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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SCREENING AND CONFIRMATION METHODS FOR CHLORAMPHENICOL RESIDUES IN MEAT WITH OFF-LINE CARTRIDGE SAMPLE CLEAN-UP AND ON-LINE DIODE ARRAY UV–VIS DETECTION

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SUMMARY

Two high-performance liquid chromatography (HPLC) methods for the analysis and confirmation of residues of the antibiotic chloramphenicol in edible animal tissues are described. The first method consists of an aqueous extraction followed by purification through an Extrelut® cartridge and toluene partition. With this simple and rapid method, meat samples can be screened at the 5 μg/kg level. The second, more comprehensive, method is based on ethyl acetate extraction, followed by purification through a silica Sep-Pak® cartridge and partition with buffer–diethyl ether and water–toluene. Confirmation of positive peaks at the 10 μg/kg level is performed by diode array UV–VIS detection. The recoveries for the two methods at the 10 μg/kg level are 58 and 85% respectively, coefficients of variation 5–6%. With the confirmation method, glucuronide and sulphate conjugates can be determined. However, in a positive reference sample (pig) none was observed.

INTRODUCTION

In veterinary practice, chloramphenicol (CAP) is extensively used as an antibiotic against bacterial infections in chickens, calves, cows and pigs. For some years it has been known that even minute amounts of CAP can cause serious health problems, *i.e.*, aplastic anaemia in man[1]. Therefore, several countries have established maximum residue levels for CAP in edible products (meat, eggs, milk). In the U.S.A. this level has been set at zero[2]. Other countries (will) establish maximum levels of 1–10 μg/kg[3].

In order to effectively monitor the occurrence of residues, specific and sensitive analytical methods are required. Often, the residue level permitted is directly related to the detection limit of the existing methods. In meat, drug residue monitoring is mainly performed by microbiological methods, together with high-voltage electrophoresis[4]. Such methods are inexpensive and capable of handling many samples simultaneously. However, they are not very specific, may produce false positive results and are not very sensitive. For CAP, a detection limit of only 1 mg/kg can be

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achieved. The immunological [radioimmunoassay, competative enzyme-linked immunoassay (CELIA)] methods are claimed to be very sensitive (1 μg/kg) and simple, but confirmation at these low concentration levels seems to be a major problem[2,5].

Thin-layer chromatography can be used for some groups of veterinary drugs[6], but is not sensitive enough for CAP. Gas chromatographic analysis can be performed only after derivatization of CAP, but often a high sensitivity (< 5 μg/kg) can be achieved[7,8]. The HPLC methods thus far published are either applicable only to blood analysis[9,10], or are very laborious and/or lack the required sensitivity[11–15]. The purpose of this study was to establish a rapid and specific HPLC method for the screening of CAP in edible tissues (pork, veal, poultry, cow) and a reliable, on-line HPLC confirmation method for positive samples (> 10 μg/kg). This confirmation method is necessary because there is always the possibility of interfering compounds that produce false positive results in screening. Recently, we described the use of diode-array UV–VIS detection as a technique for confirmation of the presence of furazolidone in eggs[16]. In the present study, the same technique was used for CAP samples (> 10 μg/kg). For confirmation purposes, a high recovery is essential, since diode array UV–VIS analysis requires a high concentration in the detection cell to produce an accurate UV spectrum. A relatively laborious clean-up procedure is acceptable, for only screening samples showing positive results are analyzed. For screening purposes a lower recovery is acceptable, provided it is reproducible, but the procedure itself must be simple. Modern, off-line solid-phase extraction techniques were used for clean-up, resulting in very clean chromatograms.

EXPERIMENTAL

Chemicals and reagents

All chemicals were of analytical grade (Merck, Darmstadt, F.R.G.). Standard chloramphenicol [D(−)-*threo*-2-dichloroacetamido-1-*p*-nitrophenyl-1,3-propanediol] was purchased from Sigma (St. Louis, MO, U.S.A.) or as a U.S.P. reference standard (Zentrallab. Deutscher Apotheker, Eschborn, F.R.G.). β-Glucuronidase/arylsulphatase was purchased from Merck (art. 4114) or Sigma (S-3009). The HPLC eluent was prepared by mixing 710 ml of 0.01 *M* sodium acetate buffer (pH 4.3) with 290 ml acetonitrile. All eluents were degassed and filtered before use.

