Determination of Sulfonamides in Meat by Liquid Chromatography Coupled with Atmospheric Pressure Chemical Ionization Mass Spectrometry

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Liquid chromatography/atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS) has been used for the determination of sulfonamides in meat. Five typical sulfonamides were selected as target compounds, and beef meat was selected as a matrix sample. As internal standards, sulfapyridine and isotope labeled sulfamethazine (13C6-SMZ) were used. Compared to the results of recent reports, our result have shown improved precision to a RSD of 1.8% for the determination of sulfamethazine spiked with 75 ng/g level in meat.

Key Words: Sulfonamides, Meat, LC-APCI-MS, IDMS, SPE

Introduction

Sulfonamides were the first antibacterial drugs, and were first used in the treatment of humans in the early 1930’s.1 Since the advent of antibiotics, sulfonamide use in human therapy has become quite limited.2 Now, sulfonamides are widely used as veterinary drugs for the treatment of infections and the promotion of growth of livestock and fish.3,4 These drugs are applied to animals or fish by various forms such as injections, additives in animal feed, and as water bathing agents for fish. For example, about 0.1 g of sulfamethazine is added per 1 kg of animal feed.

Sulfonamide residues in food are an important concern, due to the possibility of risk to human health, such as resistance development, and toxicity.5,6 Many countries, including Korea, have established allowed maximum residue limits (MRLs) of 100 ng/g for most sulfonamides in edible animal tissues, and 10 ng/mL in milk.3,5,7

In animal tissue, much of various interferences exist, but residues of sulfonamides are in very small amounts. This makes for an increasing need for analytical methods capable of rapidly and reliably assaying the presence of residual drugs in food. As a clean-up and enrichment method for sulfonamide residue in edible tissue, liquid-liquid extraction (LLE),8-11 solid phase extraction (SPE) -ion exchange,12-14 C18,15 aminopropyl silane,16 silica,17 and supercritical fluid extraction (SFE)13 have been used. For the determination of sulfonamides in tissue, gas chromatography (GC),14,15 gas chromatography/mass spectrometry (GC-MS),16-18 capillary electrophoresis (CE),19 high performance liquid chromatography (HPLC),8,10,20-22 and liquid chromatography/mass spectrometry (LC-MS)2,7,23,24 have been used. GC and GC-MS need derivatization before analysis, but LC-MS has more advantages than GC, because it can offer selectivity, structural information and sensitivity without the derivatization of sulfonamides.

The present LC-MS methods for edible tissue focus on quantification or identification of samples containing sulfonamide residue under the MRL. In general, the precisions of assays are in the range of RSD 10%.2,7,11,22,24 Even the results obtained by isotope dilution mass spectrometry (IDMS) showed similar precision.3,11,25 Another study for sulfonamides in simple matrix (animal urine) by IDMS showed more reproducible results.24 To obtain more reproducible results for a meat sample, a more sophisticated treatment and measurement of meat sample than urine was needed.

This study focused on obtaining more reproducible results than current results for the measurement sulfonamides in meat. SPE and liquid chromatography/atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS) has been used in this study for the determination of sulfonamides in meat.

Experimental Section

Chemicals and Materials. The sulfonamide standards were purchased from Sigma (sulfamethazine, SMZ, S-5632; sulfadimethoxine, SDM, S-7007; sulfathiazole, STZ, S-9876; sulfadiazine, SDZ, S-8626; sulfamethizole, SMTZ, S-6256; St. Louis, MO, USA). Sulfapyridine was used as an internal standard (SP, S-6252, 99%, St. Louis, MO, USA). As an internal standard for IDMS, isotopic enriched sulfamethazine was used (β-phenyl-13C6, atomic purity 90%, CLM-3045, 13C6-SMZ, Cambridge Isotope Laboratories, Inc., 50 Frontage Road, MA, USA). Ammonium acetate (NH4AC, 97%, Aldrich corp., St. Louis, MO, USA), sodium phosphate monobasic (NaH2PO4, Merck, P. O. Box 4119, D-6100 Darmstadt, Germany), were...
used as buffers for the HPLC solvent, or for the pH control for samples.

