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

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Received July 31, 2002

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 ($^{13}C_6$-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.[3-6] 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.[2,6] 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,[2,3,6,12] C_{18},[2-4] aminopropyl silane,[2,6] silica-,[5] 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.

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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%.[2-7,11,23,24] Even the results obtained by isotope dilution mass spectrometry (IDMS) showed similar precision.[5,11,25]

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 sophisticate 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 (-phenyl-$^{13}C_6$, atomic purity 90%, CLM-3045, $^{13}C_6$-SMZ, Cambridge Isotope Laboratories, Inc., 50 Frontage Road, MA, USA). Stock standard solutions of sulfonamides were prepared by weighing, 10 mg of sulfonamides were dissolved in 7.8 g of acetonitrile (about 10 mL) respectively. Standard solutions for LC-MS calibration were prepared by mixing stock solutions of standards, internal standards (sulfapyridine and isotope labeled SMZ).

Ammonium acetate (NH_4AC, 97%, Aldrich corp., St. Louis, MO, USA), sodium phosphate monobasic (NaH_2PO_4, 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 P5PJ047, 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 Pl 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, 10 g or 100 g of pre grind meat was weighed in 20 mL or 110 mL bottle, and spiked with five sulfonamides (about 100 mL (0.09 g) or 990 μL (0.79 g) of 10 μg/g mixture in acetonitrile), respectively. For a sample, a 10 g portion of prepared meat sample was used, and sulfapyridine (80 μL (0.06 g) of 24.5 μg/g solution in acetonitrile), and $^{13}C_6$-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 valance (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 $^{13}C_6$-SMZ spiked into the samples was determined such as to make the mass ratio of SMZ/$^{13}C_6$-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, Zefluo, 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 90501-1456, 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; $^{13}C_6$-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, $^{13}C_6$-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 $^{13}C_6$-SMZ or sulfapyridine spiked into the sample was determined to make the peak area ratio of SMZ/$^{13}C_6$-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.

$$C = \frac{W_{ISTD,\,sample} \cdot R_{sample} \cdot W_{std,\,cal} \cdot C_{std}}{W_{sample} \cdot R_{cal} \cdot W_{ISTD,\,cal}} \quad (1)$$

C: concentration of analyte in sample.

W_{sample}: weight of sample taken for analysis.

$W_{ISTD,\,sample}$: weight of $^{13}C_6$-SMZ or sulfapyridine solution spiked to sample taken for analysis.

R_{sample}: peak area ratio of the analyte to $^{13}C_6$-SMZ or sulfapyridine, from LC-MS measurement of sample.

R_{cal}: peak area ratio of the analyte to $^{13}C_6$-SMZ or sulfapyridine, from LC-MS measurement of calibration solution.

$W_{std,\,cal}$: weight of standard solution added to calibration solution.

$W_{ISTD,\,cal}$: weight of $^{13}C_6$-SMZ or sulfapyridine solution added to calibration solution.

C_{std}: concentration of standard solution.

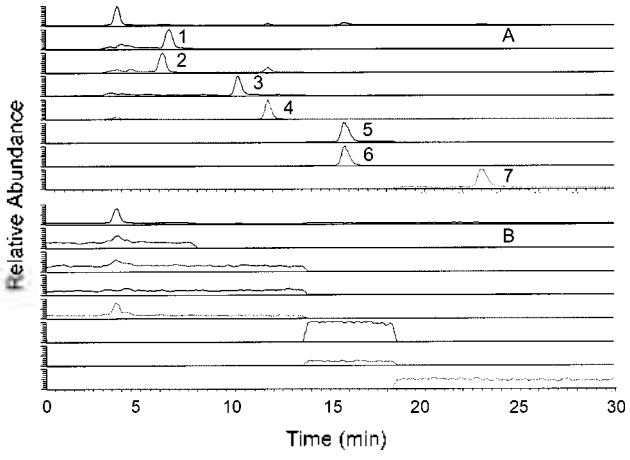

Figure 1. Extracted ion chromatograms of LC-APCI-MS for sample solution of spiked meat and blank meat separated using LC-18-DB. A, spiked meat sample; B, blank meat sample. 1, Sulfamethizole (SMTZ); 2, sulfadiazine (SDZ); 3, sulfathiazole (STZ); 4, sulfapyridine (SP, ISTD); 5, sulfamethazine (SMZ); 6, $^{13}C_6$-sulfamethazine ($^{13}C_6$-SMZ); 7, sulfadimethoxine (SDM).

