THE N-ACETYLATION OF ARSANILIC ACID IN VITRO BY MAMMALIAN ENZYMES

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ABSTRACT:
The N-acetylation of arsanilic acid was assayed in vitro by modifying a literature method for acetylation of p-aminobenzoic acid. Conditions included final concentrations of 1.0 mM dithiothreitol, 1.0 mM EDTA, 0.45 mM acetyl coenzyme A, an acetyl coenzyme A regenerating system using bacterial phosphotransacetylase and acetyl phosphate, 5.0 mM arsanic substrate, and 25 mM sodium/potassium phosphate buffer, pH 7.4, in a total volume of 0.5 ml. Incubation was at 37°C, with 0.5- to 2-mg N-acetyltransferase enzyme protein from a preparation of guinea pig liver. The reaction was terminated by heat precipitation. The resulting supernatant was put through a 4 mm 0.45 μm polysulfone membrane syringe filter. The filtrate could then be injected directly onto the HPLC. With arsanilic acid as substrate, the product N-acetylarsanilic acid (NAA) was identified by its retention time (33 min) in the HPLC system of the laboratory. The 33-min fraction collected from the HPLC was scanned and gave the characteristic UV spectrum of NAA, with peaks at 203 and 256 nm. In addition, the product comigrated in the HPLC system with standard NAA. Under comparable assay conditions, the N-acetylation of arsanilate by the guinea pig enzyme preparation is about 24% the rate of that of the model substrate p-aminobenzoic acid. Typical activity for arsanilate acetylation was 0.5 nmol/min/mg enzyme protein. Using the same assay system and HPLC detection method, the supernatant from bacterial lysates containing recombinant human N-acetyltransferase 1 exhibited acetylation activity toward arsanilate of 720 nmol/min/mg enzyme protein.

The food supply of this country is extensively regulated at the federal level and considered to be quite safe relative to most other countries. However, the food supply contains several thousand additives in trace amounts. The additives include preservatives and nutritional supplements, as well as veterinary pharmaceuticals and feed additives, in the case of animal products. Eighty percent or more of consumable animal protein in this country comes from animals exposed to “medicated” feeds for part of their lives (Hayes and Campbell, 1986).

Organoarsenicals such as roxarsone (4-hydroxy-3-nitrobenzenearsonic acid) and p-arsanilic acid (4-aminobenzenearsonic acid) can be used to control bacterial and parasitic infections and to promote growth and feed efficiency in animals. These compounds have been used as feed additives in poultry and in swine since the 1960s. The arsenicals nitarsonate (4-nitrobenzenearsonic acid) and p-ureidobenzeneearsonic acid are used therapeutically in turkeys. All four of these compounds have been approved for use by the U.S. Food and Drug Administration. U.S. Food and Drug Administration regulations require that the arsenical feed additives or medications be terminated at least 5 days before slaughter of the animal, to allow tissue levels of arsenic to drop to allowable levels of 0.5 ppm for fresh, uncooked muscle and 2.0 ppm for fresh uncooked by-products (Calvert, 1975; Calvert and Smith, 1980; Aschbacher and Feil, 1991). The allowable tissue levels listed translate to 250 μg/lb muscle and 1.0 mg/lb liver for benzenearsonate content.

Structural formulae are given for several benzenearsonic acids in Fig. 1. These benzenearsonates are chemically reactive compounds, with their phenolic, aromatic amine, and aromatic nitro groups. Chronic exposure of humans to such compounds in the food supply is a matter of serious concern because structurally similar compounds are metabolized to active electrophiles that react with protein and DNA molecules to form tissue toxins or mutagens (Long and Rickert, 1982; Vineis et al., 1994).

Older whole animal experiments (Moody and Williams, 1964) revealed excretion of N-acetylated arsanilate, and reduced, N-acetylated roxarsone (4-hydroxy-3-N-acetylenearsonic acid, acetarsone), both more toxic than arsanilate and roxarsone (Donoghue et al., 1994; Merck Index, 1996; Rath et al., 1998). The National Toxicology Program data on carcinogenicity of roxarsone and arsanilate (Abdo et al., 1989; Ashby and Tennant, 1991) raise concerns because of some pancreatic tumors in male rats exposed to roxarsone long-term, and because of positive results from arsanilate and roxarsone in Salmo nella mutagenicity tests. Inclusion of benzenearsonates in feed to enhance animal growth rate could pose a significant human risk if the potential for activation by phase I and phase II enzymes is realized. Individuals vary considerably in their capacity to metabolically activate or deactivate compounds, due to genetic differences in levels of
A structural formulae of several benzenearsonic acids.

