.V. ranged from 21% to 38.5%) [2].

The recent introduction of a new generation of ultra-pure silica, has enabled the production of reversed-phase packing materials, which allow the application of a much higher solvent pH (without degradation) and which exhibit minimal silanol interactions. These new features enabled the complete on-column separation of OPA amino acid derivatives from those of polyamines. As a result, the extraction procedure was no longer required, enabling the utilization of the proven automated pre-column derivatization procedure developed for amino acid measurements [13,14]. Despite the fact that samples now were injected immediately, the fluorescence response of especially the SPD deriva-

tive was low and variable. We suspected this to be caused by on-column degradation, and therefore attempted to increase derivative stability during the run, by decreasing the column temperature. This resulted in a large increase of the fluorescence response, reaching a maximum (3–4 fold) at about 10°C (Fig. 1) and a better reproducibility (Table 2).

The applicability of the resulting method was investigated, using three different types of sample. Plasma polyamine concentrations are low (Fig. 2B), almost reaching the limit of detection, as a result of which the C.V.s are rather large (Table 2). Homogenates from pig intestine biopsies showed a high concentration for SPM and SPD and a much lower concentration of PUT and CAD (Fig. 2C, Table 2). Urinary concentrations are sufficiently high, ensuring that there are no sensitivity problems, but the complexity of the matrix introduces a number of unknown peaks near the polyamine peaks, complicating the interpretation of the chromatogram (Fig. 2D). However, the separation is still adequate to allow a correct measurement.

The limit of detection of our method (0.1 pmol) and its C.V.-values (Table 2) are better as is described for most other OPA-applications [1–4]. Comparing our concentrations with literature values is difficult, as we study polyamine metabolism in pigs, while most references describe only human data. Nevertheless, the pig plasma polyamine concentrations we found, are in the same order of magnitude as reported human concentrations (Table 2).

The better chromatography shown in literature methods, results from the applied extraction procedures, removing all interferences and producing a more concentrated sample. However, it must be

considered that, in contrast to the present application, these methods also require validation of the extraction recovery and a correction for the rapid degradation of formed OPA derivatives.

In conclusion, the present method enables an easy and sensitive determination of polyamines in different types of sample, with minimal operator interference.

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