

Depletion of the Monoamino Metabolites of Zoalene During Frozen Storage

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3-Amino-5-nitro-*o*-toluamide and 5-amino-3-nitro-*o*-toluamide, the principal metabolites in the tissues of chickens fed a diet containing Zoalene (3,5-dinitro-*o*-toluamide), were shown to deplete in frozen liver tissues stored up to 1 year at -20°C , but not at -70°C . A slight loss of 5-amino-3-nitro-*o*-toluamide also occurred in thigh muscle at -20°C . Evidence is presented that demonstrates that the depletion of the metabolites was partially the result of their transformation to glucopyranosyl derivatives; both the α - and β -anomers of the 5-amino-3-nitro-*o*-toluamide conjugate were observed in liquid chromatograms of tissue extracts.

A previous study conducted in this laboratory (1) reported that sulfamethazine was depleted in swine liver during frozen storage. In that report, evidence was presented which showed that the depleted drug was transformed, *in situ*, to its N^4 -glucopyranosyl derivative. The conjugate had also been reported (2, 3) as a major naturally occurring metabolite in the liver, as well as the skeletal muscle, of chickens dosed with [^{14}C]-sulfamethazine. In studies on Zoalene and its metabolites in incurred chicken tissues (4), we presented evidence showing the presence of an acid-labile conjugate of 5-amino-3-nitro-*o*-toluamide, presumably the N^5 -glucoside, in chicken livers. No evidence was obtained in those studies for a conjugate of 3-amino-5-nitro-*o*-toluamide in the incurred tissues. The present study was undertaken to determine if these primary acylamino metabolites of Zoalene, structurally similar to sulfamethazine, are also depleted during frozen storage and if any observed depletion was the result of the parent compounds having been transformed to glucose conjugates.

METHOD

Reagents and Apparatus

(a) *Solvents*.—Ethyl acetate (EtOAc), hexane, and methanol (MeOH) (American Burdick and Jackson Laboratories,

Inc., Muskegon, MI 49442); chloroform N.F. (CHCl_3) (J.T. Baker, Inc., Phillipsburg NJ 08665).

(b) *Drug and metabolites*.—Zoalene (Salsbury Laboratories, Charles City, IA 50616); 3-amino-5-nitro-*o*-toluamide (3-ANOT), gift from Dow Chemical USA, Midland, MI 48640; 5-amino-3-nitro-*o*-toluamide (5-ANOT), see *Acknowledgment*.

(c) *N^3 -glucopyranosyl-5-nitro-*o*-toluamide and N^5 -glucopyranosyl-3-nitro-*o*-toluamide*.—Prepared according to the method of Paulson et al. (2). To purify glucose conjugates, dilute 0.2 mL reaction mixture (2) 2 mL with H_2O and pass through 6 mL HC Bakerbond C_{18} solid-phase extraction column (J.T. Baker, Inc.). Wash column with 6 mL H_2O and remove excess H_2O from column with vacuum. Wash column with 3 mL hexane and dry with vacuum. Elute glucosides with 2 mL MeOH. Remove solvent with stream of nitrogen and dissolve residue in 0.5 mL MeOH. For final purification of the conjugates, use preparative TLC with silica Gel G preparative Uniplate T (Analtech, Inc., Newark, DE 19714-9990) tapered plates and EtOAc–MeOH– H_2O (80 + 20 + 1) as developing solvent. After development and air drying, remove area corresponding to glucose conjugate, as determined by procedures previously described (1), from plate. Elute silica Gel G with MeOH. Evaporate to dryness to yield yellow crystals.

(d) *Tissue homogenizer*.—Polytron (Brinkmann Instruments, Inc., Westbury, NY 11590).

(e) *Centrifuge*.—Refrigerated IEC Centra, 7R; rotor No. 822A (International Equipment Co., Division of Damon Corp., Needham Heights, MA 02194).

