Application of Oxime Formation in a Radiometric Assay of Aminoxy Compounds

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Received April 22, 1992

A radiometric determination of monoaminoxy analogues of naturally occurring polyamines is described in which [2-14C]acetone is employed as reagent. The reagent is volatile while the oxime product is not allowing unreacted reagent to be removed and the oxime formation to be completed by lyophilization in vacuo. The residual radioactive compound is soluble in water and proportional to the reactive aminoxy content of the reaction mixture and can be quantified by liquid scintillation counting. The assay method is inexpensive and simple and has high specificity and flexibility in sample volume enabling reliable quantification of reactive aminoxy amines in biological extracts at concentrations exceeding 0.25 μM. Optimal pH values for oxime formation of five monoaminoxy analogues of polyamines with acetone were resolved. Reactions via reversible intermediates to irreversible oximes were sped up by removal of water. Complete oxime formation and stability was confirmed by 1H NMR studies. Tested drugs readily formed oximes with pyridoxal 5-phosphate, too. Diaminoxy analogue of cadaverine formed a volatile oxime with acetone. The method was used to monitor the stability of aminoxy analogues of putrescine and spermidine during storage and under culture conditions and to establish their scant accumulation in, but fast catabolism by, cultured baby hamster kidney cells. © 1993 Academic Press, Inc.

Polyamines are polycationic compounds which are essential for the proliferation, differentiation, and function of mammalian cells (1–3). Recently, aminoxy analogues of normally occurring polyamines have been proved very useful tools in studying the metabolism and physiology of polyamines in cultured mammalian cells. Aminoxy analogues of putrescine (APA),2 cadaverine (ABA and BAP), diaminehexane (APEA), and spermidine (AOP-P and AP-APA) (Table 1) are some of these compounds which have been shown to have potential use in the regulation and unraveling of the reaction mechanisms of enzymes involved in polyamine metabolism (4–9). One problem in these studies has been how to measure the reactive aminoxy analogues in cells and culture media. In addition to the indirect measurement of enzyme-inhibiting capacity (7), the only available methods so far have been TLC (10), HPLC with fluorescence detection (8,11), or the use of radioactive isotopes of the aminoxy analogues (11). TLC is very laborious and the aminoxy groups are interfering during postcolumn derivatization with OPA resulting in poor sensitivity with direct HPLC methods. No isotopes of aminoxy compounds are commercially available and methods for the synthesis of only 3H-labeled APA have been reported so far (10,12).

Recently, we have solved the interfering reactions of aminoxy compounds with OPA by protecting the reactive aminoxy groups with the aid of precolumn oxime derivatization with a ketone before subjecting the samples to HPLC analysis with OPA detection (13). The method enables simultaneous quantification of poly-

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2 Abbreviations used: APA, 1-aminoxy-3-aminopropane; ABA, 1-aminoxy-4-aminobutane; BAP, 1,3-bis(aminooxy)propan-2-ol; APEA, 1-aminoxy-5-amino-2-pentanone; AOP-P, 1-[2-aminoxyethyl]-1,4-diamino-2-propane; AP-APA, 1-aminoxy-3-N-[3-aminopropyl]-aminopropane; TLC, thin-layer chromatography; OPA, orthophthaldehyde; PLP, pyridoxal 5'-phosphate; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle medium; SSA, sulfosalicylic acid; TCA, trichloroacetic acid; TSP-d₅, 3-trimethylsilyl)propionic acid-d₅; NMR, nuclear magnetic resonance.
TABLE 1
Structure of Aminoxy Analogues of Polyamines at pH 7.4

<table>
<thead>
<tr>
<th>Polyamine</th>
<th>Formula (with aminoxy group)</th>
</tr>
</thead>
<tbody>
<tr>
<td>APA</td>
<td>1-Aminoxy-3-aminopropane</td>
</tr>
<tr>
<td>ABA</td>
<td>1-Aminoxy-4-aminobutane</td>
</tr>
<tr>
<td>BAP</td>
<td>1,3-Bis(aminoxy)propane</td>
</tr>
<tr>
<td>APEA</td>
<td>1-Aminoxy-5-aminopentane</td>
</tr>
<tr>
<td>AOE-PU</td>
<td>N-[(2-Aminoxyethyl)-1,4-diaminobutane]</td>
</tr>
<tr>
<td>AP-APA</td>
<td>1-Aminoxy-3-N-[3-aminopropyl]-aminopropane</td>
</tr>
</tbody>
</table>

