A Clean and Rapid Liquid Chromatographic Technique for Sulfamethazine Monitoring in Pork Tissues without Using Organic Solvents

Naoto Furusawa
Graduate School of Human Life Science, Osaka City University, Osaka 558-8585, Japan

Abstract

A rapid method for the isolation and high-performance liquid chromatographic (HPLC) determination of sulfamethazine (SMZ) in pork tissues (kidney, liver, and muscle) without using organic solvents is developed. The isolation is performed by homogenization with an acid solution using an ultrasonic-homogenizer, followed by centrifugation. The HPLC analyses are performed using a reversed-phase C_4 column (150- × 4.6-mm i.d.), a mobile phase of 0.02 mol/L citric acid solution, and a photodiode array detector. The resulting HPLC chromatograms are free from interferences for determination and identification. The proposed technique is shown to be linear ($r > 0.99$) over the concentration range 0.1–2.0 µg/g for all pork tissues. Average recoveries of SMZ (spiked 0.1–2.0 µg/g) range from 87.6% to 90.2%, with inter- and intra-assay variabilities of less than 4%. The total time required for the analysis of one sample and limit of quantitation is less than 20 min and 0.09 µg/g, respectively.

Introduction

Sulfamethazine [4-amino-*N*-(4,6-dimethyl-2-pyrimidinyl)benzensulfonamide] (SMZ), a drug for exclusive use with swine, is applied globally for growth-promoting purposes. Therefore, SMZ residues may be present in marketed pork if the drug has been improperly administered or if the withdrawal time for the treated pigs has not been observed. A report that SMZ is a possible carcinogen (1) has magnified concerns. The U.S. Food and Drug Administration; Joint Expert Committee for Food Additives under command of Codex Alimentrium Commission (CAC); and Japan Health, Labor, and Welfare Ministry (HLWM) have set a tolerance or maximum residue limit (MRL) for SMZ in pork tissues at 0.1 ppm (1–3).

The U.S. Department of Agriculture Food Safety and Inspection Service and the Agriculture, Fisheries, and Forestry of Australia have reported the results of monitoring for sulfonamide residues in marketed pork in 1998 (4) and 1999 (5). Both of these studies showed that market pork tissues had the highest incidence of SMZ residues, which is consistent with monitoring data of domestic and imported pork in 1995–2000 for the Japanese port quarantine station. The drug that was found in pork was only SMZ. A rigid residue monitoring of SMZ in pork tissues is therefore an important specific activity to guarantee the food safety.

Discharging of waste containing organic solvents is also a severe problem on the world-wide scale. From the viewpoint of the effect of organic solvents to environments and analysts, analytical methods for monitoring should help to avoid the use of organic solvents (6–9).

Presently, regardless of industrial nations or developing states, an international and harmonized method for the drug residue monitoring in pork tissues is required (10). The acceptable method must be rapid, accurate, precise, reliable, portable, economical in cost and time, capable of determining residues below the tolerance/MRL, and environmentally friendly.

There have been significant developments in recent years in techniques for the determination and identification of sulfonamide residues in animal tissues using high-performance liquid chromatography (HPLC) interfaced with UV (11–13), photodiode array detection (PDAD) (14–16), fluorescence detection (17,18), or mass spectrometry (MS) [liquid chromatography (LC)–MS] (12,13,19,20). These methods have such problems as (*a*) the extraction and clean-up involves varying analytical steps that are time consuming and do not permit the monitoring of a large number of samples, (*b*) the recoveries are sometimes low and variable, and (*c*) the methods—especially LC–MS—give low peak resolution (13,20). There is presently no acceptable and harmonized analytical method for SMZ.

This paper describes an epoch-making method that enables rapid and simple determination of SMZ residues in pork tissues without the use of any organic solvents. Determination is performed by HPLC equipped with a PDAD, which simultaneously measures the retention time and absorption spectrum. The target peak component can be instantly identified. The PDAD provides information regarding both the identity and purity of chromatographic peaks and can be readily and routinely used.

