Journal of Chromatography, 558 (1991) 175–180
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 440

Pre-column derivatization of sulfa drugs with fluorescamine and high-performance liquid chromatographic determination at their residual levels in meat and meat products

NOBUYUKI TAKEDA* and YUMI AKIYAMA
Food and Drug Division, Hyogo Prefectural Institute of Public Health, Arata-cho, Hyogo-ku, Kobe 652 (Japan)

(First received February 25th, 1991; revised manuscript received May 8th, 1991)

ABSTRACT

A rapid, sensitive and selective high-performance liquid chromatographic method is described for simultaneous determination of eight sulfa drugs in meat and meat products using pre-column derivatization with fluorescamine. The drugs are sulfisomidine, sulfadiazine, sulfamerazine, sulfadimidine, sulfamonomethoxine, sulfamethoxazole, sulfadimethoxine and sulfaquinoxaline. The method includes blender extraction of 3-g samples with chloroform, partition with 3 *M* hydrochloric acid, derivatization with fluorescamine at pH 3.0 and subsequent high-performance liquid chromatographic analysis on a C_{18} column with fluorescence detection at an excitation wavelength of 405 nm and an emission wavelength of 495 nm. The drugs were separated with a mobile phase of acetonitrile–2% acetic acid (3:5) at 55°C. The average recovery from samples fortified at 0.1 ng/g was 92.6% with a coefficient of variation of 6.2%. The detection limit was 0.01 ng/g for sulfaquinoxaline and 0.005 ng/g for the other seven drugs. The method was field-tested in a survey of 37 samples including beef (five), pork (seven), chicken (seven), ham (five), sausage (eight), bacon (two) and roast beef (three). Sulfadimidine was detected in one pork sample at the level of 0.295 ng/g and in ham at 0.178 ng/g.

INTRODUCTION

Sulfa drugs are popular antibacterial agents for livestock, however their residues in meat products are a potential danger to human health. Tolerances in feed and withdrawal periods for the drugs have been established to minimize their residual levels in the meat, nevertheless claims of food contamination have often been made. A recent report showed that 11% of pork meat imported into Japan had been contaminated with sulfadimidine at levels in the range of 0.05–1.05 ng/g [1].

Several simultaneous assay procedures for sulfa drugs have been reported [2,3], however they generally include time-consuming extraction and clean-up steps to obtain the desired sensitivity. In addition, high-performance liquid chromatographic (HPLC) determination with UV detection is not selective for sulfa drugs. Although a photodiode-array detection system or post-column derivatization has been adopted to achieve selectivity for sulfa drugs, these require expensive equipment or additional apparatus and involve setting up and optimizing the system [1,4,5].

0021-9673/91/$03.50

We applied a pre-column derivatization system with fluorescamine to develop a rapid and selective assay for sulfa drugs in meat and its products, since such a system requires no additional equipment in the post-column system and the reagent seems to be suitable for HPLC determination. Fluorescamine is specific for sulfonamide analogue in the acidic pH range and produces highly fluorescent fluorophores having essentially similar excitation–emission spectral characteristics. Also, the reagent and its hydrolysis products are non-fluorescent [6,7].

EXPERIMENTAL

Samples

A total of 37 samples, from three items of meat cut (pork, beef and chicken) and four items of meat products (ham, sausage, bacon and roast beef) was taken in the winter of 1990–1991. The products were purchased from three retailers in the Kobe area of Japan and stored in the refrigerator until analysis. Meat samples containing adipose and connective tissue were cut into small pieces with a kitchen knife and mixed manually in a beaker.

Reagents and apparatus

Sulfisomidine (ID), sulfadiazine (DZ) and sulfamethoxazole (XZ) were purchased from Sigma (St. Louis, MO, USA), sulfamonomethoxine (MX) and sulfadimethoxine (DX) from Daiichi-Seiyaku (Tokyo, Japan) and sulfaquinoxaline (SQ) from Dainihon-Seiyaku (Osaka, Japan). Sulfamerazine (MR) and sulfadimidine (DM) were generous gifts from Dr. T. Hamano of the Public Health Research Institute of Kobe City, Japan. The standard stock solution (100 μg/ml) was prepared by accurately weighing *ca.* 10 mg and dissolving in 100 ml of methanol. Fluorescamine reagent (0.2%) was prepared by dissolving 10 mg of Fluram (F. Hoffman-La Roche, Switzerland) in 50 ml of acetone. HPLC-grade methanol, acetonitrile, acetone and chloroform, and reagent-grade hydrochloric acid, acetic acid and sodium acetate were used (Wako Pure Chemicals, Japan). Water was purified by Milli-Q SP TOC (Millipore, Bedford, MA USA).