H$_2$N-O-CH$_2$-CH$_2$-CH$_2$-NH$_2$

H$_2$N-O-CH$_2$-CH$_2$-CH$_2$-NH$_2^+$

H$_2$N-O-CH$_2$-CH$_2$-CH$_2$-CH$_2$-NH$_2$

H$_2$N-O-CH$_2$-CH$_2$-CH$_2$-CH$_2$-CH$_2$-NH$_2$

N-[2-Aminoxyethyl]-1,4-diaminobutane

*H$_2$N-CH$_2$-CH$_2$-CH$_2$-NH$_2^+$-CH$_2$-CH$_2$-CH$_2$-O-NH$_2$

\[ \text{H}_2\text{N} - \text{O} - (\text{CH}_3)_2 - \text{NH}_2^+ + \text{H}_2\text{C} - \text{CO} - \text{CH}_3 \rightarrow \]

\[ (\text{CH}_3)_2 - ^{14}\text{C} - \text{N} - \text{O} - (\text{CH}_3)_2 - \text{NH}_2^+ + \text{H}_2\text{O} \]

The excess of [2-\text{\textsuperscript{14}}C]-acetone is evaporated and the oxime formation is completed during lyophilization \textit{in vaeuo}. Free [2-\text{\textsuperscript{14}}C]-acetone is completely volatile while the resulting oxime is not. The residual radioactivity is soluble in water and proportional to the reactive aminooxy content of the reaction mixture. This specific method is suitable for the determination of reactive aminooxy groups in cultured cells and tissue extracts. Sample volumes of 2 ml or more are acceptable and enable the method to quantify aminooxy compounds in more dilute solutions than the very sensitive HPLC method (13).

MATERIALS AND METHODS

Aminooxy analogues of spermidine (AOE-PU and AP-APA) were synthesized by Dr. Alex Khomutov and Dr. Radu Khomutov as previously described (10). Other aminooxy analogues were gifts from Orion Ltd. (Espoo, Finland). [2-\text{\textsuperscript{14}}C]-Acetone (8.4 mCi/mmol in batches 1 and 2 or 2.8 mCi/mmol in batch 3; purity >98% according to the supplier) was purchased from Sigma (St. Louis, MO).

\[ \text{[\text{\textsuperscript{14}}C]}\text{-Acetone reagent.} \]

[2-\text{\textsuperscript{14}}C]-Acetone (50–250 \muCi; 8.4 mCi/mmol) was diluted with 65 \muL of unlabeled acetone in 50 mL of distilled water and stored in tightly capped tubes at -20°C in 1200-\muL batches until used. [2-\text{\textsuperscript{14}}C]-Acetone (250 \muCi) of batch 3 was diluted with 82 \muL of unlabeled acetone in 60 mL of distilled water and then treated identically with batches 1 and 2. The isotope vial was cooled to -80°C, opened in the hood, and immediately rinsed several times with cold water to transfer its contents quantitatively into a capped, graduated tube. The tube was kept in ice during the preparation and fractionation of the reagent. The final concentration (and specific radioactivity) of [2-\text{\textsuperscript{14}}C]-acetone in the reagent was calculated to be 17.8 mm (56.2 \muCi/mmol) when the 50-\muCi batch 1 was used, 18.4 mm (273.6 \muCi/mmol) when the 250-\muCi batch 2 was used, and 20 mm (208.6 \muCi/mmol) when the 250-\muCi batch 3 was used, for the preparation of the reagent. When 5- to 10-\muL samples of each acetone reagent were counted in duplicate for radioactivity as described below, batch 1 gave 232 DPM/nmol (105.4 \muCi/mmol, indicating 189% yield), batch 2 gave 636 DPM/nmol (288.5 \muCi/mmol, indicating 106% yield), and batch 3 gave 559 DPM/nmol (254.1 \muCi/mmol, indicating 122% yield). Batch 1 was thus found to contain roughly twice the radioactivity indicated by the supplier. Obviously, Sigma’s information of the total or of the specific radioactivity of [2-\text{\textsuperscript{14}}C]-acetone batches may be rough estimates. However, in the quantification of aminooxy drugs, as described below, specific radioactivity of the [2-\text{\textsuperscript{14}}C]-acetone reagent plays no role provided that the standards and the samples assayed are treated with the same batch of [2-\text{\textsuperscript{14}}C]-acetone. Purities and final concentrations of acetone reagents were not determined.

