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Determination of chloramphenicol in muscle using a particle beam interface for combining liquid chromatography with negative-ion chemical ionization mass spectrometry

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ABSTRACT

A simple and rapid liquid chromatographic–mass spectrometric analysis of chloramphenicol in calf muscle is presented. A particle beam interface was used, with negative-ion chemical ionization mass spectrometry using methane as the reagent gas. The method specificity was tested for three related compounds, dehydrochloramphenicol, nitrosochloramphenicol and nitrophenylaminopropanediol. The extraction procedures require 5 g of muscle, and the quantification limit is 2 μg/kg for chloramphenicol. Residues were detected in calf muscle 48 h after intravenous administration of chloramphenicol (25 mg/kg body weight).

INTRODUCTION

Chloramphenicol is a broad-spectrum antibiotic often used in veterinary practice for treating various infectious diseases. However, adverse reactions and side-effects in humans have been extensively demonstrated. For these reasons, within the European Community, a maximum residue level of 10 μg/kg has been proposed for meat [1]. Several confirmatory methods have been reported [2], and identification methods based on gas chromatography–mass spectrometry (GC–MS) have been developed [3].

Recently, a thermospray high-performance liquid chromatographic–mass spectrometric (HPLC–MS) method for the analysis of chloramphenicol and three related compounds was described [4]. Another method using a particle beam interface was proposed. Thus, we investigated the applicability of this technique to the analysis of chloramphenicol after liquid chromatography and extraction from muscle by a method previously described [5]. The method was tested with spiked muscles as well as muscles from three animals treated with chloramphenicol.

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EXPERIMENTAL

Chemicals

Chloramphenicol (CAP) and nitrophenylaminopropanediol (NAPD) were purchased from Lepetit (Milan, Italy). Nitrosochloramphenicol (NO-CAP) and dehydrochloramphenicol (DH-CAP) were supplied by Boerhinger Mannheim

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(Mannheim, Germany). Carbon tetrachloride, hexane, methanol and acetonitrile were analytical grade (Merck, Darmstadt, Germany). Water was obtained from a reagent-grade Milli-Q system (Millipore). Ethyl acetate was obtained from Merck and was distilled with a rotary evaporator before use.

Standard curves

Stock solutions of CAP were prepared by dissolving 50 mg of the reference compound in 100 ml of water. Then 1 ml of stock solution was diluted to 100 ml with water, and 1, 0.5, 0.25 or 0.1 ml of this solution was diluted into 50 ml of water. These solutions contained 10, 25, 50 and 100 μg/l CAP, respectively. Aliquots of 5 g of ground calf muscle were fortified with 1 ml of solutions from 2 to 20 μg/kg.

Extraction from muscle

Muscle samples, spiked as stated above, were placed in 15-ml glass-stoppered tubes, followed by the addition of 2 ml of water. Samples were mixed for 1 min with a vortex stirrer and allowed to stand for 15 min. Ethyl acetate (6 ml) was added, and the samples were vortex-mixed for 1 min. After 10 min at room temperature and further vortex-stirring for 1 min, the samples were centrifuged for 5 min at 4000 *g*. The contents separated into three layers: the remaining tissue at the bottom, the aqueous layer in the middle and the organic layer at the top. A volume of 4.8 ml of the organic layer was removed, transferred to a 10-ml tube and evaporated to dryness at 60°C with nitrogen. The residue was suspended in 800 μl of hexane–CCl_4 (1:1, v/v) and 400 μl of water were added. The samples were vortex-mixed for 1 min and centrifuged for 5 min at 4000 *g*. A volume of 100 μl of the aqueous portion was injected into the chromatographic system.

Precision, accuracy and limit of quantitation

To assess the precision and accuracy of the method for CAP in muscle samples, repeated assays were carried out at four different concentrations (2, 5, 10 and 20 μg/kg). Fresh spiked muscle samples were analysed on different days at each concentration, and coefficients of variation (C.V.) and mean percentages of error were calculated.

The limit of quantitation (LOQ) was defined as the lowest concentration with an acceptable C.V. For this type of analysis, European regulatory administration defines the LOQ as the concentration with C.V. < 30%.

Specificity of determination

A standard solution containing CAP, NO-CAP, DH-CAP and NAPD at 1 μg/ml was injected into the HPLC–MS system to determine the influence of metabolites on the CAP retention time.

HPLC–MS analysis

Chromatography was performed with a Hewlett Packard (HP) pump (Series 1050) equipped with a 100-μl external loop injector (Rheodyne 7125). The chromatographic column (125 mm × 4 mm I.D.) was packed with 5 μm particle size LiChrospher RP18 endcapped (Merck), and protected by a guard column RP18e (4 mm × 4 mm I.D.). The isocratic mobile phase was methanol–0.2% formic acid in water (43:57, v/v) at a flow-rate of 0.6 ml/min.