A liquid chromatograph was composed of an LC-6AD pump, an RF-535 fluorescence monitor (Shimadzu, Kyoto, Japan) and a Chemcosorb 5-ODS-H column (150 × 4.6 mm I.D., 5 μm particle size, Chemco Scientific, Osaka, Japan). The column was put in a water bath kept a 55°C. HPLC separation was performed in a mobile phase of acetonitrile–2% acetic acid (5:3) at a flow-rate of 1 ml/min.

Extraction

A 3-g portion of the meat sample was weighed into a 50-ml centrifuge tube and blended in 30 ml of chloroform for 2 min with a Polytron homogenizer (Brinkman Instruments, Westbury, USA), then the mixture was shaken for 10 min. After centrifugation at 1600 *g* for 5 min, the chloroform phase was filtered through No. 5A filter paper (Toyo Roshi Kaisha, Japan), and 5 ml of the extract was transferred into a 10-ml test tube. A 1-ml portion of 3 *M* hydrochloric acid was added and the tube was shaken for 10 min, then centrifuged at 1600 *g* for 5 min. This acid concentration has previously been shown to give the highest recoveries for some sulfa drugs using this extraction method [2].

Derivatization with fluorescamine

For optimizing reaction conditions (pH and incubation period), a flow injection (FI) technique was used by eliminating a column from the LC system. The standard solutions of DM, MR, MX, DX and SQ were evaporated under a nitrogen gas stream and redissolved in 3 *M* hydrochloric acid to give a concentration of 1 μg/ml. Derivatization was performed by modifying the method of Sakano *et al.* [6] as follows: 2.5 ml of the standard solution were mixed with the same volume of sodium acetate solution, the concentration of which ranged from 2.5 *M* to 5.0 *M*, then with 1.0 ml of 0.2% fluorescamine acetone solution. The pH of the content was measured by a Beckman Φ 12 pH/ISE meter (Beckman Instruments, Fullerton, USA). The content was incubated for various lengths of time at room temperature. The incubate, 10 μl, was injected into the FI system and the fluorophore was detected at an excitation wavelength of 405 nm and an emission wavelength of 495 nm.

For HPLC analysis, a volume of the acidic sample extract, 3.5 *M* sodium acetate solution and the fluorescent reagent was scaled down to one-tenth, that is 250 μl of both the sample and alkaline solutions and 100 μl of the reagent were mixed in this order with a vortex mixer. The sample was ready for HPLC injection after standing for 20 min at room temperature. A 10-μl sample was injected into the system, and was equivalent to a 0.08-g sample.

RESULTS AND DISCUSSION

Optimization for fluorescamine derivatization

The first approach was to optimize pH for the derivatization in our system. Five sulfa drugs (DM, MR, MX, DX and SQ) were individually dissolved in 3 *M* hydrochloric acid and pH of the solution was varied by adding serially diluted sodium acetate-solution. Fig. 1 shows that the fluorescence intensities of the derivatives varied with the variation in pH ranging from 0.42 to 4.23. Optimal reactivity was obtained at pH 3.00 (sodium acetate concentration of 3.5 *M*) for all the drugs tested, while the intensity was very low in the strong acid range (pH 0.42 and 0.55) and decreased with an increase in pH. The pH profiles for the fluorophore were similar to those reported in the literature [6,7]. The optimal incubation period was also examined at the above buffer concentration and found to be 20–60 min (Fig. 2). In later

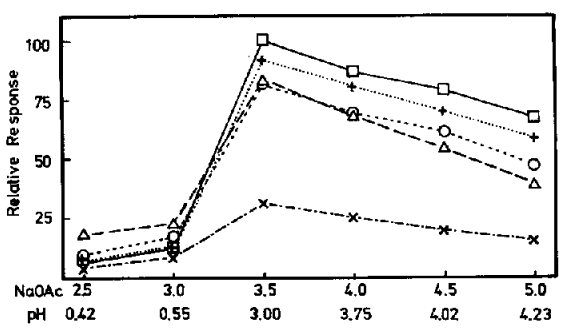

Fig. 1. Effect of pH on fluorescence intensity of fluorescamine derivatives of sulfa drugs. □ = DM; + = MR; ○ = MX; △ = DX, × = SQ. NaOAc = sodium acetate concentration in *M*.

