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Simultaneous determination of polyamines, *N*-acetylated polyamines and the polyamine analogues BE-3-3-3 and BE-4-4-4-4 by capillary gas chromatography with nitrogen–phosphorus detection

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Abstract

We describe a method for the profiling of polyamines, *N*-acetylated polyamines and the polyamine analogues N^1,N^{11}-bis(ethyl)norspermine (BE-3-3-3) and 1,19-bis(ethylamino)-5,10,15-triazanonadecane (BE-4-4-4-4) in L1210 murine leukaemia cells by capillary gas chromatography with nitrogen–phosphorus detection. The method makes use of four internal standards. Prepurification comprises deproteinization, isolation with Sep-Pak silica at pH 9.0, conversion to heptafluorobutyryl derivatives and postderivatization organic fluid extraction. Within- and between-series precisions (given as C.V.s) for analysis of $1–2\times10^6$ cells were: putrescine 5.5 and 29.4%; spermidine 1.6 and 7.1%; and spermine 3.2 and 7.6%, respectively. Recoveries relative to the respective internal standards, were in the 70.6–104.7% range. Accuracy and precision of measurements of BE-4-4-4-4 can probably be improved by the introduction of a separate pentamine internal standard. We conclude that the method can be used for studying the effect of BE-3-3-3 and BE-4-4-4-4, and possibly their metabolites, on polyamine homeostasis (biosynthesis, retroconversion, transport, terminal catabolism) and polyamine function. © 1997 Elsevier Science B.V.

Keywords: Polyamines; *N*-Acetylpolyamines; N^1,N^{11}-Bis(ethyl)norspermine; 1,19-Bis(ethylamino)-5,10,15-triazanonadecane; Putrescine; Spermidine; Spermine

1. Introduction

The polyamines putrescine (Pu), spermidine (Sd) and spermine (Sp) (Fig. 1) are ubiquitous polycationic aliphatic compounds that are present in all eukaryotic cells [1]. Cellular polyamine homeostasis is maintained by biosynthesis, retroconversion, uptake and terminal degradation [2]. Retroconversion proceeds by initial *N*-acetylation (producing *N*-acetylated polyamines), whereas terminal catabolism leads to non-α-amino acid metabolites.

Polyamines are essential for cell growth. Interference with intracellular polyamine homeostasis is, therefore, investigated as a cancer treatment modality. Perturbation of polyamine balance can be achieved either by inhibition of polyamine biosynthesis, or by polyamine analogues [1]. Polyamine analogues aim at interference with both polyamine homeostasis and function [3]. N^1,N^{11}-bis(ethyl)norspermine (BE-3-3-3) and 1,19-bis(ethylamino)-5,10,15-triazanonadecane (BE-4-4-4-4) are among

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$H_2N-^{3}CH_2-CH_2-^{1}CH_2-NH_2$	Diaminopropane
$H_2N-^{3}CH_2-CH_2-^{1}CH_2-NH-CO-CH_3$	Monoacetyl diaminopropane
$H_2N-^{4}CH_2-CH_2-CH_2-^{1}CH_2-NH_2$	Putrescine
$H_2N-^{4}CH_2-CH_2-CH_2-^{1}CH_2-NH-CO-CH_3$	Monoacetyl putrescine
$H_2N-^{5}CH_2-CH_2-CH_2-CH_2-^{1}CH_2-NH_2$	Cadaverine
$H_2N-^{5}CH_2-CH_2-CH_2-CH_2-^{1}CH_2-NH-CO-CH_3$	Monoacetyl cadaverine
$H_2N-^{8}CH_2-CH_2-CH_2-CH_2-NH-CH_2-CH_2-^{1}CH_2-NH_2$	Spermidine
$H_2N-^{8}CH_2-CH_2-CH_2-CH_2-NH-CH_2-CH_2-^{1}COOH$	Putreanine
$HOOC-CH_2-CH_2-CH_2-NH-CH_2-CH_2-CH_2-NH_2$	Isoputreanine
$H_2N-^{8}CH_2-CH_2-CH_2-CH_2-NH-CH_2-CH_2-^{1}CH_2-NH-CO-CH_3$	N^1-acetylspermidine
$CH_3-CO-HN-^{8}CH_2-CH_2-CH_2-CH_2-NH-CH_2-CH_2-^{1}CH_2-NH_2$	N^8-acetylspermidine
$H_2N-^{12}CH_2-CH_2-CH_2-HN-CH_2-CH_2-CH_2-CH_2-NH-CH_2-CH_2-^{1}CH_2-NH_2$	Spermine
$H_2N-^{12}CH_2-CH_2-CH_2-HN-CH_2-CH_2-CH_2-CH_2-NH-CH_2-CH_2-^{1}CH_2-NH-CO-CH_3$	N^1-acetylspermine
$H_3C-CH_2-HN-(CH_2)_3-HN-(CH_2)_3-NH-(CH_2)_3-NH-CH_2-CH_3$	BE-3-3-3
$H_3C-CH_2-HN-(CH_2)_4-HN-(CH_2)_4-NH-(CH_2)_4-NH-(CH_2)_4-NH-CH_2-CH_3$	BE-4-4-4-4

