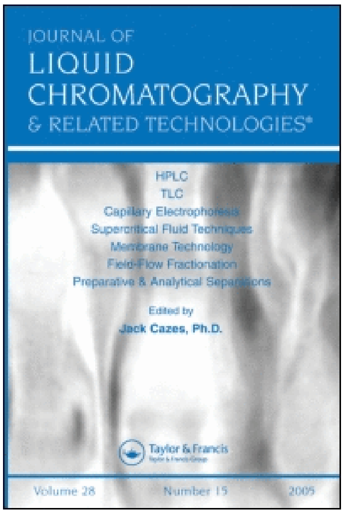

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Procedure for Liquid Chromatographic Determination of Thiamphenicol in Bovine Serum and Milk

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PROCEDURE FOR LIQUID CHROMATOGRAPHIC DETERMINATION OF THIAMPHENICOL IN BOVINE SERUM AND MILK

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ABSTRACT

We describe a method for measuring thiamphenicol (TP) by high-performance liquid chromatography (HPLC). The assay involves a simple extraction procedure using Extrelut-3 with ethyl acetate, evaporation of the extract, and redissolution in 40% acetonitrile. Wakosil-II 5C18 HG is used as a stationary phase with an eluting solvent of acetonitrile/water (40/60 by vol). TP is eluted from the column in about 2.3 min and is well separated from the other components present in serum or milk. Absorption of the eluent at 224 nm is monitored and measured. After extraction from a 1-ml sample, the antibiotic can be analyzed within 30 min. The within-day recoveries for bovine serum and milk spiked with 1 ppm TP were 92.9% and 93.5%, respectively, with coefficients of variation of 3.18% and 3.21%, respectively. The between-day recoveries for the 1 ppm samples were 91.9% and 92.3%, respectively, with coefficients of variation of 4.50% and 4.40%, respectively. The method is suitable for rapid and specific analysis of the drug in serum and milk, and will be applicable to

other biological fluids, with a quantitative sensitivity of less than 0.1 ppm. We also analyzed TP by liquid chromatography-mass spectrometry with an atmospheric-pressure chemical-ionization interface system.

INTRODUCTION

Thiamphenicol (TP) has broad spectrum activity similar to that of chloramphenicol, without any serious side effect. Therefore, TP is an attractive drug for many veterinary applications.

Several assays have been reported for TP in biological fluids and tissue. These have been compiled, and include microbiological, colorimetric (8), and a gas-chromatographic (1,2,6,7) method both electron-capture and flame-ionization detection, and high-performance liquid chromatographic (HPLC) (3,4,5) methods. However, these methods have common disadvantages including a complicated extraction and clean-up method, and lengthy analysis time. In addition, there are no reports on the qualitative analysis of TP by LC-mass spectrometry.

We describe here a rapid, specific, and sensitive chromatographic procedure for analysis of TP. The method is suitable for routine analysis of TP in serum, milk and other biological fluids.

MATERIALS and METHODS

Reagents and apparatus

TP was obtained from Sigma Chemical Co., (St. Louis, MO 63178). Water used in the HPLC eluent was Milli-Q grade (Millipore). All other reagents used were of analytical grade.

Liquid chromatography was performed with a Model 501 pump equipped with a U6K universal liquid chromatography sample injector, and a multiple-wavelength detector, Model 481 (Waters Associates, Milford, MA 01756). A chromatopak C-R1B integrator (Shimadzu Seisaku Co., Ltd., Kyoto, Japan) was used as the recorder. The chromatographic column used in the assay was a Wakosil-II 5C18 HG (15 cm x 4.6 mm I.D.; Wako Pure Chemical Co., Tokyo, Japan). The elution was carried out with acetonitrile-water (40:60, v/v) at a flow rate of 1.0 ml/min, and detection at 224 nm.

The LC-MS apparatus used was a Hitachi M-2008 mass spectrometer-computer system, equipped with a Hitachi M-8093 HPLC instrument through a Hitachi atmospheric-pressure chemical-ionization (APCI) interface system. The nebulizer and vaporizer temperatures were 260°C and 360°C, respectively.

Standard solutions and serum (milk) standards

A stock solution of TP was prepared in acetonitrile-water (40:60, v/v) at a concentration of 1 mg/ml. Serial dilution was performed to produce acetonitrile-water (40:60, v/v) spiking solutions ranging in concentration from 100 to 1 μg/ml. These solutions added to 1 ml of blank serum or milk produced final concentrations ranging from 0.1 to 10 μg/ml.

