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Rapid determination of sulphonamides in milk using liquid chromatographic separation and fluorescamine derivatization

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ABSTRACT

A simple and selective method is presented for the multiple residue determination of eight sulphonamides in consumers' milk. The drugs are sulphisomidine (ID), sulphadiazine (DZ), sulphamerazine, sulphadimidine, sulphamonomethoxine, sulphamethoxazole, sulphadimethoxine and sulphaquinoxaline (SQ). The milk sample was deproteinized with the same volume of 2 *M* hydrochloric acid and filtered. A 1-ml volume of the filtrate was mixed with 1 ml each of 1.25 *M* sodium acetate solution and a buffer (pH 3.0) for derivatization with 0.6 ml of 0.02% fluorescamine solution in acetone. A high-performance liquid chromatographic analysis was carried out on a C_{18} column with a mobile phase of acetonitrile–2% acetic acid (3:5) at 55°C using a fluorescence detector at an excitation wavelength of 405 nm and an emission wavelength of 495 nm. Average recoveries at fortification levels of 2, 5 and 10 ng/ml were 114%, 109% and 106%, respectively. Relative standard deviations were 1–4% at 10 ng/ml. The limit of determination was 10 ng/ml for ID, 5 ng/ml for DZ and SQ and 2.5 ng/ml for the other five sulphonamides. The method was applied to 25 milk samples and all appeared to be free from the drugs.

INTRODUCTION

Residues of sulphonamides, which are commonly used antibacterial agents for livestock, have been found in consumers' milk from Canada and the USA [1,2]. Although Japanese regulations prohibit their use in lactating dairy cows, possible improper use of the drugs has been reported [3]. Daily milk consumption is expected to be greater than that of meat and meat products, particularly in infants, and one of the drugs (sulphamethazine) is suspected to be carcinogenic [4]. Therefore, there is a need for a rapid, sensitive and selective method for monitoring their residual concentration in milk.

Most conventional methods for milk analysis are too tedious and time consuming for a large surveillance, as they include multiple manipulations such as extraction with an organic solvent, evaporation/concentration and clean-up/defatting steps [2,5,6]. Also, UV detection in a high-performance liquid chromatographic (HPLC) analysis seems to have insufficient selectivity for sulphonamides in milk samples, where in some instances two different mobile phases were required for isocratic separation of ten sulphonamide residues [5]. Recently, new methods have been introduced to solve the problems of the conventional technique, including a matrix solid-phase disperson (MPSD) extraction method [7], an immunochemical method [8] and a fully automated on-line dialysis–postcolumn derivatization method [9].

We have successfully introduced a fluorescamine derivatization method in the HPLC determination of residues of eight sulphonamides in meat and meat products [11]. We report here a procedure for the determination of sulphonamides in milk at ng/

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ml levels using this highly selective HPLC procedure coupled with a simple isolation method.

EXPERIMENTAL

Samples

A total of 25 samples of consumers' milk from twelve cities in Japan was collected at three retailers in Kobe and stored in a refrigerator until analysis. The samples were analysed within 2 days. The fat contents of the samples were 1.0–4.2%. Nine samples were processed milk, which consisted of raw milk, defatted–powdered milk and cream-butter.

Reagents and apparatus

Sulphisomidine (ID), sulphadiazine (DZ) and sulphamethoxazole (XZ) were purchased from Sigma (St. Louis, MO, USA), sulphamonomethoxine (MX) and sulphadimethoxine (DX) from Daiich-Seiyaku (Tokyo, Japan) and sulphaquinoxaline (SQ) from Dainihon-Seiyaku (Osaka, Japan). Sulfamerazine (MR) and sulphadimidine (DM) were generous gifts from Dr. T. Hamano of the Public Health Institute of Kobe City, Japan. Standard stock solutions (100 μg/ml) were prepared by accurately weighing 10 mg of the individual drugs and dissolving them in 100 ml of methanol. A mixture of the standards (1 μg/ml) was prepared by mixing 1 ml of the individual stock solution and diluting to 100 ml with methanol. The mixture was diluted with methanol for recovery tests. Fluorescamine reagent (0.02%) was prepared by dissolving 10 mg of Fluram (Hoffman-La Roche, Basle, Switzerland) in 50 ml of acetone. A buffer solution of pH 3.0 was prepared by mixing of 3 *M* hydrochloric acid and 3 *M* sodium acetate solution using a Beckman 12pH/ISE meter (Beckman Instruments, Fullerton, CA, USA). HPLC-grade methanol, acetonitrile and acetone and analytical-reagent grade hydrochloric acid, acetic acid and sodium acetate were used (Wako, Osaka, Japan). Water was purified with a Milli-Q SP TOC system (Millipore, Bedford, MA, USA).