(f) *Neutral alumina*.—Brockman Activity I, 80–200 mesh (Fisher Scientific Co., Fairlawn, NJ 07410). Insert 6 mm round glass bead into 5 mL pipet tip (Rainin Instrument Co., Woburn, MA 01801). Layer glass bead with 0.5 cm sand (purified, washed, and ignited for boats; J.T. Baker, Inc.) and 1.25 or 3.25 cm bed of neutral alumina. Pack firmly by gently tapping top of pipet tip. Add 0.25 cm sand. Wash 1.25 cm column with two 2 mL portions of CHCl_3 –EtOAc (1 + 1) and 3.25 cm column with three 2 mL portions of MeOH before use.

(g) *Liquid chromatography/electrochemical detection*.—ISCO LC-5000 precision pump (ISCO, Inc., Lincoln, NE 68505) connected to BAS Model LC-4B amperometric detector (Bioanalytical Systems, Inc., West Lafayette, IN 47905); glassy carbon electrode, -0.85 to -0.95 V vs Ag/AgCl, 100–

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200 nA full scale. Rheodyne sampling valve with 100 μ L loop. Recorder: Fisher Recordall Series 5000 at 10 mV full scale and chart speed 1 cm/2 min. Mobile phase: pH 6.0 phosphate buffer (0.05M monobasic potassium phosphate solution containing 0.001 mol EDTA adjusted to pH 6.0 with 1N NaOH)–MeOH (ratio variable; see figures) purged with helium. Sampling technique: Purge 2 mL sample in 9 mL vial with helium for 2 min to exclude oxygen from system. Draw ca 0.5 mL sample through capillary tubing into loop, by way of vent, with 2 mL syringe inserted in sampling valve in load position. Inject sample.

Preparation of Samples

Fresh chicken livers and thighs were purchased from local retail outlets. Livers were partially frozen and blended in a Waring blender. Thigh muscle was removed from whole thighs (excluding fatty deposits as much as possible), partially frozen, and blended. Blended tissues were frozen in dry ice and held at -20°C before fortification; 2.5 g samples were sliced (ca $2.5 \times 1.5 \times 0.5$ cm) from the blended tissues and fortified with 5 μ L MeOH solutions of 3-ANOT, 5-ANOT, and Zoalene (2.0 $\mu\text{g}/\mu\text{L}$) by injection into 2 parallel knife slits running the length of the tissue. Fortified tissues (4.0 ppm) were allowed to remain at -20°C uncovered for 1 h and then wrapped in poly-coated freezer wrap, placed in sealed plastic bags, and stored at -20° or -70°C . Control tissue samples (unfortified) were prepared and stored in the same manner. Just before analyses of the stored fortified samples, the stored control samples were fortified with 4.0 ppm 3-ANOT, 5-ANOT, and Zoalene in the manner described above.

Determination

Isolation of Zoalene, 3-ANOT, and 5-ANOT residues.—Place frozen tissue into 50 mL polypropylene centrifuge tube. Let sample partially thaw. Add 20 mL CHCl_3 –EtOAc (1 + 1) and blend 45 s with Polytron homogenizer at low speed. Centrifuge 10 min at 3500 rpm. Remove CHCl_3 –EtOAc (do not remove any aqueous layer), filter through small plug of glass wool in 4 mL disposable Pasteur pipet, and collect filtrate. Return extracted tissue in centrifuge tube to -20°C storage. Pass 2 mL CHCl_3 –EtOAc extract through 1.25 cm neutral alumina column. Wash sides of column and alumina with 2.5 mL CHCl_3 in 0.75, 0.75, and 1.0 mL increments. Remove excess CHCl_3 from column with vacuum and continue until column dries, as evidenced by the disappearance of moisture on outside of column. Elute column with LC mobile phase, collecting first 2 mL effluent in a 2 mL volumetric flask. Shake stoppered flask and transfer contents with disposable Pasteur pipet to 9 mL screw-capped specimen vial. Inject 100 μ L of this solution onto LC column as described above. To determine concentration of residues in stored, fortified tissues, compare peak heights to those of drug controls; fortified control samples; and fortified, stored control samples.

