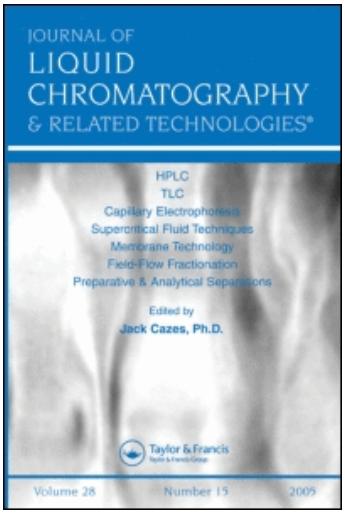

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LIQUID CHROMATOGRAPHIC ANALYSIS OF THIAMPHENICOL RESIDUES IN MILK

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ABSTRACT

The present paper describes a modified high-performance liquid chromatographic (HPLC) method for the determination of thiamphenicol (THA) residues in spiked milk at levels as low as 30 ppb. Milk spiked with THA was extracted with ethyl acetate. Following addition of hexane, the extract was cleaned up with a silica cartridge. Analysis was performed on a reversed-phase C_{18}, 5 μm, column using water-methanol (70:30) as mobile phase. Recovery was found to range from 68.0 to 90.0 % and precision data suggested that relative standard deviation ranged from 7 to 9.4 %.

INTRODUCTION

Thiamphenicol (THA) is a synthetic broad-spectrum antibiotic, analog of chloramphenicol (CAP). With the prohibition of CAP in food producing animals, since it is known to produce irreversible aplastic anaemia in humans (1), THA appears to be a very viable substitute for CAP. Thiamphenicol is currently marketed in many European countries including France, Italy, Spain, Germany, Belgium and also in Central and

South America, Africa and Asia including Japan. In EEC provisional Maximum Residue Levels (MRLs) have been established for THA residues.

Both gas chromatographic (2-5) as well as high-performance liquid chromatographic methods (6-9) have been described for the determination of THA in tissues and body fluids (plasma, serum, urine). HPLC analysis of body fluids involves either the direct injection of the sample (serum) mixed with methanol, or employs first a solvent extraction of the sample, followed by evaporation and reconstitution in the mobile phase (8). Previously reported HPLC methods for THA determination in serum and plasma when applied to milk yield chromatograms with many interference peaks, as these procedures lack the necessary sample clean up step.

This paper describes an HPLC method particularly suitable for the determination of THA residues in milk. As far as the authors know there are no data available on this subject.

EXPERIMENTAL

Instrumentation

HPLC was carried out on a Gilson system consisting of a Model 802 manometric module, a Model 302 piston pump, a model HM/HPLC dual-beam variable wavelength UV-Vis spectrophotometer set at 224 nm and a Model NI variable-span recorder. A HPLC technology Model TC 831 column oven set at 35 °C permitted temperature regulation. Injections were made on a Hichrom 25X0.46 cm (excel range) column prepacked with Nucleosil 120, C_{18}, 5 μm, through a Rheodyne 7125 sample injector equipped with a 100 μl loop.

HPLC procedure

The mobile phase used was methanol-water (30:70 v/v). The mobile phase, after filtration through a 0.2 μm filter (Nylon-Rainin), was degassed under vacuum and delivered at a flow rate of 1 ml/min.

Chemicals

Thiamphenicol (THA) was obtained from Sigma (St. Louis, MO, USA). Methanol, ethyl acetate, water (all Lichrosolv), n-hexane (analytical grade), citric acid (extra pure) were purchased from Merck (Munchen, FRG).

Stock solution was prepared by weighing accurately and dissolving THA in 10 % v/v methanol in water (0.5 mg/ml). Each day an aliquot of the stock solution was further diluted to give working solutions containing THA, in the range 0.05-1.0 μg/ml.

Silica gel disposable SPE columns (3 ml; 500 mg) were from Analytichem International (Harbor City, USA). Just before use, the column was pretreated by passing 8 ml of ethyl acetate-hexane (2:3).

Extraction procedure

2 g of milk was weighed in a tube containing 0.08 g of citric acid monohydrate. A volume (6 ml) of ethyl acetate was added and the tube was vortexed at moderate speed. After centrifugation at 2300 g, the organic layer was pipetted to another tube. The extraction procedure was repeated with another 4 ml ethyl acetate. After addition of 15 ml of n-hexane to the total extraction solvent, the solution was centrifuged and passed through the pretreated silica gel column at a flow rate of ca 8-10 ml/min. The column was washed with 2 ml of hexane and dried in a stream of nitrogen for 10 min. Thiamphenicol was eluted from the column with 3 ml of methanol (2 ml using vacuum off and 1 ml using vacuum on). The eluate was collected and evaporated in a stream of nitrogen at 30 °C. The residue was dissolved in 1 ml of the mobile phase using Vortex mixer for 30 s. The solution was used for HPLC analysis.

Aliquots of the sample and standard solutions were injected by means of the loop injector (100 μl). Samples were eluted isocratically at a flow rate of 1 ml/min.

RESULTS AND DISCUSSION

Chromatography

Typical chromatograms from standard, blank and spiked milk extracts are shown in Fig. 1. The chromatographic conditions are the same as those proposed by Felice et al. (8) for the determination of THA residues in bovine plasma

Working solutions and milk samples were monitored at 224 nm. THA was eluted in 6.35 min. Regression analysis of the data obtained by running a series of working solutions of THA showed the response to be linear through the range 0.05-1.0 μg/ml ($y=0.16+1.3x$; $r=0.999$; where y represents peak height in mm and x the quantity of the compound injected in nanograms).

