Confirmation of Azaperone and Its Metabolically Reduced Form, Azaperol, in Swine Liver by Gas Chromatography/Mass Spectrometry

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The method described confirms the use of the tranquilizer azaperone by detecting the parent compound and the metabolically reduced form, azaperol. Both are confirmed in swine liver at a target concentration of 10 ppb by gas chromatography/mass spectrometry (GC/MS) with electron ionization in the selected-ion-monitoring mode. Swine liver tissue is ground with dry ice. Acetonitrile is added to extract the drug from the tissue. Sodium chloride buffer is added to prepare for solid-phase extraction (SPE). The aqueous extract is loaded onto an SPE cartridge designed to extract acidic and neutral drug residues from biological matrices. The cartridge is washed with methanol and conditioned with sodium phosphate buffer. Azaperone and azaperol residues are eluted with a 2% ammonium hydroxide in ethyl acetate. The extracts are evaporated to dryness under a stream of nitrogen and reconstituted in ethyl acetate for GC/MS analysis. A DB-1 analytical column is used to separate the compounds prior to electron ionization. The parent ion, the base peak ion, and one diagnostic fragment ion are monitored for both compounds. The method was validated with fortified tissue samples containing both azaperone and azaperol. Azaperone-incurred tissues also were analyzed, and the presence of the parent drug and the metabolically reduced form, azaperol, was confirmed.

Azaperone is a neuroleptic tranquilizer belonging to the class of butyrophenones. The current literature for azaperone is limited, but information is available for other related butyrophenones (1–4). The antipsychotic butyrophenones also inhibit motor activity in animals (2). These tranquilizers may be used therapeutically in veterinary medicine to reduce aggressiveness and activity during livestock breeding (5). Azaperone is approved by the U.S. Food and Drug Administration (FDA) for use at 2.2 mg/kg (CFR 21, 522.150) to control aggressiveness when mixing or regrouping weanling or feeder pigs weighing up to 80 pounds (6).

Azaperone is not approved by the FDA for use in market-weight swine, although it is known to be used in an extra-label manner and given prophylactically to prevent stress in market-weight pigs during transport to the slaughterhouse. Market-weight pigs are sensitive to stress due to transport, and various veterinary tranquilizers are used to prevent mortality and loss of meat quality caused by this stress. These tranquilizers may be administered only a few hours before slaughter and may then give rise to residues in the animal (7, 8).

Azaperone is one of the most widely used veterinary tranquilizers (9). It is active at low doses (0.5 to 2.0 mg/kg), the incidence of side effects is low, and it is very effective in preventing traumatic shock (10). It is a short-acting drug; 16 h after administration, it is essentially completely removed from pig tissues (11–13).

Published analytical methods exist for azaperone and related butyrophenone tranquilizers with varying detection capabilities. In addition to residue analysis, methods have been designed for analytical forensic toxicology and clinical chemistry applications. These methods use various chromatographic techniques such as thin-layer chromatography (5, 14, 15), liquid chromatography (LC; 5, 6, 16–20), and gas chromatography (GC; 21, 22). Various mass spectrometric (MS) techniques (23, 24) such as LC/MS (4, 16), GC/MS (25–28), and LC-tandem MS (LC-MS/MS; 29–32) also have been reported. However, none of these meet the Center for Veterinary Medicine (CVM) requirements for confirmation of azaperone residues.

CVM requires that a method be validated with both known negative and fortified control samples. The method must show specific criteria for analyte retention time matching and relative abundance matching for at least 3 diagnostic ion fragments for each target. Furthermore, many of the published procedures rely on chlorinated solvents to extract the drug from the matrix. Use of chlorinated solvents is not desirable for health, safety, and environmental reasons.

After our approach had been validated and found to conform to our criteria, azaperone-incurred tissues were generated at our facility and analyzed. The presence of both the par-
ent drug and the metabolically reduced form, azaperol, were confirmed in the incurred tissue.

**METHOD**

**Apparatus**

Unless noted otherwise, equivalent apparatus and reagents may be substituted.

(a) *Centrifuge.*—Beckman GPR centrifuge equipped with a Model CH 3.7 swinging bucket rotor (Beckman/Spinco Division, Palo Alto, CA).

(b) *Nitrogen evaporator.*—Meyer N-EVAP analytical evaporator, Model 111, equipped with Luer adapters for Pasteur pipettes (Organomation Associates, South Berlin, MA).

(c) *Sample processor.*—Robot Coupe sample processor, Model RSI BX6V, equipped with stainless steel bowl and cutting blades (Robot Coupe USA, Ridgian, MS).

(d) *Solid-phase extraction (SPE) cartridges.*—Bond Elut Certify HF cartridges for rapid extraction of drugs of abuse, Varian Cat. No. 1410-2081, 3 cc/300 mg (Varian, Harbor City, CA).

