

Investigations on Nonsulfonamide Bratton-Marshall-Positive Compounds in Porcine Liver

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An unknown Bratton-Marshall-positive compound was observed when liver extracts were screened by a procedure for the simultaneous determination of sulfamethazine and sulfathiazole in swine livers. The unknown has been conclusively identified as *o*-aminobenzoic acid (anthranilic acid), a tryptophan metabolite, by gas-liquid chromatography-mass spectrometry of swine liver extracts obtained by a modified Tishler procedure for sulfonamide residues in animal tissues.

In a previous publication (1), we described a rapid thin layer chromatographic screening procedure for the simultaneous determination of sulfamethazine and sulfathiazole in swine livers. Application of this procedure to a number of liver samples resulted in the consistent observation of an unknown Bratton-Marshall-positive (2) compound. Although the unknown did not interfere with determining the presence or confirming the absence of the drugs, its potential was recognized for contributing to false positive results in the modified Tishler method (3) which is used by regulatory agencies for monitoring sulfonamide residues in swine livers. This communication presents evidence which identifies the unknown and establishes its presence in liver extracts obtained by a modified Tishler method.

METHOD

Reagents and Materials

(a) *Solvents*.—Ethyl acetate, dichloromethane (DCM), acetone, and methanol (Distilled-in-Glass®, Burdick & Jackson Laboratories, Inc., Muskegon, MI 49442). Chloroform (Baker Analyzed reagent, J. T. Baker Chemical Co., Phillipsburg, NJ 08665).

(b) *Tetrabutylammonium hydroxide (TBAH)*.—40% aqueous solution (Aldrich Chemical Co., Inc., Milwaukee, WI 53233).

(c) *Methylating reagent*.—0.2M iodomethane (Aldrich Chemical Co., Inc.) in DCM.

(d) *0.1M Carbonate buffer, pH 10*.—Prepared from 0.1M solutions of sodium carbonate and sodium bicarbonate.

(e) *N-(1-Naphthyl)ethylenediamine dihydrochloride (NEDA)*.—Sigma Chemical Co., St. Louis, MO 63178. 0.4% methanol solution.

(f) *Benzoic acids and benzoates*.—*o*-Aminobenzoic acid, methyl *p*-aminobenzoate (Eastman Kodak, Rochester, NY 14650); *p*-aminobenzoic acid (Chem Service, Media, PA 19063); methyl *m*-aminobenzoate, methyl *o*-aminobenzoate (Chemalog, South Plainfield, NJ 07080).

(g) *Chromatographic support*.—100–120 mesh Chromosorb 102 (Sigma Chemical Co.).

Slurry 0.6 g in 10 mL methanol. Add slurry to super Pasteur disposable pipet (Curtin Matheson Scientific, Inc., Houston, TX 77001) plugged with glass wool. Add small wad of glass wool to top of column. Wash resin consecutively with 15 mL portions of methanol, acetone, and water before use. Note: Do not let column run dry.

(h) *Resin*.—Dowex-1, chloride form, 2% cross-linked, 200–400 mesh (Sigma Chemical Co.). Slurry sufficient resin in water to prepare 2 cm column in disposable Pasteur pipet plugged with glass wool. Add plug of glass wool to top of column and wash resin with 3 mL 1N NaOH. Rinse column with water until effluent is neutral, then wash column with 5 mL 1N HCl. Rinse column with water until effluent is neutral. Rinse column with 5 mL methanol before use.

Apparatus

(a) *Tissue grinder*.—Brinkmann Polytron® homogenizer (Brinkmann Instruments Inc., Westbury, NY 11590).

(b) *Centrifuges*.—International clinical centrifuge, Rotor 213 (International Equipment Co., Needham Heights, MA 02194); Servall Super-speed centrifuge, type SS-1 rotor. (Ivan Sorvall, Inc., Norwalk, CT).

(c) *UV source*.—Chromato-Vue (Ultra-Violet Products, Inc., San Gabriel, CA 91778).