Isolation of modified residues.—After removing CHCl_3 –EtOAc solvent as described above, store extracted tissues at -20°C or immediately re-extract. Add 18.25 mL MeOH and blend 30 s with Polytron homogenizer at low speed. Centrifuge

10 min at 3500 rpm. Recover solvent layer and filter through small plug of glass wool packed in 4 mL disposable Pasteur pipet, collecting filtrate. Pass 15 mL filtrate through 3.25 cm column of neutral alumina. Wash sides of column and alumina with 5 mL MeOH in 1.0, 1.0, 1.0, and 2 mL increments. Remove excess MeOH from column with vacuum and continue until column dries. Elute column with mobile phase, collecting first 2 mL effluent in 2 mL volumetric flask. Transfer contents of flask to 9 mL screw-capped specimen vial and inject 100 μ L onto LC column as described above.

Limited studies on tissues fortified with the glucose conjugates of 3-ANOT and 5-ANOT showed $>80\%$ recovery by this method.

Results and Discussion

Table 1 summarizes the average concentrations of Zoalene, 3-ANOT, and 5-ANOT in fortified (4.0 ppm) liver and thigh muscle after 1 year of tissue storage at -20° and -70°C . For all practical purposes, Zoalene and its monoamino metabolites were stable in both liver and thigh muscle at -70°C . With the exception of 5-ANOT, which decreased about 15%, the compounds were stable in thigh muscle at -20°C . Slightly lower values for Zoalene in livers at both storage temperatures may indicate losses during the spiking procedure, although all precautions were taken to prevent warming of the tissues. Zoalene is rapidly metabolized by fresh liver tissues at room temperature. In -20°C livers, 3-ANOT concentrations decreased about 20%, whereas about 60% depletion of 5-ANOT was observed. Preliminary studies of the fortified tissues after 3 months storage showed that 5-ANOT concentrations in -20°C liver samples had decreased 20%. No other depletion of these compounds in either tissue or at either storage temperature was observed at that time.

Figure 1 compares LC chromatograms of MeOH extracts of liver samples stored at -20°C , controls stored at -20°C and then fortified at the time of analysis with 4 ppm Zoalene, 3-ANOT, 5-ANOT, and the synthesized glucosides of 3-ANOT and 5-ANOT. As can be observed in the liquid chromatograms of the glucosides (A), both anomers were present, with one (presumably the α) anomer dominating. Matusik et al. (5) reported the formation of both anomers of N^4 -glucopyranosylsulfamethazine when prepared according to the reaction procedure of Paulson et al. (2). A comparison of the liver tissues shows 3 distinct LC peaks in MeOH extracts of the stored sample (C) that are absent in the extracts of the fortified stored controls (B). The peaks have the retention times of both anomers of the 5-ANOT glucoside (5.7 and 6.3 min) and the parent 5-ANOT (7.3 min). The presence of 5-ANOT in the extract of the stored liver sample suggests that hydrolysis of the conjugate occurred on the alumina during drying or after removal from the column because neither 3-ANOT, 5-ANOT, nor Zoalene are retained on alumina from a MeOH solution (see LC of extract of fortified stored control). However, LC recovery studies on the synthesized glucosides in spiked liver tissues did not reveal the presence of the parent compound in MeOH extracts of the tissue. These results suggest that 5-ANOT arose

Table 1. Concentration in tissues fortified with 4.0 ppm 3-ANOT, 5-ANOT, and Zoalene following frozen storage for 1 year^a

Tissue	Temp, °C	Concentration (ppm ± SD)		
		3-ANOT	5-ANOT	Zoalene
Thigh	-20	4.15 ± 0.16	3.47 ± 0.30	3.86 ± 0.25
Thigh	-70	4.08 ± 0.16	4.11 ± 0.31	3.82 ± 0.22
Liver	-20	3.19 ± 0.16	1.70 ± 0.17	3.63 ± 0.14
Liver	-70	3.90 ± 0.20	3.67 ± 0.21	3.75 ± 0.06