![Structural formulae of several benzenearsonic acids.](image)

The long-term objectives of this project are to characterize the metabolism and assess the potential toxic effects on humans of the benzenearsonic acids. There is more individual variation in levels of metabolites generated from xenobiotics because many of these enzymes are inducible (Parkinson, 1996). In spite of these considerations, a Federal Register summary notice dated April 1998 gives approval for continued use of arsanilate (21 CFR 558.62, 1998) and roxarsone (21 CFR 558.530, 1998) as feed additives.

The N-acetyltransferase (NAT) enzyme (EC 2.3.1.5.) represents one of the phase II conjugation enzymes (Evans, 1992) that catalyze N-acetylation of aromatic amine substrates, structurally similar to arsanic acid. The enzyme is cytosolic, acetyl coenzyme A (acetylCoA)-dependent and has sensitive sulfhydryl groups. There are substantial species differences in levels of this enzyme. Humans have two distinct but related forms, NAT1 and NAT2, and exhibit polymorphism, with “slow-acetylators” and “rapid-acetylators” of both forms identifiable in the population (Evans, 1992; Bell et al., 1995).

Numerous whole animal studies have been published on arsanilic acid and similar feed additives, but an in vitro system to measure the biotransformation of these compounds has not yet been reported. Because purified human source NAT enzyme is not available commercially, a commercially available source for pigeon liver was used, and in addition, the supernatant fraction from guinea pig liver homogenate was isolated as a source of mammalian enzyme (Weber and King, 1981). Recombinant human NAT1 and NAT2, kindly supplied by a colleague, were tested as well.

The long-term objectives of this project are to characterize the metabolism and assess the potential toxic effects on humans of the benzenearsonic acids in poultry and swine feed. Such information, currently lacking in the literature, would provide data for consideration of potential metabolic or disease impact in humans on ingestion of these compounds. With an HPLC separation system and the in vitro assay conditions now defined, enzyme preparations from human and other mammalian sources can be tested for NAT activity toward arsanilic acid and related feed additives.

**Materials and Methods**

**Chemicals.** AcetylCoA, arsanilic acid, roxarsone, acetarsone, p-aminobenzoic acid (PABA), 4-N-acetyl PABA, dithiothreitol (DTT), EDTA, phosphotransacetylase, acetyl phosphate, and pigeon liver arylamine acetyltransferase were obtained from Sigma Chemical Co. (St. Louis, MO). Nitosone was purchased from Pfaltz and Bauer, Inc. (Waterbury, CT). Bradford reagent was purchased from Bio-Rad Laboratories (Hercules, CA). 4-N-acetylarsanilic acid (NAA) was synthesized by Dr. Cornelia Gillyard and her research group at Spelman College, by reacting acetic anhydride with arsanilic acid, in a modification of a method for acetaldehyde preparation (Moore et al., 1982). Dr. Eyerie Armstrong and Mr. Michael Halfhill at DuPont Central Research and Development evaluated the recrystallized product both by proton NMR and by $^{13}$C NMR on a 400-mHz instrument, and concluded that the synthesized product (NAA) is 96% pure. Chemicals were dissolved in line-demineralized water, unless otherwise indicated. Phosphate buffer and sulfuric acid were obtained from Fisher Scientific Co. (Norcross, GA). Water used for the HPLC mobile phase of 5.0 mM sulfuric acid was HPLC grade from Jackson and Burdick (Muskegon, MI).

**Supplies.** Polysulfone 0.45 µm membrane syringe filters were obtained from Whatman, Inc. (Clifton, NJ).

**Equipment.** The HPLC system was obtained from ThermoSeparation Products (Riviera Beach, FL) and used a 100-µl injection loop and a variable wavelength UV detector. The LC Talk software was mounted on a Dell 486 P/50 computer with a Hewlett-Packard LaserJet 5L printer.

