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Short communication

Polyamine analysis using N-hydroxysuccinimidyl-6-aminoquinoyl carbamate for pre-column derivatization

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Abstract

N-Hydroxysuccinimidyl-6-aminoquinoyl carbamate (AccQ.Fluor) was used as a polyamine pre-column derivatization reagent prior to HPLC analysis using a 5-μm C_8 reversed-phase column. The fluorescence detector excitation wavelength was set at 250 nm and emission at 395 nm. Quantitation, reproducibility, linearity, recovery and stability were demonstrated. The lower limit of detection was 660 fmol. This method is 45 and 61 times more sensitive than those using the pre-column derivatizing agents dansyl chloride and orthophthalaldehyde, respectively. Applicability to biological samples was demonstrated by analyses of polyamines in extracts of mouse erythrocytes and *Trypanosoma brucei brucei*.

Keywords: Polyamines; N-Hydroxysuccinimidyl-6-aminoquinoyl carbamate

1. Introduction

Polyamines are ubiquitous in all living cells and play an important role in cell growth and differentiation [1–3]. Malignant cell proliferation is associated with increased cellular polyamine metabolism [4]. Several diagnostic assays based on polyamine detection have been developed to screen for cancer, to evaluate efficacy of therapy and to detect relapse [5]. For example, in mice bearing lung carcinomas or melanomas, the concentration of spermidine in erythrocytes has been shown to be an index of tumor growth [6,7]. Deliberate interruption of polyamine biosynthesis has been used as an approach to treating several diseases; this has been notably successful in the case of African sleeping sickness caused by the protozoan parasite *Trypanosoma brucei gambiense* [8]. Improved polyamine analytical techniques will be useful both for diagnostic purposes and for metabolic studies intended to produce new anti-polyamine therapies.

A variety of methods for HPLC analysis of polyamines have been developed and most involve a pre-column derivatization of the amino groups of these molecules [9,10]. Although pre-column derivatization enhances sensitivity and speed of analysis, existing methods are not optimal for several reasons [11]. Dansyl chloride pre-column derivatization produces numerous side-products by reacting with itself and with imidazoles, phenolic hydroxyls, and some alcohols thus producing significant baseline noise level and decreasing selectivity.

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Orthophthalaldehyde reacts only with primary amino groups and the derivatives are stable for only a few hours. A derivatization method that would label both primary and secondary amines and also provide highly fluorescent and stable adducts would improve HPLC analysis of polyamines.

Here we report a single-step method utilizing an activated carbamate, N-hydroxysuccinimidyl-6-aminoquinoyl carbamate (AccQ.Fluor) that derivatizes all the amines of the three most important polyamines and provides stable and highly fluorescent adducts. Using polyamine standards, we demonstrate that analysis based on this pre-column derivatization method is quantitative, reproducible, linear and sensitive to 660 fmole. We applied this method to two biological systems measuring the polyamine content of *Trypanosoma brucei brucei* and mouse erythrocytes.

2. Experimental

2.1. Chemicals

An AccQ.Fluor kit, which included the AccQ.Fluor derivatizing agent N-hydroxysuccinimidyl-6-aminoquinoyl carbamate, was donated by Waters (Milford, MA, USA). Putrescine, spermidine and spermine were from Sigma (St. Louis, MO, USA). The internal standard, 1,7-diaminoheptane, was from Aldrich (Milwaukee, WI, USA). Eluent A (140 m*M* acetate, 17 m*M* triethanolamine, pH 5.05) was used as supplied in the AccQ.Fluor kit. Water was from a Millipore (New Bedford, MA, USA) Milli-Q system. HPLC-grade acetonitrile was from Fisher Scientific (Springfield, NJ, USA).