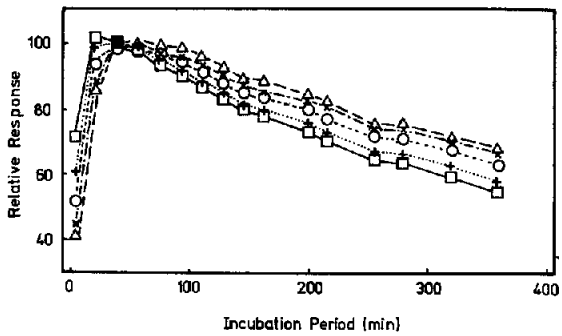

Fig. 2. Effect of incubation period on fluorescence intensity of fluorescamine derivatives of sulfa drugs. □ = DM; + = MR; ○ = MX; △ = DX; × = SQ.

experiments the reaction was performed for 20 min, since much longer incubation lowered the intensity. In practice, a reproducible reaction period was achieved as follows: the stop time of the integrator was set at 20 min (HPLC separation was completed within the time) and the sample solution for the next injection was prepared immediately after the previous sample had been injected for HPLC. In such a manner, the interval was measured for twelve injection and found to be 19.98 ± 0.95 min.

HPLC separation

HPLC conditions for separating eight sulfa drugs were examined by varying column temperature and the ratio of acetonitrile to 2% acetic acid in the mobile phase. As shown in Fig. 3A, the best separation was obtained within 20 min at 55°C

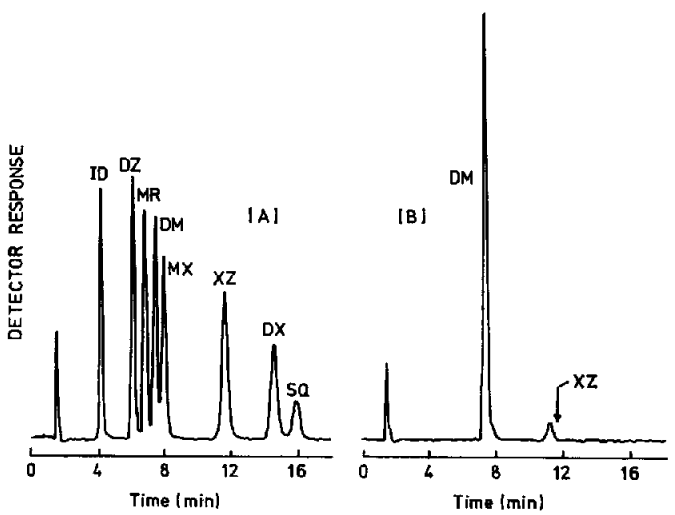

Fig. 3. Chromatograms of standard sulfa drugs (A) and positive pork sample (B). ID = Sulfisomidine; DZ = sulfadiazine; MR = sulfamerazine; DM = sulfadimidine; MX = sulfamonomethoxine; XZ = sulfamethoxazole; DX = sulfadimethoxine; SQ = sulfaquinoxaline.

and at a composition ratio of 3:5 (37.5% acetonitrile in 2% acetic acid). A dose–response relation was tested at the above optimal reaction conditions with the highest HPLC detector sensitivity, and found to be linear over the range tested (16.6–167 ng per milliter of final solution). The fluorescent peak height of the derivative of SQ was the lowest of the drug compounds examined, giving a detection limit of 0.05 ng on the chromatogram. Those of other derivatives were as low as 0.02 ng. These values correspond to 0.006 ng/g and 0.003 ng/g, respectively. In a practical analysis, the detection limit was set at 0.01 ng/g for SQ and 0.005 ng/g for the other seven sulfa drugs.

Sample analysis

The method was applied to residue analysis for sulfa drugs in 37 samples of meat and meat products including beef, pork, chicken, ham, sausage, bacon and roast beef. The results are summarized in Table I. Sulfadimidine (DM) was detected in two samples: pork at 0.30 ng/g (Fig. 3B) and ham at 0.18 ng/g. No sulfa drug was detected in beef, chicken, sausage, bacon or roast beef (Table I), however all the samples showed a fluorscent peak at retention time 11.2 min, which cause ambiguity of identification of XZ (11.6 min) (Fig. 3B). The peak (equivalent to 0.003–0.011 ng/g XZ) may be derived from some usual but unidentified meat component. Although the other seven sulfa drugs except DM were not detected in all the samples tested, a large peak having retention time 8.6 min appeared in one of the eight sausage samples. This could not be identified in a limited number of our authentic standards but may be a sulfa drug, since other sausage samples exhibited no fluorescent peak at this retention time.