Instrumentation and chromatographic conditions

A Model 6000 A pump (Waters Assoc., Milford, MA, U.S.A.) equipped with a Model 7125 fixed-loop injector (Rheodyne, Berkeley, CA, U.S.A.) and an HP 1040 A photodiode array detector (Hewlett-Packard, Palo Alto, CA, U.S.A.) was used. Alternatively, a Model PU 4020 detector (Pye Unicam, Cambridge, U.K.) was employed. The column was a CpTM Spher C_{18} cartridge (200 × 3 mm), 8 μm (Chrompack, Middelburg, The Netherlands), and the precolumn contained Bondapak C_{18} (20 × 3.9 mm) (Waters). The flow-rate was 0.6 ml/min, and the detection wavelength 278 nm.

Sample preparation

Screening. To an accurately weighed amount (*ca.* 10 g) of homogenized meat was added 40 ml of water. After vigorous homogenization for 3 min, the sample was

filtered off and 20 ml of the filtrate were applied to an Extrelut® cartridge (Merck 11737). After 15 min, CAP was eluted from the cartridge with 50 ml dichloromethane. The organic extract was evaporated under a gentle stream of nitrogen at 40°C and the residue transferred to a centrifuge-tube with 15 ml of dichloromethane. After evaporation, 300 μl of water and 2 ml of toluene were added to the residue. After gentle mixing, the phases were separated by centrifugation. The organic phase was discarded and the partition repeated with 1.5 ml of fresh toluene. The aqueous phase was filtered through a Millex HV-filter (Millipore, Bedford, MA, U.S.A.). A maximum of 200 μl were injected into the column when using the diode array detection. When a normal variable UV–VIS detector was used, injection of 100 μl was sufficient.

Confirmation. To an accurately weighed homogenized meat sample (50 g) were added 100 ml sodium acetate buffer. After vigorous mixing for 3 min, 200 μl of β-glucuronidase–arylsulphatase were added if conjugates were to be analyzed. The solution was placed in an incubator at 37°C for 16 h. Then, 200.0 ml ethyl acetate and 20 g potassium chloride were added. After vigorous mixing for 3 min followed by shaking for 20 min on a mechanical shaker, 150.0 ml organic phase were isolated by centrifugation. The organic phase was evaporated in a rotary evaporator and the residue was dissolved in 25 ml of dichloromethane–light petroleum (b.p., 40–60°C), (1:1).

A silica Sep-Pak cartridge (Waters) was washed with ethyl acetate–hexane (70:30) and light petroleum, then dried by forcing a gentle stream of nitrogen through the column. The sample extract was gently pressed through the cartridge with a disposable syringe and flushed with 5 ml light petroleum and 5 ml ethyl acetate–hexane (50:50) solution. After drying with a stream of nitrogen, CAP was eluted from the column with 25 ml ethyl acetate–hexane (70:30) solution. The effluent was evaporated and 2 ml of Tris buffer (pH 10.4) were added. This solution was extracted three times with 5 ml of diethyl ether. The combined organic phases were evaporated, the residue was dissolved in 1.0 ml water and 3 ml of toluene were added. After thorough mixing, the aqueous phase was isolated and filtered, and 200 μl of the final solution were injected.

The HPLC system was coupled to a UV–VIS diode array detector. The amount of CAP present was calculated by comparison of the peak height or peak area of the sample with that of standard CAP. The confirmation was based on a comparison of the retention time and of the UV spectrum of the sample with those of standard CAP.

RESULTS AND DISCUSSION

Chromatography

The objective was to use isocratic HPLC. Therefore the clean-up procedure had to be very effective. A number of reversed-phase columns were tested and compared with regard to the separation of CAP from interfering peaks and the peak heights obtained upon injection of equal amounts of CAP. The peak height is particularly important, for confirmation by comparison of UV spectra requires as high a concentration in the diode array detection cell as possible. The results are presented in Table I. Columns with an inner diameter of 3 mm clearly yielded higher peak heights compared to columns with a diameter of 4.6 mm. The use of CpTM Spher

TABLE I

COMPARISON OF COLUMN MATERIALS WITH REGARD TO SEPARATION OF CAP FROM MATRIX COMPONENTS (I) AND SENSITIVITY EXPRESSED AS RELATIVE PEAK HEIGHT (II)

Chromatographic conditions as described in text. +, Good separation from matrix components or good peak height/concentration ratio; ±, some interference from matrix or moderate peak height/concentration ratio; −, strong matrix interference or low peak height/concentration ratio (peak broadening).