2.2. Extraction of biological samples

For isolation of erythrocytes, blood was collected from mice, using EDTA to prevent coagulation, and diluted 4-fold in buffer (58.5 m*M* disodium phosphate, 1.5 m*M* monopotassium phosphate, 43.5 m*M* sodium chloride, 10 m*M* trisodium citrate, 10 m*M* dithiothreitol, and 2.7 m*M* potassium chloride, pH 7.4). The diluted blood was centrifuged at 800 *g* for 5 min to sediment the erythrocytes leaving the plasma and most other formed elements in the supernatant. The erythrocyte pellet was washed three times by resuspension in buffer and centrifugation at 2000 *g* for 10 min. The final suspension volume was twice the packed cell volume. The suspended cells were sonicated for 10 min at 40 W and a 70% duty cycle (Heat System, Ultrasonics, Plainview, NY, USA). Cell ghosts were removed by centrifugation at 2000 *g* for 5 min. An aliquot of the supernatant was retained for protein assay and the balance was deproteinized by heating in a boiling water bath for 2 min. The sample was clarified by centrifugation at 2000 *g* for 5 min and the final supernatant was defined as the biological extract. Protein assays were done with the BioRad (Melville, NY, USA) dye-binding assay using bovine serum albumin as the standard.

Purified *T. brucei brucei* cells were obtained as previously described [12] and suspended in buffer (73 m*M* NaCl, 100 m*M* $NaPO_4$, pH 7.2) with a total volume twice the packed cell volume. The suspended cells were sonicated as described above. Cell ghosts and nuclei were removed by centrifugation at 2000 *g* for 5 min. The trypanosome lysate was processed as described above for mouse erythrocyte lysate.

2.3. Pre-column derivatization of samples

An internal standard (10 μl of 2 μM 1,7-diaminoheptane) was added to 10–60 μl of mixtures of polyamine standards (putrescine, spermine, spermidine) or biological extracts; borate buffer (0.2 *M* sodium borate, 1 m*M* EDTA, pH 8.8) was added to produce final volumes of 90 μl. The AccQ.Fluor reagent (10 μl) was added and the sample was mixed and incubated in a 55°C water bath for 20 min to promote derivatization. The 20-min incubation period was used to assure that the reaction was complete but control experiments demonstrated no gain in signal after a 10-min incubation. Derivatized samples were analyzed within 24 h.

2.4. High-performance liquid chromatography

The Waters HPLC system included a quaternary pump (Model 625), a system controller (Model 600E), an autosampler (Model 715), and a fluorescence detector (Model 470). The system was controlled and data were collected using Waters Millennium software. The peak-area values reported here were calculated by the Millennium software. A 5-

Table 1
Elution gradient

Time (min)	Flow-rate (ml/min)	Eluent A (%)	Acetonitrile (%)	Water (%)
0	1.0	100	0	0
1	1.0	99	1	0
18	1.0	95	5	0
19	1.0	91	9	0
29.50	1.0	83	17	0
33	1.0	60	40	0
42	1.0	0	60	40
50	1.0	100	0	0

μm silica particle C_8 Microsorb-MV column (150× 4.6 mm I.D.) with a 100-Å pore size (Rainin Instrument, Woburn, MA, USA) was used for separations. Fluorescence excitation was at 250 nm and emission was detected at 395 nm. The mobile-phase elution gradient is shown in Table 1. All changes in mobile phase were linear from one composition to the next. The flow-rate was 1.0 ml min^{-1}. All the analyses were done at room temperature.

2.5. Method validation

The suitability of the analytical system was assured by demonstrating that putrescine, spermidine, spermine and the internal standard were resolved from each other and from all other potentially interfering peaks in the biological sample extract. Instrument precision was monitored by making triplicate injections into the HPLC system from a single pooled standard. To demonstrate linearity, a series of standard solutions of the three polyamines in water were used to construct calibration curves; the polyamine content of these standards ranged from 0.66 to 40 pmol and 10 pmol of the internal standard, 1,7-diaminoheptane, was included in every injection. To demonstrate recovery of polyamines from a biological matrix, 20 pmol of polyamine standard was added to an erythrocyte extract and the results were compared to an unspiked sample.

