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Monitoring of the uptake and metabolism of aminooxy analogues of polyamines in cultured cells by high-performance liquid chromatography

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ABSTRACT

A high-performance liquid chromatographic method for the determination of polyamines and their aminooxy analogues is described. Oxime derivatization with a ketone is used to protect the aminooxy group during post-column reaction with *o*-phthalaldehyde. The amount of the polyamines and of the oximes of their aminooxy analogues can be determined simultaneously in cultured cells and cell culture media. The limit of detection is 20–30 pmol, and the response of the fluorescence detection is linear up to 4 nmol. The separation of the aminooxy analogues from the naturally occurring polyamines can be varied by using different ketones for oxime formation. The method was used to measure the stability of aminooxy analogues of putrescine (1-aminooxy-3-aminopropane) and spermidine [N-(2-aminooxyethyl)-1,4-diaminobutane and 1-aminooxy-3-N-(3-aminopropyl)aminopropane] in cell culture media and the uptake into cultured baby hamster kidney (BHK21/C13) cells.

INTRODUCTION

Polyamines are polycationic compounds that are essential constituents of living cells. The importance of polyamines in the proliferation, differentiation and function of mammalian cells is well studied [1–3]. The biosynthesis of polyamines is enhanced during growth, and growth-promoting stimuli usually enhance polyamine synthesis. Hence, the pathway of polyamine biosynthesis is an interesting target for the design of anti-neoplastic agents [4,5]. Recently, aminooxy analogues of polyamines have been proved very useful tools in studying the physiology of polyamines. 1-Aminooxy-3-aminopropane (APA), an aminooxy analogue of putrescine, and N-(2-aminooxyethyl)-1,4-diaminobutane (AOE-PU) and 1-aminooxy-3-N-(3-aminopropyl)aminopropane (AP-APA), aminooxy analogues of spermidine, are some of these compounds shown to have potential use in unravelling the regulation and the reaction mechanisms of enzymes involved in po-

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lyamine metabolism [6–11]. APA reversibly prevents the proliferation of cultured baby hamster kidney cells (BHK21/C13) [7]. As a result of APA treatment, the cellular spermidine level is rapidly reduced and excretion of putrescine, spermidine and acetylated polyamines into the culture medium stops. One problem in these studies has been how to measure the amounts of the aminooxy analogues in cells and culture media. In this paper we describe a high-performance liquid chromatographic (HPLC) method by which polyamines and their aminooxy analogues can be determined simultaneously.

EXPERIMENTAL

Chemicals

Putrescine, spermidine and spermine (Boehringer Mannheim, Germany, or Calbiochem, San Diego, CA, USA) were crystallized from hot 6 *M* HCl–ethanol solution. Spermine was further purified on a Dowex 50 (H^+) column [12]. Acetylated polyamines and [2-^{14}C]acetone (specific activity 56.2 μCi/mmol) were obtained from Sigma (St. Louis, MO, USA). 1-Aminooxy-3-aminopropane (APA, $H_2NOCH_2CH_2CH_2NH_2$) was from Orion Oy (Espoo, Finland). The aminooxy analogues of spermidine, N-(2-aminooxyethyl)-1,4-diaminobutane (AOE-PU, $H_2NOCH_2CH_2NHCH_2CH_2CH_2CH_2NH_2$) and 1-aminooxy-3-N-(3-aminopropyl)aminopropane, (AP-APA, $H_2NCH_2CH_2CH_2NHCH_2CH_2CH_2ONH_2$) were synthesized as described previously [13]. Other chemicals were of analytical grade (Merck, Darmstadt, Germany).

Cell culture and sample preparation

BHK21/C13, a continuous cell line derived from baby hamster kidney, was maintained as described earlier but in DMEM and without antibiotics [7]. The media were supplemented with 10% foetal calf serum (Gibco, Grand Island, NY, USA) guaranteed to be free of any mycoplasmas or nanobacteria (common contaminants of commercial sterile serum products) by Abcell (Kuopio, Finland). The experiments were started from confluent cultures by subculturing $1 \cdot 10^6$ cells in 6-cm diameter dishes and letting the cells adhere for 24 h before experiments. The cells were detached into phosphate-buffered saline (PBS; 116 m*M* NaCl, 20.8 m*M* Na_2HPO_4, 2.9 m*M* KH_2PO_4), centrifuged and disrupted in cold 5% sulphosalicylic acid. The pellet was used for the DNA measurement [14]. The supernatant was neutralized, and at least 40-fold molar excess of acetone or ethyl methyl ketone was added (10 μl/ml of sample) in 100 m*M* potassium phosphate buffer (pH 7.4). The derivatization of the aminooxy group is quantitative if an excess of acetone or ethyl methyl ketone is used at neutral pH. After incubation for 1 h at room temperature (20–22°C), a 20- or 30-μl aliquot was directly subjected to HPLC analysis.

