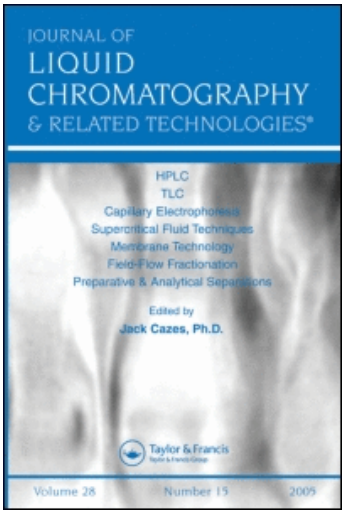

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF RESIDUES OF THIAMPENICOL IN BEEF MUSCLE

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ABSTRACT

The present paper describes a high-performance liquid chromatographic (HPLC) method for the determination of thiamphenicol (THA) residues in spiked meat at levels as low as 10 ppb. Meat spiked with THA was extracted twice with ethyl acetate. The extract was washed with 4% w/v NaCl solution saturated with ethyl acetate and then was evaporated to dryness. The oily residue was dissolved in 1 ml of a mixture of n-hexane-chloroform (1:1, v/v). Then 1 ml of water was added to the tube and after stirring and centrifugation, an aliquot of the upper layer was analyzed on a reversed-phase C_{18}, 5 μm column. Recovery was found to range from 64.8% to 75.5%. The linearity, in the range examined, was good (r=0.995, 5-80 ppb). Precision data suggested relative standard deviation ranged from 2.1 to 3.9%.

INTRODUCTION

Thiamphenicol (THA) is a synthetic broad-spectrum antibiotic, analog of chloramphenicol (CAP) in which the nitro (-NO_2) group of the benzene ring of CAP has been replaced with a methyl-sulphonyl (CH_3-SO_2) group. Although CAP has found widespread application in veterinary practice, its use in food producing animals is prohibited because it is known to produce irreversible aplastic anaemia(1) in humans. No

case of aplastic anaemia can be attributed to THA(2). As such, THA shows promise to be a very viable substitute for CAP, especially in food producing animal practice. THA has been marketed in many countries in Europe including France, Italy, Spain, Germany, Greece.

As THA is a new antibiotic being developed for use in veterinary practice, analytical methods with high sensitivity are needed for residue analysis. THA has been measured in plasma and tissues by gas chromatographic methods using electron capture or flame ionization detectors (3,4,5,6).

A few high-performance liquid methods have been also reported for THA. Nagata et al.(7) and Otsuka et al.(8) developed methods for the determination of THA in chicken and yellow tails, respectively. They used a column for the clean up of the meat extract. Felice et al(9) presented a method for the measurement of THA in bovine plasma. At the same time, Zambon Group(10) proposed a high-performance liquid chromatographic method for the analysis of THA in tissues with a sensitivity equal to 20 ppb and a six-step scheme for the clean up of the meat extract. The aim of this paper is to develop a HPLC method with a rapid and simple clean up of the meat extract and, also, with an improved sensitivity.

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 40^{o} C permitted temperature regulation. Injections were made on a Hichrom 25 X 0.46 cm (excel range) column

prepacked with Nucleosil 120, C_{18} 5 μm (Hichrom), through a Rheodyne 7125 sample injector equipped with a 100 μL loop.

HPLC procedure

The mobile phase used was methanol-water (25:75 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 purchased from Sigma (St. Louis, MO, USA). Ethyl acetate (Lichrosolv), methanol, n-hexane, chloroform, sodium chloride (all analytical grade) were obtained from Merck (Munchen, FRG). Deionized water that had been distilled was used. Stock solution was prepared by weighing accurately and dissolving THA in 10% v/v methanol in water (0.5 mg/mL). It was stable for several weeks at -25° C. Aliquot of the stock solution was further diluted to give working solutions containing THA, in the range 0.05-0.8 μg/mL, each day. All solutions were protected from direct sun throughout the analysis.