(d) *TLC plates*.—2.5 × 10 cm precoated (250 μm layer) silica gel G glass plates (Analtech, Inc., Newark, DE 19711). Free acid solvent system:

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ethyl acetate-methanol (4 + 1); methyl ester system: hexane-ethyl acetate (3 + 1). Develop plates 0.5 cm from origin, dry in forced air oven at room temperature, and redevelop to height of 6 cm from origin. Partially air-dry developed plates and view under longwave (365 nm) UV light to locate blue-white fluorescent spot. Completely dry plates in forced air oven at room temperature and visualize aromatic primary amines with NEDA following diazotization (1).

(e) *GLC-MS system*.—70 eV electron impact mass spectra were obtained on Hewlett-Packard Model 5992-B combination GLC-low resolution quadrupole MS system interfaced to Hewlett-Packard Model 9825-A data system. GLC conditions: 1.83 m × 0.64 cm od glass column packed with 3% OV-17 on 100-120 mesh Gas-Chrom Q; injection port 150°C; column programmed from 70 to 280°C at 6°/min; helium carrier gas flow rate 20 mL/min.

Liver Samples

Swine livers were obtained from a local slaughter house and portions were extracted within 3 h of being excised from the animals.

Procedures

(a) *Methylation of isolated unknown-TBAH ion pair*.—Combine DCM extracts from two 2.5 g liver samples obtained by screening procedure previously described (1). Evaporate combined extracts to dryness in 9 mL screw-cap specimen vial. Add 2 mL 0.2M iodomethane in DCM and stir mixture magnetically 30 min. Remove iodomethane-DCM under stream of nitrogen at 40°C. Dissolve residue in 0.4 mL DCM and spot 10 μL on thin layer chromatographic (TLC) plate. Develop plates in hexane-ethyl acetate (3 + 1).

(b) *Isolation of unknown by modified Tishler procedure (3)*.—Add 20 mL chloroform-acetone (1 + 1) to 5 g swine liver in 50 mL polypropylene centrifuge tube and homogenize mixture 1 min at low speed. Centrifuge homogenate 2 min at 3500 rpm. Use Pasteur pipet to remove solvent, and filter solvent through plug of glass wool packed in super Pasteur pipet into 50 mL polypropylene screw-cap centrifuge tube. Just remove solvent under stream of nitrogen at 60°C and dissolve residue in 10 mL hexane. Add 2 mL 1N HCl and shake mixture 3 min in rocking motion and then centrifuge 2 min at 3500 rpm. Pour contents of centrifuge tube into 60 mL separatory funnel containing 8 mL water in manner that prevents mixing aqueous and organic phases. Recover acid phase (0.2N) and

pass it through column of Chromosorb 102. Wash resin with 15 mL water and elute column with 5 mL methanol. Combine methanol effluents from 4 isolations (20 g liver) and evaporate to dryness in 9 mL screw-cap specimen vial under stream of nitrogen at 60°C. Dissolve residue in 0.5 mL methanol. Spot 10 μL aliquot on TLC plate and develop in ethyl acetate-methanol (4 + 1).

Carry out methylation by exhaustive alkylation procedure of Gyllenhaal et al. (4). Remove methanol under stream of nitrogen and add 2 mL pH 10 buffer and 0.05 mL TBAH solution to residue. Vortex-stir solution 30 s. Add 2 mL 0.2M iodomethane in DCM and magnetically stir mixture 4 h to complete methylation. Centrifuge reaction vial 2 min at 2500 rpm and slowly pour contents (less magnetic stirrer) into 60 mL separatory funnel containing 5 mL DCM. Rinse reaction vial with 6 mL DCM, centrifuge, and add solvent to separatory funnel. Recover DCM and evaporate it to ca 0.5 mL in 9 mL screw-cap specimen vial under stream of nitrogen. Spot 20 μL aliquot on TLC plate and develop plate in hexane-ethyl acetate (3 + 1). Evaporate sample to dryness.