A Marathon Products 22KBT refrigerated centrifuge from Fisher Scientific Co. (Fairlawn, NJ), with a microfuge rotor, was used at 5000g to pellet heat-precipitated protein from the reaction mixtures. A Diode Array Spectrophotometer (model 8453; Hewlett-Packard, Palo Alto, CA) was used to determine lambda maxima and mM absorptivity values for the standard benzenearsonic acids.

**Spectral Data.** Benzenearsonic acids were dried overnight at 110°C, then prepared analytically as duplicate 1.0 mM aqueous solutions. Duplicate dilutions to 0.05 mM were made with the HPLC mobile phase, and the spectra were determined in quartz cells of 1-cm light path, using mobile phase as a blank.

**HPLC.** The column was obtained from Interaction Chromatography, Inc. (San Jose, CA), now Transgenic (Omaha, NE) and was an ARH-601 Weak Organic Acids column, 6.5 mm × 100 mm heated at 45°C. An isotropic mobile phase of 5.0 mM sulfuric acid was used at a flow rate of 0.6 ml/min. NAA was detected at 256 nm with a retention time of 33 min. 4-N-acetyl PABA was detected at 269 nm with a retention time of 42 min. NAA and acetylated PABA are well separated from other assay components. The retention times in minutes for the coenzymes/cofactors are EDTA, 1.6; acetylCoA, 1.6, 1.8; and DTT, 10.1.

Standard curves of absorbance versus concentration were performed on analytically prepared solutions of 4-N-acetyl PABA and NAA, diluted over the 0.05 to 0.5 mM range, then injected into the HPLC system. Each compound exhibited a linear relationship for absorbance versus concentration. Low pH maximum wavelengths used for detection of 4-N-acetyl PABA and NAA are 269 and 256 nm, respectively. At these wavelengths, the experimentally determined millimolar absorptivity for injected 4-N-acetyl PABA is 1.6 and for injected NAA is 1.8. A standard was run with each set of experimental samples to confirm consistency of column conditions from day to day.

**Guinea Pig Liver NAT Activity.** NAT activity was determined in guinea pig liver in the supernatant fraction from homogenate prepared by a literature method (Weber and King, 1981). The livers were provided by Dr. Pamela Gunter-Smith of the Biology Department at Spelman College, and were obtained immediately from animals sacrificed by CO2 asphyxiation. The enzyme was stored at −80°C. The proportion of NAT1 and NAT2 in the preparation is not known. Protein concentration was determined by the Bradford method (Bradford, 1976) using BSA as a standard.

**Recombinant Human NAT1 and NAT2.** *Escherichia coli* lysates containing recombinant human NAT1 and NAT2 were kindly provided by Dr. David W. Hein, Department of Pharmacology and Toxicology, University of Louisville School of Medicine (Louisville, KY). These human enzymes were expressed in pKK223–3 vector and *E. coli* strain JM1055. Lysates were centri-
fuged at 15,000g for 20 min. The resulting supernatant was used as a source of NAT activity.

**NAT Assay Conditions.** The N-acetylation of arsanilic acid was assayed in vitro by modifying a literature method (Ward et al., 1995) for acetylation of PABA. Conditions included final concentrations of 1.0 mM DTT, 1.0 mM EDTA, 0.45 mM acetylCoA, an acetylCoA regenerating system using 0.1 U bacterial phosphotransacetylase with 5.0 mM acetyl phosphate, and 5.0 mM arsanilate substrate, in 25 mM sodium/potassium phosphate buffer, pH 7.4, in a total volume of 0.5 ml. Incubation was at 37°C for 45 to 60 min, with 0.5 to 2.0 mg of NAT protein from a preparation of guinea pig liver. With commercial pigeon liver NAT (arylamine acetyltransferase), 0.5 U was used. Using recombinant human NAT1 and NAT2, incubation was for 30 min with 0.2 to 2 µg, and 30 to 300 µg, respectively.

When PABA was used as substrate, incubation time was 15 to 30 min. With commercial pigeon liver NAT as enzyme, 0.25 to 0.5 U was used per assay, and 4 mM substrate. Otherwise, assay conditions were identical with those described in the previous paragraph. For both substrates, conditions of linearity with time and enzyme concentration were met, as well as saturation with substrate, for each enzyme source tested.

Reactions were terminated by heat precipitation. The centrifuged reaction mixture supernatant was passed through a 0.45-µm membrane syringe filter. The resulting filtrate could be injected directly onto the HPLC. Samples were stored frozen until HPLC analyses could be completed. With arsanilic acid as substrate, the product NAA was identified by its retention time of 33 min in the laboratory HPLC system. Acetylated PABA was identified by its retention time of 42 min.