The organic solvents, acetonitrile were pesticide grade (Burdick & Jackson, Muskegon, MI, USA). For SPE, LiChrolut EN (200 mg, Merck KGaA, D-64293 Darmstadt, Germany) was used. 2.0 µm membrane filter (47 mm, Zefluor, P/N PSP9047, Pall Gelman Lab, 600S, Wagner Rd., Ann Arbor, MI 48103-9019, USA) and 0.2 µm disposable filter (4 mm Nylon, Whatman Inc., 9 Bridewell PI Clifton, NJ 07014, USA) were used for sample treatments.

Preparation of Fortified Sample, and Clean Up. As a sample matrix, beef meat was purchased from a market and preserved at -20 °C until use. For the preparation of sample, sample matrix, beef meat was purchased from a market and from the difference between the weight of the syringe before of internal standard added to the samples was calculated.

The entire syringe, including solution and septum, was weighed in a chemical balance (AT-201, Mettler-Toledo, GmbH, CH-8603 Schwerzenbach, Switzerland). The amount of 13C6-SMZ (70 µL (0.05 g) of 15 µg/g solution in acetonitrile), and 13C6-SMZ (70 µL (0.05 g) of 15 µg/g solution in acetonitrile), were spiked as internal standards.

The internal standard was filled in a 250 µL gas-tight syringe (ILS, GmbH, Mittelstrasse 37, D-98714, Stutzerbach, Germany), and the end of a needle was closed with a small septum. The entire syringe, including solution and septum, was weighed in a chemical balance (AT-201, Mettler-Toledo, GmbH, CH-8603 Schwerzenbach, Switzerland). The amount of internal standard added to the samples was calculated from the difference between the weight of the syringe before and after injecting the internal standard solution. For IDMS, the amount of 13C6-SMZ spiked into the samples was determined such as to make the mass ratio of SMZ/13C6-SMZ in the meat sample near 1 : 1.

The sample was cleaned-up by the procedures previously described.26 The sample (10 g) was transferred into a mini container (12-37 mL) of a high-speed blender (MC1-12-37 mL, Waring Commercial, 283 Main St. New Hartford, CT 06057, USA), and blended with 20 mL of acetonitrile and 2 g of sodium phosphate for 2 min. The extract was filtered with suction through a 2.0 µm membrane filter (47 mm, Zefluor, Pall Gelman Lab) using glass filter holder (VWR Scientific, 1310 Goshen Pkwy. West Chester, PA 19380, USA), and the volume of the filtrate was reduced to 5 mL by reduced pressure rotary evaporation at 40 °C. Into the concentrated solution, 100 mL of pure water was added and passed through an SPE cartridge (LiChrolut EN, flow rate 3 mL/min) preconditioned with 15 mL of acetonitrile and water. The SPE cartridges, washed with 2 mL of pure water and enriched sulfonamides, were eluted using 20 mL of acetonitrile. Then the solvent was removed to dryness by rotary evaporation. The residue was reconstituted with 250 µL of HPLC eluent and filtered through a 0.2 µm syringe filter (4 mm, Nylon) to a 300 µL vial insert (part No. 5181-1270, Agilent, 2850 Centerville Rd., Wilmington, DE 19808, USA) for a 2 mL autosampler vial.