^a Average of 5 fortified tissues.

from some unknown entity not detected under the conditions of this study. In this respect, the isolated glucosides accounted for about 40% of the depleted 5-ANOT in the liver tissues

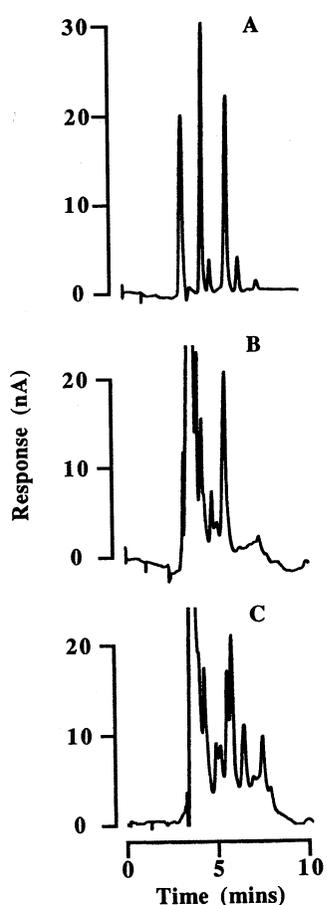


Figure 1. Liquid chromatograms of (A) synthesized glucopyranosyl derivatives of 3-ANOT and 5-ANOT; (B) MeOH extract of liver control stored at -20°C and then fortified with 4.0 ppm 3-ANOT, 5-ANOT, and Zoalene; and (C) MeOH extract of fortified liver sample stored at -20°C. Retention times (min) of standards: 3-ANOT α -glucoside = 4.3; 3-ANOT β -glucoside = 4.8; 3-ANOT = 5.5; 5-ANOT α -glucoside = 5.7; 5-ANOT β -glucoside = 6.3; 5-ANOT = 7.3. Mobile phase: pH 6.0 phosphate buffer-MeOH (7 + 3). Electrochemical detection: potential, -0.95 V. Attenuation: 100 nA full scale.

stored at -20°. No evidence was observed for the presence of the glucoside or fortified compounds in MeOH extracts of the liver samples stored at -70°C.

Indirect evidence is presented in Figure 2 for the presence of the 3-ANOT glucoside in the liver tissues stored at -20°C; MeOH extracts of a fortified control stored at -20°C and a liver sample stored at -20°C were compared before and after acidification and heating for 1 min at 100°C. Although the presence of the 3-ANOT glucosides was not observed because of co-chromatographing contaminants, a substantial increase in the 3-ANOT peak, as well as the 5-ANOT peak, after acidification of the 2 mL sample with 0.25 mL 4 M HCl and heating suggests their presence in the extract.

Figure 3 compares chromatograms of MeOH extracts of thigh samples stored at -20°C (B) and thigh controls stored at -20°C (A) fortified with 4 ppm each of 3-ANOT, 5-ANOT, and Zoalene at time of analysis. The chromatograms of (B) are characterized by the presence of 2 small peaks at the retention times of the α -anomer of 5-ANOT glucoside (5.0 min) and 5-ANOT (6.5 min). A larger peak at the retention time (5.7 min) of the β -anomer and an unknown peak (retention time, 7.1 min) were observed in all 5 tissues analyzed. No peaks were present in thigh samples stored at -70°C or their fortified stored controls. It was not within the scope of this study to attempt to identify the unknown peak, but LC studies demonstrated that it did not have the retention time of N⁵-acetyl-3-nitro-*o*-toluamide, which had been determined (unpublished data) to coincide with an unknown metabolite previously observed in chromatograms of extracts of incurred tissues (4).