Experimental

Reagents and materials

The SMZ standard was obtained from Sigma (St. Louis, MO). Other chemicals were purchased from Wako Pure Chem (Osaka, Japan). Distilled water was of HPLC grade. A stock standard solution of SMZ was prepared by accurately weighing 10 mg and dissolving it in 100 mL distilled water. The working standard solutions were prepared by diluting the stock solution with distilled water. A 10% (v/v) perchloric acid (PCA) solution that was diluted with distilled water was used as the extraction/deproteinzation solution. The following apparatuses were used in the sample preparation: a model HOM-100 ultrasonic-homogenizer with a 2-mm-i.d. probe (Iwaki Glass Co., Funabashi, Japan), a Biofuge fresco microcentrifuge (Kendo Lab Products, Hanau, Germany), and a Dismic 13HP 0.45-µm hydrophilic disposable syringe filter unit (Advantec Toyo Roshi, Tokyo, Japan). The following three Mightysil RP GP (5 µm) reversed-phase columns were used: column A, an RP-18 GP Aqua (C_{18}) (250- × 4.6-mm i.d.); column B, an RP-4 GP (C_4) (250- × 4.6-mm i.d.); and column C, an RP-4 GP (5 µm, 150- × 4.6-mm i.d.). The columns were purchased from Kanto Chem. (Tokyo, Japan).

HPLC

The HPLC system consisted of a Model PU-980 pump and DG-980-50 degasser (JASCO, Tokyo, Japan) equipped with an SPD-M10A VP PDAD (Shimadzu, Kyoto, Japan). The operating conditions were as follow: analytical column, column C (described previously) equipped with a guard column (5- × 4.6-mm i.d.) (Kanto Chem.) containing the same packing material; isocratic mobile phase, 0.02-mol/L citric acid solution (in distilled water); flow rate, 1.0 mL/min; column temperature, ambient; and injection volume, 20 µL.

Procedure

Pork tissues (kidney, liver, and muscle) were minced fully and used as the blank samples. An accurately weighed 0.2-g sample was inserted into a microcentrifuge tube and homogenized in 0.4 mL of 10% (v/v) PCA solution with an ultrasonic homogenizer for 1 min. After 1 min, the capped tube was centrifuged at 12,000-g for 5 min. The supernatant liquid was filtrated through a 0.45-µm disposable syringe filter unit, and the filtrate was injected into the HPLC system.

Recovery test

The recoveries of SMZ from blank samples spiked at 0.1, 0.2, 0.4, 1.0, and 2.0 µg/g, respectively, were determined. These fortification concentrations were prepared by adding 10 µL of five mixed standard solutions of the mentioned drug (20, 40, 80, 200, and 400 µg/mL, respectively) to separated 2-g portions of the sample. Fortified samples were allowed to stand at 4°C for 12 h after the standards addition, and they were then mixed prior to workup. The average relative standard deviation (RSD) determined for each spiked concentration was then calculated, which resulted in a mean ± standard deviaition (SD). This was defined as interassay variability. Intra-assay variability was defined as the RSD for the mean of five replicates of an identical sample and represents the variability associated with the analytical procedure used.

Results and Discussion

One aim of this work was to develop a technique that SMZ in pork tissues can be to analyzed without the use of any organic solvents.

Extraction

In the bioanalytical and analytical chemistry fields, a strong solution of trichloroacetic acid (TCA) or PCA is more commonly used as the protein precipitant in biological matrices, which results in protein removal. Because TCA (an organic halogenated compound) is an environmental pollutant and interfered with UV absorption at the wavelength used for HPLC analysis of SDZ, PCA was used as a solution in the extraction and deproteinzation.

The extraction operation using an ultrasonic homogenizer, which is easy-to-use and portable, yielded rapid and easy extraction of target compound in pork tissues. This operation did not lead to residue loss caused by the "flying off" of the tube content. Furthermore, the extract did not form an emulsion that would hinder SMZ recoveries. After centrifugation, SMZ was completely recovered in the supernatant liquid. The extract did not need further purification for the HPLC analysis, as can be seen in Figure 1.