Fig. 1. Chemical structures of the polyamines and their derivatives.

the bis(ethyl)polyamine analogues [4]. They cause profound growth inhibition in experimental systems and are currently evaluated in clinical phase I trials. BE-3-3-3 and BE-4-4-4-4 inhibit polyamine biosynthesis through inhibition of the key enzymes ornithine decarboxylase (BE-3-3-3 [5] and BE-4-4-4-4 [6]) and *S*-adenosylmethionine decarboxylase (BE-3-3-3 [5]). BE-3-3-3 may also deplete polyamines by augmentation of the retroconversion pathway [5,7]. Mitochondrial damage and ATP depletion are features that may contribute to the commonly observed cytotoxicity of polyamine analogues [4].

Measurement of intracellular analogue contents is important, since analogue accumulation is a determinant for their antitumour efficacy [4]. BE-3-3-3 metabolites were observed in the liver and kidney of dogs [8]. These metabolites seem to derive from deethylation and retroconversion. Profiling of the naturally occurring polyamines, *N*-acetylated polyamines and their non-α-amino acid metabolites, together with polyamine analogues and their metabolites may, therefore, be of importance to the design of rational cancer therapy strategies.

Cation-exchange and reversed-phase HPLC, followed by on-line UV–Vis spectrophotometric or fluorimetric detection, are commonly applied methods for the analysis of polyamines [2]. At present, simultaneous determination of polyamines and polyamine analogues is usually performed by initial dansylation followed by reversed-phase HPLC with fluorimetric detection [4,5,7–9]. We present a gas chromatographic profiling method for polyamines, their *N*-acetylated conjugates and the polyamine analogues BE-3-3-3 and BE-4-4-4-4. The method makes use of capillary gas-chromatography with nitrogen–phosphorus detection and is essentially as described by Van den Berg et al. [10]. It was applied to measurements in murine L1210 leukaemia cells.

2. Experimental

2.1. Chemicals

BE-3-3-3 was a gift from Dr. R. Schipper (Department of Pathology, University Hospital Nijmegen, The Netherlands) and BE-4-4-4-4 was a gift from the National Institutes of Health (Bethesda, MD, USA). Di- and polyamines [diaminopropane (DAP), Pu, cadaverine (Cad), 1,6-diaminohexane (Internal standard 1; I.S.-1), Sd, Sp], *N*-acetylated di- and polyamines [monoacetyl diaminopropane (acDAP), monoacetyl putrescine (acPu), monoacetyl cadaverine (acCad), N^8-acetyl spermidine (N^8-acSd), N^1-acetylspermidine (N^1-acSd), N^1-acetyl spermine (N^1-acSp)] and the non-α-amino acid putreanine (Putr) were from Sigma (St. Louis, MO, USA). Isoputreanine γ-lactam (Isoga) was from Aldrich (Beerse, Belgium). Isoga was converted to the non-α-amino acid isoputreanine (Isoputr) by heating 2.5 mmol Isoga in 2 ml of 6 *M* HCl during 18 h at 120°C and adjustment of the volume to 100 ml with water. Monoacetyl-1,6-diaminohexane (Internal standard 2; I.S.-2), non symmetrical homospermidine (internal standard 3; I.S.-3) and symmetrical homospermine (internal standard 4; I.S.-4) were synthesized as described by Van den Berg et al. [10].