The HPLC system was connected to a Hewlett Packard 59980B particle beam interface. The column effluent was converted into an aerosol in a pneumatic nebulizer, into which helium was introduced coaxially at a pressure of 413 kPa. The aerosol was sprayed into a desolvation chamber, in which the pressure was maintained at 26.6 kPa and the temperature was held at 70°C. Finally, a virtually solvent-free particle stream was introduced into the ion source of a Hewlett Packard 5989A mass spectrometer. The MS system was operated in the negative-ion chemical ionization (NICI) mode with an electron energy of 230 eV, an emission current of 300 μA and an ion source temperature of 200°C. Prior to the analysis, the instrument was tuned in the NICI mode, using the fragment *m/z* 302, 452 and 633 from the perfluorotributylamine calibrant gas.

The reagent gas (methane) was admitted via a gas-flow controller to an indicated ion source

pressure of 0.226 kPa. The NICI mass spectra of CAP was recorded during an HPLC run by repeatedly scanning the quadripole mass filter every 1.4 s from *m/z* 121 to *m/z* 350. Quantitation was performed by focusing the instrument in the selected-ion monitoring (SIM) mode in order to measure the fragments *m/z* 151, 250, 286, 288, 322 and 324 for CAP, with a dwell time of 200 ms for each mass range.

Ruggedness

A ruggedness test was provided with the method to test several parameters (HPLC flow-rate, percentage of methanol, methane pressure, helium pressure, temperature of particle beam, temperature of ion source and electron energy). Two levels were fixed for each parameter and then tested in various combinations [6].

Drug administration

Three one-month-old calves received one intravenous dose of CAP (25 mg/kg body weight). Animals were slaughtered 48 h later and muscles were collected and frozen at −20°C until analysis.

RESULTS AND DISCUSSION

The extraction method is the same as that previously described for the HPLC–UV analysis [5], where the stability of stored samples was studied. Elution conditions in the HPLC system were

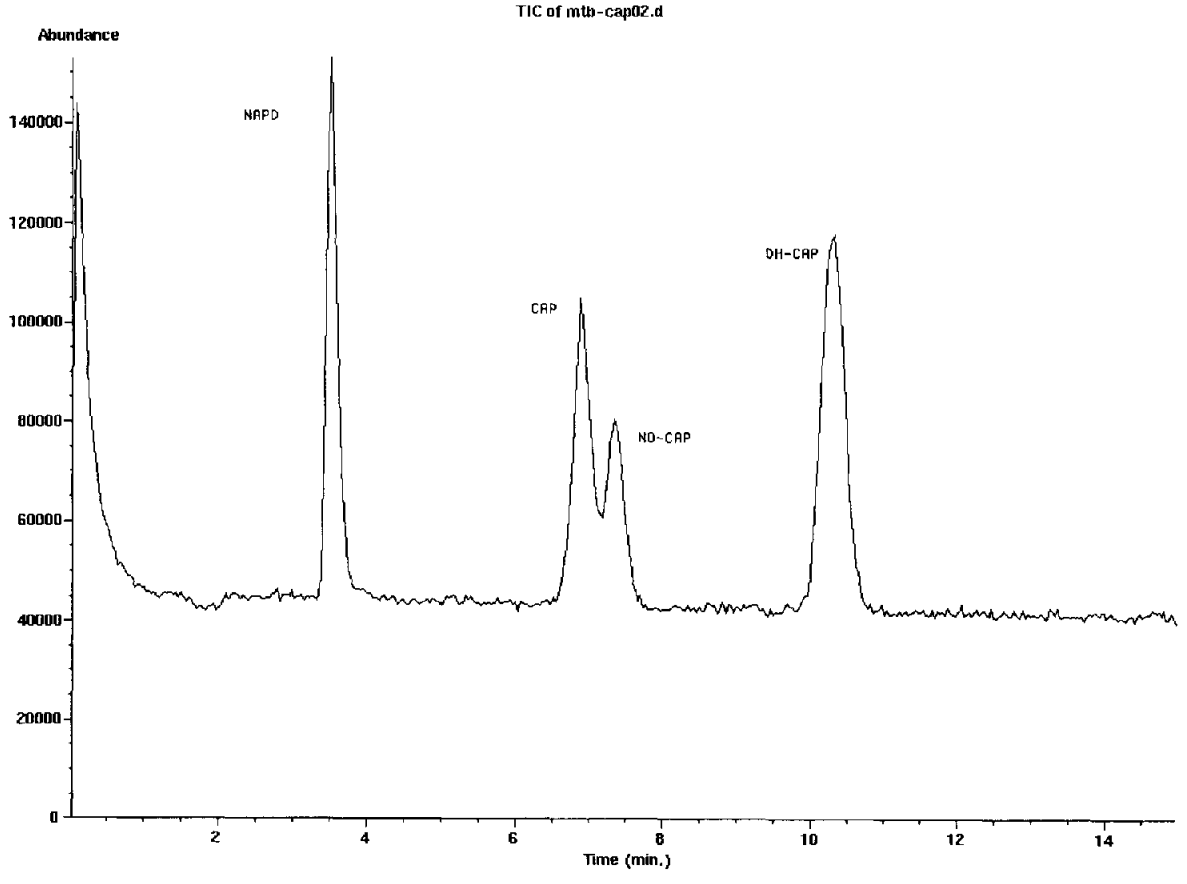


Fig. 1. HPLC mass chromatogram of NAPD (peak 1), CAP (peak 2), NO-CAP (peak 3) and DH-CAP (peak 4) at a concentration of 1 μg/ml.

modified only to take into account the optimum of the particle beam interface at mobile phase flow-rates close to 0.6 ml/min. The column was similar to that used in HPLC–UV method [5]. CAP was resolved from the three metabolites tested; the retention times were: 3.5 min for NAPD; 7 min for CAP; 7.5 min for NO-CAP; and 10.3 min for DH-CAP (Fig. 1).