Preparation of cell and medium samples. BHK 21/C13-cells were cultured as previously described (5) in the presence of 1 mM aminooxy compound. Cells were disrupted with distilled water, macromolecules were precipitated with cold 0.5 M HCl or 5% (w/v) sulfosaliclyc acid (SSA), and removed by centrifugation for 5 min at 16,000g. Precipitation with SSA instead of HCl is recommended due to much better capacity to precipitate macromolecules in medium samples. After centrifugation, the supernatant was neutralized with NaOH or KOH and assayed for aminooxy groups as described below. Medium samples of 20–100 \muL were treated identically. Acid treatment and salt formation as a result of neutralization had no effect on the recovery (data not shown) but decreased the counting efficiency in liquid scintillation counting (see below).
Procedure for the aminooxy group assay. The proper amount (containing 1–40 nmol of the drug) of neutralized cell extract or other sample was added to 37–125 μl of 1 M potassium phosphate buffer, pH 7.4, and supplied with 50 μl of [2-14C]acetone reagent. Distilled water was added up to 1.5 ml, the tube was closed with a cap, and the reaction mixture was allowed to stand at room temperature for 1–2 h. Then, it was lyophilized three times to remove excess of [2-14C]acetone and to complete the oxime formation. Water was added before each lyophilization. The dried residue was dissolved in 0.5 ml of water. Then, 40 μl of 6 M HCl and 5 ml of liquid scintillation cocktail (ACS, Amersham) were added and the radioactivity was determined by a liquid scintillation counter (LKB Wallac 1214 Rack Beta, Turku, Finland). Counting efficiency for standards and acetone reagents was 92.6% and for cell and medium samples varied from 83 ± 2 to 89 ± 2%, as determined by recounting the vials after adding 9138 ± 292 DPM of [14C]toluene (Packard S.A., Zurich, Switzerland).

NMR studies. 1H NMR spectra were recorded on a Bruker AM 400 WB spectrometer using a 5-mm 1H/13C dual probehead. Typical parameters for the 1H measurements were as follows: 16 K data points, 5 kHz spectral width, 90° pulse angle, and 50 scans at 300 K with low power irradiation at HOD frequency for water suppression. TSP-d4 [(CH3)2Si(CD3)2COONa] (0.00 ppm) was used as an internal reference.

RESULTS

Effect of pH on the oxime formation. Formation of radioactive oximes from 20 nmol of aminooxy compounds in solution containing 15 or 100 mM potassium phosphate buffer and 50 μl of [2-14C]acetone at varied pH is shown in Figs. 1 A and 1B. The reactions were strongly dependent on pH and each aminooxy analogue had its own optimal pH value for oxime formation. Apparently, the reaction went to completion during lyophilization when the reactive aminooxy end of the molecule was uncharged but the molecule carried a positive net charge [H2N-O-(CH2)3-CH2-O-NH2]. If either one of these demands was not fulfilled the rate of oxime formation was drastically retarded.

At pH 7.4, BAP (H6N-O-CH3-CH3-CH2-O-NH2) did not form any nonvolatile oxime and no nonvolatile radioactivity was formed during the 1 to 24-h reaction time. To find out whether a volatile oxime was formed, 100 μM BAP was incubated for 1.5 h with 50 μl of [2-14C]acetone reagent in 1 ml of 30 mM K phosphate buffer (pH 7.0 or 7.5). Then, the reaction mixture either was first lyophilized and redissolved in 1 ml of water or was directly subjected to HPLC (20-μl samples) as previously described (11,13). The eluate was collected in 1-min fractions and counted for radioactivity as above. A 20-μl aliquot of the nonlyophilized reaction mixture (containing 2 nmol of BAP or an oxime formed from it) revealed no fluorescence-detectable compound following postcolumn derivatization with OPA (11,13) but gave 1840 DPM in fractions eluting at the retention time expected for the possible oxime. The total amount of radioactivity in these fractions indicated that both of the aminooxy moieties had reacted with acetone. Lyophilized samples did not contain any radioactivity in any of the HPLC fractions further implying that BAP forms a volatile oxime with acetone.

The physiological pH 7.4, being sufficiently close to the optimal for each of the monoaminooxy analogues of the natural polyamines (see Figs. 1 A and B), was chosen for convenience for routine determinations of all aminooxy drugs. Using a single pH, common sample blanks could be used for the whole assay series no matter how many different aminooxy drugs were included.
Extreme pH values could not be used since the aminooxy compounds did not tolerate basic conditions and the oxime formation was negligible at too acidic pH. Only with huge excess of ketone (e.g., 5 μl of undiluted acetone, ethylmethyl ketone, or diethyl ketone in 100 μl of reaction mixture containing 100 μM aminooxy drug), quantitative oxime formation could be attained in very acidic solutions (5% SSA or 5% TCA) without neutralization, as judged by determining the oxime formation by the HPLC method (13). Furthermore, buffers containing carbonyl or charged amino groups (i.e., carbonate, Tris/HCl) could not be used since they interfered with the oxime formation. At pH 7.4, recovery of 20 nmol of APA as an oxime with acetone was only 6% in 16 mM KHCO₃ and 10% in 16 mM Tris/HCl, under conditions giving 100% recovery in potassium phosphate buffer.