Extraction procedure

Biological samples (serum or milk, 1 ml) were diluted with 2 ml of distilled water and vortex-mixed. The total sample was applied to Extrelut-3 (Merck) and allowed completely enter the sorbent matrix. Next, a 25G x 1-needle (Terumo Co., Tokyo) was placed on the end of the Extrelut-3 and elution was performed with 10 ml of ethyl acetate. The organic phases were collected, evaporated under a stream of nitrogen at 40°C, and redissolved in 1 ml of mobile phase. A 20-μl volume was injected into the HPLC column. For quantitative analysis, the injection volume was increased to 50 μl. These all extraction procedures were completed within only 30 min.

RESULTS AND DISCUSSION

It was observed frequently that TP-containing serum or milk (pH 7.0) after deproteinization produced early interfering peaks on the chromatograms. To avoid this

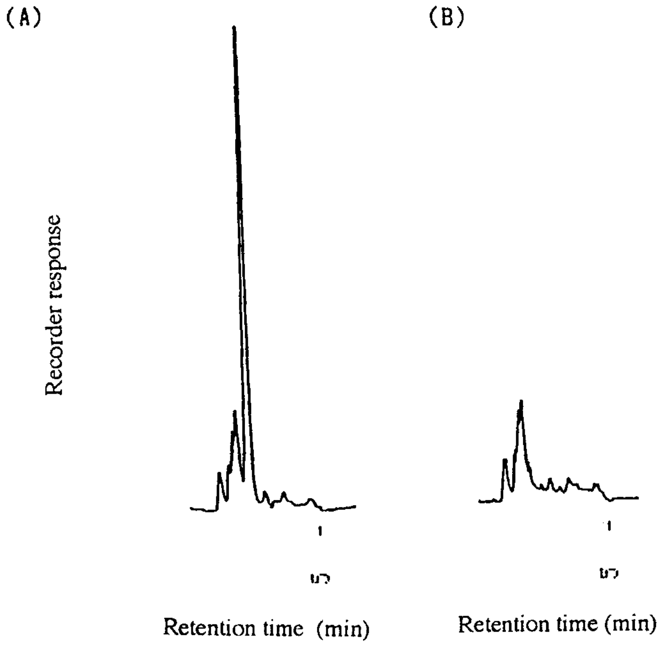

FIGURE 1. Typical chromatograms of (A) bovine serum spiked with 1.0 µg/ml thiamphenicol; and (B) bovine serum blank.

problem, we selected Extrelut-3 to extract TP from serum and milk. Extrelut-3 contains porous diatomaceous earth (Kieselguhr) and allows liquid-liquid extraction between the sample and organic solvent.

Typical chromatograms of serum blanks with and without TP supplementation are shown in Figure 1, and milk blanks with and without TP are shown in Figure 2. TP was identified by its retention time (2.3 min). Even though no internal standard was used in this method, there was a good relationship between peak heght and TP concentration. The limit of sensitivity of this method is about 0.1 µg/ml. Analytical recovery of TP added to serum and milk was determined by comparing the peak heights for serum and milk containing 1.0 µg of TP per ml with the results obtained from an

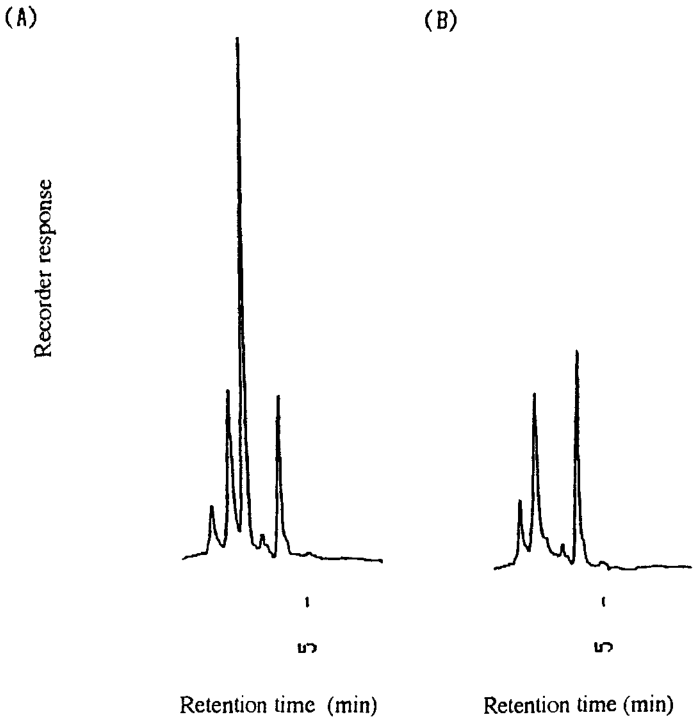

FIGURE 2. Typical chromatograms of (A) bovine milk spiked with 1.0 µg/ml thiamphenicol; and (B) bovine milk blank.