Following the injection of each pure reference standard, the NICI mass spectra of CAP, NO-CAP, NAPD and DH-CAP were obtained by on-line HPLC–MS with methane as reagent gas. The different mass spectra exhibit ions in the high mass region, as shown in Fig. 2a–d. The molecular ion of CAP at m/z 322 and the peak at m/z 324 due to the chlorine-37 isotope were characteristic. Under NICI conditions, the dominant mechanism is an electron-capture process. In the mass spectra of CAP, the fragments are derived by loss of one (m/z 286 and 288) or two (m/z 250) HCl from the molecular ion (Fig. 2a).

The mass spectrum of NO-CAP was characterized by a molecular ion at m/z 306 with its chlorine isotope at m/z 308, and fragments produced by loss of one HCl (m/z 270 and 272) and two HCl (m/z 234). The mass spectrum of DH-CAP showed fragments with mass values two less than those of chloramphenicol, owing to the loss of two hydrogens (m/z 320, 322, 284, 286 and 248). For NAPD, only the molecular ion at m/z 212 was observed.

The usefulness of this technique is that the molecular ions of the compounds can be obtained.

Precision and accuracy

The C.V. of the assay were less than 15% for

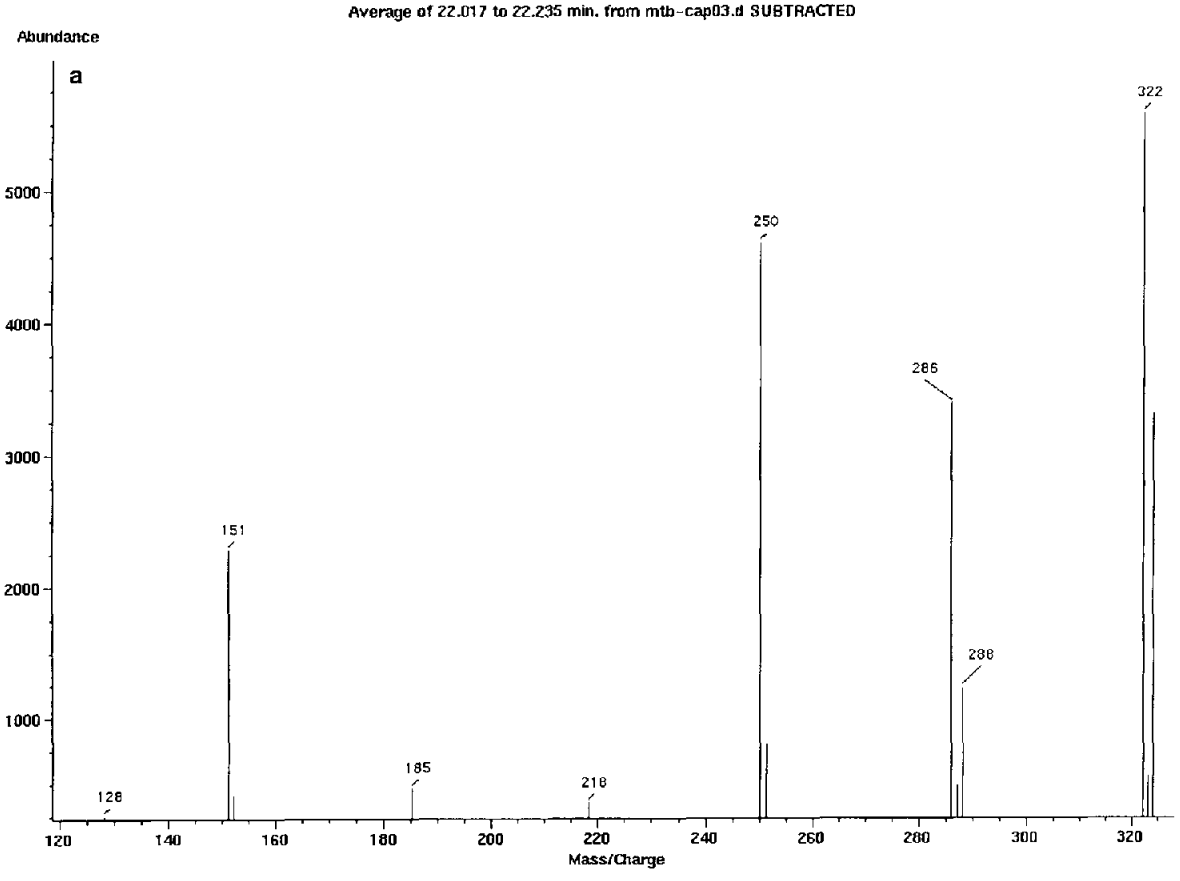
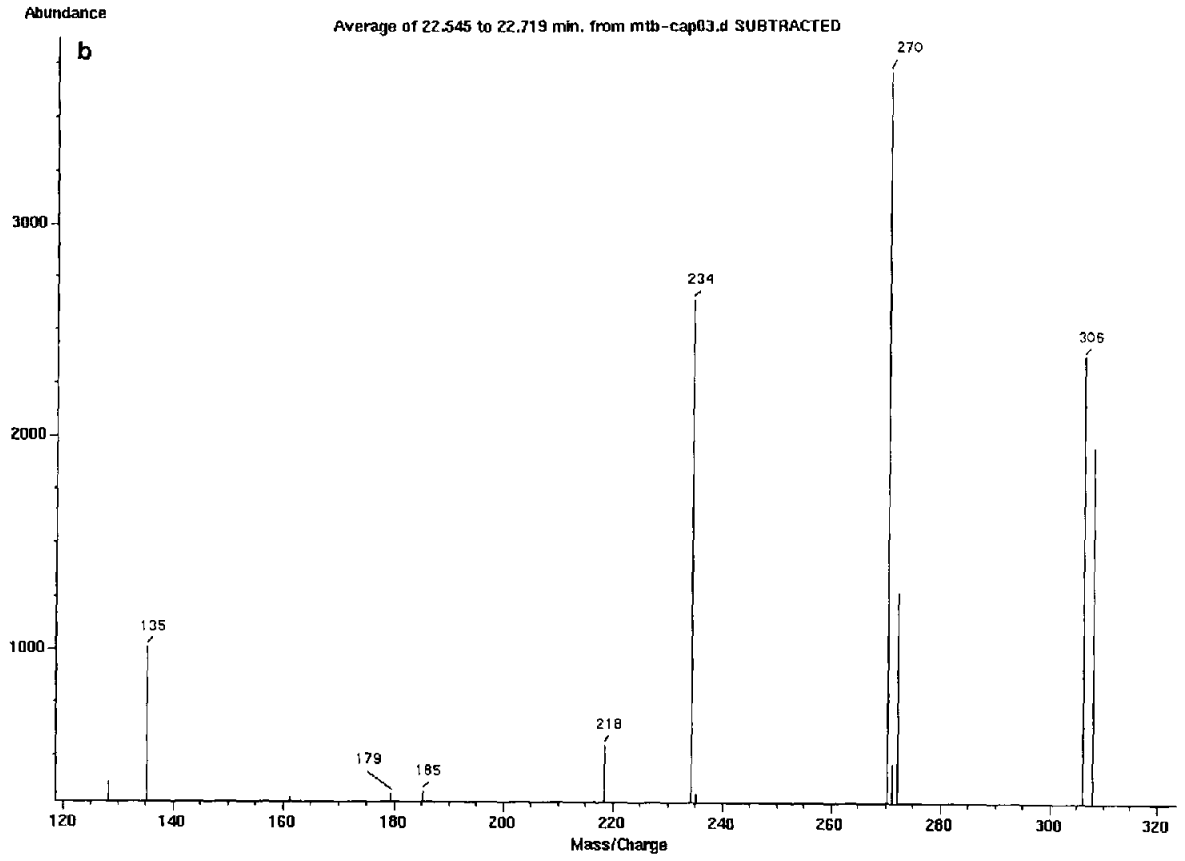


