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RAPID SAMPLE PREPARATION METHOD FOR THE DETERMINATION OF CHLORAMPHENICOL IN SWINE MUSCLE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A simple and rapid sample preparation method for the determination of chloramphenicol in swine muscle tissue at the 10 μg/kg level is described. The method comprises sonication-aided extraction with ethyl acetate, addition of hexane to the extract and cleaning up and concentration of the extract on a small column packed with silica gel. Analysis was performed by high-performance liquid chromatography on a ChromSep column with ChromSpher C_8 using acetonitrile–sodium acetate buffer as the mobile phase. Detection was performed at 280 nm. Mean recoveries from spiked muscle samples were 79 ± 3% (10–50 μg/kg). The distribution of chloramphenicol in different muscle and fatty tissues from a pig to which a single dose of chloramphenicol was administered was also investigated.

INTRODUCTION

Chloramphenicol (CAP) is a very effective broad-spectrum antibiotic. However, because of its toxic properties[1,2] the policy in several European Community countries is to diminish its use in food-producing animals by setting limits on CAP residues of 10 μg/kg in edible tissues. In the U.S.A., the use of CAP was never authorized in food-producing animals. In case of illegal use, however, tissue residues are also determined at the 10 μg/kg level[3]. As microbiological methods are insufficient to meet this level, various chemical methods were developed to detect or quantitate CAP in animal tissues at levels of 5–10 μg/kg. These include not only high-performance liquid chromatographic (HPLC) procedures[4–8], but also gas chromatographic methods[9–11,13] and radioimmunoassays[12–14]. Recently, a review of the chromatographic methods for CAP residues in milk, eggs and tissues was presented by Allen[2].

The usual extractant in quantitative HPLC procedures is an organic solvent, predominantly ethyl acetate. This extractant is always evaporated in a rotavapor followed by a varying number of liquid–liquid extractions in the majority of methods. In two HPLC methods[6,8] a solid-phase extraction column is used for further clean-

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up and concentration after ethyl acetate evaporation. Due to the involvement of a rotavapor step of large volume, these procedures are also somewhat laborious. For this reason, the use of solid-phase extraction for rapid and efficient sample clean-up directly after ethyl acetate extraction of CAP from the tissue was investigated. This paper describes such a procedure, in which the principles of solid-phase extraction are directly applied to the extract solution, *i.e.* extraction of CAP from relatively large volumes of sample extract solution by the sorbent bed in a solid-phase extraction column and elution of CAP from the column in a small volume[15,16]. In this way a very clean extract is obtained, which is suitable for CAP analysis by means of HPLC and detection at 280 nm.

For inspection purposes it is desirable to be informed about the distribution of CAP over different muscle and fatty tissues. Therefore, a pig was treated with a single dose of CAP and the contents in all tissue parts of the carcass were determined according to the procedure described.

MATERIALS AND METHODS

Reagents and chemicals

Water was purified via Milli-Q® (Millipore, Bedford, MA, U.S.A.). Methanol, acetonitrile, ethyl acetate, hexane, acetic acid (99%), sodium acetate trihydrate (all HPLC grade) and anhydrous sodium sulphate were from Baker (Phillipsburgh, NY, U.S.A.).

Filter-paper circles (S & S 589.1, diameter 90 mm and S & S 589.3, diameter 110 mm) were from Schleicher and Schüll (Dassel, F.R.G.) and silica gel (average particle diameter 40 μm, for flash chromatography) was from Baker. Chloramphenicol was from Sigma (St. Louis, MO, U.S.A.).

A chloramphenicol standard solution was prepared by dissolving 100 mg of CAP in 100 ml of methanol. Working standards for HPLC were prepared in the range of 50–500 ng/ml HPLC eluent by diluting the standard solution in the mobile phase solvent. Spiking solutions containing 0.50, 0.75, 1.25 and 2.50 μg/ml methanol were prepared by diluting the standard solution in methanol.

Silica gel disposable extraction columns were prepared by filling 3-ml filtration columns (Baker) with 1.2 g of silica gel each. After the material is tightly packed, a fritted disc was placed over the sorbent bed. Just before use, the column was pretreated by passing about 8 ml of ethyl acetate–hexane (4:6). After this pretreatment, the column should not be allowed to run dry.

The mobile phase solvent for HPLC was acetonitrile–sodium acetate buffer (0.01 mol/l, pH 4.3) (25:75).

Samples

For spiking studies, ham muscle was used. Visible fat and collagen were removed as far as possible. Ground tissue samples were spiked at levels of 10, 15, 25 and 50 μg/kg at least 15 min before extraction by the procedure described below.

For studying the distribution of CAP in a pig (weight 85 kg), a single intramuscular injection of 50 mg of CAP per kg body weight was administered. The animal was slaughtered 42 h after administration. The carcass was dissected, by the Dutch method, into 22 pieces of muscles, lean and streaky, and fatty tissues. Before

analysis, visible fat and connective tissue was removed from the pieces of lean meat as far as possible.