The rate, completeness, and (ir)reversibility of oxime formation. The time course of oxime formation from 20 nmol of aminooxy compounds (AIBA, APEA, APA, AOE-PU, AP-APA) in a solution (total volume 1.5 ml) containing 15 mM potassium phosphate buffer (pH 7.4) and 50 μl of [2-14C]acetone reagent batch 1 was studied both at room temperature and at 37°C. At both temperatures, the tested drugs were completely converted to stable oximes within 15 min incubation followed by lyophilization in vacuo. No changes were found in the amounts of nonvolatile radioactive products when the reactions were followed for 24 h. Identical results were obtained when the reactions were run with 100 μl of [2-14C]acetone reagent and no significant increase was found in the blank values either.

1H NMR spectra were used to study the rate and completeness of oxime formation of APA with acetone. Firstly, the 1H NMR spectra of 5 μmol of APA were measured in potassium phosphate buffer (pH 7.4) containing 0.1 μmol of TSP-d₄ shift standard. Secondly, 10 μl (68 μmol) of acetone reagent (10 μl of acetone, 20 μl of acetone-d₆, and 40 μl of D₂O) was added to the mixture and the reaction was monitored by measuring the 1H NMR spectra at 6, 16, and 24 min following the addition. As shown in Fig. 2, APA rapidly reacted with acetone and the irreversible oxime formation was almost completed within 24 min. Lyophilized, APA-acetone oxime was used as a reference for completed oxime formation.

In order to study whether the irreversible oxime formation could proceed to completion without lyophilization at the acetone concentration used, 20 μM solutions of APA and AOE-PU were incubated in 48 mM potassium phosphate buffer (pH 7.4) containing 613 μM acetone reagent (batch 2). At the indicated time intervals (Table 2), samples were taken from the reaction mixtures and treated for an additional period of 1 or 24 h with unlabeled acetone or pyridoxal 5'-phosphate (PLP), in amounts equimolar to that of labeled acetone in the sample, before subjected to detection of nonvolatile radioactivity. Samples diluted to the treatment volume by water were treated identically and served as controls of the total oxime formation in the sample. As shown in Table 2, unlabeled acetone added to the mixture 1 h after labeled acetone could still replace 50% of labeled acetone in the final oxime product with both APA and AOE-PU indicating that only reversible intermediates were formed during the first hour. With more prolonged incubation, the intermediates were irreversibly converted to oximes but longer than 48 h treatment was apparently needed to guarantee the completion of oxime formation in buffered water solution. Removal of water (by lyophilization) is thus crucial to speed up the irreversible oxime production. Apparently, both APA and AOE-PU reacted with PLP more readily than with acetone, but PLP could no more replace labeled acetone when the oxime had been formed (Table 2). The apparent decrease of nonvolatile radioactivity in the control samples during the 24-h treatment (Table 2; B vs A) was due to increased blank values resulting from a slow binding of acetone by the disposable scintillation vials in which the treatments were performed.

To further study the stability of the oximes, preformed oxime of APA with labeled acetone, at 20 μM concentration, was incubated with varied concentrations of unlabeled acetone at ambient temperature. Samples were taken at 0-, 1-, 2-, 6-, and 24-h time points and assayed for nonvolatile radioactivity. No time-dependent reduction of nonvolatile radioactivity occurred during incubation with unlabeled acetone at concentrations varied from 0- to 112.5-fold that of the oxime. Thus, no significant dissociation of the labeled oxime to volatile acetone or degradation to other volatile radioactive components took place during lyophilizations or the 24-h incubation in buffered water solution. When the lyophilized labeled oxime was directly dissolved into the counting scintillant, no radioactivity was lost within 1 week storage at ambient temperature indicating that any acetone released from the oxime did not escape the scintillation vial.

Since biological samples have to be acid-treated prior to aminooxy assays, the stability of APA-acetone oxime in strong acid was confirmed by 1H NMR. After lyophilization, APA-acetone oxime was dissolved in 500 μl of 1 M HCl (in D₂O) and subjected to prolonged incubation at ambient temperature. 1H NMR spectra were recorded before and after the 48-h incubation period. The data showed that less than 5% of APA-acetone oxime had dissociated during acid treatment indicating that the stability in acid was comparable to that shown above at pH 7.4.

Stability of oximes with pyridoxal 5'-phosphate. As described earlier (14), aminooxy compounds do form
FIG. 2.  

oximes also with aldehydes, e.g., with PLP that is an essential cofactor of several enzymes. AOE-PU, or APA-APA (50 μM) were converted to the corresponding PLP-oximes by incubating with 150 μM PLP in 20 mM potassium phosphate buffer at 37°C for 2 h (14). Then, the preformed oximes were incubated with 150 or 300 μM [2-13C]acetone reagent, in the presence of 100 μM PLP that remained unreacted, for 24 h at 37°C and subjected to counting both volatile and nonvolatile radioactivity. Samples treated identically in the absence of aminooxy
TABLE 2

