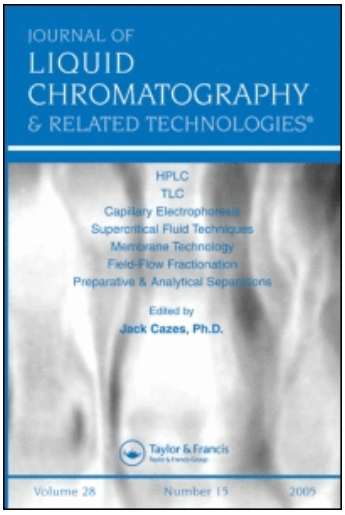

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH UV DETECTION AND SCANNING UV CONFIRMATION OF CHLORAMPHENICOL IN FATTY LIVER

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ABSTRACT

A liquid chromatographic (HPLC) method has been developed to detect chloramphenicol (CAP) in fatty liver. After addition of water, samples were defatted with hexane partition and CAP was extracted by ethyl acetate. A solid-phase extraction protocol was used to recover CAP from the fatty liver extract. CAP was analyzed using a mobile phase of acetonitrile-0.005 M phosphate buffer, pH 7.9 (19:81), an ultraviolet detection wavelength of 278 nm and confirmation by scanning. The column used was a Nova-Pak C_{18} (150 x 3.9 mm). The limit of detection was < 1 ng/g in fatty liver and the mean recovery of CAP was found to be 71 % over the concentration range 5 - 20 ng/g. The analyte identity can be confirmed by the use of scanning detection and by LC/MS method for concentrations less than 10 ng/g.

INTRODUCTION

The breeding of palmipedes for fattened goose or duck liver is well established in France. Antibiotics may be used to treat diseases in palmipedes and the possible health risk to humans, presented by traces of antibiotics in fatty liver, has generated serious concern in the export and domestic markets.

To ensure that levels of antibiotics in fatty liver are within acceptable limits, monitoring of antimicrobial residues is realized by agar diffussion microbiological method.[1] But this method is not very sensitive for detection of CAP in fatty liver. CAP is one of the most controversial antibiotics because of its risk of provoking aplastic anaemia not related to the absorbed dose. The risk has led for several years to severe restrictions in the use of CAP. Since June 1994, CAP is not approved for food producing animals in the European Union.[2] Thus, there is a demand for development of techniques which are rapid, specific and sensitive for quantitative detection of CAP residues. Chromatographic methods are methods of choice for monitoring many antimicrobial agents and many high performance liquid chromatographic methods have been reported for the analysis of CAP in a number of matrices.[3-9] To date, there have been no published reports of HPLC analysis of CAP in fatty liver. So, the aim of this study was to develop a protocol to determine CAP in fatty liver from previous works.[10,11] For confirmation purposes, scanning UV detection may be used, but the analysis requires a high concentration in the detection cell to produce an accurate UV spectrum.

EXPERIMENTAL

Reagents and Chemicals

Acetonitrile (Merck, Darmstadt, Germany) was HPLC grade. Ethyl acetate distilled before use, methanol, chloroform, n-hexane and hydrochloride acid (Merck) were analytical grade. Diammonium hydrogen phosphate, anhydrous sodium sulfate and dipotassium hydrogen phosphate were obtained from Merck. HPLC-grade water was produced using a Milli-Q water purification system (Millipore, St Quentin en Yvelines, France). Chloramphenicol was obtained from Lepetit (Milan, Italy).

Bond Elut SPE cartridges (Silica gel, 3 cc), reservoirs 75 mL, adaptors for reservoirs and SPE vacuum manifold were obtained from Varian, Harbor City, CA, USA.

Diammonium buffer (pH 7.9) contained 0.005 M (0.66 g/L) of diammonium hydrogen phosphate. Phosphate buffer pH 10 contained 0.05 M dipotassium hydrogen phosphate.