The established procedure did not require complicated extraction and cleanup operations, was easy to use, did not require the use of organic solvents, and had a shorter operation time, which resulted in high recovery and reproducibility with considerable saving of analysis costs. The time required for one sample preparation was less than 10 min.

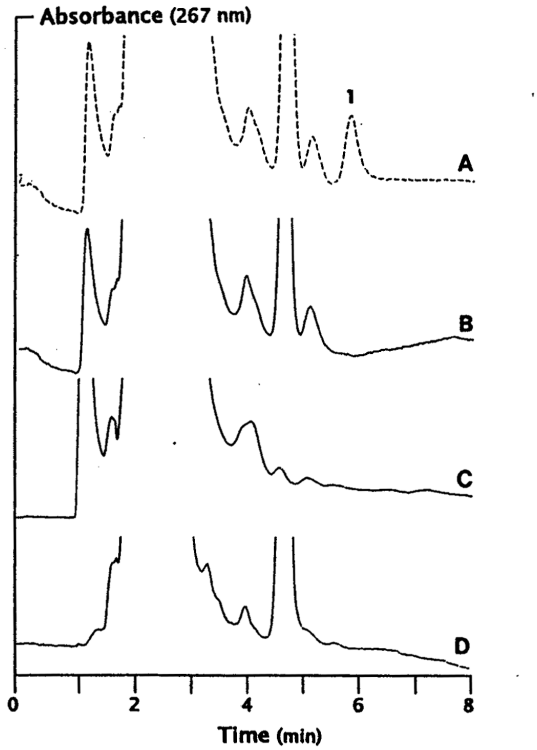

Figure 1. HPLC chromatograms obtained from pork tissue samples (PDAD set at 267 nm). (A) spiked kidney (SMZ, 0.4 µg/g); (B) blank kidney; (C) blank liver; and (D) blank muscle. Peak 1 is SMZ (retention time = 5.9 min).

HPLC optimal conditions

Considering the reversed-phase C_{18}, C_8, and C_4 nonpolar sorbents, which were the columns for the HPLC separation, the C_4 was less retentive than C_{18} and C_8 sorbents when retention was based on nonpolar interactions alone. When the C_{18} and C_8 sorbents require larger volume of strong elution solvents as the mobile phase, the C_4 would remarkably reduce the volume of elution solvents required and provide a more concentrated and cleaner separation.

The author has previously reported acceptable determination of SMZ, or sulfadimethoxine, by HPLC using RP C_{18} (Mightysil RP-18 GP Aqua 250- × 4.6-mm i.d.) (column A) and RP C_4 (Mightysil RP-4 GP, 250- × 4.6-mm i.d.) (column B) columns and ethanol–water as the mobile phase (16,21). In order to optimize the separation, the mentioned columns and a Mightysil RP-4 GP column (150- × 4.6-mm i.d.) (column C) with a citric acid solution as the mobile phase were tested and compared with regard to the separations: SMZ from interfering peaks of resulting extract origin and a sharp peak obtained upon injection of equal amounts. Mobile phases with molarities of citric acid between 0.005 and 0.2 mol/L were tested. Under the mobile phase examined over the range, SMZ was hardly eluted from column A, the SMZ peak was easy to detect as a significant broadening peak on column B, SMZ peak was eluted from column C, and its retention decreased with increasing molarities of citric acid used as the mobile phase.

A chromatogram with the complete separation of the target compound, its clear–sharp peak, and its short retention time (5.9 min, Figure 1A) was obtained using column C, the Mightysil RP-4 GP column (150- × 4.6-mm i.d.), and an isocratic mobile phase of 0.02 mol/L citric acid solution. The monitoring wavelength was set at 267 nm because the maximum absorption of SMZ dissolved in the mobile phase was 267 nm, which was determined by PDAD.

Figure 1 indicates that the resulting chromatograms are free from interfering compounds for quantitation and identification by HPLC with a PDAD. HPLC combined with the PDAD easily confirms peak identity. The SMZ examined was identified in the sample with its retention time and absorption spectrum. The present sample preparation allowed for a reliable confirmation.