Sep-Pak silica cartridges were from Waters (Milford, MA, USA), heptafluorobutyric anhydride from Pierce (Rockford, Il, USA) and Carbowax 1000M from Chrompack (Middelburg, The Netherlands). All other reagents were from Merck (Darmstadt, Germany).

2.2. L1210 cell suspension

The murine L1210 lymphocytic leukaemia cell line was kindly donated by Mr. P.K. Wieringa (Department of Radiobiology, Groningen State University, The Netherlands). Three times weekly, cells were routinely cultured in 25-cm^2 flasks (Nunc, Roskilde, Denmark) at a density of 5.10^4/ml in 10 ml RPMI-1640 medium (BioWhitaker, Verviers, Belgium), containing 10% fetal calf serum (GIBCO Life Technologies, Merelbeke, Belgium), 300 mg/l glutamine and 50 μ*M* mercaptoethanol. Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2.

For the assessment of precision and recovery (see below), we grew the cells in 9 flasks at an initial density of 1×10^4 cells per ml in 10 ml of medium per flask. They were harvested after three days and washed three times in ice-cold phosphate buffered saline (PBS). The cell suspensions were counted in a haemocytometer, divided into 1.0-ml aliquots ($1–2\times10^6$ L1210 cells per ml), frozen in liquid nitrogen and stored at −20°C until analysis.

2.3. Analytical method

2.3.1. Prepurification

Frozen samples, containing $1–2\times10^6$ L1210 cells in 1 ml of PBS, were thawed. A 400-μl aliquot of an internal standard solution (containing 31.25 μmol/l each of internal standards 1–4 in 0.1 *M* HCl solution) was added to each sample. Samples were deproteinized by adding 1.8 ml 12% sulphosalicylic acid and subsequent freezing at −20°C for 1 h. After thawing they were centrifuged at 2000 *g* for 10 min at 4°C. The supernatant was transferred to a polyethylene tube. 8 ml of 50 m*M* borate buffer (pH 9.0) were added. The pH was adjusted to 9.0 with 4 *M* NaOH, if necessary. The analytes were adsorbed to a Sep-Pak silica cartridge, that was prewashed with 30 ml of 0.02 *M* acetic acid, 30 ml of water, 10 ml of 0.1 *M* HCl in methanol and 30 ml of water. After sample application the column was washed with 30 ml of water, and subsequently eluted with 10 ml of 2 *M* HCl in methanol (i.e. 12 *M* HCl diluted to 2 *M* in methanol). Five ml of eluate were evaporated to dryness overnight in a Savant Speedvac AS260 vacuum evaporator (Savant Instruments, Bierbeek, Belgium) at 30°C.

2.3.2. Derivatization

The compounds were derivatized by adding a mixture of 200 μl each of acetonitril and heptafluorobutyric anhydride and heating at 60°C for 1 h. 1 ml of methanol was added after cooling, and the sample was evaporated to dryness under a stream of nitrogen at room temperature. The residue was dissolved in 1 ml 0.5 *M* phosphate buffer (pH 7.0) and the derivatives were extracted with 3 ml dichloromethane. The dichloromethane layer was collected, dehydrated with a small amount of anhydrous sodium sulphate and evaporated to dryness under a

stream of nitrogen at room temperature. The residue was finally dissolved in 100 μl ethyl acetate containing 0.2% v/v Carbowax 1000 M. Aliquots of 1.5 μl were injected into the gas chromatograph with an autosampler.