Extraction Procedure

Five g of ground meat sample was weighed in a centrifuge tube. Fifteen mL of ethyl acetate was added and the sample was homogenized using an Ultra-turrax homogenizer. After the tube was centrifuged for 10 min at 7000 g, the supernatant phase of ethyl acetate was pipetted in separatory funnel. This extraction procedure was repeated with another 10 ml of ethyl acetate.

The combined ethyl acetate layers were washed twice with 20 ml (2 X 20ml) of 4% NaCl solution saturated with ethyl acetate. The NaCl washes before discarding were

extracted with 40 ml of ethyl acetate. All ethyl acetate layers were transferred to a round-bottom flask and evaporated under vacuum at 30° C to a few ml which were pipetted quantitatively to a tube and evaporated to dryness under a nitrogen stream at 30° C. The oily residue was dissolved in 1 ml of a mixture of hexane-chloroform (1:1 v/v). Then 1 ml of water was added and the tube was vortex mixed for 1 min at high speed and centrifuged for 10 min at 14000 g. The centrifugation was repeated if the partitioning was not completed. The upper water phase was used for analysis.

Calibration curve was constructed by plotting peak heights versus concentration from 100 μl injections of each of the prepared working solutions 0.05-0.8 μg/ml. The concentration of THA in samples were calculated by reference to the calibration curve.

RESULTS AND DISCUSSION

Extraction solvents reported in the literature were ethyl acetate (4,6,9,10) and acetonitrile (7,8). We found ethyl acetate to be a suitable extractant. The use of Lichrosolv-ethyl acetate was imperative; alternatively distilled analytical grade ethyl acetate could be used.Otherwise ghost peaks, which were not derived from componets of the muscle tissue, were observed in the chromatograms.

It was observed that the loss of the ethyl acetate solvent, after centrifugation, was 20% since 20 ml of ethyl acetate, of the 25 ml added,were recovered each time. THA residues were chromatographed well on the reversed-phase column and did not require a buffered mobile phase(10). The chromatographic conditions were the same as those proposed by Felice et al.(9) for the determination of THA residue in bovine plasma. The eluting mixture was only slightly more polar (75% v/v water instead of 70% v/v) to allow complete elution of interfering substances before THA peak appearance. It was found that after an overnight column conditioning with water at a flow rate of 0.1 ml/min, peak responses were consistently high.This was probably due to well wetting of