Dissolve residue in 0.5 mL methanol and pass solution through Dowex 1 column, collecting effluent in 15 mL screw-cap centrifuge tube. Rinse vial with three 0.5 mL portions of methanol. Add rinses to ion exchange resin individually and collect total effluent. Remove methanol under stream of nitrogen at 50°C and dissolve residue in 10 mL DCM. Add 5 mL water and shake mixture vigorously 2 min. Centrifuge mixture 2 min at 2500 rpm, and then slowly add separated mixture to 60 mL separatory funnel containing 5 mL DCM. Recover DCM in 23 mL screw-cap specimen vial and evaporate to ca 3 mL at 40°C under stream of nitrogen. Transfer DCM by using disposable Pasteur pipet, in ca 0.5 mL increments, to 2 mL screw-cap specimen vial and evaporate to ca 75 μL at room temperature. Inject 15 μL into gas-liquid chromatographic-mass spectrometric (GLC-MS) system.

Results and Discussion

The unknown compound was tentatively identified in liver extracts obtained by the screening procedure which limits the extraction of Bratton-Marshall-positive compounds to amphoteric compounds (1). Hence, a variety of agricultural chemicals and drugs, and/or their metabolites (5), were eliminated as the possible source of the Bratton-Marshall-positive compound in the liver extracts. In addition, the

Table 1. Comparison of relative intensities (%) of the 10 most abundant mass fragment ions of methylated unknown Bratton-Marshall-positive compound with authentic methyl aminobenzoate isomers^a

m/z	Unknown (10.6) ^b	<i>ortho</i> - (10.5)	<i>meta</i> - (13.6)	<i>para</i> - (15.2)
119	100.0	100.0	<1.0	<1.0
92	73.4	68.3	99.1	34.9
65	53.3	50.1	75.3	42.6
151	41.1	44.9	85.7	38.6
120	31.0	31.3	100.0	100.0
39	29.6	30.2	38.5	21.2
64	19.6	16.8	14.8	8.1
52	17.3	13.4	13.1	6.9
63	15.7	16.0	18.8	10.1
91	12.8	12.5	9.9	4.4

^a Data corrected for background by data system.

^b Values in parentheses are GLC retention times (min).

unknown was present to varying degrees in nearly all livers subjected to the screening procedure, thus suggesting to us that the unknown was a naturally occurring compound. Among such compounds, *p*-aminobenzoic acid (PABA) has been considered a potential source of false positive results in the Tishler procedure for sulfonamides (6). However, our comparative TLC studies of the unknown with PABA revealed 3 differences which eliminated this compound as the unknown: (a) R_f values were slightly different (PABA, 0.7; unknown, 0.6); (b) the unknown exhibited a blue-white fluorescence when viewed under longwave UV light whereas PABA did not fluoresce; and (c) the diazotized PABA reacted almost immediately with NEDA whereas the unknown developed color slowly.

Methylation of the isolated unknown-TBAH ion pair with iodomethane presented the first evidence as to the identity of the unknown. The methylated compound had a grape-like odor which we recognized as that of the *ortho*-isomer of aminobenzoic acid (anthranilic acid). TLC studies on authentic *o*-aminobenzoic acid and methyl *o*-aminobenzoate revealed the same R_f

values, and the same fluorescence and color development characteristics with NEDA, as those for the unknown and its methyl ester. Based on this information, the unknown was tentatively identified as *o*-aminobenzoic acid.

This compound was also present in extracts obtained by the Tishler procedure. TLC results were positive on these extracts, and the identity of the unknown, as its methyl ester, was unequivocally established by GLC-MS. Table 1 presents GLC retention times and the relative intensities of the 10 most abundant MS ions of the methylated unknown and methyl *o*-aminobenzoate. The retention times and ion intensities of methyl *p*-aminobenzoate and methyl *m*-aminobenzoate are presented for comparison. Based on TLC and GLC-MS studies, we conclude that the unknown is *o*-aminobenzoic acid, a naturally occurring metabolite of tryptophan in animal tissues.

The specific contribution of *o*-aminobenzoic acid to the results obtained by the Tishler method for sulfonamide residues in swine liver is not precisely known. Our knowledge of the actual sampling, storage, and other conditions that occur before analysis in the regulatory monitoring program suggest that *o*-aminobenzoic acid contributes to the sulfa drug "background" level frequently encountered. Further investigations on this effect will be carried out.

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