HPLC methods

The HPLC method used for separation of the naturally occurring polyamines and the oximes of aminooxy analogues of polyamines was the same as previously described [15]. A 20- or 30-μl sample was applied to a reversed-phase column (Hewlett-Packard Hypersil ODS 5 μm, 100 × 2.1 mm I.D., Hewlett-Packard, Waldbronn, Germany) [15] equilibrated for 5 min with buffer A [8 m*M* octanesulphonate and 0.1 m*M* EDTA in a 98:2 (v/v) mixture of 0.1 *M* potassium phosphate, (pH 2.5) and acetonitrile]. The column was then eluted with buffer A for 5 min, followed by a linear gradient to 75% buffer B [8 m*M* octanesulphonate in a 7:3 (v/v) mixture of 0.2 *M* potassium phosphate (pH 3.1) and acetonitrile] over 15 min (*i.e.* 5%/min), then holding this mixture, 25% buffer A, 75% buffer B for 5 min, and back to the starting conditions over 3 min at a flow-rate of 0.5 ml/min at 37°C, using an HP 1090 liquid chromatograph (Hewlett-Packard). The polyamines and the oximes of the aminooxy drugs were detected by a fluorescence detector after post-column derivatization with *o*-phthalaldehyde [16]. The *o*-phthalaldehyde solution (2 g of *o*-phthalaldehyde, 15 ml of methanol, 0.5 ml of mercaptoethanol) was mixed under nitrogen with 1 l of 1 *M* borate buffer, pH 10.4 (61.83 g of boric acid, 45.6 g of potassium hydroxide, 6 ml of 30% Brij 35, in 1 l of water). The mixture was allowed to stabilize under nitrogen for 1 h. The fluorescence reagent was pumped at a flow-rate of 0.4 ml/min and the detection was carried out with a 340-nm excitation filter and a 425-nm emission

filter (LKB 4460 fluorescence detector, LKB Biochrom, Cambridge, UK).

RESULTS AND DISCUSSION

The acetone oxime of APA eluted with N^1-acetylspermidine, and the oximes of AOE-PU and AP-APA did not separate completely from spermidine and spermine (not shown). Because the amount of N^1-acetylspermidine in BHK21/C13 cells is usually below the detection limit, the acetone oxime of APA can be used to monitor the amount of APA in the cells. The presence or the absence of N^1-acetylspermidine can still be reliably measured from samples that are not treated with acetone.

The ethyl methyl ketone oximes of AOE-PU and AP-APA eluted after spermine and are suitable for the determination of these analogues. The ethyl methyl ketone oxime of APA eluted with spermidine (Fig. 1A and B).

The oximes formed with diethyl ketone eluted after spermine in the following order: APA partially overlapping with spermine, AOE-PU and AP-APA (data not shown). Acetone and ethyl methyl ketone were routinely used to study the stability of the aminooxy drugs in cell culture media and the uptake into the cells.

When the oximes were formed with [^{14}C]acetone, the radioactivity coeluted with the fluorescent peaks and was proportional to the amount of the oxime (Fig. 2). The fluorescence of the *o*-phthalaldehyde derivatives was proportional to the amount of aminooxy analogues and polyamines, and was linear up to 4 nmol. When the lines (nmol *vs.* peak area) were calculated by the least-squares method, the coefficients of correlation (r^2) varied from 0.9944 to 0.9999 and all the lines passed through the origin. The slopes of lin-