Apparatus and chromatographic conditions

The instruments used were a Moulinette homogenizer (Moulinex, Gouda, The Netherlands), a Bransonic® B-221 ultrasonic bath, frequency of operation: 50–60 kHz (Branson Europe, Soest, The Netherlands) equipped with a tray insert and filled with water, and a Vortex mixer (Scientific Industries, Bohemia, NY, U.S.A.). In order to operate several cartridges simultaneously, a vacuum manifold (Baker) was used. It was connected via a filtration flask to a water aspirator. The extraction column was connected to a 75-ml reservoir equipped with an adaptor (Baker). For sample elution, a collection rack was inserted in the vacuum manifold basin. For HPLC, a LKB 2150 pump (Bromma, Sweden) equipped with a 50-μl Rheodyne 7125 sampling valve (LKB) and a 2151 variable-wavelength detector operated at 280 nm and equipped with a 10-μl HPLC flow cell (LKB) was used. A Chrompack (Middelburg, The Netherlands) ChromSep HPLC system (21 cm) with two glass columns (100 × 3 mm each) containing 5-μm ChromSpher C_8 was used, with a guard column (10 × 2.1 mm) packed with reversed-phase material (Chrompack). The chromatograph was operated at ambient temperature. Peak heights were measured with an SP4270 printer/plotter integrator (Spectra-Physics, San Jose, CA, U.S.A.).

Sample preparation

Extraction. Approximately 10 g of ground tissue were accurately weighed in a 100-ml beaker. A 20-ml volume of ethyl acetate was added. After stirring thoroughly with a glass rod, the beaker was covered and placed on the tray insert in the ultrasonic bath for 15 min. The temperature of the water in the bath was kept below 40°C. After the solids were allowed to settle from the solution, the solvent was decanted through filter paper (S & S 589.1) over 5 g of anhydrous sodium sulphate.

This extraction procedure was repeated with another 20 ml of ethyl acetate. After this, the solids were rinsed with an additional 10 ml of ethyl acetate. The ethyl acetate was decanted through the filter paper with sodium sulphate.

Clean-up. To the combined extracts and wash liquid (total volume *ca.* 40 ml), 60 ml of hexane were added (the ethyl acetate–hexane ratio should be *ca.* 4:6). After stirring thoroughly, the solution was allowed to stand for 5 min to allow the precipitate to settle from the milky solution. The total solvent solution was poured off through filter paper (S & S 589.3). The filter was washed with 10 ml of hexane and the hexane was combined with the filtrate. The combined filtrate and wash hexane was passed through the pretreated silica gel column (connected with the 75-ml reservoir) at a flow-rate of *ca.* 8–10 ml/min through the manifold. The column was washed with 10 ml of hexane. The column was removed from the manifold and the 75-ml reservoir and was dried in a stream of nitrogen for 20 min. The column was replaced directly on the manifold containing the collection rack with a 3.5-ml glass collection vessel. Immediately after drying, 3 × 1 ml of methanol were added to the column and CAP was eluted from the column with vacuum off. Another 1 ml of methanol was aspirated through the column using vacuum on. The eluate was collected in the collection vessel.

The eluate was evaporated to dryness in a stream of nitrogen at room tem-

perature. The residue was dissolved in 1.0 ml of the mobile phase solvent using a Vortex mixer for 30 s. This solution was used for HPLC analysis.

Chromatography. Aliquots of the sample and standard solutions (50 μl) were injected by means of the loop injector. Samples were eluted isocratically at a flow-rate of 0.8 ml/min.

RESULTS AND DISCUSSION

Spiking studies

Recovery experiments were carried out on swine muscle tissues at 10, 15, 25 and 50 μg/kg spiking levels. Each amount was added in seven-fold to the ground muscle tissue. The samples were submitted to HPLC analysis in duplicate according to the procedure described. The blank samples were analysed in seven-fold. The results are presented in Table I. A good recovery at all levels investigated and a low standard deviation for the repeatability was attained.

Typical chromatograms from spiked and control muscle tissues are shown in Fig. 1. The HPLC method used was based on methods normally used for CAP chromatography with slight but essential modifications. The use of the 5-μm Chrom-Sep columns was particularly essential with respect to sensitivity of CAP detection and separation of CAP from endogenous compounds present in the tissue. With the aid of this column, and the UV detector used, the signal for 5 ng of CAP (in a standard solution) was found to be about 30 times as high as the noise level. Results like this were impossible, for example, by using a conventional 8-μm CP-Spher C_8 column (250 × 4.6 mm I.D., Chrompack). When applying the described system to blank tissues, a small peak is sometimes observed having the same retention time as CAP. When analysing more tissue samples in practice, it was found that these signals never exceeded values corresponding to 1–2 μg/kg CAP. It should be a matter of further investigation whether this small peak is caused by the presence of a trace of CAP, or not.

Extraction and clean-up

For quantitative purposes, ethyl acetate was found to be a suitable extractant in our sonication-aided extraction. In comparison to techniques in which a conventional blender or an Ultra Turrax is used, this techniques has the advantage that several samples can be handled simultaneously and the risk of cross-contamination is prevented[17].