Apparatus and Chromatographic Conditions

The HPLC system constisted of a Kratos Spectroflow 400 pump (Applied Biosystems, Foster City, CA, USA) connected to a Kratos Spectroflow 773 variable-wavelength detector operated at 278 nm and a Spectra-Physics SP 4290 integrator (Spectra-Physics, Fremont, CA, USA) coupled to a computer for data handling (Spectra-Physics Winner Software). A Focus multiwavelength scanning detector (Spectra-Physics) was also used. The analytical column was a 4 μm Nova-Pak C_{18} stainless-steel column (150 x 3.9 mm I.D., Waters Milipore) with a guard column (4 x 4 mm) packed with reverse phase material (Merck). The samples were injected with a Rheodyne 7125 injector with a 200 μL loop. The mobile phase consisted of acetonitrile - 0.005 M diammonium hydrogen phosphate buffer, pH 7.9 (19:81,v/v) and was delivered isocratically at a flow - rate of 1.0 mL/min. The mobile phase was filtered prior to use with a Millipore HPLC solvent system and 0.45 μm membrane Millipore filter.

Preparation of Standard Solutions

A 500 μg/mL CAP stock solution was prepared by dissolving 50 mg in 2 mL of methanol, then adjusting to 100 mL with water. After a 5 μg/mL intermediate solution, working standards (15, 30, and 60 ng/mL) were prepared by diluting the intermediate solution in water.

Sample Preparation

For spiking studies, fatty livers from untreated crammed ducks were used. Partially thawed fatty livers were homogenized with a scalpel. Approximately 3 g of fatty liver were accurately weighed in a centrifuge tube. To each control fatty liver, 1 mL of the working solution were added, resulting in a final concentration of 5, 10 and 20 ng/g. The sample was vortex-mixed and allowed to stand for 15 min before addition of 2 mL of water and extraction.

Extraction Procedure and Cleanup

Three g of cutted fatty liver were weighed in a 50-mL glass-stoppered centrifuge tube. A 3-mL volume of water was added and the tube was vortex-mixed for 1 min. Then, 15 mL of hexane was added and the tube was placed between the plates of a rotary stirrer Reax 2 (Heidolph, Germany) and stirred at 40 rpm for 10 min. After centrifugation for 5 min at 2400 g, the supernatant was discarded. A 12-mL volume of ethyl acetate was added and the tube was vortex-mixed for 2 x 1 min at 15 min interval. After centrifugation for 5 min at 4000 g, aliquot of the organic phase (8 mL) was decanted through glass wool topped by 1 g of anhydrous sodium sulphate which were then washed with 2 x 2 mL of ethyl acetate. To the combined extracts, 30 mL of hexane were added.

The combined filtrate was passed through the pretreated silica cartridge (10 mL of a mixture ethyl acetate-hexane) connected with a 75-mL reservoir. The flask and sodium sulfate were washed with 10 mL ethyl acetate-hexane (4:10, v/v). The cartridge was washed with 10 mL of hexane, then left under vacuum for 2 min in order to dry it. CAP was eluted from the cartridge with 5 mL of phosphate buffer pH 10, then extracted from the eluate with 20 mL ethyl acetate. After vortex-mixing and centrifugation for 10 min at 2400 g, 18 mL of the ethyl acetate phase was transfered into a round bottomed flask and evaporated to dryness with a rotary evaporator (Büchi, Switzerland). The oily residue was resuspended in 1.2 mL mixture of hexane - chloroform (1:1, v/v). After addition of 0.8 mL of water, the flask was stirred between the plates of the rotary stirrer for 5 min at 35 rpm. The mixture was transfered into a vial and centrifuged for 10 min at 3300 g.

Chromatography

The supernatant (aqueous phase) was injected by means of the loop injector for HPLC analysis. For confirmation, the HPLC system was coupled to a UV-VIS scanning detector. It was based on a comparison of the retention time and of the UV spectrum of the sample with those of standard CAP.