The high-performance liquid chromatograph (HPLC) was composed of an LC-6AD pump, an RF-535 fluorescence monitor set at the highest sensitivity, a C-R4A integrator set at attenuation 1 and an Inertsil ODS-2 column (150 mm × 4.6 mm I.D., 5-μm particle size) (GL Sciences, Tokyo, Japan) placed in a CTO-6A column oven set at 55°C (Shimadzu, Kyoto, Japan). Although an unusually high column temperature (55°C) was used for obtaining a better separation in a shorter period, no problems with the column performance were found after separating more than 100 samples. The mobile phase was acetonitrile–2% acetic acid (5:3) at a flow-rate of 1 ml/min.

Deproteinization/extraction and derivatization

A 2-ml sample of milk was placed into a 10-ml test-tube containing 2 ml of 2 *M* hydrochloric acid and mixed thoroughly, then allowed to stand for 5 min. The mixture was filtered through a No. 5A filter-paper (Toyo Roshi Kaisha, Japan). To obtain a clearer filtrate, it is recommended to discard the first 0.5–1 ml of the filtrate or to filter again with the same filter. A 1-ml volume of the filtrate was mixed with 1 ml of 1.25 *M* sodium acetate solution, then 1 ml of 3 *M* buffer (pH 3.0), followed by 0.6 ml of the fluorescamine solution. The mixture was incubated for 20 min at room temperature, then 100 μl were injected into the HPLC system.

RESULTS AND DISCUSSION

Sample pretreatment is often the time-limiting step in most procedures for residue analysis. A number of new techniques for saving time and solvent have already been applied to extract residual sulphonamides from milk, including a solid-phase extraction method (SPE) with a commercially available C_{18} cartridge, a matrix solid-phase dispersion method (MPSD) with C_{18}-bonded phase material [7] and an on-line dialysis/sample enrichment method [9].

Highly selective methods such as enzyme-linked immunosorbent assay (ELISA) [8], postcolumn derivatization [9] and photodiode-array detection [10] have also been developed for determining sulphonamide residues in food. An ELISA system provides excellent rapidity and specifity. However, apart from the difficulty in preparing anti-sulpha drug antibodies for the usual chemical analysis, a multiple sulphonamide residue analysis is impossible. On the other hand, on-line dialysis/concentration in combination with an HPLC separation and a postcolumn derivatization method [9] seemed very promising for the determination of residual sulphona-

mides in milk. However, it required additional apparatus to set up the overall instrumentation.

Therefore, the SPE and MPSD methods were first tried for extracting sulphonamides from milk. However, the former required keeping a high positive pressure for an adequate flow and gave poor recovieres and the latter required time-consuming manipulation to prepare a column containing the C_{18}–milk mixture, making them unattractive methods for the pretreatment of milk samples. In the method development, direct derivatization of the acid-deproteinized milk sample with fluorescamine was considered to be a simple and selective procedure which requires no evaporation/concentration and clean-up steps, because all the sulphonamides having amino groups dissolve in an aqueous acid and the reagent reacts selectively with sulphonamides under acidic conditions to give highly fluorescent products [11–13].

In a preliminary experiment, a milk sample fortified with sulphonamides at 10 ng/ml was deproteinized with 6 *M* hydrochloric acid. A 1-ml volume of the filtrate, with an acid concentration was 3 *M*, was mixed with 1 ml of 3.5 *M* sodium acetate, then incubated with 0.5 ml of the fluorescamine reagent for 20 min [11]. However, no reproducible results were obtained. As the derivatization had been found to be pH dependent [11–13], the pH of the incubation mixture was checked. Some samples that had shown smaller fluorescent peaks on the HPLC trace were found to have a pH of less than 1.0. This result showed that the acid concentration (6 *M*) was too high to keep the pH of the incubation mixture at the optimum value of 3.0 [11]. Therefore, an attempt to obtain an incubation mixture of pH 3.0 was made by mixing 1 ml of 1 *M* hydrochloric acid with 1 ml of sodium acetate solution of concentration ranging from 1.0 tot 1.4 *M* and 0.6 ml of the reagent. The pH of the mixture was checked with and without the addition of 1 ml of 3 *M* hydrochloric acid–sodium acetate buffer (pH 3.0). Fig. 1 shows that 1 *M* hydrochloric acid mixed with 1.2 and 1.3 *M* sodium acetate gave mixtures of pH 3.1 and 3.0 with and without the buffer, respectively. In subsequent milk analyses, 2 *M* hydrochloric acid as a deproteinization agent (the acid concentration of the filtrate was 1 *M*) and 1.25 *M* sodium acetate solution were used. Buffer solution was further added, because the milk extract was expected to contain