Column material	*Length (cm)*	*Inner diameter (mm)*	*Particle size (μm)*	*I*	*II*
LiChrosorb RP-18	10	3	7	−	+
LiChrosorb RP-18	15	4.6	5	+	−
Supelcosil LC-18	15	4.6	5	±	±
Cptm Spher RP-18	20	3	8	+	+

cartridges gave the best results in the analysis of CAP. The use of a sodium acetate–acetonitrile mixture as eluent in RP-HPLC analysis of CAP has previously been described by Bécheiraz *et al.*[11] and Petz[13]. Buffers with pH between 4 and 5 were tested. At pH 4.3 the optimum peak shape and peak height was observed. A number of buffer–acetonitrile mixtures were investigated. Small changes in the eluent composition caused substantial changes in retention time. The injection of a 200-μl sample is not common in modern HPLC analysis, since severe peak broadening usually results. However, dissolution of the final extract in water instead of the eluent proved to be very effective in minimizing peak broadening, as pre-concentration takes place on top of the column.

Extraction and sample clean-up

Screening. The extraction of CAP with ethyl acetate has frequently been described[9,11,12,14,15,17,18]. High recoveries are obtained but, because of the polarity characteristics of this solvent, many matrix components are also extracted. Thus a very extensive sample clean-up after the initial extraction is needed in order to remove these components. Furthermore, troublesome emulsions are often encountered. Therefore, for screening purposes, the use of ethyl acetate is not recommended. It may work for lean meat samples, *i.e.*, steak but chicken, veal or pork contains amounts of fat that complicate the analyses by yielding interfering peaks (false positives). For the confirmation analysis the recovery must be as high as possible, and extensive clean-up is not a major concern. Johannes *et al.*[12] observed that muscle-drip contains a relatively high amount of CAP and relatively few matrix components. We have found aqueous extraction to be very effective, resulting in a recovery of over 90% at the 10 and 110 μg/kg levels. All extraction experiments were monitored by analyzing a reference pork sample, obtained from homogenized frozen or freeze-dried meat containing 110 μg/kg CAP (dosed pig). After aqueous extraction, additional clean-up was still necessary. Extrelut cartridges can be used with aqueous samples which are applied on the solid-phase material (diatomaceous earth) and extracted with an organic solvent after equilibration (15 min). No emulsions occur and many interfering compounds remain on the column.

In our study, extraction with diethyl ether gave very low recoveries. This was

surprising, since extraction of standard CAP solutions, applied to Extrelut, gave 100% recovery. Furthermore, it was noticed that increasing the amount of sample extracted with water resulted in lower recoveries. Apparently, CAP is firmly bound to meat components. With ethyl acetate, high recoveries were obtained, but many interfering compounds are also eluted. Extraction with dichloromethane gave an acceptable recovery of about 65% and showed only two minor interfering peaks just before and after CAP. These interferences were quantitatively removed by toluene extraction.

Confirmation. With the linear diode array UV–VIS detector, about 50 ng CAP are required to produce an accurate UV spectrum. Considering an 80% recovery, 200-μl injection and use of 75% of the extract, this implies a sample of 50 g containing 10 μg/kg CAP. For effective clean-up, it was necessary to perform both a solid-phase extraction and a liquid–liquid extraction. Emulsions formed after ethyl acetate extraction were minimized by addition of potassium chloride, centrifugation and using only 75% of the extract. It proved essential to prewash the silica cartridge before use to obviate irreproducible ghost peaks in the chromatogram. After the Sep-Pak clean-up, an ether extraction was performed according to Najolia[10]. This extraction, together with a final toluene partition, removed almost all interfering peaks, and allowed a reliable confirmation by plotting of UV spectra taken at the apex of the peak.