**Results**

**UV Absorption Characteristics and HPLC Retention Times.** Table 1 gives the low pH λ maxima values and millimolar absorptivity values for five benzenearsonic acids. Arsanilate and two potential arsanilate metabolites, one oxidized (nitarsonone), the other acetylated (NAA), are included. Values are given as well for roxarsone and its reduced, acetylated metabolite, acetarsone. Table 1 also gives HPLC retention times for the benzenearsonic acids. NAA is well separated from other assay components, (EDTA, acetylCoA/CoA, DTT) although its 33-min retention time is relatively long. The nature of the column material precludes a higher flow rate. Both NAT substrates, arsanilic acid and PABA, are noneluting in this system. However, both ortho amino derivatives do elute from the column.

Standard curves of absorbance versus concentration were performed on analytically prepared solutions of 4-N-acetyl PABA and NAA, diluted over the 0.1- to 1.0-mM range, then injected into the HPLC system. Each compound exhibited a linear relationship for absorbance versus concentration. Low pH λ maximum wavelengths used for detection of 4-N-acetyl PABA and NAA are 269 and 256 nm, respectively. At these wavelengths, the experimentally determined millimolar absorptivity for injected 4-N-acetyl PABA is 1.6 and for injected NAA is 1.8. A standard was run with each set of experimental samples.

**Arsanilate (5 mM) was incubated in phosphate buffer at pH 7.4 with an acetyl-CoA regenerating system and the guinea pig liver NAT preparation (2.1 mg). Product NAA is plotted as the mean of duplicate reaction mixtures versus time.**

**Guinea Pig Liver NAT.** Pigeon liver NAT activity was assayed by a literature method (Ward et al., 1995) using PABA as substrate. Arsanilic acid was unreactive as a substrate in this system.

**Guinea Pig Liver NAT Activity.** Using the assay conditions outlined in Materials and Methods for acetylation of arsanilic acid, the production of NAA with this mammalian enzyme source was linear with time over a 1.0-h incubation period, and linear with enzyme concentration at least to 2 mg of enzyme protein per assay. These results are shown in Figs. 2 and 3, respectively. Preliminary experiments indicate a pH optimum of 7.4 and a substrate saturation level of 5 mM for arsanilate acetylation.

**Fig. 2. NAA produced from arsanilate versus time.** Arsanilate (5 mM) was incubated in phosphate buffer at pH 7.4 with an acetyl-CoA regenerating system and the guinea pig liver NAT preparation (0.5–2.1 mg). Product NAA is plotted as the mean of duplicate reaction mixtures versus mg of enzyme protein.

**Fig. 3. NAA produced from arsanilate versus enzyme concentration.** Arsanilate (5 mM) was incubated for 45 min in phosphate buffer at pH 7.4 with an acetyl-CoA regenerating system and the guinea pig liver NAT preparation (0.5–2.1 mg). Product NAA is plotted as the mean of duplicate reaction mixtures versus mg of enzyme protein.

![Image](https://via.placeholder.com/150)

**TABLE 1**

**UV absorption and HPLC characteristics of several benzenearsonic acids**

<table>
<thead>
<tr>
<th>Compound</th>
<th>λ&lt;sub&gt;max&lt;/sub&gt; (nm)</th>
<th>ε&lt;sub&gt;max&lt;/sub&gt; (mM&lt;sup&gt;-1&lt;/sup&gt; cm&lt;sup&gt;2&lt;/sup&gt;)</th>
<th>Retention Time (min)</th>
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<tr>
<td>Arsanilic acid</td>
<td>258</td>
<td>11.27</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>N-Acetylasranilic acid</td>
<td>203</td>
<td>23.08</td>
<td>33</td>
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<tr>
<td>Nitarsonone</td>
<td>256</td>
<td>19.16</td>
<td></td>
</tr>
<tr>
<td>Roxarsone</td>
<td>254</td>
<td>11.08</td>
<td>13</td>
</tr>
<tr>
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</tr>
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<td></td>
<td>281</td>
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<sup>a</sup> ND, not detected.
spectrum of NAA at low pH, with peaks at 203 and 256 nm. In addition, the product comigrates in the HPLC system with standard NAA. Controls including coenzyme, but substituting phosphate buffer for enzyme, gave minimal product formation. Correction for these coenzyme-catalyzed controls was always made in calculating NAA product concentration. The small chromatograph peaks between acetylCoA/CoA and DTT are also observed in a NAT-only control, indicating that they are not additional arsanilate reaction products, but UV-absorbing components in the enzyme preparation.