3. Results and discussion

The resolution between each of the polyamines throughout this study was at least 1.2. Instrument precision was indicated by a <5% R.S.D. (standard deviation expressed as a percentage of the mean) for peak areas resulting from triplicate injections from a single pooled standard with each injection containing 10 pmol of putrescine, spermidine and spermine. Linearity was demonstrated by standard curves generated by injecting varying amounts of the three polyamine standards; the response was linear from 0.66 to 40 pmol with r^2 values >0.99 for all three polyamines. This linearity was independent of the volume of biological sample analyzed as demonstrated by r^2 values of >0.999 produced by comparing the peak areas for the three polyamines and the volumes of erythrocyte extract (15, 30 and 60 μl) derivatized and analyzed. Sensitivity was demonstrated by the ability to detect 660 fmol of each of the three polyamines in three independent analyses with R.S.D. values of 9%, 12% and 7% for putrescine, spermidine and spermine, respectively. The reported sensitivities of dansyl chloride and orthophthalaldehyde derivatizations are 30 and 40 pmol, respectively [9,10]; therefore these methods are 45- and 61-fold less sensitive, respectively. The calculated recoveries (±S.D.) for putrescine, spermidine and spermine added to an erythrocyte extract were 97 (±4)%, 94 (±5)%, 98 (±3)%, respectively. The stability of the adducts was demonstrated by analyses performed on samples held at room temperature for three days after derivatization; these produced peak areas 95 (±2)% of that obtained with samples analyzed immediately after derivatization.

Fig. 1 shows a chromatogram of a mixture containing 20 pmol of each of the three polyamine standards. Reproducibility was demonstrated by chromatograms done on four different days which showed mean retention times (±R.S.D.) of 35.2 (±1.2%), 36.2 (±1.0%), 36.9 (±0.4%) and 37.9

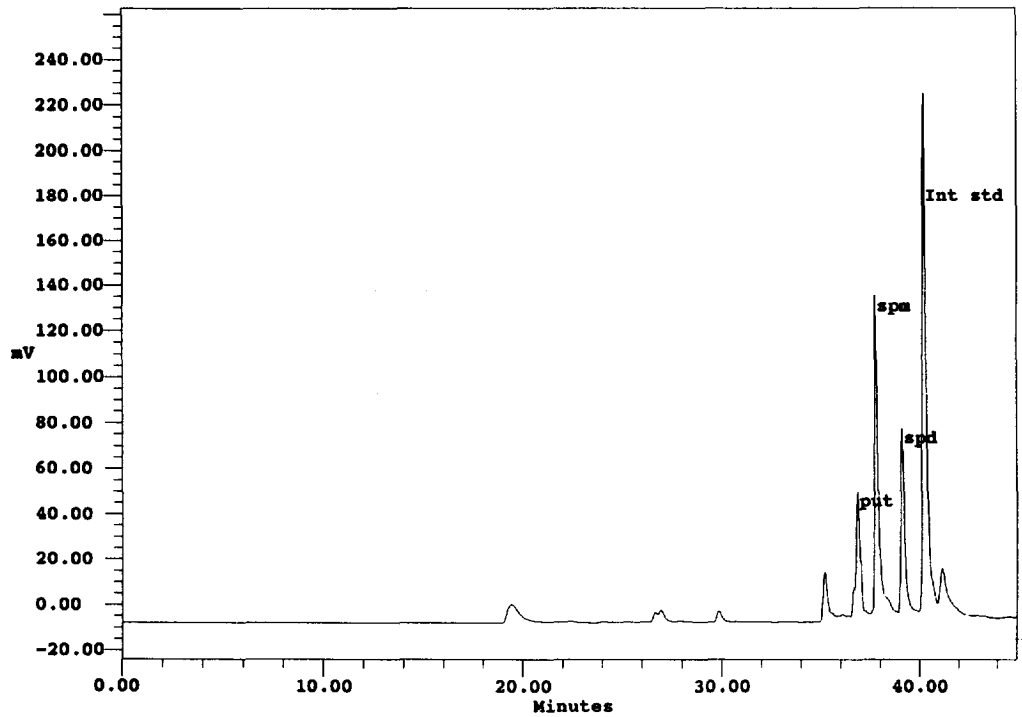

Fig. 1. Reversed-phase HPLC separation of 20 pmol of AccQ.Fluor derivatized polyamine standards. Put=putrescine; Spm=spermine; Spd=spermidine; Int std=internal standard. Chromatographic conditions are given in the text.