Application to meat samples

Using the methods described, many samples varying in species and fat content

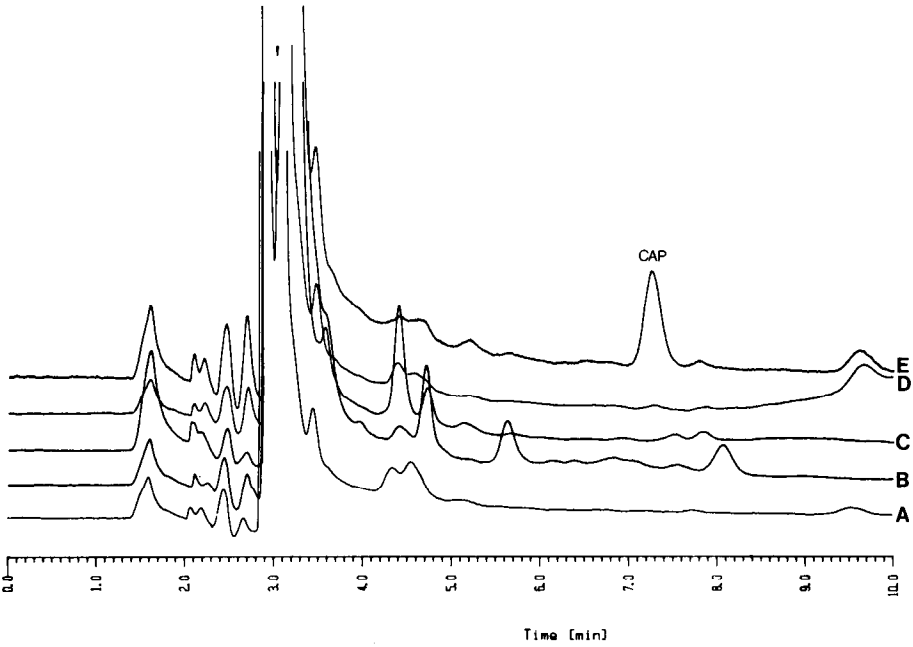

Fig. 1. Typical chromatograms obtained in screening analysis for meat samples from the cow (A), chicken (B), calf (C), pig (D) and a pork sample (E), spiked at the 10 μg/kg level. HPLC conditions as described in the text.

(steak or minced pork meat) obtained from slaughter-houses have been analyzed. In Fig. 1 a few examples of negative (A–D) and positive (E) results for screening samples are shown. Very clean final extracts were obtained for the different species. The recovery was 58% (C.V. = 6%, n = 10) at the 10 μg/kg level. For the reference sample (110 μg/kg) the recoveries were similar. Concentrations of 5 μg/kg can easily be detected. It is also possible to produce a reasonable UV spectrum at the 10 μg/kg level using the diode array UV–VIS detector (Fig. 2A).

Fig. 3 shows some examples of samples analyzed by the confirmation method. The recovery was 85% (C.V. = 5%, n = 10) at the 10 μg/kg level.

For each sample series, a sample spiked at the 10 μg/kg level and the reference sample were analyzed for quality control. When corrected for recovery, the CAP content of the reference sample obtained with both methods was identical. In Fig. 2B confirmation of a 10-μg/kg sample is shown.

The difference between deep-frozen and freeze-dried material was investigated using the reference sample. Freeze-dried material has the advantage of being easy to store and homogenize. Furthermore, it does not release water during extraction. No difference was observed regarding the CAP contents of the two forms. On the other hand, when using the screening method, the extracts resulting from fresh (deep-frozen) samples were much cleaner (Fig. 4). With the confirmation method, no dif-

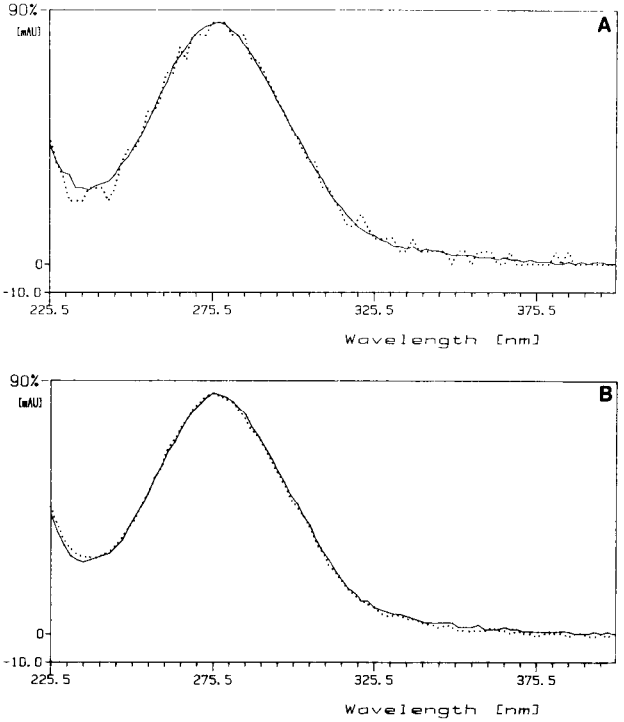

Fig. 2. Diode array UV–VIS confirmation of positive peaks obtained with the screening procedure (A) and the confirmation procedure (B) from a spiked (10 μg/kg) pig-meat sample. The sample spectrum (dotted line) is taken at the peak apex and compared with a standard CAP spectrum (full line). HPLC conditions as described in the text.