Identical assay conditions were used with 0.5 to 1.0 mM PABA as substrate, except that incubation time was less for this more active substrate. Activity of the guinea pig NAT preparation with PABA as substrate was about four times the activity toward arsanilate as substrate. In the absence of added acetylCoA, no activity toward either substrate was observed. With both substrates, the activity was significantly better with the acetylCoA regenerating system than with acetylCoA alone.

**Recombinant Human NAT 1 and NAT2.** The supernatant from bacterial lysates containing recombinant NAT 1 and NAT 2 was incubated with arsanilate and PABA under conditions given in the NAT assay procedure. Figure 5, gives the chromatograph for arsanilate acetylation by recombinant human NAT1, with a nonenzyme control (top) and a NAT1-containing reaction mixture (bottom). Table 2, summarizes the specific activities of the NAT enzyme sources tested with arsanilate as substrate. Activity toward PABA is included for comparison.

**Discussion**

UV spectral characteristics for five benzenearsanic acids were determined at low pH (2.2), the pH of the HPLC mobile phase used. These compounds undergo a red shift with increasing pH and thus should be reassessed for wavelength maxima and molar absorptivity when used at other pH values. This allows for choice of the most sensitive/feasible wavelength and concentration for use in the HPLC system.

The HPLC system presented here with the ARH-601 column separates several benzenearsanic acids and their metabolites, making it valuable in general for the study of the benzenearsenate feed additives. With the NAT assay components, coenzymes and cofactors elute early, giving excellent separation from the acetylated products NAA and N-acetyl PABA.

An in vitro assay system was modified from the literature (Ward et al., 1995) and used to follow N-acetylation of arsanilate by NAT activity in the supernatant fraction of guinea pig liver. The NAA enzymatic product identity was confirmed by comparison of HPLC retention time with standard NAA. UV spectral analysis of the col-
lected product and comigration in the HPLC system with standard NAA gave additional proof of product identity.

Arsanilate was also acetylated by recombinant human NAT1, at about 15% the rate at which PABA was acetylated, as shown in Table 2. Both arsanilate and PABA were unreactive as substrates with NAT2. Arsanilate does not serve as a substrate for pigeon liver NAT. This result is in agreement with the work on colostomized roosters reported by Aschbacher and Feil in 1991. Roosters were fed ring-labeled \([^{14}\text{C}]\)arsanilate, and excreted about 90% of the arsanilate dose unchanged. Highest tissue levels of the remaining radioactivity were found in liver, but identification of the specific metabolites was not performed. Avian species, then, appear unable to acetylate arsanilate.

In this study, pigeon liver NAT, the guinea pig liver cytosol preparation, and recombinant human NAT1 all were able to acetylate PABA. The guinea pig preparation and human NAT1 were able to acetylate arsanilate. Human NAT2 was unreactive toward both substrates.

Although arylamine acetylation is considered a detoxification reaction, subsequent \(N\)-hydroxylation by cytochrome P-450 or flavin monooxygenase (FMO) enzymes, followed by deacetylation, would produce a reactive nitrone, which can bind to DNA and protein. Likewise, \(N\)-hydroxylation of arsanilate, followed by \(N\)-acetylation, then deacylation, would produce the reactive nitrenium ion (Parkinson, 1996).

Investigators are actively searching for linkages between arylamine exposure from environmental and dietary sources, and increased risk of cancer due to genetic makeup (Hein, 1988; Kirlin et al., 1991; Bell et al., 1995). NAT1 polymorphism is now recognized in the population and variant alleles continue to be described (Doll et al., 1997;
Payton and Sim, 1998). With the HPLC system and the in vitro assay system for acetylation described here, other human NAT1 variants can be tested for acetylation activity toward arsanilate.

Acknowledgments. We are indebted to Dr. David W. Hein (University of Louisville School of Medicine, Louisville, KY) for the generous gift of recombinant human NAT1 and NAT2.

References