<table>
<thead>
<tr>
<th>Preincubation time (h)</th>
<th>APA</th>
<th></th>
<th>AOE-PU</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[Control]</td>
<td>[Acetone]</td>
<td>[PLP]</td>
<td>[Control]</td>
</tr>
<tr>
<td>1</td>
<td>2160 ± 40</td>
<td>820 ± 80</td>
<td>110 ± 10</td>
<td>1020 ± 40</td>
</tr>
<tr>
<td>8</td>
<td>1620 ± 160</td>
<td>730 ± 30</td>
<td>70 ± 50</td>
<td>1670 ± 30</td>
</tr>
<tr>
<td>24</td>
<td>2380 ± 120</td>
<td>1220 ± 10</td>
<td>90 ± 10</td>
<td>2070 ± 20</td>
</tr>
<tr>
<td>48</td>
<td>1970 ± 160</td>
<td>990 ± 10</td>
<td>670 ± 20</td>
<td>1690 ± 180</td>
</tr>
<tr>
<td></td>
<td>2200 ± 20</td>
<td>1620 ± 20</td>
<td>1460 ± 10</td>
<td>1870 ± 30</td>
</tr>
<tr>
<td></td>
<td>1910 ± 180</td>
<td>1520 ± 40</td>
<td>1520 ± 60</td>
<td>1670 ± 70</td>
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<tr>
<td></td>
<td>2240 ± 20</td>
<td>2090 ± 40</td>
<td>2120 ± 30</td>
<td>1850 ± 10</td>
</tr>
<tr>
<td></td>
<td>1690 ± 70</td>
<td>1680 ± 190</td>
<td>2110 ± 20</td>
<td>1510 ± 20</td>
</tr>
</tbody>
</table>

*20 μM Aminoxy drugs (APA or AOE-PU) were preincubated in 20 ml of 48 mM potassium phosphate buffer (pH 7.4) containing 613 μM acetone reagent (batch 2). At the indicated time intervals, 0.5 ml of H2O [control], 610 μM unlabeled acetone [acetone], or 610 μM pyridoxal 5'-phosphate [PLP] was added to 0.5 ml samples of the preincubated mixtures. Then, the samples were incubated for 1 h (A) or 24 h (B) at ambient temperature before being subjected to detection of nonvolatile radioactivity. For blank determinations, samples were taken from a reaction mixture without APA or AOE-PU and treated as above. Each blank was made in triplicate and the mean blank values have been subtracted from the data shown. Values are expressed as DPM and represent means ± SD of triplicate assays.

Drugs were used as controls and all experiments were performed in duplicate. The volatile radioactivity was fully recovered and no accumulation of nonvolatile radioactivity was found. Thus, there was no incorporation of radioactivity to any of the preformed PLP-oximes indicating that the PLP-oximes were very stable. Slight dissociation of the PLP-oximes might, however, have remained undetectable if the aminoxy compounds had much higher affinity to PLP than to acetone, as seems to be the case according to the results in Table 2.

Standard curves. Standard curves for each of the aminoxy compounds obtained under identical conditions are shown in Fig. 3. The reaction mixtures containing 3–20 nmol of the analogues of putrescine and spermidine were incubated at room temperature for 1.5 h before subjected to detection of nonvolatile radioactivity. With each drug, a linear relationship was obtained (APA; \( r^2 = 0.99989, y = -50.97 + 0.5424x; \) AOE-PU; \( r^2 = 0.99946, y = 139.60 + 0.6094x; \) AP-APA; \( r^2 = 0.99943, y = -133.05 + 0.5696x \)) between the formation of nonvolatile radioactivity (DPM) and the amount of aminoxy compound (pmol). Standards for each individual aminoxy compound in concern were routinely included in every series of quantitative assays from biological samples.

Stability of aminoxy compounds during storage. The stability of 5–9 mM stock solutions of APA, AOE-PU, and AP-APA at physiological pH (7.4) and at pH 5 was studied in response to repeated freezings and thaws. Freshly made stock solutions of all three drugs were freeze with liquid nitrogen and thawed in a warm water bath. After 0, 5, 10, and 15 freezings and thaws, triplicate samples were taken to the radiometric aminoxy group assay. All of the drugs tested retained unchanged ability to form nonvolatile oximes with \( [2\text{-}^{14}\text{C}]\)acetone, even after 15 repeated freezings and thaws. No difference was found between buffered and nonbuffered stock solutions. Thus, it is recommended to store the stock solutions of the aminoxy compounds in mild acid at -20°C.