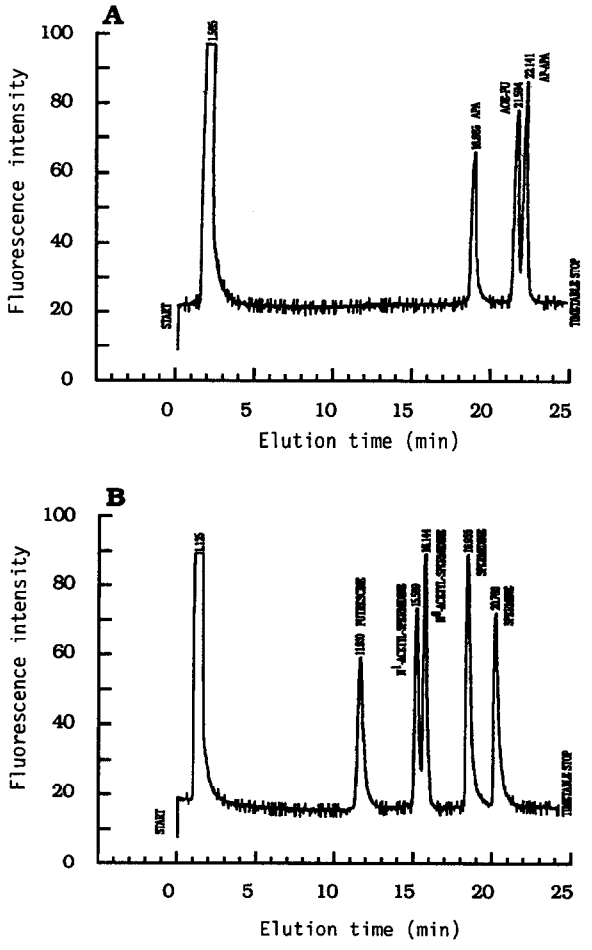

Fig. 1. HPLC elution profiles of ethyl methyl ketone oximes of aminooxy compounds (A) and polyamines (B): (A) standard mixture containing 1 nmol each of 1-aminooxy-3-aminopropane (APA), N-(2-aminooxyethyl)-1,4-diaminobutane (AOE-PU) and 1-aminooxy-3-N-(3-aminopropyl)aminopropane (AP-APA); (B) standard mixture containing 1 nmol each of putrescine, N^1-acetylspermidine, N^8-acetylspermidine, spermidine and spermine.

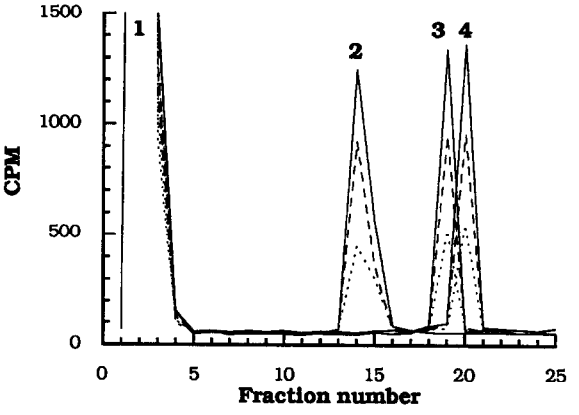

Fig. 2. HPCL elution profiles of [2-^{14}C]acetone oximes of aminooxy compounds. Amounts of 1, 2 and 3 nmol of oximes of 1-aminooxy-3-aminopropane (peak 2), N-(2-aminooxyethyl)-1,4-diaminobutane (peak 3) and 1-aminooxy-3-N-(3-aminopropyl)aminopropane (peak 4) formed with [2-^{14}C]acetone (specific acitivity 0.274 μCi/μmol or 460 cpm/nmol) were subjected to HPLC; 1-min fractions were collected and subjected to liquid scintillation counting. Peak 1 is free [2-^{14}C]acetone. For clarity, points have been omitted. The lines are means of duplicate analyses.

ear regression lines from the least to the most sensitive drug were 774 for APA, 822 for AOE-PU, 852 for putrescine, 868 for AP-APA, 876 for N^1-acetylspermidine, 976 for spermine, 1100 for N^8-acetylspermidine, and 1218 for spermidine. The detection limit is *ca.* 20–30 pmol for polyamines and their aminooxy analogues. The linearity and the fluorescence response of the oximes did not depend on the ketone used, *i.e.* the responses of the acetone and ethyl methyl ketone oximes were identical. These results show that the oximes, unlike the intact aminooxy compounds, are stable during HPLC separation followed by the derivatization with *o*-phthalaldehyde. The retention time of each aminooxy derivative can be varied by forming the oxime with a proper ketone to achieve a complete separation from the naturally occurring polyamines and their acetylated catabolites.