2.3.3. Gas chromatography

For gas chromatography with nitrogen–phosphorus detection, we used a Model 6890 gas chromatograph equipped with a Model 6890 automated sampler (both from Hewlett-Packard, Amstelveen, The Netherlands). The gas chromatograph was interfaced with a ChromPerfect analytical processing system (Justice Innovations, Mountain View, CA, USA). We used a 37.5 m×0.2 mm (I.D.) fused-silica capillary column that was coated with cross-linked methyl silicone (film thickness 0.11 μm), and deactivated with siloxane (Hewlett-Packard, Amstelveen, The Netherlands). Gas flow-rate (helium) was 0.6 ml/min, split ratio 1:65, detector temperature 300°C, and injector temperature 260°C. The oven temperature programme was: initial temperature 120°C, increase at 5°C/min to 260°C, then increase at 2°C/min to 280°C and constant at 280°C for 15 min.

2.3.4. Quantification

Quantification was performed by comparing the peak area ratio of each analyte and its internal standard with the corresponding ratio of a standard. The latter contained 12.5 nmol each of all polyamines, *N*-acetylated polyamines and their non-α-amino acid derivatives (see above), together with 6.25 nmol each of BE-3-3-3 and BE-4-4-4-4, and 12.5 nmol each of the internal standards 1–4. Prior to its analysis, this standard was evaporated to dryness in the Speedvac and derivatized as described above.

We quantified the diamines DAP, Pu, Cad, Isoputr and Putr by using 1,6-diaminohexane (internal standard 1) as an internal standard; the monoacetylated diamines acDAP, acPu and acCad on the basis of monoacetyl-1,6-diaminohexane (Internal standard 2); the triamines Sd, N^8-acSd and N^1-acSd on the basis of non-symmetrical homospermidine (internal standard 3); and BE-3-3-3, Sp, N^1-acSp and BE-4-4-4-4 on the basis of symmetrical homospermine (internal standard 4).

2.4. Quality control

2.4.1. Within-series precision and recovery

Aliquots of 1 ml of cell suspension were spiked with 0.781, 1.563, 3.125 or 6.25 nmol each of BE-3-3-3 and BE-4-4-4-4 ($n=6$ for each concentration). To the samples that were enriched with 1.563 nmol each of BE-3-3-3 and BE-4-4-4-4, we additionally added 200 μl of a solution containing 62.5 μmol/l each of DAP, acDAP, Pu, Cad, acPu, acCad, Isoputr, Putr, Sd, N^8-acSd, N^1-acSd, Sp and N^1-acSp. Six unspiked aliquots of 1 ml cell suspension were used for the assessment of the endogenous contents. All samples ($n=30$) were prepurified in a single series and subsequently analyzed together with 3 standards by gas chromatography within 40 h.

2.4.2. Between-series precision and recovery

Aliquots of 1 ml of another cell suspension were spiked with 1.563 or 6.25 nmol each of BE-3-3-3 and BE-4-4-4-4 ($n=3$ for each concentration). To the samples that were enriched with 1.563 nmol each of BE-3-3-3 and BE-4-4-4-4, we additionally added 200 μl of a solution containing 62.5 μmol/l each of DAP, acDAP, Pu, Cad, acPu, acCad, Isoputr, Putr, Sd, N^8-acSd, N^1-acSd, Sp and N^1-acSp. Three unspiked aliquots of 1 ml cell suspension were used for the assessment of the endogenous contents. The 3 sets of samples, each set composed of 3 samples with 0, 1.563 and 6.25 nmol BE-3-3-3 and BE-4-4-4-4, were prepurified and subsequently analyzed together with one standard at 3 different occasions in a period of 2 weeks.