(e) *Vacuum manifold.*—Visiprep DL SPE vacuum manifold equipped with disposable flow control liners (Supelco, Bellefonte, PA).

(f) *Vortex mixer.*—Vortex Genie 2, Model G-560 (Scientific Industries, Bohemia, NY).

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**Figure 1.** Structure and main fragment ions of azaperone.

**Figure 2.** Structure and main fragment ions of reduced azaperone: azaperol.
**Reagents**

(a) *Ammonium hydroxide, 28.0–30.0%.*—J.T. Baker (Phillipsburg, NJ).

(b) *Azaperol.*—Research Diagnostics (Flanders, NJ).

(c) *Azaperone.*—Research Diagnostics.

(d) *Hydrochloric acid (HCl), concentrated.*—Fisher Scientific (Fairlawn, NJ). Used to adjust pH of phosphate buffer solution.

(e) *Potassium hydroxide.*—J.T. Baker. Used to adjust pH of phosphate buffer solution.

(f) *Sodium phosphate, monobasic, monohydrate, crystal.*—J.T. Baker.

(g) *Sodium sulfate, anhydrous powder.*—J.T. Baker.

(h) *Solvents.*—UV spectrophotometric grade ethyl acetate, acetonitrile, and methanol (Burdick & Jackson, Muskegon, MI).

(i) *Stearic acid methyl ester (methyl stearate).*—Sigma Chemical (St. Louis, MO).

(j) *Deionized water.*—Purified through the Millipore (Bedford, MA) Milli-Q UV plus system to a purity of >17 MΩ/cm or equivalent. Use for all following references to water.

(k) *Dry ice pellets.*—Clean pellets or chunks for grinding tissues.

**Solutions**

Stability periods are noted in parentheses.

(a) *Sodium chloride solution, 10% (w/v).*—Store at room temperature in a screw-capped bottle (6 months).

(b) *2% Ammonium hydroxide in ethyl acetate, elution solution.*—Prepare fresh daily.

(c) *Phosphate buffer solution, 0.1M, pH 6.0.*—Store at ambient temperature. Inspect prior to use for any signs of contamination or growth (30 days).

(d) *Acetic acid 1.0M.*—Store at room temperature in glass or plastic (2 months).

(e) *Azaperol stock standard, 1000 μg/mL (RAZA-1000).*—Store protected from light at 0°C or below (6 months).

(f) *Azaperone stock standard, 1000 μg/mL (AZA-1000).*—Store protected from light at 0°C or below (6 months).

(g) *AZA/RAZA mixed standard, 10 μg/mL (AZA/RAZA-10).*—Combine equal volumes of AZA-1000 and RAZA-1000. Dilute with ethyl acetate to yield a solution containing 10 μg/mL each of AZA and RAZA. Store protected from light at 0°C or below (2 months).

(h) *AZA/RAZA mixed standard, 1 μg/mL (AZA/RAZA-1).*—Dilute mixed standard AZA/RAZA-10 solution to yield a solution containing 1.0 μg/mL each of AZA and RAZA. Store protected from light at 0°C or below (2 months). This solution is used to fortify tissue samples.

(i) *Potassium hydroxide, 1.0M.*—Store at ambient temperature (3 months).

**Animal Treatment**

To generate a tissue sample containing azaperone residues and metabolites, a male pig weighing 79 kg was intramuscularly injected with 32 mg azaperone U.S.P. dissolved in ethanol. The animal displayed no unusual behavior after injection. After 2 h, the pig was sacrificed, and the liver tissue was collected for analysis.
Sample Preparation

Cut fresh livers into chunks. Pregrind dry ice to a fine powder in a sample processor. Quickly drop individual liver chunks into processor and grind into a fine powder at high speed. Allow dry ice to sublime in a –20°C freezer, leaving a fine powder of frozen liver.

Extraction of Samples

It is convenient to prepare 6 to 9 samples in a batch depending on positions available in the centrifuge or the evaporator. Include at least one known negative control and one fortified sample with each day’s test samples. Begin by weighing 10 g portions of the uniformly ground powder. To prepare fortified samples, add 100 µL AZA/RAZA-1. Mix on a Vortex mixer briefly. Add 10 mL acetonitrile to each sample tube, cap the tube tightly, and mix on a Vortex mixer for 30 s. Sonicate for 10 min. Repeat the mixing and sonication once. Centrifuge sample at ambient temperature for 30 min at 3300 RCF (relative centrifugal force). Pour the upper acetonitrile layer into a new tube that contains 40 mL 10% NaCl solution. Discard the liver tissue pellet. Mix the sample tubes on a Vortex mixer. Condition the Bond Elut Certify SPE cartridges (33) with 6 mL methanol followed by 6 mL 0.1M sodium phosphate buffer. Transfer the aqueous extracts directly to the conditioned SPE cartridge. Reduce vacuum and slowly draw the extract through the SPE until all the extract has been loaded (at