Fig. 2.



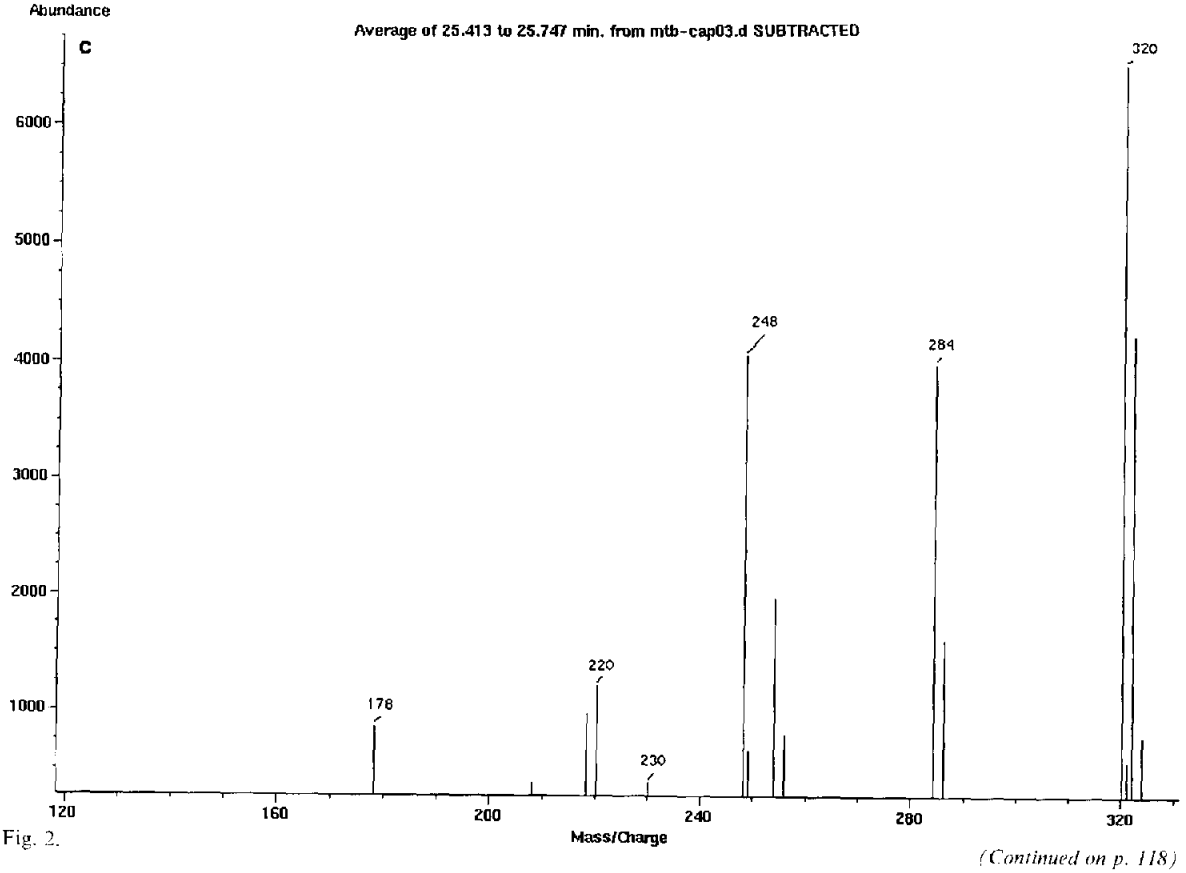


Fig. 2.

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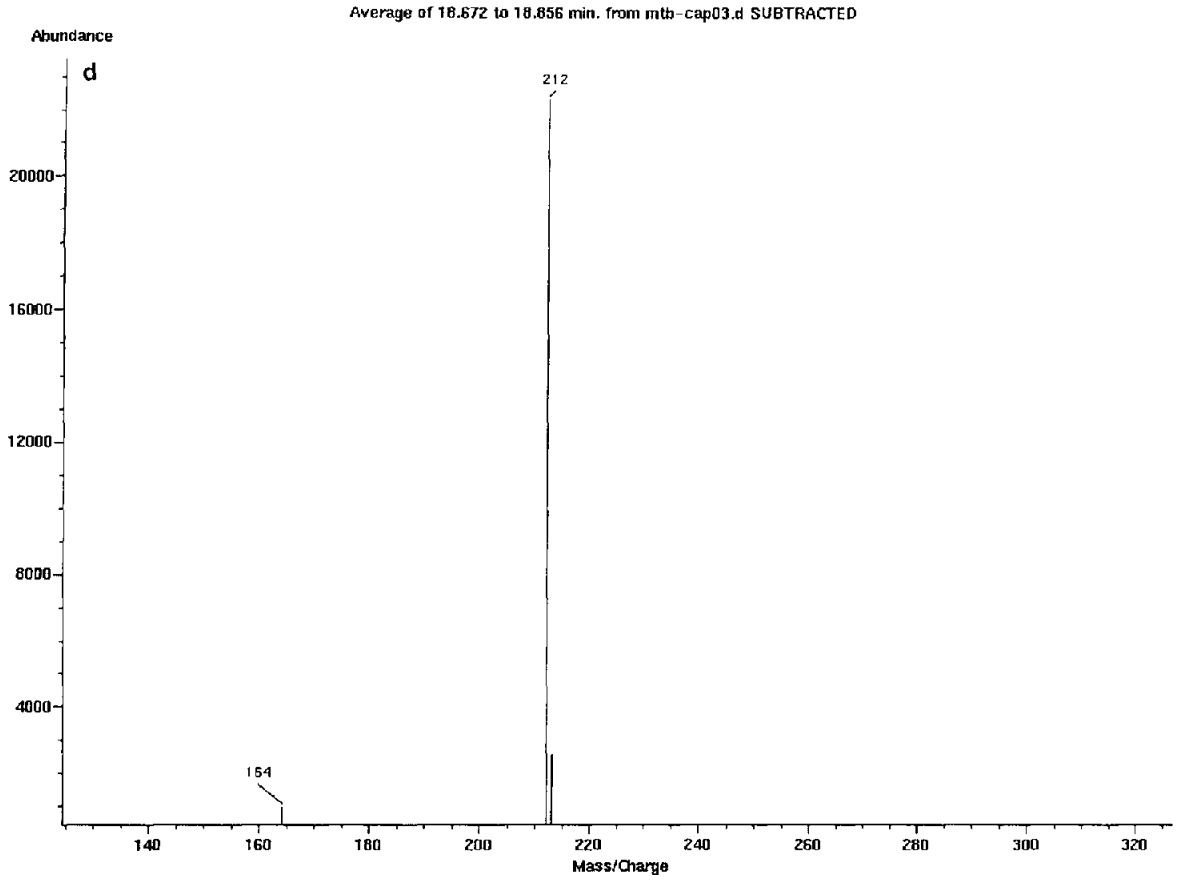


Fig. 2. Mass spectra of CAP (a), NO-CAP (b), DH-CAP (c) and NAPD (d) in standard solution.