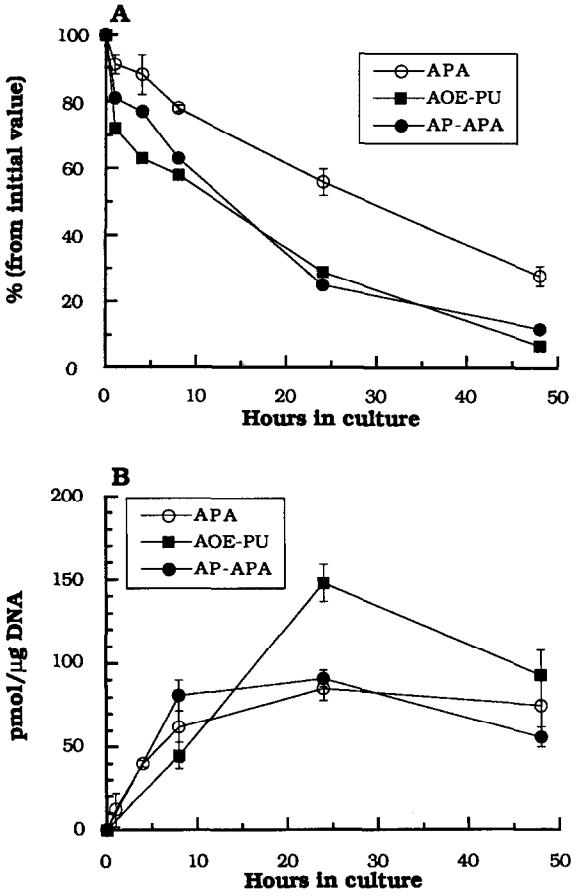

Fig. 3. (A) Stability of APA, AOE-PU and AP-APA in cell culture media; (B) uptake into BHK21/C13 cells. About $1 \cdot 10^6$ BHK21/C13 cells were cultured in the presence of 1 m*M* aminooxy analogue for 48 h. At the indicated time-points, the cells and the media were collected and the aminooxy analogues were measured as described in Experimental. All aminooxy analogues from the media (A) were determined as ethyl methyl ketone oximes. From the cell samples (B), APA (1-aminooxy-3-aminopropane) was determined as its acetone oxime and AOE-PU [N-(2-aminooxyethyl)-1,4-diaminobutane] and AP-APA [1-aminooxy-3-N-(3-aminopropyl)aminopropane] as their ethyl methyl ketone oximes. Points are mean ± S.D. of triplicate cultures. When not shown, the S.D. bars fall within the symbols.

This new method was used to measure the stability of aminooxy analogues of polyamines in cell culture media and to monitor the uptake of these compounds into BHK21/C13 cells. The stability of 1 m*M* APA, AOE-PU or AP-APA in the culture media was measured over a 48-h period (Fig. 3A). Because BHK21/C13 cells are known to excrete N^1-acetylspermidine into culture media [7,17] we used ethyl methyl ketone derivatization for all compounds. The stabilities of AOE-PU and AP-APA were similar and, after 48 h in culture, only *ca.* 10% remained intact in the media. APA was more stable and, after 48 h in culture, *ca.* 30% was still intact. No significant accumulation of any detectable compounds occurred in the media samples, showing that the catabolites either did not react with ketones or *o*-phthalaldehyde or were not retained by the column and eluted in the front peak.

In the uptake studies, APA was measured as its acetone oxime and AOE-PU and AP-APA as their ethyl methyl ketone derivatives. The uptake of APA occurred more rapidly than that of AOE-PU or AP-APA (Fig. 3B). APA was measurable in the cells after 1 h and reached a maximum at 8 h. The uptake kinetics measured by this HPLC method closely agreed with the results previously obtained by measuring the uptake of the radioactivity of ^{3}H-labelled APA [15]. The uptake of AP-APA occurred more rapidly than that of AOE-PU, but the latter attained higher intracellular concentrations. The amounts of APA and AP-APA in the cells remained fairly constant in the period from 8 to 48 h, although the level in the media declined drastically. The aminooxy analogues of spermidine did not have any effect on the cell growth, contrary to APA which markedly inhibited the proliferation (Table I). The data show that when the cellular effects of these aminooxy compounds are studied it is nec-

TABLE I

EFFECT OF APA, AOE-PU AND AP-APA ON THE GROWTH OF BHK21/C13 CELLS

$1 \cdot 10^6$ cells were subcultured onto 6-cm diameter dishes and let adhere for 24 h. Then the media containing 1 m*M* APA, AOE-PU and AP-APA were changed to dishes. The cells and media were collected at the indicated time-points. The amounts of aminooxy compounds in the media and cells are presented in Fig. 3A and B.