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 (≡ 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

Sample	Compound	Prepared concentration (μg g)	Measured concentration (μg/g)[a]	RSD (%)	Difference (%)
A-1	SMTZ	0.076	–	–	–
	SDZ	0.073	0.082	12.6	11.8
	STZ	0.066	0.053	21.9	-19.5
	SMZ	0.074	0.082	8.0	10.1
	SDM	0.074	0.053	30.7	-28.7
A-2	SMTZ	0.078			
	SDZ	0.075	0.088	5.5	16.5
	STZ	0.067	0.055	17.1	-17.7
	SMZ	0.076	0.086	3.0	12.1
	SDM	0.076	0.072	23.5	-6.0
A-3	SMTZ	0.078	0.058	4.7	-24.8
	SDZ	0.075	0.090	5.1	20.1
	STZ	0.067	0.058	5.4	-13.6
	SMZ	0.076	0.086	2.2	13.7
	SDM	0.076	0.060	5.9	-21.4
A-4	SMTZ	0.077	0.051	6.0	-33.8
	SDZ	0.074	0.102	4.7	37.9
	STZ	0.066	0.054	4.6	-18.4
	SMZ	0.075	0.100	4.7	33.1
	SDM	0.075	0.068	5.0	-8.6
A-5	SMTZ	0.078	0.056	9.1	-28.1
	SDZ	0.075	0.102	4.5	34.9
	STZ	0.067	0.053	3.9	-21.1
	SMZ	0.077	0.096	6.8	25.4
	SDM	0.076	0.070	5.8	-8.7

[a]average of three. SMTZ, sulfamethizole; SDZ, sulfadiazine; STZ, sulfathiazole; SMZ, sulfamethazine; SDM, sulfadimethoxine. Sample solutions were separated on a LC-18-DB column.

Table 2. Results of determination of sulfamethazine in meat sample by isotope dilution method

Sample	Prepared concentration (μg/g)	Measured concentration (μg g)			Avg.	STDEV	RSD (%)	Difference (%)
		1	2	3				
A-1	0.074	0.071	0.071	0.071	0.071	0	0	-4.1
A-2	0.076	0.073	0.071	0.075	0.073	0.002	2.7	-3.9
A-3	0.076	0.072	0.071	0.070	0.071	0.001	1.4	-6.6
A-4	0.075	0.072	0.073	0.072	0.072	0.0006	0.8	-3.6
A-5	0.077	0.074	0.074	0.075	0.074	0.0006	0.8	-3.5
B-1	0.078	0.064	0.064	0.064	0.064	0	0	-17.9
B-2	0.078	0.063	0.063	0.065	0.064	0.001	1.8	-18.4
B-3	0.078	0.063	0.065	0.065	0.064	0.001	1.8	-17.5
B-4	0.078	0.060	0.064	0.066	0.063	0.003	4.8	-18.8
B-5	0.078	0.065	0.065	0.065	0.065	0	0	-16.7

A: Five sulfonamides were spiked to meat, and $^{13}C_6$-SMZ was added immediately, then left at room temperature. B: Five sulfonamides were spiked to meat, then left at room temperature. After 12 hrs $^{13}C_6$-SMZ was added, and sample was cleaned up. Sample solutions were separated on a ODS2 column.

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.

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 the 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 -16.7~-18.8%. When comparing the measured concentrations of sample A to B, although the reproducibility of results was similar, the measured concentrations of sample B deviated more from the prepared concentration than sample A. This may result from insufficient equilibration between the matrix and $^{13}C_6$-SMZ.

Balizs *et al*. used IDMS by liquid chromatography/thermospray-mass spectrometry (LC-TSP-MS) for the determination of sulfonamides in meat.[5] The precision of the LC-MS assay was a RSD of 2-27% at the concentrations of 80-100 μg/kg.

Fuh *et al*. used IDMS by liquid chromatography/electrospray-mass spectrometry (LC-ESI-MS) for the determination of sulfonamides in meat.[11] They spiked the isotope labeled internal standard to the extract of sample instead of spiking to the sample matrix. Although the concentrations of sulfonamides were higher than in our study, the precision of the LC-MS assay was a RSD of 1.3-9.7%. If they had spiked the internal standard to sample matrix the precision of the assay might have lowered. In our study, the internal standard was spiked to the sample matrix. After enough equilibration, samples were extracted with acetonitrile and cleaned up.

For the determination of sulfonamides in milk, van Rhijn *et al*. used a highly specific tandem mass spectrometry (LC-ESI-MS-MS) to reduce the influence of interference caused by simple cleanup procedures.[25] The precision of the LC-MS assay was a RSD of 2-17%, at the 50-150 μg/kg concentration levels. The unsatisfactory precision may have resulted from an ionization suppression caused by a matrix co-extractant and the poor precision of MS-MS. To improve precision, we used SPE for sample cleanup and LC-APCI-MS instead of LC-ESI-MS-MS.