Assay Precision and Recovery

The recovery study was performed by adding 1 mL of CAP solution to 3 g of fatty liver. Six samples were prepared at each concentration (5, 10 and 20 ng/kg) and were not frozen before extraction. The area of the CAP peak was

Table 1

Recovery Data of CAP from Fatty Liver Fortified with CAP at Various Levels (n=6) and Repeatability of the Method

Concentration Added (ng/g)	Concentration Found (ng/g)	Mean Recovery	SD %	CV %
5	3.5	68.9	2.0	2.8
10	7.3	72.6	5.0	6.8
20	14.3	71.6	6.0	8.4

compared to the area of the peaks for identical amounts of standards solutions. A linear regression equation was obtained by plotting the peak areas corrected by the recovery against concentrations.

RESULTS AND DISCUSSION

The previous method employed to dose CAP in muscle, kidney and liver[10] cannot be used to determine CAP in fatty liver because interfering peaks appeared in blank sample chromatograms. A solid phase extraction step in silica cartridge was introduced to remove major interfering components. An additional washing step (liquid-liquid extraction with hexane) was also necessary to eliminate other lipophilic interfering components. Indeed, fat content in fatty liver represents about 50 to 60 % of the total weight. Dissolution of the final residue in water instead of the eluent allowed to increase the volume injected to 200 µl to improve the limit of detection. Indeed, the preconcentration which takes place on the top of the column prevents peak broadening. The chromatographic conditions specified here are the same as those proposed and discussed for the determination of CAP residues in muscle.[10] The mobile phase is only slightly more polar (81 % aqueous phase) to allow complete elution of interfering substances before CAP peak appearance. Figure 1 shows representative chromatograms of (A) a standard of CAP, (B) a blank fatty liver extract and (C) a CAP fortified fatty liver. They do not reveal significant interference at the retention time of chloramphenicol.

Table 1 gives the concentration examined, standard deviations, coefficients of variation and recoveries for CAP fortified fatty liver samples. A good recovery at all levels investigated and a low standard deviation for the

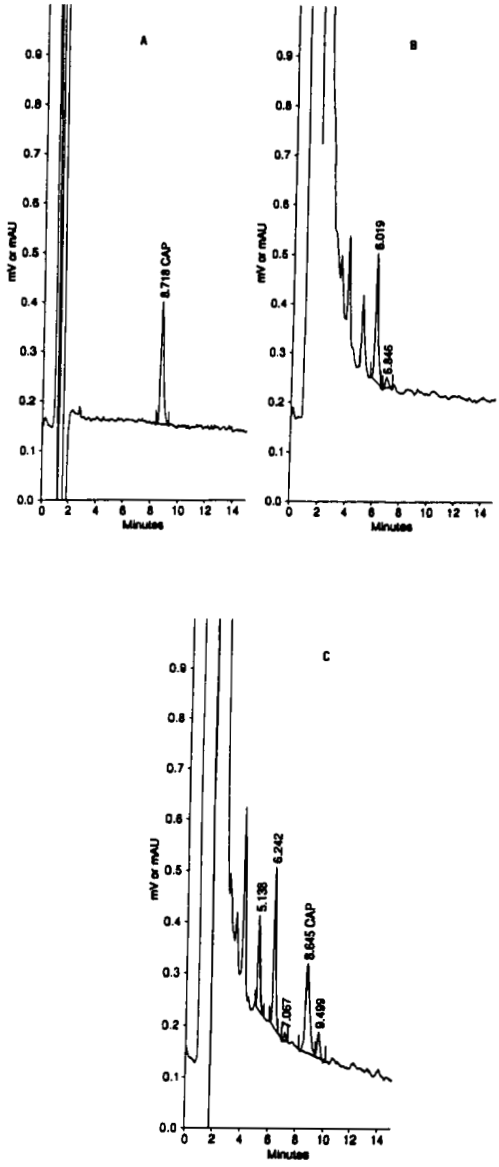

Figure 1. Chromatograms of (A) CAP standard solution (15 ng/mL), (B) a blank fatty liver sample and (C) a fatty liver sample spiked with 5 ng/g of CAP.