TABLE I

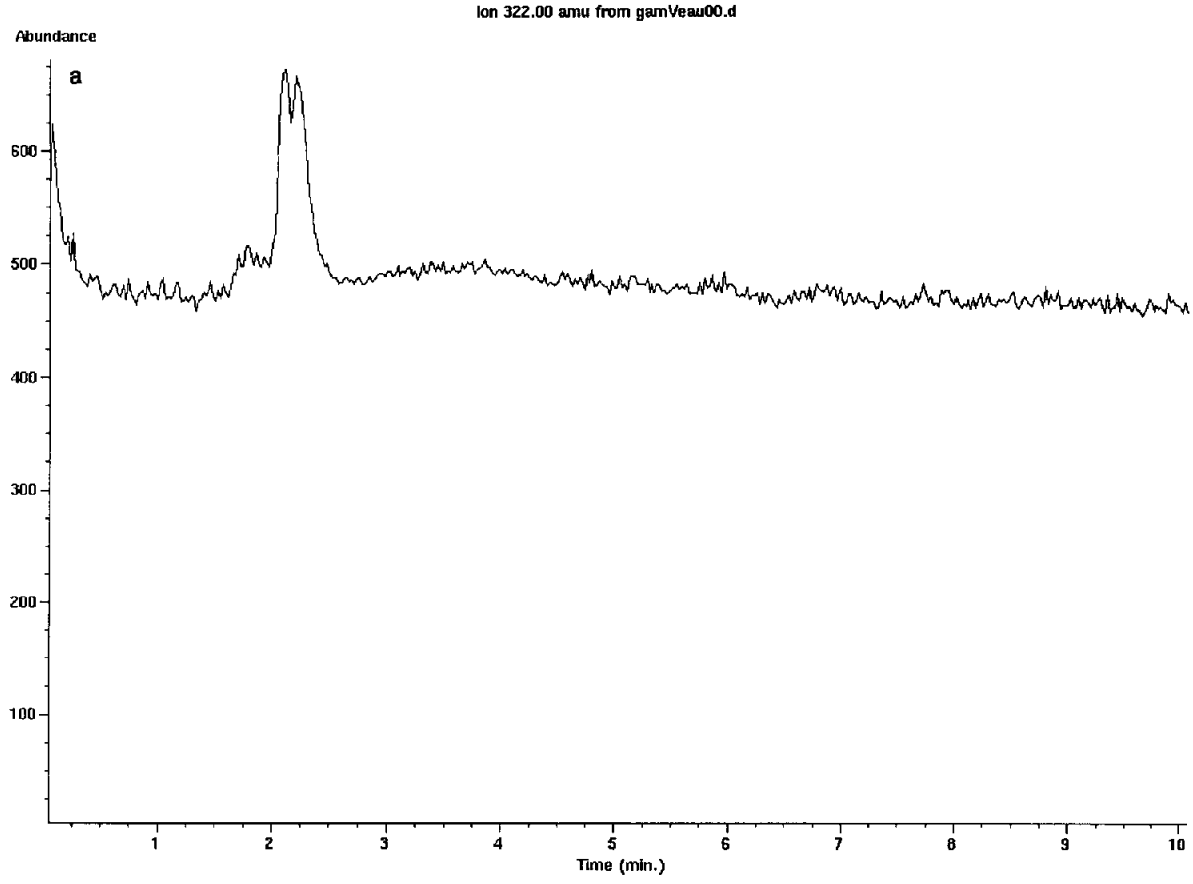
PRECISION AND ACCURACY OF THE HPLC–MS METHOD FOR CHLORAMPHENICOL IN SPIKED SAMPLES OF CALF MUSCLE

Added concentration (μg/kg)	Measured concentration, (mean ± S.D., n = 8) (μg/kg)	C.V. (%)	Error (%)
2	2.01 ± 0.2497	12.41	+0.5
5	4.96 ± 0.3294	6.64	−0.8
10	10.04 ± 0.5708	5.68	+0.4
20	19.99 ± 0.2218	1.11	−0.05

the four tested levels. The mean percentage error ranged from 0.05 to 0.8 for muscle concentrations varying between 2 and 20 μg/kg (Table I).

Standard curves

The four-point calibration graphs obtained on each day of the assay by plotting the peak-area ratio of m/z 322 *versus* the known muscle concentrations were straight lines (r^2 = 0.995) over the studied range of concentrations. The slopes (14 755 ± 294.26) varied between days; the daily calibration curve was used to determine the concentrations in test samples.