TABLE I

ANALYSIS OF SULFA DRUGS IN COMMERCIAL MEATS AND MEAT PRODUCTS

Sample analyzed		Sulfadimidine (DM)	
Item	Number	Detected	Level (ng/g)
Beef	5	n.d.[a]	
Chicken	7	n.d.	
Pork	7	1	0.295 ± 0.011[b]
Ham	5	1	0.180
Sausage	8	n.d.	
Bacon	2	n.d.	
Roast beef	3	n.d.	

[a] n.d. = Not detected (<0.005 ng/g).
[b] Average ± S.D. of four determinations.

Recovery test

The recovery study was done at the level of 0.1 ng/g through the entire procedure using samples which had not showed any peak. The percentage recoveries are summarized in Table II. The highest recovery was 102% with a coefficient of variation (C.V.) of 2.6% for DM and the lowest 82.3% with a C.V. of 3.6% for DZ.

TABLE II

RECOVERY (%) OF EIGHT SULFA DRUGS FROM MEAT AND MEAT PRODUCTS FORTIFIED AT 0.1 ng/g

Sample	Sulfa drug										
	ID	DZ	MR	DM	MX	XZ	DX	SQ	Mean	S.D.	C.V.(%)
Beef	87.9	83.4	94.8	98.8	90.6	89.0	89.5	79.3	89.2	5.7	6.4
Chicken	89.8	77.8	97.6	102.9	88.9	107.3	92.9	85.2	92.7	9.0	9.7
Pork	91.1	80.0	102.4	105.2	93.1	90.8	97.2	91.7	93.9	7.3	7.8
Ham	87.4	85.0	97.2	104.1	93.4	101.0	94.7	92.3	94.4	6.0	6.4
Sausage	88.3	85.4	96.8	99.0	91.6	94.5	93.9	94.4	93.0	4.1	4.5
Mean	88.8	82.3	97.7	102.0	91.5	96.5	93.6	88.6	92.6	5.6	6.2
S.D.	1.3	3.0	2.5	2.6	1.7	6.8	2.5	5.6			
C.V.(%)	1.5	3.6	2.6	2.6	1.8	7.0	2.7	6.3			

Every meat sample gave high recoveries for the eight drugs ranging from 89.2% in the beef sample to 94.4% in ham. The average mean recovery was 92.6% with a C.V. of 6.2%. The contaminated pork sample had the level of 0.295 ± 0.011 ng/g with a C.V. of 3.8%.

The high recovery rate and low C.V. value indicate that the method is reproducible and accurate. The method is also rapid and simple, since it requires little glassware and solvent and no laborious clean-up and solvent evaporation. In conclusion, the method is applicable to a highly specific and sensitive routine analysis for sulfa drugs in various kinds of meat and meat products.

REFERENCES

1 M. Horie, K. Saito, Y. Hoshino, N. Nose, N. Hamada and H. Nakazawa, *J. Food Hyg. Soc. Japan*, 31 (1990) 171.
2 H. Terada, M. Asanoma, H. Tsubouchi, T. Ishihara and Y. Sakabe, *Eisei Kagaku*, 29 (1983) 226.
3 T. Nagata and M. Saeki, *J. Food Hyg. Soc. Japan*, 29 (1988) 13.
4 R. L. Smallidge, E. J. Kentzer, K. R. Stringham, E. H. Kim, C. Lehe, R. W. Stringham and E. C. Mundell, *J. Asoc. Off. Anal. Chem.*, 71 (1988) 710.
5 M. N. L. Aerts, W. M. J. Beek and U. A. Th. Brinkman, *J. Chromatogr.*, 435 (1988) 97.
6 T. Sakano, S. Masuda, A. Yamaji, T. Amano, H. Oikawa and K. Nakamoto, *Yakugaku Zasshi*, 97 (1977) 464.
7 J. A. F. de Silva and N. Strojny, *Anal. Chem.*, 47 (1975) 714.