Recovery and stability of aminoxy compounds in culture medium. Aminoxy analogues of putrescine and spermidine were added to fresh culture medium at 1.0 mM concentration and incubated under normal culture conditions without cells. At the indicated time intervals, 20-μl samples of each medium were taken to aminoxy

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FIG. 3. Standard curves. Fresh stock solutions of APA, AOE-PU, and AP-APA were made and samples (containing 3–20 nmol of the drugs) were treated as described for aminoxy groups. A linear relationship was obtained. The lines were drawn by the least-squares method. Assays were done with batch 3 [2\text{-}^{14}\text{C}]acetone reagent (specific radioactivity 550 DPM/pmol). The DPM values are means ±SD (shown as error bars) of triplicate determinations. Blank values have been subtracted from the data shown.
TABLE 3

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>APA (µM)</th>
<th>AOE-PU (µM)</th>
<th>AP-APA (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1109 ± 52.4</td>
<td>947 ± 10.1</td>
<td>878 ± 10.2</td>
</tr>
<tr>
<td>48</td>
<td>897 ± 54.9</td>
<td>793 ± 18.4</td>
<td>648 ± 3.5</td>
</tr>
<tr>
<td>72</td>
<td>882 ± 46.0</td>
<td>718 ± 34.3</td>
<td>696 ± 8.5</td>
</tr>
</tbody>
</table>

* Fresh culture media, 6 ml of each, containing 1.0 mM APA, AOE-PU, or AP-APA were incubated in cell culture dishes (10 cm diameter) under normal culture conditions without cells. At the indicated time points, 20 µl samples were taken and assayed for aminooxy groups. Blanks were determined identically using medium incubated without aminooxy drug addition and the blank values have been subtracted from the data shown. The values are means ± SD of three determinations. Acetone reagent of batch 3, specific radioactivity 659 DPM/mmol, was used in all determinations. Incubation time had no effect on the blanks made in triplicate at each time point. The mean blank value ± SD was 1650 ± 56 DPM (n = 9) and 11,180 DPM/sample corresponded to 1000 µM drug concentration.

assay as described above. After 48 h incubation, 81% of APA, 84% of AOE-PU, and 74% of AP-APA were recovered in the medium by the assay method (Table 3). With more prolonged incubation, the recovery of AOE-PU and AP-APA was further decreased being 77 and 69% at 72 h, respectively. The putrescine analogue APA seemed to be more stable than the spermidine analogues (Table 3).

Stability of aminooxy compounds in lysed cell extracts. Recovery and stability of aminooxy compounds in cell lysates was measured using BHK 21/C13 cell homogenate. Cells (260 x 10⁶) were homogenized in 50 ml of 50 mM potassium phosphate buffer (pH 7.4) containing 1 mM dithiothreitol and 0.1 mM EDTA. To 1-ml portions of the homogenate, 20 or 40 nmol of the drug was added and the mixtures were incubated in duplicates at ambient temperature for 0, 1, or 4 h before being subjected to the aminooxy group assay. Blanks were treated identically without drug addition. The drugs were added to the homogenate in amounts corresponding to 1.5 and 3.0 mM intracellular concentrations. At such high levels, all the drugs tested (APA, AOE-PU, AP-APA) were stable for up to 4 h and the recoveries varied between 90 and 103%. Blank values for the homogenates were roughly twice those of the standard mixtures and decreased slightly upon incubation. The results imply that the analogues were not irreversibly bound in significant amounts to cellular structures and that BHK cell homogenate did not contain any prominent enzyme activities or cellular components that could interfere with the oxime formation or destroy the functional aminooxy groups of the drugs at ambient temperature. Cells were stored frozen (at −20°C) before being used in this experiment.

Uptake experiments with BHK 21/C13 cells. BHK 21/C13-cells were cultured, as previously described (5), in the presence of 1 mM aminooxy compounds. As shown in Table 4, only APA reduced the growth rate of the cells markedly within the 72-h culture period. During the 72-h culture, concentrations of APA, AOE-PU, and AP-APA in the culture media were decreased by 96–98%, APA being somewhat more stable than the spermidine analogues (Table 4). While only 2–7% of the aminooxy drugs was still present in the cell culture medium after the 48-h culture period (Table 4), in fresh medium 74–84% of the drugs were still recovered at that time point (Table 3). Thus, the disappearance of the drugs from the media must be attributable to cellular functions.