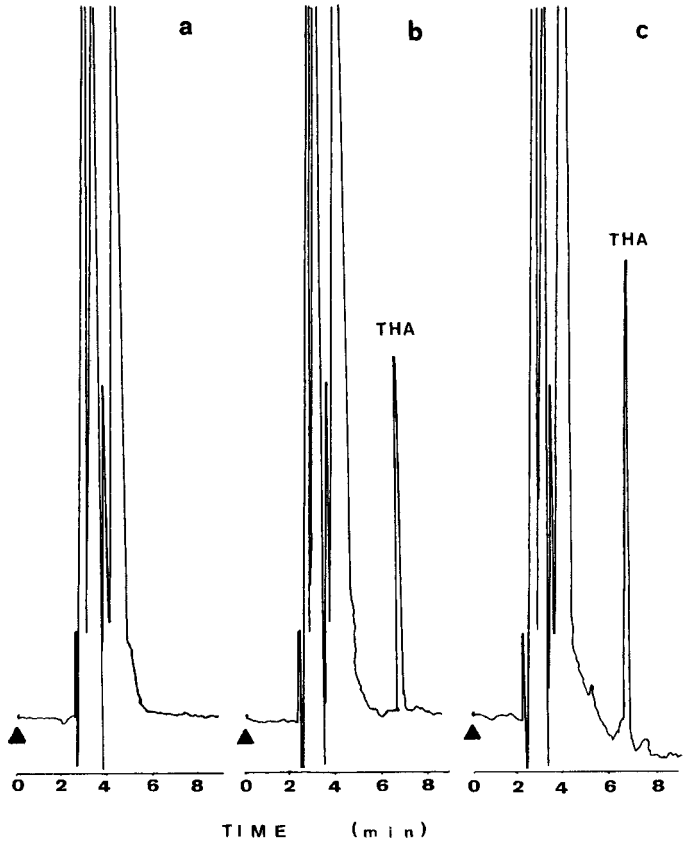

FIGURE 1. Typical chromatograms of a THA free meat sample (a), a sample spiked with 100 ppb THA (b), and a real sample from a calve injected intramuscularly once with 20 mg of THA/kg of B.W. (c). Conditions: mobile phase, MeOH-H_2O (25:75 v/v); column, 25 X 0.46 cm; C_{18} (5 μm); temperature 40° C; flow rate 1 mL/min; wavelength 224 nm; recorder sensitivity 0.02 AUFS; chart speed 5 mm/min; injection volume 100 μL.

the packing and consequently to an equilibrium across the interface of the two dissimilar phases: the stationary phase (C_{18}), which is especially hydrophobic, and the mobile phase (25% v/v MeOH-H_20).

Working solutions and meat extract were monitored at 224 nm. THA was eluted in 6.4 min (Fig.1). Regression analysis of the data obtained by running a series of working

TABLE 1.

Raw Data and Regression Equation of Calibration Curve for THA Determination by HPLC.

Concen. of working solution (ng/100 μL)[a]	Peak height[b], mm {mean ± SD (n=6)}
80	86.2 ± 2.7
60	76.2 ± 2.9
40	52.2 ± 3.7
20	27.7 ± 2.9
10	13.5 ± 1.4
5	8.5 ± 0.5

a= Volume injected.
b= Regression equation, $y=2.53+1.17x$, $r=0.995$

solutions of THA (Table 1) showed the responses to be linear ($y=2.53+1.17x$; $r=0.995$; where y represents peak height in mm and x the quantity of the compound injected in nanograms. It was estimated by weighed linear regression).

This method allows the determination of THA residues in bovine meat with a quantitation limit of 10 ppb. This limit is much lower than that reported by Zambon Group(10) which is 20 ppb.

The accuracy of the method was studied by spiking meat samples at 4 different levels (160, 100, 50, 25 ng/g) with standard THA (1.6, 1.0, 0.5 and 0.15 ml of a 0.5 ppm solution) and analyzing four replicates. The recoveries ranged from 64.8% to 75.5% (Table 2). The precision of the proposed method was studied by assaying on each of three different days several meat samples with THA at 100 ppb level. The data are presented in Table 3.

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

TABLE 2.

Recovery Data for THA Analysis in Meat

Concen. of THA added (ppb)	Mean concen. found[a] (ppb)	Mean recovery %
160	118.7 ± 4.6	74.2
100	75.5 ± 1.6	75.5
50	32.4 ± 1.4	64.8
25	17.9 ± 0.3	71.6

a= Mean of four replicates ± SD

TABLE 3.

Precision data for the determination of THA in meat samples spiked with 100 ppb.

Day	Mean value of THA found[a] (ppb)	SD	Rel.SD %
1	76.3	2.5	3.2
2	72.0	2.8	3.9
3	75.5	1.6	2.1

a=Mean of four replicates

To validate the method with real samples, a trial was undertaken with a calve which was given a single intramuscular injection of 20 mg of THA per kg of body weight. The animal was slaughtered 15 h after injection and samples were taken from the injected site in order to have relatively high levels of THA, thus making it possible to detect THA. A chromatogram is shown in Fig.1.

In conclusion the proposed HPLC method is more sensitive than that proposed by Zambon Group(10). It is also easier than those HPLC methods proposed by Otsuka et al.(8) and Nagata et al.(7), since it involves a three step scheme for the clean up of the

extract of the meat, without the use of a column. The required time for a four sample analysis is about 90 min. Consequently, this method combines the advantages of sensitivity, simplicity and speed and it should be useful for routine analysis.

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