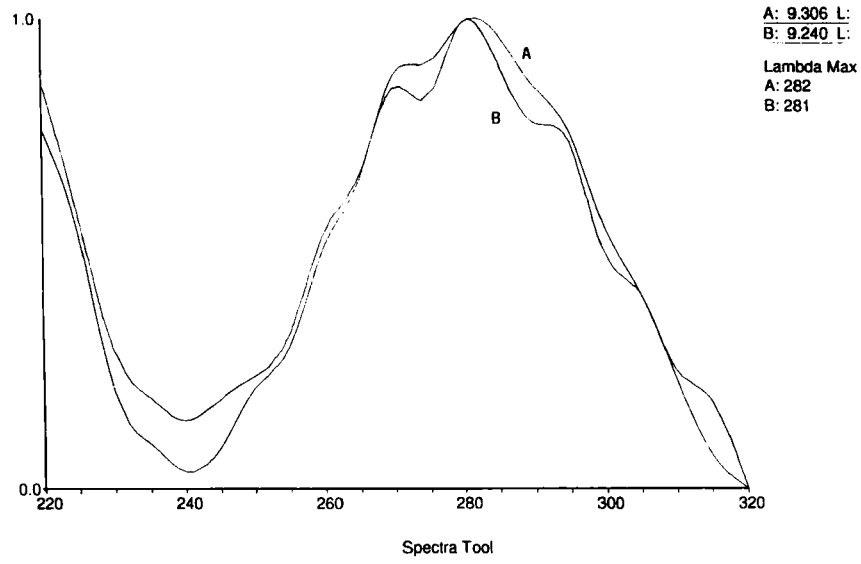

Figure 2. Ultraviolet spectra of (A) CAP standard solution (60 ng/mL) and (B) fatty liver extract spiked with 10 ng/g of CAP.

repeatability were attained. The mean recovery of CAP was found to be 71 % (SD = 4.5%) over the concentration range 5 - 20 ng/g. With this method, the peak area was linear against the dose added in the fatty liver (r = 0.998, n = 17). The coefficients of variation were less than 8.4 % (Table 1). The method can be considered as reliable for the intended application. However, some precautions have to be taken to avoid decrease of CAP concentration as it has been noted for calf liver.[10] The samples have to be stored at -20°C until analysis and never be frozen another time.

The limit of detection in the HPLC system using the UV detector was estimated from representative blank samples. It is equal to three times the peak-to-peak noise, i.e. 0.4 ng/g. The limit of quantification is estimated at the 0.8 ng/g level. The HPLC was also performed using scanning detection. CAP could be identified in samples with a CAP content above 8 ng/g. The CAP spectra obtained from liver samples are practically identical with that of standard CAP, including the wavelength of maximum absorption (in a range of 2 nm). A spectrum of the CAP peak of a fatty liver obtained with the scanning detector is presented in Figure 2.

The method was applied to control about 30 samples of fatty livers from different origin and species. All samples were found negative (< 0.8 ng/g). Some of these extracts were also analyzed by a particle beam HPLC - mass spectrometric method with negative ion chemical ionization.[12] This method which is applied with success at the laboratory for meat with a limit of detection of 0.5 ng/g has confirmed that samples were negative. CAP is metabolized in liver with a phase I metabolism involving oxidation, catalyzed by cytochrome P-450. Factors affecting liver function can, therefore, affect drug disposition as it can be noted by Soback[13] for some antibiotics. The metabolic pathways for CAP in the duck was recently reported by Cravedi[14] but no study was published about fattened duck.

In conclusion, this method has proved to be reliable and robust after testing by a second analyst. The procedure satisfies the quality criteria specified in Commission Decision 93/256/EEC[15] and possesses the required sensitivity to detect the improper use of CAP in the fatty liver-production industry in France and monitoring of CAP residues in imported livers. Further investigations will be programmed to validate the LC/MS method for the identification of CAP in fatty liver.

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