TABLE I

RECOVERY OF CHLORAMPHENICOL FROM SPIKED SWINE MUSCLE

Added (µg/kg)	*Recovery (%)*	*Standard deviation (n = 7)*
10	85	3.3
15	79	2.8
25	77	1.8
50	78	3.1

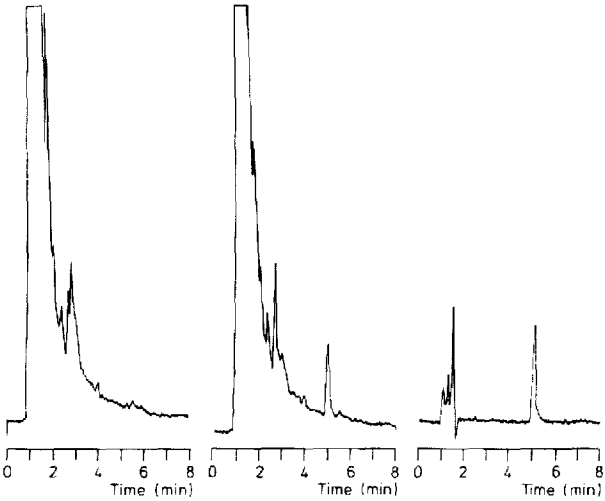

Fig. 1. Chromatograms of extracts of blank and spiked (10 μg/kg CAP) swine muscle tissue and of a standard solution of CAP. For conditions, see text. Absorbance range setting 0.02 a.u.f.s.

The clean-up of the ethyl acetate extract and the concentration of CAP from this extract prior to HPLC deserves some additional attention. This ethyl acetate extract, after removal of tissue solids by filtration, is moderately polar and unsuitable as such for CAP extraction from this solution by a silica column. The sample solution has to be diluted with a non-polar solvent, *e.g.* hexane, to improve the conditions for CAP retention on the silica column. In preliminary experiments, the silica column was shown to be the most suitable for extraction of CAP from ethyl acetate–*n*-hexane; for quantitative purposes, however, the amount of 0.5 g of silica sorbent in the standard silica columns has to be enlarged to 1.2 g.

For complete retention of CAP from the sample solution, the proportion of ethyl acetate to hexane has to be at least 4:6. Moreover, the maximum volume of this diluted solvent solution should not exceed 110 ml. This limits the volume of ethyl acetate used for extraction. After this retention, CAP is easily removed from the silica column by means of a few milliliters of methanol.

An adventitious circumstance of this clean-up procedure is that after hexane addition to the ethyl acetate sample solution, matrix components precipitate, which can easily be removed by filtration.

In this clean-up procedure, the use of HPLC-grade solvents for extraction is imperative. Originally, analytical-reagent-grade ethyl acetate was used for extraction. However, when using analytical-reagent-grade ethyl acetate, impurities from this solvent were found to accumulate on the column, resulting in a number of peaks on the chromatogram that were not derived from components of the muscle tissue.

Distribution of CAP in swine tissues

All muscle (lean and streaky) and fatty tissue parts of the carcass of a CAP-treated pig were analysed for CAP content according to the procedure described. The results are shown in Table II. All values were determined in duplicate for each part and corrected for recovery. The recovery values were all in the range of 70–90%,

TABLE II

CAP CONTENT OF MUSCLE (LEAN AND STREAKY) AND FATTY TISSUES FROM CAP-TREATED SWINE

Name of tissue	*Weight (g)*	*CAP content (μg/kg)*
Lean tissue		
Pillar of the diaphragm	85	247
Diaphragm	81	230
Outside round*	1034	207
Eye of round	410	224
Heel	458	243
Top round*	1512	265
Knuckle*	1194	222
Sirloin*	984	269
Bottom butt loinside	960	246
Loin centre cut	1450	218
Loin	1130	212
Tenderloin	474	272
Shoulder picnic	3782	225
Streaky tissue		
Bottom butt frontside	630	173
Shank	412	185
Belly	3230	146
Fatty tissue		
Ventral part of the belly	366	32
Ham fat	612	64
Jaw	913	44
Flare	257	27
Back fat	665	59
Shoulder fat	303	40

* Comparable name for beef tissues.

including the fatty tissues. As in swine muscle tissue, CAP-glucuronide was reported to be absent[8]; no glucuronidase was added during this procedure. The animal under investigation was slaughtered 42 h after CAP administration in order to ensure the presence of relatively high levels in the tissue, thus making it possible to establish any differences in content between the parts, more precisely and accurately.

Table II shows a variation in the CAP content of the lean meat tissues between 212 and 272 μg/kg. In fatty tissues, the values are considerably lower, whilst in the streaky tissues intermediate values were found. As in lean tissues the highest levels were found with relatively small variations, the pillar of the diaphragm can be considered as representative for the inspection of swine carcasses regarding CAP residues. (Generally this tissue is desirable as the target tissue for investigation because it is easily attainable in an early stage of the slaughtering process with little economical damage to the carcass.) However, it should still be checked as to whether the pillar of the diaphragm is also representative for screening at the 10 μg/kg level.

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