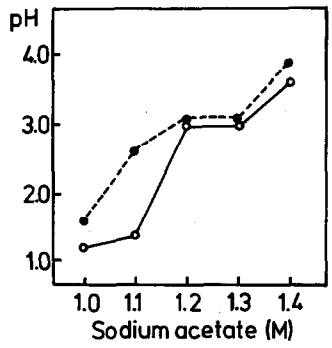

Fig. 1. Effect of sodium acetate concentration on pH of the incubation mixture with (●) and without (○) 3 *M* buffer (pH 3.0).

many components which would affect the pH of the mixture. The pH of the incubation mixture of real samples was found to be 3.0 ± 0.2 [n = 12, relative standard deviation (R.S.D.) = 7.0%]. The dose–response relationship was tested under the optimum reaction conditions and was found to be linear over the range tested, which was equivalent to 2–100 ng/ml sulphonamides in milk.

This method was applied to residual analysis for eight sulphonamides in 25 consumer milk samples. A typical chromatogram and the data for interfering peaks are shown in Fig. 2 and Table I. All the samples showed three large peaks at retention times

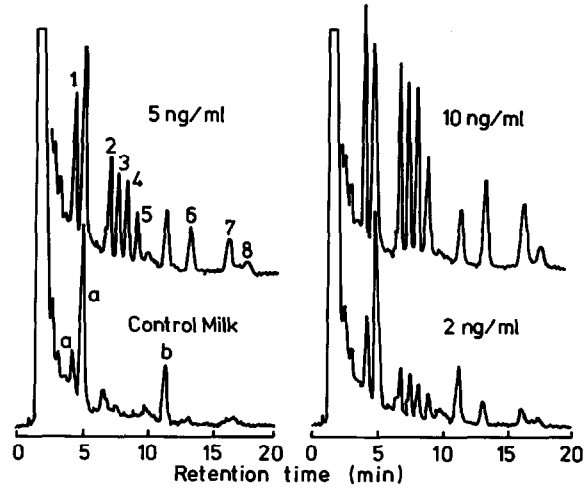

Fig. 2. Chromatograms of control milk and milk fortified at 2, 5 and 10 ng/ml. 1 = ID; 2 = DZ; 3 = MR; 4 = DM; 5 = MX; 6 = XZ; 7 = DX; 8 = SQ. a = Endogenous peak; b = reagent-derived peak.

TABLE I

DATA ON INTERFERING PEAKS AND LIMIT OF DETECTION (LOD), LIMIT OF QUANTIFICATION (LOQ) AND LIMIT OF DETERMINATION (L.Dtm) FOR THE HPLC METHOD FOR SULPHONAMIDE DETERMINATION

Data were obtained from 25 consumer milk samples.

Interfering peaks t_R (min)	Corresponding drugs		ng equivalent/ml milk		LOD[a] (ng/ml)	LOQ[a] (ng/ml)	L.Dtm.[a] (ng/ml)
	Drug	t_R	Av.	S.D.			
4.4	ID	4.4	2.46	0.42	3.7	6.7	10.0
6.7	DZ	7.0	0.96	0.15	1.4	2.5	5.0
7.6	MR	7.7	0.43	0.08	0.7	1.2	2.5
8.2	DM	8.3	0.30	0.05	0.4	0.8	2.5
9.0	MX	9.1	0.48	0.08	0.7	1.3	2.5
13.2	XZ	13.2	0.56	0.11	0.9	1.6	2.5
16.8	DX	16.2	0.47	0.12	0.8	1.7	2.5
17.3	SQ	17.5	1.17	0.12	1.5	2.3	5.0