Spiking Studies

Recovery experiments were carried out in cow milk spiked at 30, 62.5, 125 and 250 ng/g. In four replicates each amount was added to milk. The results are presented in Table 1. The precision of the method was studied by assaying on each of three different days several milk samples with THA at 250 ppb level. The data are presented in Table 2.

This modified developed method allows the determination of THA residues in milk with a quantitation limit of 30 ppb. This limit can not, unfortunately, compared to, since there are no data in the literature. On the other hand, EEC established the level of 40 μg/kg as provisional MRLs only for bovine and poultry tissues (13).

Sample Pretreatment

Ethyl acetate was used for the extraction of THA from milk as was also the case for the extraction of THA from meat (9). The mixing was

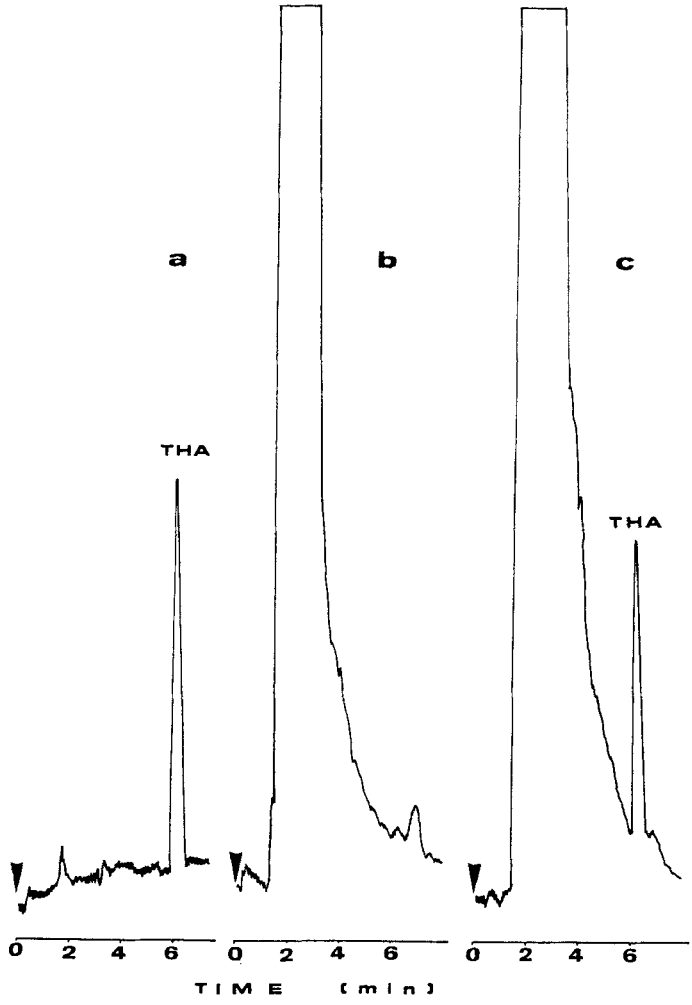

FIGURE 1. Typical chromatograms of (a) a standard solution of thiamphenicol (THA) (0.5 μg/ml), (b) a blank milk sample extract, and (c) a spiked (0.25 μg/g) milk sample extract. Conditions: mobile phase, MeOH-H_2O (30:70 v/v); column, 25X0.46 cm; C_{18} (5 μm); temperature 35 °C; flow rate 1 ml/min; wavelength 224 nm; recorder sensitivity 0.02 AUFS; chart speed 5 mm/min; injection volume 100 μl.

TABLE 1

Recovery Data for THA Analysis in Spiked Milk (n=4)

Thiamphenicol added (ng/g)	Mean Concentraton found ± SD (ng/g)	Mean Recovery %
30	27 ± 1.9	90.0
62.5	48.8 ± 4.4	78.2
125	94.8 ± 18.4	75.8
250	177.4 ± 16.7	70.7

TABLE 2

Precision Data for the Determination of THA in Milk Samples Spiked with 250 ng/g (n=4)

Day	Mean Concentration found ± SD (ng/g)	Rel SD %
1	177.4 ± 16.7	9.4
2	170.2 ± 8.6	5.4
3	171.4 ±12.0	7

performed on a vortex mixer in order to eliminate the emulsion formation observed, sometimes, during the mixing using ultra sonic energy.

The clean up procedure of the ethyl acetate extract was based on that scheme described by Haagsma et al.(10-11) for the determination of CAP in swine tissue and milk. The method comprises addition of hexane to the ethyl acetate extract and SPE using a small silica gel column. Elution was performed with methanol. The modification made concerned the removal of matrix components precipitate from the ethyl acetate-hexane layer. The centrifugation of the ethyl acetate-hexane layer, have been found to give better recovery than the filtration that Haagsma and her co-workers described(10-11). Moreover, in trace analysis, centrifugation should be preferred to filtration, since the sample does not come into contact with filter material that might cause recovery problems (12).

Characterization of the recorded peak was based solely on the retention behaviour of THA

In conclusion, the proposed modified HPLC method is a sensitive, rapid, easy and simple method and it should be useful for the routine analysis.

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