LC-MS Analysis. Reversed-phase liquid chromatographic experiments for the separation of the sulfonamides were performed on a chromatograph equipped with a HP 1050 autosampler and pump (Hewlett-Packard, Washington, DC, USA), Phenomenex ODS2 (250 mm × 2.5 mm × 5 µm, Phenomenex, 2320 W. 205th Street Torrance, CA 90050-1456, U.S.A) and LC-18-DB (250 mm × 2.5 mm × 5 µm, Supelco, Supelco Park, Bellefonte, PA 16823-0048, USA) were used as a stationary phase. Samples were separated in an isocratic condition, and the eluents were acetonitrile: water: 0.1 M aqueous ammonium acetate solution (13 : 35 : 50, v/v).27 The HPLC conditions were as follows: volume injected, 2 µL; column pressure, 600 psi; temperature, 25 °C; and flow rate, 200 µL/min.27

A Finnigan LCQ iontrap LC-MS system (Finnigan, San Jose, CA, USA), equipped with an APCI source was used. Nitrogen was used for the sheath gas at 45 (an arbitrary value used in LCQ). The LC-MS system was operated at a high resolution MS scan (Zoom Scan) and a positive-ion modes. In this modes LCQ conducts a high-resolution scan of 10 u width, so the protonated positive ions of sulfonamides ([M + H]+; SDZ, m/z = 251; STZ, 256; SMTZ, 271; SP, 250; SMZ, 279; 13C6-SMZ, 285; SDM, 311) were scanned within a 10 u window; and the scan rages were changed as time passed for the acquisition of different compounds. APCI conditions were: sheath gas flow rate, 50 arb (arbitrary unit of LCQ instrument); vaporizer temperature, 450 °C; discharge voltage, 5.5 kV; tube lens offset voltage, 25 V; capillary temperature, 150 °C; and, capillary voltage, 10 V.

Calculation. For the measurement of sulfonamides, 13C6-SMZ, and sulfapyridine were used as internal standards for IDMS and internal standard (ISTD) method, respectively. For a one-point calibration, a similar concentration of the calibration mixture to the final sample solution was prepared. The amount of 13C6-SMZ or sulfapyridine spiked into the sample was determined to make the peak area ratio of SMZ/13C6-SMZ, or the concentration ratio of target sulfonamides/sulfapyridine in the meat sample, near to 1:1. The concentration of sulfonamides in the sample was calculated by the following equation.

C = \frac{W_{\text{STD, sample}} \cdot R_{\text{sample}} \cdot W_{\text{std, cal}} \cdot C_{\text{std}}}{W_{\text{sample}} \cdot R_{\text{cal}} \cdot W_{\text{STD, cal}}}

C: concentration of analyte in sample.
W_{\text{sample}}: weight of sample taken for analysis.
W_{\text{STD, sample}}: weight of 13C6-SMZ or sulfapyridine solution spiked to sample taken for analysis.
R_{\text{sample}}: peak area ratio of the analyte to 13C6-SMZ or sulfapyridine, from LC-MS measurement of sample.
R_{\text{cal}}: peak area ratio of the analyte to 13C6-SMZ or sulfapyridine, from LC-MS measurement of calibration solution.
W_{\text{STD, cal}}: weight of standard solution added to calibration solution.
W_{\text{std, cal}}: weight of 13C6-SMZ or sulfapyridine solution added to calibration solution.
C_{\text{cal}}: concentration of standard solution.
Results and Discussion

The LC-APCI-MS extracted ion chromatograms of the sample solution of blank meat and spiked meat separated on the LC-18-DB are shown in Figure 1. For this experiment an acetonitrile : water : 0.1 M aqueous ammonium acetate solution (13 : 37 : 50, v/v) was used as an eluent. From the blank meat sample we found no interfering substances were present in the sample matrix and reagents.

The results of the quantification for five sulfonamides by the ISTD method, and the results of the quantification for SMZ by IDMS, for spiked meat samples, are shown in Table 1 and 2, respectively. We assessed the precision of the method for the five samples by using a three-repeated measurement of each sample.