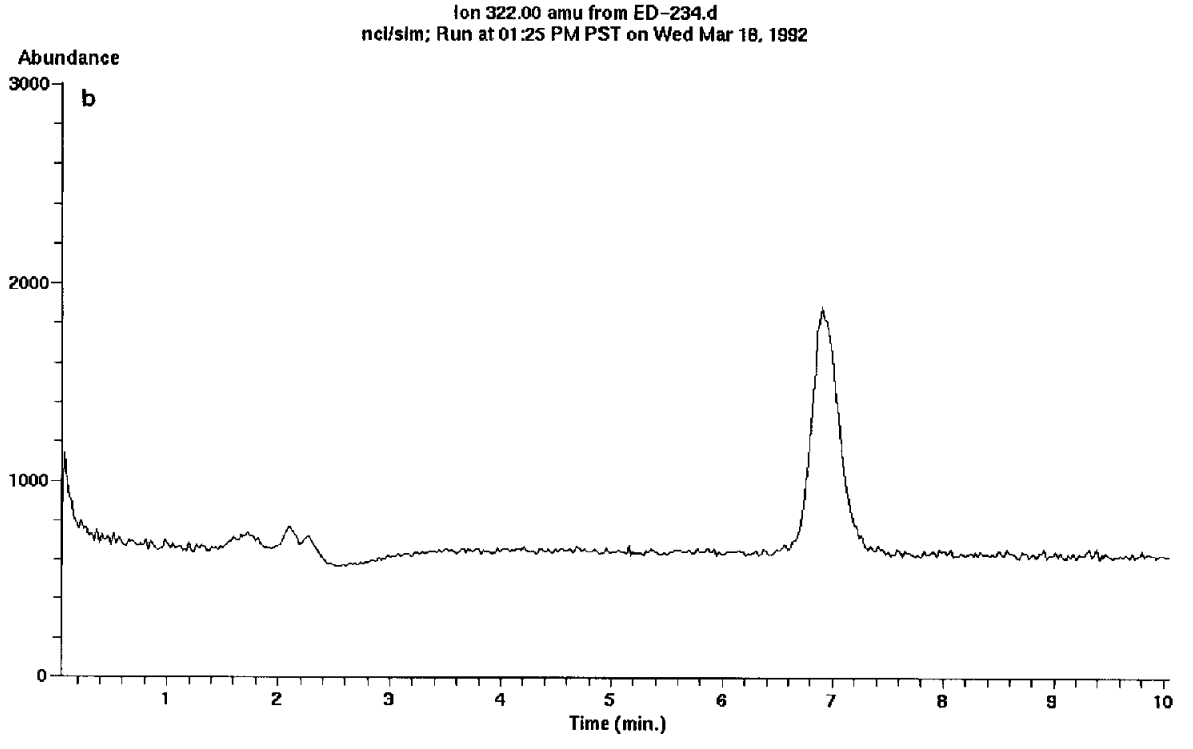


Fig. 3.

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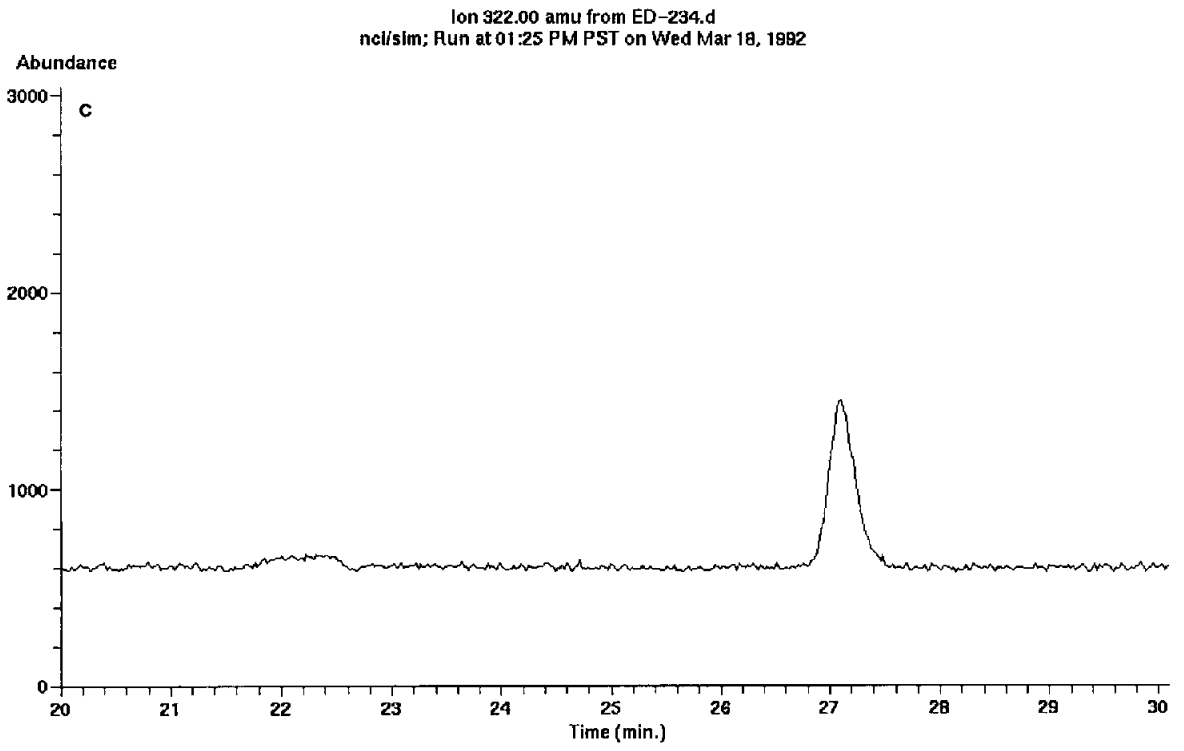

Fig. 3. LC mass chromatograms (SIM m/z 322) of (a) muscle blank sample, (b) muscle spiked at 10 μg/kg and (c) muscle of treated animals (6.91 μg/kg). Injection time, 20 min.