As compared to the results of other reseachers,[5,11,25] our study has shown a more reproducible and reliable result. This may be the result of a more sophisticated treatment and measurement of weighing, spiking of internal standard, SPE clean up, separation, and detection (LC-APCI-MS).[26,27]

Conclusions

Liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS) has been used in this study for the determination of sulfonamides in meat. Compared to the results of recent research, our result have shown the improved precision of a RSD of 1.8% for the determination of SMZ spiked with 75 ng/g level in meat. The combination of IDMS by LC-APCI-MS and SPE can be utilized for the accurate determination of sulfonamides from biological matrices. Further work is required for the application to real world samples, and on the employment of isotope labeled internal standards for all sulfonamides.

References

1. Kennedy, D. G.; McCracken, R. J. A.; Cannavan, A.; Hewitt, S. A. *J. Chromatogr. A* **1998**, *812*, 77.
2. Poter, S. *Analyst* **1994**, *119*, 2753.
3. Gehring, T. A.; Rushing, L. G.; Churchwell, M. I. *J. Agric. Food Chem.* **1996**, *44*, 3164.
4. Boison, J. O.; Keng, L. J.-Y. *J. AOAC Int.* **1995**, *78*, 651.
5. Balizs, G.; Benesch-Girke, L.; Borner, S.; Hewitt, S. A. *J. Chromatogr. B* **1994**, *661*, 75.
6. Ito, Y.; Oka, H.; Ikai, Y.; Matsumoto, H. *J. Chromatogr. A* **2000**,

898, 95.
7. Kristiansen, G. K.; Brock, R.; Bojesen, G. *Anal. Chem.* **1994**, *66*, 3253.
8. Bui, L. V. *J. AOAC Int.* **1993**, *76*, 966.
9. Stoev, G.; Michailova, A. *J. Chromtogr. A* **2000**, *871*, 37.
10. Gehring, T. A.; Rushing, L. G.; Thompson, H. C. *J. AOAC International* **1995**, *78*(5), 1161.
11. Fuh, M. S.; Chan, S. *Talanta* **2001**, *55*, 1127.
12. Tarbin, J. A.; Clarke, P.; Shearer, G. *J. Chromtogr. B* **1999**, *729*, 127.
13. Combs, M. T.; Ashraf-Khorassan, M.; Taylor, L. T. *J. Pharmaceutical and Biomedical Anal.* **1999**, *25*, 301.
14. Goodspeed, D. P.; Simpson, R. M.; Ashworth, R. B.; Shafer, J. W.; Cook, H. R. *J. Assoc. Off. Anal. Chem.* **1978**, *61*, 1050.
15. Okamoto, J.; Matsubara, T. *Anal. Chem.* **2000**, *72*, 634.
16. Takatsuki, K.; Kikuchi, T. *J. Assoc. Off. Anal. Chem.* **1990**, *73*, 886.
17. Carignan, G.; Carrier, K. *J. Assoc. Off. Anal. Chem.* **1991**, *74*, 479.
18. Simpson, R. M.; Suhre, F. B.; Shafer, J. W. *J. Assoc. Off. Anal. Chem.* **1985**, *68*, 23.
19. Lin, C.-E.; Lin, W.-C.; Chiou, W.-C. *J. Chromtogr. A* **1996**, *755*, 261.
20. Leveque, D.; Gallion-Renault, C.; Monteil, H. *J. Chromtogr. A* **1998**, *815*, 163.
21. Meinertz, J. R.; Schmidt, L. J. *J. AOAC International* **1999**, *82*(5), 1064.
22. Gehring, T. A.; Rushing, L. G.; Thompson, H. C. *J. Assoc. Off. Anal. Chem.* **1995**, *78*, 1161.
23. Pleasance, S.; Blay, P.; Quilliam, M. A. *J. Chromtogra.* **1991**, *119*, 2753.
24. Bartolucci, G.; Pieraccini, G.; Villanelli, F.; Moneti, G.; Triolo, A. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 967.
25. Rhijn, J. A.; Lasaroms, J. J. P.; Berendsen, B. J. A.; Brinkman, U. A. Th. *J. Chromtogr. A* **2002**, *960*, 121.
26. Kim, D. H.; Lee, D. W. *J. Chromatogr. A*, in print.
27. Kim, D. H.; Lee, D. W. *Analyst*, submitted.