For samples of A in Table 1, five sulfonamides were fortified into 10 g of ground meat to prepare the sample, and $^{13}$C$_6$-SMZ and sulfapyridine were spiked immediately, then left at room temperature for 12 hours. For sample B, five sulfonamides were fortified into 100 g of ground meat. These samples were homogenized and left in the same environment as sample A. After 12 hours, 10 g portions of samples were weighed, and $^{13}$C$_6$-SMZ and sulfapyridine were spiked to each sample. Then, within one hour these samples were analyzed. The fortified level used in this study ($\approx$ 75 ng/g) was lower than the allowed maximum residue limits (MRLs) of 100 ng/g in edible animal tissues.

The results of the quantification of sulfonamides by the ISTD (sulfapyridine) method are shown in Table 1. The precision of instrument measurement from the three measurement of each sample was an RSD of 2.0-31% and the precisions of results for the five independent samples was an RSD of 3.8-12.3%. The measured concentrations showed about -33.8-

Table 1. Results of determination of sulfonamides in meat sample-A by internal standard (sulfapyridine) method

<table>
<thead>
<tr>
<th>Sample</th>
<th>Compound</th>
<th>Prepared concentration (µg/g)</th>
<th>Measured concentration (µg/g)$^a$</th>
<th>RSD (%)</th>
<th>Difference (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-1</td>
<td>SMTZ</td>
<td>0.076</td>
<td>$-$</td>
<td>$-$</td>
<td>$-$</td>
</tr>
<tr>
<td></td>
<td>SDZ</td>
<td>0.073</td>
<td>0.082</td>
<td>12.6</td>
<td>11.8</td>
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<tr>
<td></td>
<td>STZ</td>
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<td>0.053</td>
<td>21.9</td>
<td>-19.5</td>
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<tr>
<td></td>
<td>SMZ</td>
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<td>0.082</td>
<td>8.0</td>
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<tr>
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<td>SDM</td>
<td>0.074</td>
<td>0.053</td>
<td>30.7</td>
<td>-28.7</td>
</tr>
<tr>
<td>A-2</td>
<td>SMTZ</td>
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<td>0.088</td>
<td>5.5</td>
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</tr>
<tr>
<td></td>
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<td>0.088</td>
<td>17.1</td>
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</tr>
<tr>
<td></td>
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<td>0.055</td>
<td>3.0</td>
<td>12.1</td>
</tr>
<tr>
<td></td>
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<td>0.086</td>
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</tr>
<tr>
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<td>0.072</td>
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<tr>
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<td>0.058</td>
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<td>0.090</td>
<td>5.1</td>
<td>20.1</td>
</tr>
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<td></td>
<td>STZ</td>
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<td>0.058</td>
<td>5.4</td>
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</tr>
<tr>
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<td>0.060</td>
<td>5.9</td>
<td>-21.4</td>
</tr>
<tr>
<td>A-4</td>
<td>SMTZ</td>
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<td>0.051</td>
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<tr>
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<td>0.102</td>
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<td>4.6</td>
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<tr>
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<td>0.056</td>
<td>9.1</td>
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<td>5.8</td>
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</tr>
</tbody>
</table>

$^a$average of three. SMTZ, sulfamethizole; SDZ, sulfadiazine; STZ, sulfathiazole; SMZ, sulfamethazine; SDM, sulfadimethoxine. Sample solutions were separated on a LC-18-DB column.
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For SMZ, the variation of measured concentration was overcome by use of the isotope labeled internal standard (\(^{13}\)C\(_6\)-SMZ). The results of the quantification for SMZ by IDMS are shown in Table 2. For sample A, the reproducibility of the instrument measurement for the three replications was an RSD of 0-2.7%, and the reproducibility of results for the five independent samples was an RSD of 1.8%. The measured concentrations of “sample A” showed a -3.5-6.6% deviation from the prepared concentrations. However for sample B the deviation from the prepared concentrations was -33.1% of a deviation from the prepared concentrations. The measured concentrations were variable. This may result from instability of the spray characteristics of the APCI probe. Also, this may result from the different properties (such as pKa value) of the internal standard (sulfapyridine) to the target sulfonamides.

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