3. Results

Fig. 2 shows a chromatogram of the endogenous polyamines in an L1210 cell suspension (panel A), together with a chromatogram of the same cell suspension after enrichment with 12.5 nmol each of the various polyamines, *N*-acetylated polyamines and their non-α-amino acid metabolites, and with 1.563 nmol each of BE-3-3-3 and BE-4-4-4-4 (panel B). The cells contained notably Sd and Sp, and 5–10 times lower amounts of Pu. BE-3-3-3 eluted close to Sp, but was nevertheless baseline separated. The

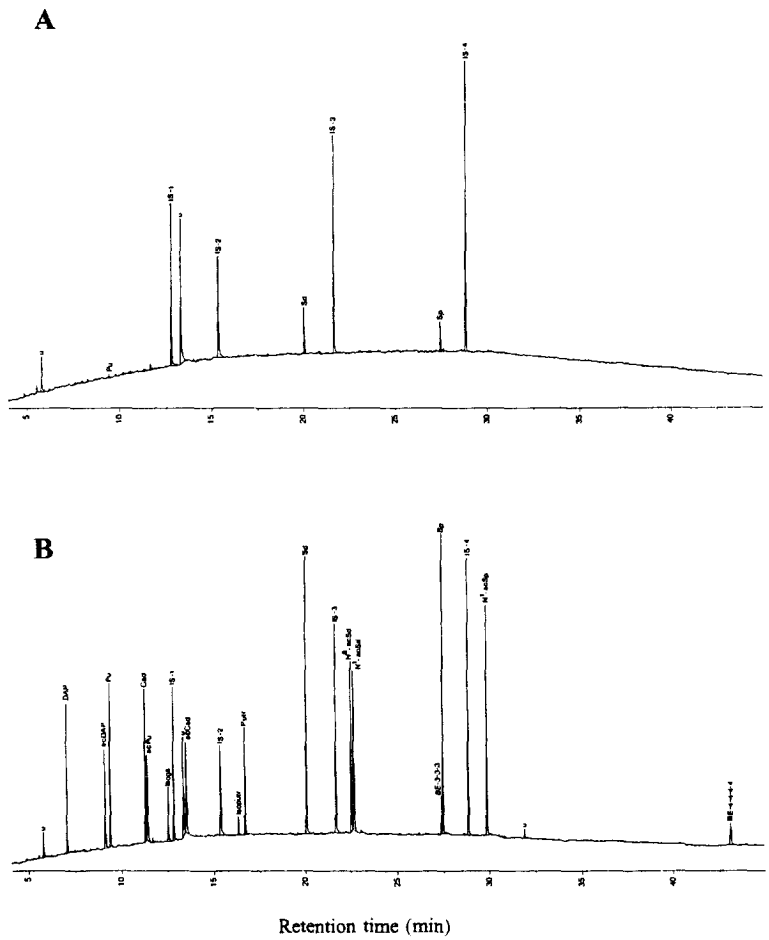

Fig. 2. Capillary gas chromatograms of heptafluorobutyryl derivatives of polyamines in L1210 cells. Panel A shows endogenous L1210 polyamines; panel B is prepared from L1210 cells that were enriched with 12.5 nmol each of the polyamines, *N*-acetylated polyamines, non-α-amino acid metabolites and 1.563 nmol each of BE-3-3-3 and BE-4-4-4-4. In both panels, the cells were enriched with 12.5 nmol each of the internal standards 1-4. DAP, 1,3-diaminopropane; acDAP, monoacetyl diaminopropane; Pu, putrescine (1,4-diaminobutane); Cad, cadaverine (1,5-diaminopentane); acPu, monoacetyl putrescine (*N*-acetyl-1,4-diaminobutane); Isoga, isoputreanine-γ-lactam [*N*-(3-aminopropyl)pyrrolidin-2-one]; I.S.-1, internal standard 1 (1,6-diaminohexane); acCad, monoacetyl cadaverine (*N*-acetyl-1,5-diaminopentane); I.S.-2, internal standard 2 (monoacetyl 1,6-diaminohexane); Isoputr, isoputreanine [*N*-(3-aminopropyl)-4-aminobutyric acid]; Putr, putreanine [(4-aminobutyl)-3-aminopropionic acid]; Sd, spermidine [*N*-(3-aminopropyl)-1,4-diaminobutane]; I.S.-3, internal standard 3 (non symmetrical homospermidine); N^8-acSd, N^8-acetyl spermidine, N^1-acSd, N^1-acetyl spermidine; BE-3-3-3, N^1, N^{11}-bis(ethyl)norspermine; Sp, spermine [N^1,N^{11}-bis(3-aminopropyl)-1,4-diaminobutane]; I.S.-4, internal standard 4 (symmetrical homospermine); N^1-acSp, N^1-acetyl spermine; BE-4-4-4-4, [1,19-bis(ethylamino)-5,10,15-triazanonadecane]; u, unknown contaminants.

pentamine BE-4-4-4-4 eluted about 5 min after the final temperature of 280°C.