Using a spiked (SMZ 0.4 µg/g) pork kidney tissue sample obtained under the established procedure, the chromatographic repeatability was obtained from the RSDs of areas and retention times calculated for ten replicate injections of the spiked sample. The values were 0.08% for area and 0.54% for retention time, respectively.

The total time required for the analysis of one sample was less than 20 min. The rapid and harmless method without the use of organic solvents could be achieved.

Method qualification

Table I summarizes the recoveries from pork tissue samples at five different spiking levels, the correlation coefficient (*r*) of calibration curve, and inter- and intra-assay variabilities of target compound isolated from spiked pork tissue samples. Overall, excellent recoveries and assay variabilities were obtained.

Average recoveries were 87.6–90.2%, with SDs between 3.0% and 3.6%. Inter- and intra-assay variabilities ranged from 2.6% to 3.8%. These values are well within acceptable criteria for the analysis methods that the Codex setup (i.e., average recoveries of 80–110% with RSDs < 15% when the MRL for the analyte is ≥ 0.1 ppm) (10).

Calibration was performed by linear regression analysis of peak areas of spiked sample extracts ranging from 0.1 to 2.0 µg/g versus their concentrations. The curve was constructed from five points, and each point represented the mean of five injections. The resulting curves were linear in the concentration ranges 0.1–2.0 µg/g for SMZ in the kidney, liver, and muscle, with *r* values of greater than 0.99 ($P < 0.01$).

Using the peak areas in HPLC chromatograms obtained from blank and spiked pork tissue samples, the limits of quantitation (LOQs) for pork kidney, liver, and muscle, respectively, were calculated as follow: ten times the SD obtained by replicate analysis at a sufficiently low spike concentration. Five different blank pork samples known to be near the LOQ were analyzed in duplicate. In a practical analysis for the residue monitoring, the LOQ was calculated to be 0.09 µg/g for all of the pork tissues (Table I).

Table I. Accuracy, Precision, and Sensitivity Data for SMZ-Fortified Pork Tissues

Sample	Calibration curve*	%Recovery†	%Assay variability: Interassay (*n* = 25)	%Assay variability: Intra-assay (*n* = 5)	LOQ (µg/g)‡
Kidney	0.998 ± 0.002	88.8 ± 3.3	3.1 ± 0.7	3.0	0.09
Liver	0.999 ± 0.001	90.2 ± 3.6	3.4 ± 0.6	2.6	0.09
Muscle	0.998 ± 0.001	87.6 ± 3.0	2.7 ± 0.4	3.8	0.09

* *r* ± SD, *n* = 5. Mean of five determinations using spiked pork samples for calibration curve (range of concentration was 0.1 – 2.0 µg/g), and *r* is the correlation coefficient.
† Mean ± SD, *n* = 25. Five replicates at concentrations ranging from 0.1 to 2.0 µg/g (at 5 levels).
‡ Limit of quantitation.

Conclusion

The present study has developed a clean and rapid method without using organic solvents for determination and identification of SMZ in pork tissues (muscle, liver, and kidney) using HPLC interfaced with a PDAD. The main advantages of proposed procedure are summarized as follows: (*a*) by extraction using an ultrasonic homogenizer followed by high-speed centrifugation, the sample preparation is especially easy and rapid and is able to recover SMZ effectively; (*b*) shorter analysis time, for which the total time required for the analysis of one sample was < 20 min and economical; (*c*) the proving that the recoveries were reproducible , repeatable (the inter- and intra-assay variabilities were 2.6–3.8%), and economical; and (*d*) no organic solvents were used at all, which means that this is harmless to the environment and to humans. The present procedure may be useful as an international and harmonized analytical method for routine residue monitoring of SMZ in pork tissues.