(±0.7%) for putrescine, spermidine, spermine and the internal standard, respectively. Fig. 2A is a chromatogram of polyamines in a mouse erythrocyte lysate. Identification of the peaks were confirmed by spiking the lysate with 30 pmol of polyamine standards, Fig. 2B. The mean concentrations from three separate analyses of mouse erythrocytes were: 38 (±5), 31 (±3) and 1.4 (±0.5) nmol per 10^9 erythrocytes for putrescine, spermidine and spermine, respectively. Values calculated from previously reported dansyl chloride-based analyses of mouse erythrocytes are: 0 (below detectable level), 24 (±3), and 6 (±2) nmol per 10^9 erythrocytes, respectively [13]. To demonstrate the versatility of this method, an extract of *T. brucei brucei* was prepared and analyzed, Fig. 3. The mean concentrations from three separate analyses (±S.D.) were found to be 16.38 (±1.4), 6.96 (±0.85) and 1.31 (±0.21) nmol mg^{-1} protein for putrescine, spermidine and spermine, respectively. Previously reported values for *T. brucei brucei* obtained with orthophthalaldehyde derivatized samples are 11.7 (±7.2), 5.9 (±4.8), and 1.2 (±1.3) nmol mg^{-1} protein, respectively [14]. Our trypanosome analyses agree more closely with previous data than our mouse erythrocyte analyses. The differences between our results and the previous reports may relate to the improved methodology or to the physiological state of the cells at the time the extracts were made.

In summary, the AccQ.Fluor system, originally developed for amino acid analyses [15], provides a pre-column derivatization method for polyamine analysis in biological samples that is quicker to perform, produces a more stable derivative and is more sensitive than previously described methods.

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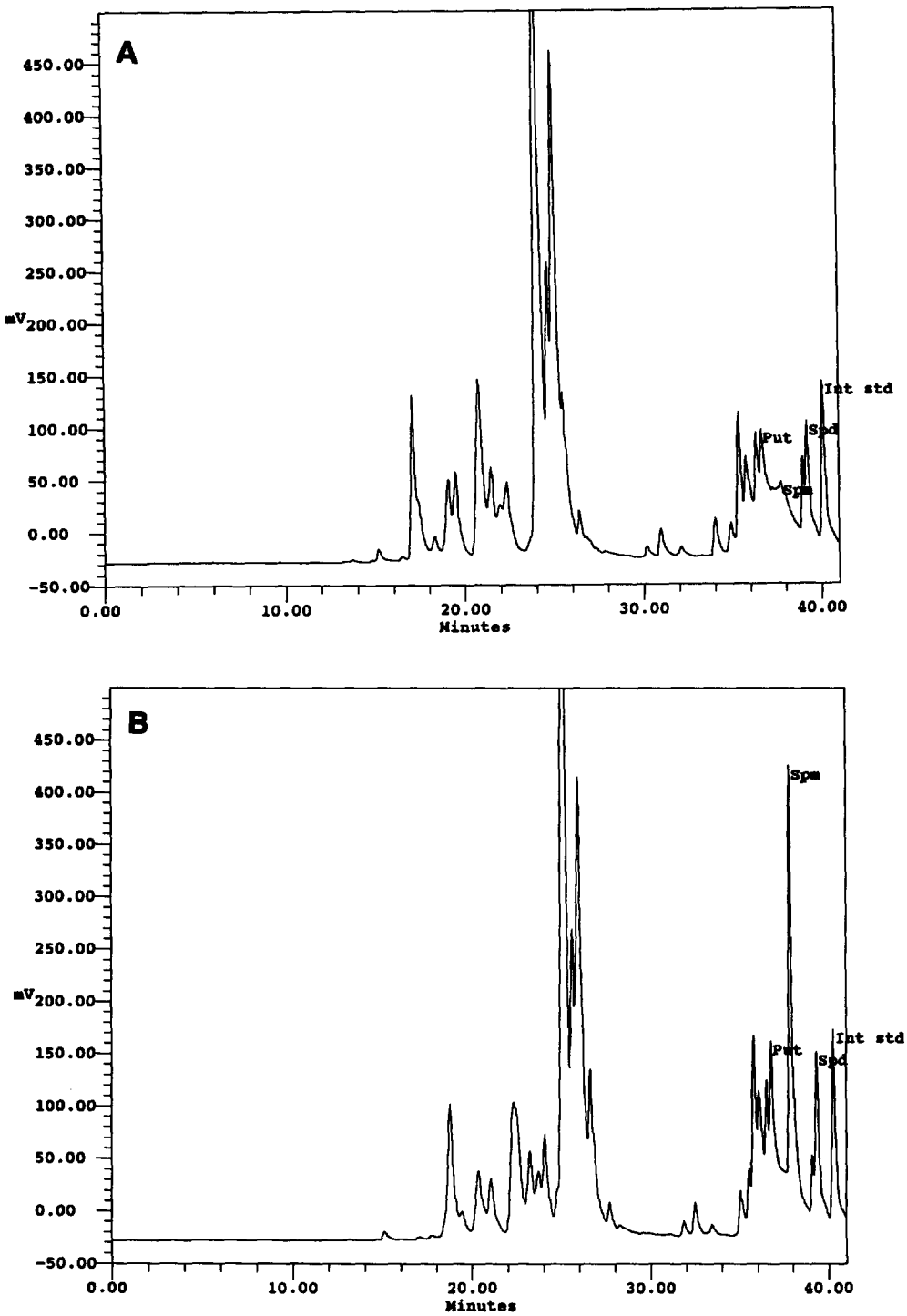