Cellular accumulation of the aminooxy compounds was determined with the aid of standard curves made simultaneously with the cell sample assays for all the drugs and assuming that 5.7 µg of DNA corresponded to 10⁶ BHK cells, each having an approximate volume of 2.5 pl. The DPM values were corrected for the slight oxime formation with normal cellular constituents showing a linear correlation with cell growth \[\gamma = 0.9997; \gamma(DPM) = 713.51 + 6.56x(µg DNA)\]. As seen in Table 4, intracellular concentrations of APA were lower

TABLE 4

<table>
<thead>
<tr>
<th>Culture time (h)</th>
<th>0</th>
<th>48</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control culture</td>
<td>DNA (µg)</td>
<td>5.66 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>Intracellular Blank (DPM)</td>
<td>745 ± 56</td>
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<tr>
<td></td>
<td>Extracellular Blank (DPM)</td>
<td>961 ± 66</td>
</tr>
<tr>
<td>Culture with 1.0 mM APA</td>
<td>DNA (µg)</td>
<td>nd</td>
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<tr>
<td></td>
<td>Intracellular APA (µM)</td>
<td>77.7 ± 9.0</td>
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<tr>
<td></td>
<td>Extracellular APA (µM)</td>
<td>78.9 ± 11.5</td>
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<tr>
<td>Culture with 1.0 mM AOE-PU</td>
<td>DNA (µg)</td>
<td>nd</td>
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<tr>
<td></td>
<td>Intracellular AOE-PU (µM)</td>
<td>415.0 ± 120.0</td>
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<tr>
<td></td>
<td>Extracellular AOE-PU (µM)</td>
<td>37.1 ± 4.4</td>
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* BHK 21/C13 cells were cultured in the presence of 1.0 mM APA, AOE-PU, or AP-APA in culture medium containing 2 mM glutamine and 10% FCS in DMEM under normal culture conditions. Cell and medium samples were treated as described under Materials and Methods. The values are means ± SD of triplicate determinations. Control cells (n = 4) cultured without aminooxy drug addition were used for blank determinations and the values obtained by least-squares method \[\gamma = 0.9997; \gamma(DPM) = 713.51 + 6.56x(µg DNA)\] have been subtracted before calculating drug concentrations. Acetone reagent of batch 3 was used in all determinations. Intracellular drug concentrations were calculated assuming that 10⁶ cells = 5.7 µg of DNA = 2.5 µl, nd, Not determined.
than those of AOE-PU and AP-APA already after the 48-h culture period, but were not further decreased during the next day of culture, unlike those of AOE-PU and AP-APA. Apparently, 100 \( \mu \text{M} \) intracellular concentration of APA could efficiently retard proliferation, whereas AOE-PU and AP-APA seemed to have no effect even at higher concentration levels. In general, the intracellular concentrations of all drugs tended to remain higher than the extracellular ones during prolonged incubation due to fairly slow response to rapidly decreased extracellular concentrations. Further details of the uptake, accumulation, function, and metabolism of AOE-PU and AP-APA are currently under study.

**DISCUSSION**

Aldehydes and ketones react with a variety of aminooxy derivatives to give corresponding adducts, imines, shift bases, oximes, etc. Formation of these adducts obeys typical equilibrium reaction kinetics. Thus, stabilities and reactivities of these adducts vary markedly. Shift bases and oximes are generally quite stable and can be used for different synthetic and analytical purposes (16).

Oxime formation of L-canaline with [2-\(^{14}\)C]acetone was initially introduced for the quantification of L-canaline in plant extracts (14). The present paper extends this radiometric method for the detection of a variety of aminooxy derivatives having potential use in cell culture experiments. This method is suitable for all aminooxy compounds that form nonvolatile and water-soluble oximes with acetone or other labeled ketones. Since the ketones also react with a variety of normal cellular constituents, formation of stable and nonvolatile shift bases in such reactions have to be controlled in the quantification of aminooxy drugs in biological samples by the method described. However, such interfering reactions seem to be rather insignificant in the assays of aminooxy drugs in cell culture experiments.

The present data confirm that oxime formation offers reliable and sensitive means for the quantification of aminooxy compounds by methods involving either HPLC with fluorescence detection (13) or lyophilization and scintillation counter. According to the above NMR and radioactivity exchange data, 30-fold molar excess of acetone at pH 7.4 is sufficient to push the reaction within 1 h at ambient temperature to complete conversion of any of the aminooxy drugs to the corresponding hemiaminals that are quantitatively converted to stable oximes during water removal by lyophilization (16). The detection limit of the HPLC method is about 20 pmol in the maximal loading volume of 40 \( \mu \text{l} \) (13), whereas the detection limit of the radiometric assay with batch 2 acetone reagent is about 500 pmol in 2 ml sample volume. Thus, both methods can detect aminooxy compounds present in assay samples at concentrations exceeding 0.5 \( \mu \text{M} \). HPLC is the best choice when sample amounts are limited, whereas the radiometric method allows flexibility as to the cost and sample volume of the assay. The sensitivity of the radiometric assay can be improved up to 30-fold by costly increases in the specific radioactivity of the acetone reagent and further by increasing the sample volume. Roughly equal amounts of samples can be analyzed daily by both methods provided that the HPLC system is equipped with an automatic sample injector. Nonvolatile ketones can be applied in the HPLC detection, whereas the radiometric assay does not suffer from the presence of any polyamines or other metabolites forming OPA derivatives overlapping with those of aminooxy compounds in HPLC.