Limit of quantification and limit of detection

The limit of detection is the lowest concentration of an analyte that can be reliably differentiated from background levels. During the HPLC MS of four different blank samples, the SIM traces were free of endogenous interference at the retention time of CAP (Fig. 3a and b). The limit of quantification is 2 μg/kg, because the accuracy, precision and variability are acceptable for the objective of the method as defined by EC recommendations.

Ruggedness

Variation of the percentage of methanol in LC eluent modified the retention time. High electron energy and methane pressure increased peak m/z 322, whereas a high value of the helium pressure decreased this peak. CAP was more fragmented if the source temperature was increased; the m/z 322 abundance decreased and so did the sensitivity, because the method is based on SIM.

Residue data

The slaughter time of calves was calculated on the basis of a previous pharmacokinetic study after a similar dose: 48 h after an intravenous injection of 25 mg/kg CAP, concentrations in muscle were between 5 and 10 μg/kg for the three calves (Table II). The C.V. was less than 30%, as recommended by EC guidelines. The mass spec-

TABLE II

CHLORAMPHENICOL CONCENTRATIONS IN MUSCLE OF VEAL SLAUGHTERED 48 h AFTER INTRAVENOUS ADMINISTRATION (25 mg/kg)

Animal	Number of analyses	Concentration found, (mean ± S.D. (μg/kg)	C.V. (%)
I	6	11.19 ± 0.571	5.1
II	8	7.74 ± 1.565	20.2
III	8	5.90 ± 0.682	11.56

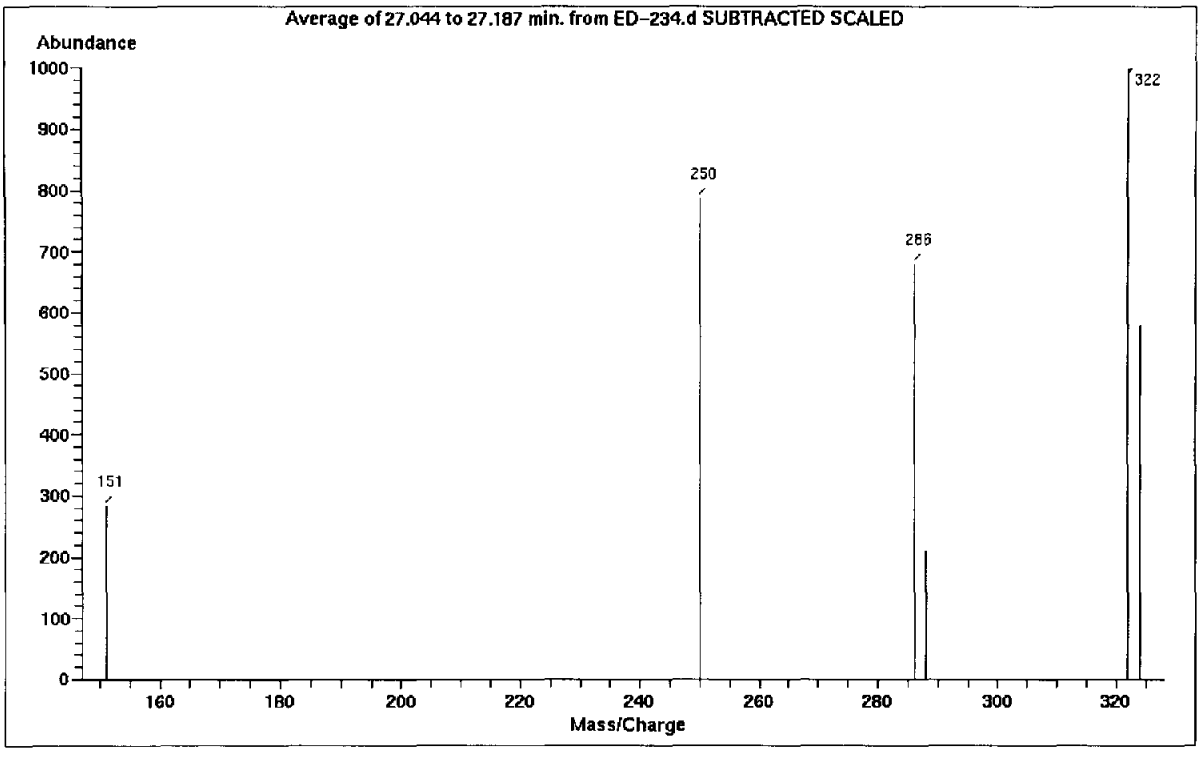

Fig. 4. Mass spectrum of CAP in muscle of a treated animal.

trum was similar to those of spiked samples (Fig. 4), and permitted the identification of the compounds in treated animals (Fig. 3c).

CONCLUSION

The particle beam interface was successfully used for the determination and quantitation of CAP in veal muscle at levels below 10 μg/kg. The sample preparation and extraction steps were those previously described for an HPLC–UV method [5]. The terminal recovery step and the HPLC flow-rate were modified only for the use of the particle beam and MS. For a positive sample, confirmation can be obtained more rapidly with this method than with a GC–MS method that needed a silylation step [3].

This HPLC–MS technique provides both good separation and good mass spectral data for CAP and three of its metabolites.

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