References

1. NCTR Technical Report Experiment No.418. *Fed. Reg.* **1988,** 53, 9492.
2. World Health Organization. *36th Report of the Joint FAO/WHO Expert Committee on Food Additives*. Report 799. WHO, Geneva, Switzerland, 1990.
3. Food Additives Organization. Food and Nutrition Papers 41/4. 38th Meeting of the Joint FAO/WHO Expert Committee on Food Additives. FAO, Rome, Italy, 1991.
4. Food Safety and Inspection Service. *National Residue Program Domestic Residue Data Book*. United States Department of Agriculture, Office of Public Health and Science, Washington, D.C., 1998.
5. Agriculture, Fisheries and Forestry Australia National Office of Food Safety. Report on the Australian National Residue Survey Results, Jan. 1–June 30,1990.
6. M. Ogawa. The selection of organic solvents for food analyses that considered the influence to environment. *J. Food Hyg. Soc. Jpn.* **37:** J289–90 (1996).
7. M. Ishibashi. The selection of analytical solvents by the Japanese pharmacy. *J. Food Hyg. Soc. Jpn.* **38:** J194–95 (1997).
8. R. Malish, B. Bourgeois, and R. Lippold. Multiresidue analysis of selected chemotherapeutics and antiparasitics. *Dtsch. Lebensm-Rundsch.* **88:** 205–16 (1992).
9. P.T. Anastas and J.C. Warner. *Green Chemistry—Theory and Practice*. Oxford University Press, Oxford, U.K., 1998.
10. Codex Alimentarius Commission. *Joint FAO/WHO Food Standards Program, Residues of Veterinary Drugs in Food*. Vol. 3, 2nd ed. Codex Alimentarius Commission, Rome, Italy, 1993.
11. P. Shearan, M. O'keeffe, and M.R. Smyth. Comparison of matrix solid phase dispersion (MSPD) with a standard solvent extraction method for sulphamethazine in pork muscle using high performance liquid and thin-layer chromatography. *Food Addit. Contam.* **11:** 7–15 (1994).
12. J.O. Boison and L.J. Keng. Determination of sulfadimethoxine and sulfamethazine residues in animal tissues by liquid chromatography and thermospray mass spectrometry. *J. AOAC Int.* **78:** 651–58 (1995).
13. J.F. Jen, H.L. Lee, and B.N. Lee. Simultaneous determination of seven sulfonamide residues in swine wastewater by high-performance liquid chromatography. *J. Chromatogr. A* **793:** 378–82 (1998).
14. S.L. Boulaire, J.C. Baudure, and F. Andre. Veterinary drug residues survey in meat: an HPLC method with a matrix solid phase dispersion extraction. *J. Agric. Food Chem.* **45:** 2134–42 (1997).
15. N. Furusawa. HPLC determination of sulfadimethoxine and its hydroxyl metabolics following SPE of edible chicken tissues. *J. Liq. Chromatogr. & R.T.* **23:** 1413–22 (2000).
16. N. Furusawa. Determining the procedure for routine residue monitoring of sulfamethazine in edible animal tissues. *Biomed. Chromatogr.* **15:** 235–39 (2001).
17. M.H. Akhtar, M. Wong, S.R. Crooks, and A. Sauve. Extraction of incurred sulphamethazine in swine tissue by microwave assisted extraction and quantification without clean up by high performance liquid chromatography following derivatization with dimetylaminobenzaldehyde. *Food Addit. Contam.* **15:** 542–49 (1998).
18. G. Stoev and Al Michailova. Quantitative determination of sulfonamide residues in foods of animal origin by high-performance liquid chromatography with fluorescence detection. *J. Chromatogr. A* **871:** 37–42 (2000).
19. M.R.S. Fuh and S.A. Chan. Quantitative determination of sulfonamide in meat by liquid chromatography-electrospray-mass spectrometry. *Talanta* **55:** 1127–39 (2001).
20. G. Balizs, L. Beneschgirke, S. Borner, and S.A. Hewitt. Comparison of the determination of four sulphonamides and their N4-acetyl metabolites in swine muscle tissue using liquid chromatography with ultraviolet and mass spectral detection. *J. Chromatogr. B* **661:** 75–84 (1994).
21. N. Furusawa. Simplified determining procedure for routine residue monitoring of sulphamethazine and sulphadimethoxine in milk. *J. Chromatogr. A* **898:** 185–91 (2001).

Manuscript accepted June 3, 2003.