Table 1 shows the within-series and between-series precision and recovery for BE-3-3-3 and BE-4-4-4-4, together with those of the naturally occurring polyamines and their *N*-acetylated conjugates. Within- and between-series precision (given as C.V.s) at indicated endogenous levels were: Pu 5.5 and 29.4%; Sd 1.6 and 7.1%; and Sp 3.2 and 7.6%, respectively. Within-series recovery, relative to the respective internal standards, was in the 70.6–104.7% range. Within-series and between-series recoveries did not show major differences, with the exception of BE-4-4-4-4. The C.V.s of the recoveries

Table 1
Within- and between-series precisions and recoveries for polyamines, *N*-acetylated polyamines and the polyamine analogues BE-3-3-3 and BE-4-4-4-4 in L1210 murine leukaemia cells

	Spiked (nmol)	Within series ($n=6$)			Between series ($n=3$)		
		Endogenous (nmol)	Recovery (%)	C.V. (%)	Endogenous (nmol)	Recovery (%)	C.V. (%)
BE-3-3-3	0.781	nd[a]	89.4	3.3			
	1.563	nd	93.7	2.8	nd	95.6	5.2
	3.125	nd	97.7	2.0			
	6.25	nd	97.0	2.3	nd	99.2	3.7
BE-4-4-4-4	0.781	nd	70.6	5.8			
	1.563	nd	82.9	2.7	nd	91.5	4.5
	3.125	nd	87.0	3.3			
	6.25	nd	87.1	6.8	nd	95.3	4.0
DAP	12.5	nd	92.1	1.7	nd	92.4	0.8
acDAP	12.5	nd	83.0	15.6	nd	87.7	3.3
Pu	12.5	0.46±0.03	93.9	2.8	0.28±0.08	95.4	3.7
Cad	12.5	nd	101.6	1.2	nd	98.2	1.9
acPu	12.5	nd	92.9	4.4	nd	86.9	4.3
acCad	12.5	nd	104.5	1.9	nd	96.6	6.9
Sd	12.5	4.65±0.08	104.7	1.4	2.54±0.18	102.5	6.5
N^8-acSd	12.5	nd	103.3	2.7	nd	96.0	6.4
N^1-acSd	12.5	nd	104.5	1.5	nd	97.6	4.8
Sp	12.5	2.90±0.09	99.4	3.2	1.45±0.11	101.9	2.4
N^1-acSp	12.5	nd	99.5	2.8	nd	96.4	0.9

[a] nd=not detectable. L1210 cell numbers were 1.6×10^6 cells/ml (within-series) and 1.7×10^6 cells per ml (between-series).

were generally below 7%, with their within-series precisions generally being somewhat lower than their between-series precisions. An exception was acDAP, which showed a 15.6% within-series C.V..

The detection limit (peak/noise ratio=2) for the polyamine analogues proved to be 0.4–0.6 pmol when 1 μl is injected with a split ratio of 1:65. The detection limits for Pu, Sd and Sp were 0.7 pmol, 0.4 pmol and 0.5 pmol, respectively.