In summary, the results of this study demonstrate that the depletion of the monoamino metabolites of Zoalene in chicken liver and thigh muscle occurred at -20°C but not at -70°C during a 1 year storage period. Although the transformation of the depleted monoamino metabolites to their glucopyranosyl derivatives was not unexpected, the presence of both anomers in the tissue was not anticipated on the basis of similar studies of depleted incurred sulfamethazine residues in chicken livers (1). The reason for these differences is unclear; perhaps they are due to differences in the isolation procedures or LC conditions used.

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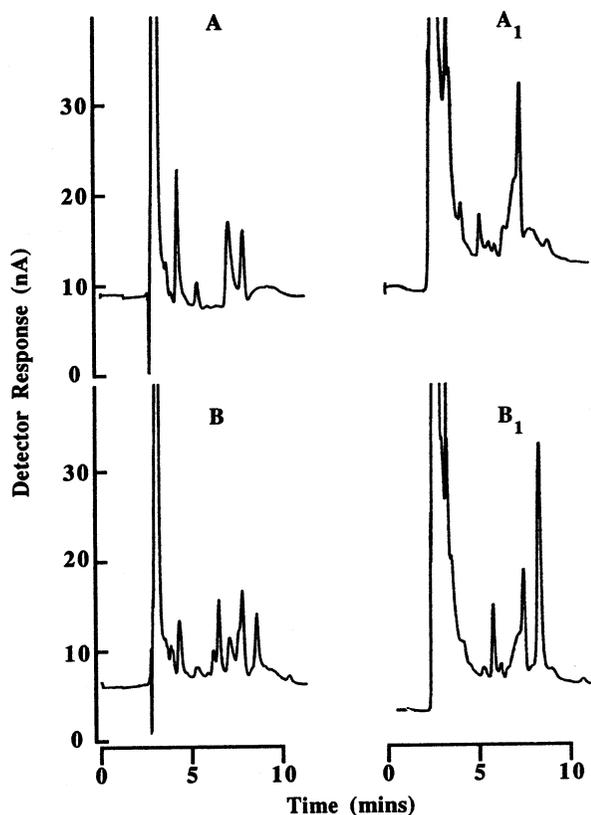


Figure 2. Liquid chromatograms of (A) MeOH extract of liver control stored at -20°C and then fortified with 4.0 ppm 3-ANOT, 5-ANOT, and Zoalene; (A₁) acidified and heated 1 min at 100°C ; (B) MeOH extract of fortified liver sample stored at -20°C ; (B₁) acidified and heated 1 min at 100°C . Retention times (min) of standards: 3-ANOT α -glucoside = 4.4; 3-ANOT β -glucoside = 5.1; 3-ANOT = 6.0; 5-ANOT α -glucoside = 6.4; 5-ANOT β -glucoside = 7.6; 5-ANOT = 8.4. Mobile phase: pH 6.0 phosphate buffer–MeOH (7.5 + 2.5). Electrochemical detection: potential, -0.95 V . Attenuation: 100 nA full scale.

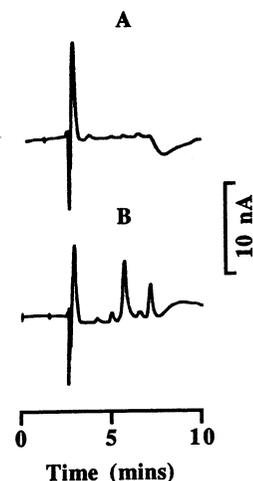


Figure 3. Liquid chromatograms of (A) MeOH extract of thigh muscle control stored at -20°C and then fortified with 4.0 ppm 3-ANOT, 5-ANOT, and Zoalene; (B) MeOH extract of fortified thigh muscle sample stored at -20°C . Retention times (min) of standards: 5-ANOT α -glucoside = 5.0; 5-ANOT β -glucoside = 5.7; 5-ANOT = 6.5. Mobile phase: pH 6.0 phosphate buffer–MeOH (6.5 + 3.5). Electrochemical detection: potential, -0.95 V . Attenuation 100: nA full scale.

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