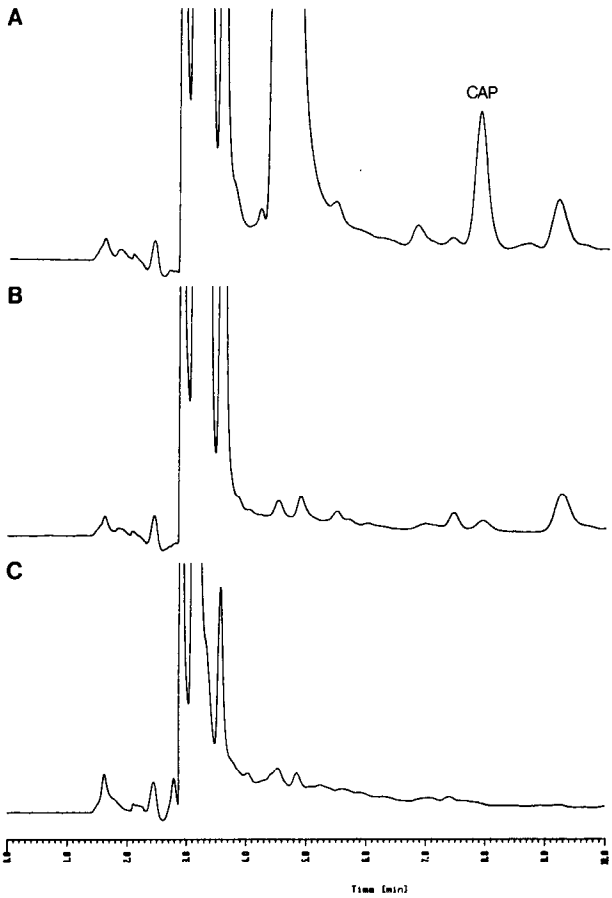

Fig. 3. Typical chromatograms obtained in confirmation analysis for a spiked (10 μg/kg) pork sample (A) and control meat samples from the pig (B) and cow (C). HPLC conditions as described in the text.

ference was observed between freeze-dried or deep-frozen material. We therefore preferred to analyse fresh (or deep-frozen) meat samples. It is well known that CAP is rapidly transformed to its glucuronide conjugate in the liver and subsequently eliminated in the urine. In pig liver and kidney, free CAP represents only a fraction of the total CAP present[12]; in plasma, about 50% CAP is present as free CAP. Because glucuronides or sulphates can be transformed back to CAP upon intake by man, we included a deglucuronidation/desulphatation step. Under various deglucuronidation conditions, effective in transforming glucuronides of other veterinary drugs, no increase in CAP content of the reference sample was observed. Apparently, the CAP conjugates are not transported from plasma to muscle.

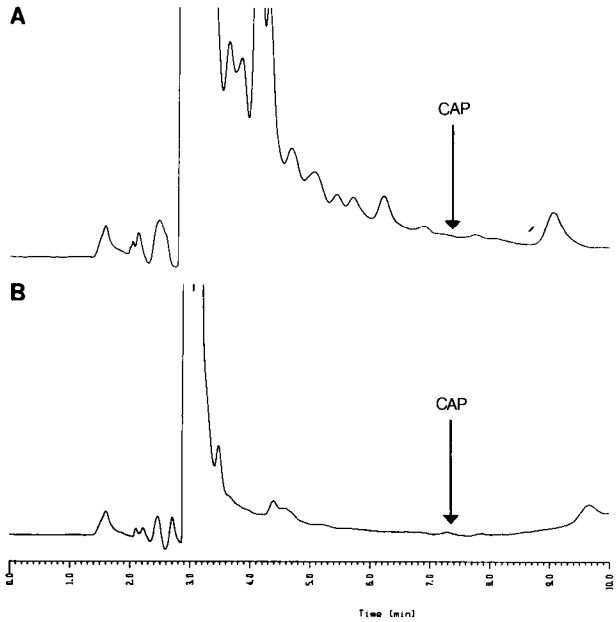

Fig. 4. HPLC elution patterns observed after screening analysis of freeze-dried (A) and deep-frozen (B) pork samples.

CONCLUSIONS

A rapid, sensitive and specific HPLC screening procedure for CAP, applicable to different meat species, has been developed. An aqueous extraction is combined with off-line clean-up on an Extrelut cartridge. This method permits detection of >5 μg/kg. No false positive results have been observed in routine analyses up to now. For official residue analysis, a confirmation method is essential to detect and prevent false positive results. An on-line HPLC method, based on RP-HPLC, together with diode array UV–VIS detection, has been developed. Residues of CAP, including possible glucuronide/sulphate conjugates, can be confirmed at the 10 μg/kg level. This confirmation technique, being much simpler and less expensive than the often used gas chromatography–mass spectrometry, may well prove to be as reliable in the confirmation of positive peaks and indication of false positive ones, in a number of applications. The use of freeze-dried material may cause confusing chromatograms, compared with fresh or deep-frozen samples. In a positive CAP sample, obtained from a CAP-treated pig, no CAP conjugates were observed under conditions where complete transformation of these conjugates should have taken place.

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