Time in culture (h)	Concentration (mean ± S.D., $n=3$) (μg DNA per dish)			
	Control	APA	AOE-PU	AP-APA
1	9.2 ± 0.6	10.7 ± 1.1	10.8 ± 0.3	10.9 ± 1.2
4	10.6 ± 0.9	11.2 ± 0.6	9.8 ± 0.6	10.7 ± 0.4
8	14.2 ± 0.8	13.4 ± 0.6	12.9 ± 0.3	13.8 ± 0.5
24	26.3 ± 0.7	16.9 ± 1.4	26.2 ± 0.7	26.2 ± 1.1
48	39.4 ± 1.0	19.6 ± 0.4	40.6 ± 0.8	34.0 ± 1.0

essary to add the drugs to the media at regular intervals, not exceeding 48 h in the case of APA and 24 h in the case of AOE-PU and AP-APA.

This method can be used for all aminooxy analogues that react with *o*-phthalaldehyde after oxime derivatization. Aminooxy compounds that do not contain primary amino group(s) after ketone derivatization, and therefore do not react with *o*-phthalaldehyde, can most likely be measured after reaction with ketone-containing primary amino group(s), *i.e.* 1,4-diamino-2-butenone or 1,5-diamino-3-pentanone. By choosing a proper ketone for derivatization it is possible to achieve a good separation from naturally occurring polyamines. The protection of the aminooxy group by oxime formation is quantitative if an excess of acetone or ethyl methyl ketone is used at neutral pH. Acetone and ethyl methyl ketone are water-soluble and form the oxime rapidly and quantitatively, and are suitable for the determination of APA, AOE-PU and AP-APA. Diethyl ketone can also be used to measure AOE-PU and AP-APA. The method makes it possible to study the metabolism of the aminooxy analogues.

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REFERENCES

1 O. Heby, *Differentiation*, 19 (1981) 1.
2 A. E. Pegg, *Biochem. J.*, 234 (1986) 249.
3 C. W. Tabor and H.Tabor, *Ann. Rev. Biochem.*, 53 (1984) 749.
4 P. P. McCann, A. E. Pegg and A. Sjoerdsma, *Inhibition of Polyamine Metabolism. Biological Significance and Basis for New Therapies*, Academic Press, Orlando, FL, 1987.
5 A. E. Pegg, *Cancer Res.*, 48 (1988) 759.
6 R. M. Khomutov, T. Hyvönen, E. Karvonen, L. Kauppinen, T. Paalanen, L. Paulin, T. Eloranta, R.-L. Pajula, L. C. Andersson and H. Pösö, *Biochem. Biophys. Res. Commun.*, 130 (1985) 56.
7 T. Hyvönen, L. Alakuijala, L. Andersson, A. R. Khomutov, R. M. Khomutov and T. O. Eloranta, *J. Biol. Chem.*, 263 (1988) 11138.
8 L. Persson, A. R. Khomutov and R. M. Khomutov, *Biochem. J.*, 257 (1989) 929.
9 R. Poulin, J. A. Secrist III and A. E. Pegg, *Biochem. J.*, 263 (1989) 215.
10 T. O. Eloranta, A. R. Khomutov, R. M. Khomutov and T. Hyvönen, *J. Biochem.*, 108 (1990) 593.
11 T. O. Eloranta, S. Lapinjoki, T. Hyvönen, T. Keinänen, V.-P. Korhonen, A. R. Khomutov and R. M. Khomutov, *Life Chem. Rep.*, (1991) in press.
12 R.-L. Pajula, A. Raina and T. Eloranta, *Eur. J. Biochem.*, 101 (1979) 619.
13 A. R. Khomutov and R. M. Khomutov, *Bioorg. Khim.*, 15 (1989) 698.
14 K. W. Giles and A. Myers, *Nature (London)*, 206 (1965) 93.
15 T. Hyvönen, A. R. Khomutov, R. M. Khomutov, S. Lapinjoki and T. O. Eloranta, *J. Biochem.*, 107 (1990) 817.
16 R. P. Andrews and N. A. Baldar, *Sci. Tools*, 30 (1983) 8.
17 H. M. Wallace and H. M. Keir, *Biochem. J.*, 202 (1982) 785.