4. Discussion

The present capillary gas chromatographic profiling method for polyamines, their *N*-acetylated conjugates, and the polyamine analogues BE-3-3-3 and BE-4-4-4-4 in cells or tissues is based on the method of Van den Berg et al. [10], who applied it to the analysis of polyamines, *N*-acetylated polyamines and their non-α-amino acid metabolites in urine. From the results (Table 1) we conclude that precision and recovery of polyamines and *N*-acetylated polyamines are comparable to the previously reported data [10]. The endogenous polyamine contents in the within-series experiment were different from those in the between-series experiment, due to the fact that the cell suspensions, though equal in cell numbers per milliliter, were from different cultures with different viabilities. Comeasurement of BE-3-3-3 can be performed with comparable accuracy (as derived from blanks and recovery) and precision, but quality control of BE-4-4-4-4 may be subject to future improvement (see below). As we spiked after thawing, we did not measure intracellular analogue levels. However, our quality control data will probably also apply for cells, which have taken up these compounds from the extracellular matrix, since addition of 12% sulphosalicylic acid is generally considered to be sufficient to release polyamines from intracellular binding sites.

A major difference with respect to our previous method is elution of the Sep-Pak silica column with 2 *M* HCl in methanol, instead of 0.1 *M* HCl in methanol [10]. This proved necessary to recover

BE-4-4-4-4 to a reasonable extent. The interaction of polyamines with the silica matrix at pH 9.0 is known to increase with the number of nitrogen atoms, necessitating the use of stronger acid to remove the pentamine BE-4-4-4-4. Elution with 1 *M* HCl in methanol resulted in a 65–74% recovery, relative to the internal standard (data not shown), and was further improved to 71–87% (Table 1) by using 2 *M*. Use of higher HCl concentrations to further improve recovery might not be advisable because of the eventual risk of the hydrolysis of *N*-acetylated conjugates. The suboptimal recovery of BE-4-4-4-4 and its resulting suboptimal quality control (Table 1) will undoubtedly be improved by the use of a separate internal standard for pentamines, such as the linear polyamines from thermophilic eubacteria and hyperthermophilic archaebacteria [11]. These include caldopentamine (3-3-3-3) and homocaldopentamine (3-3-3-4), but these compounds were not available to us during the study. The use of a pentamine internal standard also eliminates the differences in nitrogen–phosphorus detection response factors between BE-4-4-4-4 and its present tetramine internal standard symmetrical homospermine.

We employed derivatization with heptafluorobutyric anhydride, instead of methylation/heptafluorobutyration [12]. It precludes accurate and precise analysis of the non-α-amino acid metabolites with the present method. Aggressive methylation with acetylchloride/methanol [12] jeopardizes the integrity of the *N*-acetylated polyamines, whereas non-α-amino acid metabolises are usually not detectable in cells. Moreover, the Sep-Pak silica co-isolation and subsequent methylation/heptafluorobutyration of dibasic amino acids (like lysine) may cause high peaks in the area of interest, under conditions that these amino acids are abundant. That Isoputr and Putr are, nevertheless, demonstrable in the sample after their addition (panel B) is caused by the elution of the Sep-Pak silica cartridge with the relatively anhydrous methanolic HCl solution and its subsequent evaporation at 30°C. These conditions cause incomplete methylation (Isoputr and Putr) and partial γ-lactamization of Isoputr [13]. The resulting (methyl)heptafluorobutyryl [(Me)HFB] derivatives elute in the sequence Isoga-(HFB), Isoputr-Me$(HFB)_2$ and Putr-Me$(HFB)_2$ (Panel B).

We conclude that the present method can be used for studies of the polyamine analogues BE-3-3-3 and BE-4-4-4-4. Because of the effects of polyamine analogues on polyamine biosynthesis, retroconversion and transport, and their interference with polyamine function, these studies may benefit from simultaneous determination of polyamines, *N*-acetylated polyamines, analogues and most probably also analogue metabolites. Bergeron et al. [8] found that BE-3-3-3 is metabolized in dogs by deethylation [to mono(ethyl)norspermine and norspermine] and subsequent further degradation [to mono(ethyl)norspermidine, norspermidine, mono(ethyl)DAP and DAP]. It is conceivable that BE-4-4-4-4 is subject to similar metabolism, and that some of these metabolites will add to the effect of the parent analogue, or even explain their main mode of action. These metabolites are undoubtedly co-isolated and derivatized, whereas the high number of theoretical plates of capillary gas chromatography and the selective detection with nitrogen–phosphorus detection, if necessary combined with mass spectrometry, will enable their identification and quantification.

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