Figure 4. Electron ionization mass spectrum of azaperone showing the molecular ion at m/z 327 and the characteristic fragment ions at m/z 233 and 107.
least 10 min). Rinse the charged SPE cartridges with 3 mL 1.0M acetic acid. Dry the cartridge under full vacuum for 5 min. Rinse cartridges again with 3.0 mL methanol and again dry the cartridge. Elute with 3.0 mL ethyl acetate–ammonium hydroxide (98 + 2) and evaporate to dryness under a stream of nitrogen at ambient temperature. Reconstitute the dry extracts in 50 µL ethyl acetate, briefly mix on a Vortex mixer, and transfer to base-treated GC vials containing glass inserts to accommodate the small volume. Adjust final volume to accommodate the sensitivity of the instrument. Inject and analyze samples within 24 h.

**Instrumental Operating Conditions**

(a) **GC/MS system.**—Hewlett-Packard 5890 Series II gas chromatograph equipped with a Series 5970 mass selective detector and a Series 7673A automatic sampler (Hewlett-Packard, Avondale, PA).

(b) **Column.**—DB-1 column (30 m, 0.25 µm film, and 0.25 mm od; J&W Scientific, Folsom, CA) baked at 250°C for 8 h before the daily analytical run.

(c) **Injector.**—Quartz 2 mm id, 250 µL, deactivated, splitless injector liner; injector temperature, 240°C.

(d) **Carrier gas.**—Ultra-high-purity helium at a linear velocity of 30 cm/s.

![Abundance vs Time](image1)

**Figure 5.** Electron ionization mass spectrum of azaperol, the reduced form of azaperone, showing the molecular ion at m/z 329 and the characteristic fragment ions at m/z 235 and 107.
Operating temperatures.—40°C for 1 min, rise to 140°C at 30°C/min and to 190°C at 6°C/min, maintain at 190°C for 3 min, rise to 250°C at 30°C/min, maintain for 12.3 min; total run time for each analysis, 30 min; interface transfer line temperature, 280°C.

MS analysis.—Obtain electron ionization (EI) spectra of analytes at 70 eV. Monitor sample extracts for ions at m/z 329, 327, 309, 233, 235, 123, 125, and 107. Monitor ion ratios m/z 329/107 and 235/107 for azaperol and m/z 327/107 and 233/107 for azaperone. The ion at m/z 309 corresponds to loss of water from azaperone. This ion typically is not seen in fresh azaperol standards.

System Suitability

Conduct these tests when first establishing the analytical system and during evaluation, to verify system suitability. Acceptable criteria for actual assays follow:

(a) Ethyl acetate blanks.—Inject a rinse of ethyl acetate to verify baseline stability at the start of each analytical run and after each sample.

(b) Method check.—To establish that reagents and other aspects of the laboratory procedure are performing within acceptable limits, calculate the signal-to-noise (S/N) ratio with the 1.0 ng/µL standard. The minimum peak-to-peak S/N ratio must be greater than 3.

(c) Resolution and tailing.—Calculate per the current method (34) based on the 1.0 g/µL mixed standard containing AZA and RAZA. Resolution between the 2 peaks should be greater than 2.0, and the tailing factors for both peaks should be 1.2 or less. When system suitability criteria have been met, begin analysis sequence with standards containing 1.0–4.0 g/µL azaperone and azaperol to verify the daily instrument performance. Inject a solvent rinse before analysis of known controls and before analysis of suspected samples. Analyze fortified samples last to avoid analyte carryover in the

(e) Operating temperatures.—40°C for 1 min, rise to 140°C at 30°C/min and to 190°C at 6°C/min, maintain at 190°C for 3 min, rise to 250°C at 30°C/min, maintain for 12.3 min; total run time for each analysis, 30 min; interface transfer line temperature, 280°C.

(f) MS analysis.—Obtain electron ionization (EI) spectra of analytes at 70 eV. Monitor sample extracts for ions at m/z 329, 327, 309, 233, 235, 123, 125, and 107. Monitor ion ratios m/z 329/107 and 235/107 for azaperol and m/z 327/107 and 233/107 for azaperone. The ion at m/z 309 corresponds to loss of water from azaperone. This ion typically is not seen in fresh azaperol standards.
GC inlet. Reanalyze standards at the end of the run, as the GC column is sensitive to column loading. The second set of standard injections may show greater detector response because of carryover and column loading. Inject 1 L of the extracts onto the column.