[a] LOD = Av. + 3S.D.; LOQ = Av. + 10S.D; L.Dtm. ≈ 2LOQ.

t_R of 4.3, 5.1 and 11.3 min. The first two peaks might be endogenous peaks and the last one was derived from the reagent. The first peak interfered with the analysis of ID (t_R 4.4 min), which was equivalent to 2.5 ± 0.4 ng/ml I.D. in milk. The remaining two large peaks were well separated from the drugs of interest. All the chromatograms showed another smaller interfering peak (t_R 6.7 min) which was poorly resolved from DZ (t_R 7.0 min). It was equivalent to 1.0 ng/ml of DZ in milk. More than half of the samples gave slight but positive instrumental readings and nearly the same retention times of other drugs. These interferences were equivalent to 0.3–1 ng/ml of the corresponding sulphonamide in milk (Table I). The limits of detection (LOD) and quantification (LOQ) were determined using the data from the actual analysis ($n = 25$), and the values are summarized in Table I. LOD and LOQ are defined as the average of the background plus three standard deviations and ten standard deviations, respectively [5,14]. The LODs and LOQs of ID and DZ were higher owing to the interfering peaks described above, and those of other drugs were 0.4–2.3 ng/ml. All the milk samples tested were found to contain no sulphonamide above the LOQs.

Recovery studies were done at fortification levels of 2, 5 and 10 ng/ml using milk that had been found free from sulphonamides. The sample was prepared by adding 100 μl of a standard methanolic solution (0.2, 0.5 or 1.0 μg/ml) per 10 ml of the milk. A control sample was prepared similarly by adding 100 μl of methanol. Average recoveries of four replicate analyses, standard deviations (S.D.s), R.S.D.s and retention times (t_R) are presented in Table II. At the 2 ng/ml level DZ had the lowest recovery (61.6%) and the highest R.S.D. (39%), owing to the poorly resolved peak at t_R 6.7 min. The average recoveries were 106–114% for the three fortification levels. These values indicated that all the sulphonamides in milk were completely extracted into the acidic medium but at lower levels the instrumental readings gave slightly higher positive results. The R.S.D.s for the six drugs other than of DZ and SQ were in the range of 0.7–4.9% (average 2.4%). These recoveries and R.S.D.s were satisfactory for the surveillance. Taking the LOQs and visual inspection of the chromatograms into consideration, where SQ gave the smallest peak among the drugs, the limit of determination (L.Dtm.) was set at 10 ng/ml for ID, 5 ng/ml for DZ and SQ and 2.5 ng/ml for other sulphonamides (Table I). The L.Dtm. were set at about twice the LOQs to avoid "false positive" due to the interfering peaks in the actual routine analysis. They are, in other words, practical LOQs.

Compared with published HPLC methods for determining multiple sulphonamide residues in milk [5,7,9], the present method requires less sample manipulation, giving high precision and recovery with

TABLE II

RECOVERIES OF SULPHONAMIDES IN MILK FORTIFIED AT 2, 5 AND 10 ng/ml.

Values represent the averages of four analyses.

Drug	Fortification level								
	2 ng/ml			5 ng/ml			10 ng/ml		
	Av. recovery (%)	S.D. (%)	R.S.D. (%)	Av. recovery (%)	S.D. (%)	R.S.D. (%)	Av. recovery (%)	S.D. (%)	R.S.D. (%)
ID	106.7	2.0	1.9	113.7	5.6	4.9	111.8	2.7	2.4
DZ	61.6	24.2	39.3	93.8	14.1	15.0	98.4	4.0	4.1
MR	117.5	3.0	2.5	113.3	0.8	0.7	108.3	2.2	2.0
DM	126.5	2.7	2.2	115.8	1.2	1.0	109.5	2.6	2.4
MX	148.5	5.4	3.6	113.8	3.2	2.8	109.6	0.9	0.9
XZ	123.7	4.8	3.9	106.5	1.9	1.8	109.6	2.3	2.1
DX	106.1	5.2	4.9	107.9	2.9	2.7	107.5	1.9	1.8
SQ	123.3	3.3	2.7	104.0	14.1	18.5	94.2	1.5	1.6
Average	114.2			108.6			106.1		

lower limits of determination. In conclusion, this simple, rapid and selective method is considered to be applicable to the multiple determination of sulphonamide residues in milk for large surveillance projects.

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