Fig. 2. Chromatograms of AccQ.Fluor derivatized polyamines from: (A) mouse erythrocyte extract and (B) mouse erythrocyte extract with 30 pmol of putrescine, spermidine and spermine added to the extract. Chromatographic conditions are given in the text. Abbreviations as in Fig. 1.

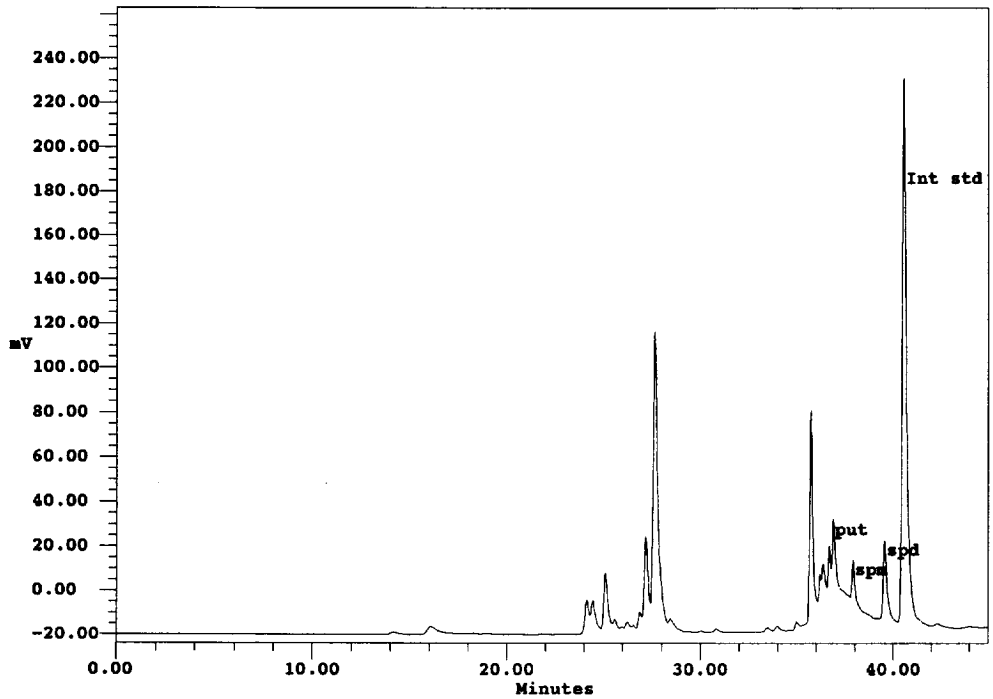

Fig. 3. Chromatogram of AccQ.Fluor derivatized polyamines from an extract of *Trypanosoma brucei brucei*. Chromatographic conditions are given in the text. Abbreviations as in Fig. 1.

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