**Results and Discussion**

Rapid extraction is achieved by Vortex mixing and sonication of samples in acetonitrile. To eliminate a time-consuming evaporation step, the acetonitrile extract is diluted in concentrated salt solution and applied to the SPE. The Bond Elut Certify SPE, which is marketed for routine testing of drugs, contains a mixed sorbent bed designed to reliably extract drug residues from complex biological matrices. It has been used successfully, for example, to extract haloperidol from serum or urine. It performs well with our mixed extract, which contains high concentrations of salt and acetonitrile, giving a clean final extract.

This method was validated by analyzing control samples fortified with azaperone and azaperol at 10 ppb. Figures 1–3 show the possible fragmentation and structures of the diagnostic ions of azaperone and azaperol. Figures 4 and 5 are the EI mass spectra of azaperone and azaperol, respectively. With clean control tissue obtained from a U.S. Department of Agriculture market pig, fortified samples were prepared at the tar-
get concentration of 10 ppb. All fortified samples were confirmed, and all of the companion negative control samples failed to confirm.

For a sample to be confirmed, the retention times must fall within 10% of the acceptable retention time range of each standard, based on the average of the standards included with each batch. The ion ratios of a sample must match the corresponding average ratios of the standards included in the analysis batch within 10% absolute. Finally, the presence of both the parent drug azaperone and the metabolically reduced form must be confirmed to distinguish between actual misuse of azaperone and cases of carryover from the GC inlet.

Tables 1 and 2 list ion abundances from the selective-ion-monitoring (SIM) analysis of the fortified samples. Method performance was validated with incurred tissues from an azaperone-dosed pig. Tissues were tested immediately af-

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Figure 7. Chromatogram of extract from a known incurred swine liver sample containing confirmed azaperol.
after slaughter and after freezing at –80°C for 6 weeks. In all cases, azaperone use was confirmed. Figures 6 and 7 show chromatograms of extracts from the same sample of known incurred liver tissue that confirmed for both azaperone and azaperol. Tables 3 and 4 list ion abundances from the SIM analysis of fresh and frozen incurred tissues.

Conclusions

The method reliably confirmed presence of azaperone in swine liver tissue by identifying both the parent drug compound and the metabolically reduced target compound, azaperol. By using SPE cartridges designed for drug-of-abuse testing, we were able to keep costs down and ensure specificity toward the target compound. To save time and to reduce the number of transfer steps, the organic sample extracts were dissolved in a large volume of salt solution. This step precluded the need for a long evaporation step and reduced sample losses due to transfer. By relying on common instrumentation to detect 2 compounds simultaneously, the method is well suited for confirming cases of suspected extra label use of azaperone because it reliably identifies both the parent drug and the metabolite, azaperol, at a concentration of 10 ppb.

Acknowledgments

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References


| Table 3. Validation of azaperol in incurred swine liver tissue a |
|-----------------|-----------------|-----------------|
| Replicate No.   | m/z 329 | m/z 235 | m/z 107 |
| Day 1           |         |         |         |
| 1               | 5.3     | 14.3    | 100     |
| 2               | 5.8     | 12.9    | 100     |
| 3               | 4.9     | 13.5    | 100     |
| 4               | 3.4     | 15.2    | 100     |
| 5               | 4.6     | 9.3     | 100     |
| 6               | 6.0     | 15.0    | 100     |
| Averaged standards | 5.4     | 12.5    | 100     |
| Daily limits    | ≤15.4   | 2.5–22.5 | Base peak |
| Day 2           |         |         |         |
| 7               | 5.7     | 13.5    | 100     |
| 8               | 6.0     | 13.6    | 100     |
| 9               | 6.5     | 15.4    | 100     |
| 10              | 3.6     | 14.2    | 100     |
| 11              | 6.0     | 14.3    | 100     |
| Averaged standards | 3.5     | 11.8    | 100     |
| Daily limits    | ≤13.5   | 1.8–21.8 | Base peak |
| Day 3           |         |         |         |
| 12              | 1.0     | 8.9     | 100     |
| 13              | 4.6     | 11.6    | 100     |
| 14              | 4.1     | 17.1    | 100     |
| 15              | 2.1     | 14.9    | 100     |
| Averaged standards | 3.8     | 12.6    | 100     |
| Daily limits    | ≤13.8   | 2.6–22.6 | Base peak |

a The columns correspond to the relative abundance percentages at each of the diagnostic ion ratios. Abundance matching limits were calculated by averaging the daily responses. Data for day 1 were collected from fresh tissue samples. The samples had been frozen prior to analysis for days 2 and 3.
(34) U.S. Pharmacopea XXIII (1995) U.S. Pharmacopeal Convention, Rockville, MD