The use of the same stock solutions in the standards and cell culture work is extremely important in order to get reliable results. The volatility of acetone obviously creates problems rendering the volume measurements by the supplier suspect. Furthermore, aminooxy analogues, although insensitive to repeated freezeings and thawings, seem to lose their reactive aminooxy groups to some extent during prolonged storage even at \(-20^\circ\text{C}\). The aminooxy analogues used in the experiments have been synthetized by different laboratories and by different methods and may thus have some differences in their purity. However, as shown in Fig. 3, standard curves made with fresh stock solutions of APA, AOE-PU, and AP-APA are almost identical.

The most time-consuming part of the assay is lyophilization. The capacity and drying speed of the lyophilizer limits the amount of samples that can be assayed, but with an efficient lyophilizer up to 50 samples can be handled by a person in a day. Due to the volatility of acetone, it is important to use the same reagent solution for standards and analyzed samples. To take precautions against gradual accumulation of radioactive acetone in the laboratory air, working in the hood is recommended. As a substitute for acetone, several less volatile \(^{14}\)C-labeled ketones may be used in the assay. Ethylmethyl ketone or diethyl ketone can be used in the oxime formation identically with acetone (13). However, under the assay conditions we used, [2-\(^{14}\)C]acetone proved the most convenient ketone due to its superior solubility in water.

The degradation of AOE-PU and AP-APA is rapid under the cell culture conditions. After 4 h incubation, only about 60\% of 1 mM AOE-PU is found intact in the growth medium (13) indicating a degradation rate exceeding 250 nmol/h per \( 10^6 \) cells. As reported above, aminooxy drugs are quite stable in cell homogenates implying that cellular functions, i.e., whole living cells, in addition to the action of serum enzymes, are responsible for the fast degradation. Provided that the degradation is primarily intracellular, uptake rates of aminooxy compounds by BHK cells are markedly higher than pre-
viously estimated by measuring their cellular accumulation (11,13). Although AOE-FU and AP-APA are good substrates of polyamine acetylating enzyme(s) (8) and thus also of polyamine oxidase, it seems likely that also other degrading mechanisms must be involved to explain the huge degrading capacity during cell culture. Details of the degrading machinery are under current study.

The present results of the uptake and accumulation of APA by BHK cells closely agree with the data obtained by HPLC (13) but differ significantly from the data previously published with L1210 and D-R cells (7). While we found 77.7 ± 9.0 μM intracellular APA concentration in BHK cells cultured for 48 h with 1.0 mM drug (Table 4), Poulin et al. report only 0.5 μM intracellular concentration in both L1210 and D-R cells cultured for 48 h with 0.2 mM drug (7). Poulin et al. did not, however, monitor the stability of the drug in the culture medium, and marked reduction of medium APA concentration may have occurred by 48 h (see Table 4) which would explain the discrepancy in cellular APA levels. On the other hand, BHK cells seem to attain maximal APA level within 10–12 h and to retain it fairly constantly, in spite of continued decrease in the extracellular concentration, for a prolonged period of retarded growth (13). Even though the extracellular concentration was decreased from 80 to 40 μM, no change was found in the cellular accumulation during the third day in culture (Table 4). Considering that L1210 cells and their variant subline D-R cells are, unlike BHK cells, of malignant origin and seem to be very sensitive to APA (7), the possible differences in the uptake and metabolism of APA by different cell lines merit clarification.

The high reactivity of aminooxy groups with PLP should be kept in mind when carrying out cell culture experiments with aminooxy compounds. The present method cannot determine any oximes formed by the drugs with cellular or media constituents. Although aminooxy compounds readily form hemiaminals with ketones and aldehydes, these may not in general, with the possible exception of PLP, be efficiently converted to stable oximes under cell culture conditions. No detectable accumulation of such oximes has been noticed by the HPLC method (13) during prolonged culture of BHK cells. Since hemiaminals formed during cell culture are likely to lose their aminooxy portion to the vast excess of labeled acetone in the treatment before lyophilization, the radiometric assay makes no distinction between free and reversibly reacted aminooxy groups. PLP might be able to compete efficiently even with vast excesses of ketones in the oxime formation. However, PLP is not present in culture media and need not to accumulate in the cells, since the pyridoxamine form that does not react with aminooxy groups is equally effective as a coenzyme. Long-chain aminooxy drugs may not reach easily the active sites of enzymes and PLP formed there may not be attacked. APA, on the other hand, may be small enough and reacts with PLP with very high affinity forming a stable, fluorescing oxime. Application of APA in the detection of PLP in biological samples is currently under study.

ACKNOWLEDGMENTS

The authors thank Miss. Riia Korhonen for technical assistance and the National Research Council for Natural Sciences, Finland, for financial support.

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