Table 1. Recovery rate of thiamphenicol in bovine serum and milk

Sample	Added (ppm)	Mean (%) (n=5)	
		Within-day	Between-day
Serum	10	93.9	-
	1.0	92.9	91.9
Milk	1.0	93.5	92.3

Table 2. Precision of assays for thiamphenicol in serum and milk

Sample	Concn (ppm)	Precision (n=5)			
		Within-day		Between-day	
		±SD (ppm)	CV (%)	±SD (ppm)	CV (%)
Serum	1.0	2.95	3.18	4.13	4.50
Milk	1.0	3.00	3.21	4.06	4.40

CV: Coefficient of variation.

aqueous standard of the same concentration. The within-day recoveries of serum and milk were 92.9% and 93.5%, respectively.

The precision of this method is summarized in Table 2. Precision for this assay was calculated for serum samples containing TP at concentrations of 1.0 and 10 μg/ml. Five samples at each concentration were extracted separately and chromatographed on the same day. The coefficient of variation (relative standard deviations) for TP at 1.0 μg/ml was 3.18%; at 10 μg/ml, the value was 2.77%. Between-day variations in recovery from serum samples containing 1.0 μg/ml was 4.5%. For five milk samples containing 1.0 μg/ml, the coefficients of within-day and between-day variation were 3.21% and 4.40%, respectively.

Our assay for TP is sufficiently sensitive for routine use. Its major advantages over other assays are its rapidity (assay can be performed in less than 30 min), accuracy, reproducibility and specificity. Preliminary studies suggest that it is highly specific for TP, and that tetracyclines such as oxytetracycline and chlortetracycline, sulfonamide, oxolinic acid and tylosin, do not interfere with the assay.

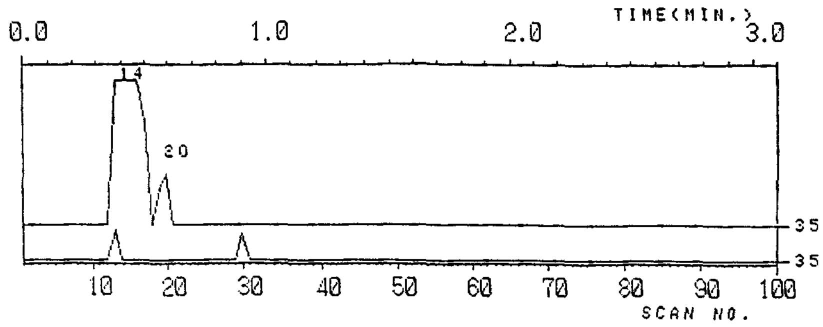

FIGURE 3. Mass chromatograms of thiamphenicol (m/z 356) and $[M+H]^+$ ions. The chromatographic conditions were: mobile phase, CH_3CN-H_2O (40:60, v/v); flow rate, 1.0 ml/min; injection volume 10 µl.

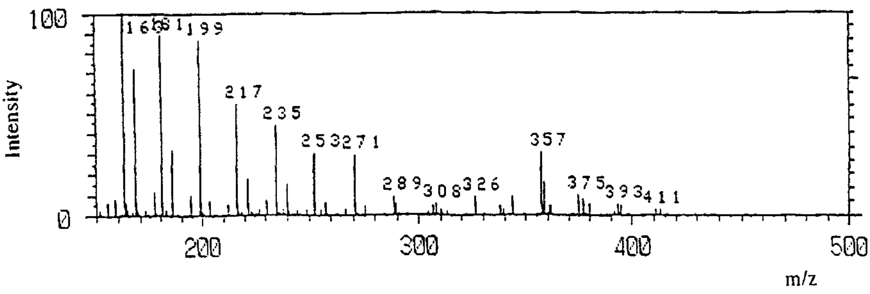

FIGURE 4. Mass spectra of thiamphenicol as scanned at the peak tops of the mass chromatograms in Figure 3. The mass spectrometer scanned from m/z 150 to 500.

Mass chromatograms and mass spectra of TP, obtained using the LC-APCI-MS system, are shown in Figure 3. In the LC-APCI-MS system, TP was determined by scanning the quasi-molecular ion $[M+H]^+$, and m/z 357 was specific for TP when selected ion monitoring was performed. The results suggest the possibility of qualitative analysis of other antibiotics and antibacterial agents. This is the first report of TP analysis using a LC-MS system.

In conclusion, this procedure appears to be potentially applicable for quantifying TP residue in stock farm products and the LC-APCI-MS method is applicable for qualitative analysis.

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