ELSEVIER

Journal of Chromatography B, 694 (1997) 401–407

JOURNAL OF CHROMATOGRAPHY B

Rapid method for the determination of ampicillin residues in animal muscle tissues by high-performance liquid chromatography with fluorescence detection

Wenhong Luo*, Catharina Y.W. Ang, Harold C. Thompson Jr.

Department of Health and Human Services, Food and Drug Administration, National Center for Toxicological Research, Division of Chemistry, Jefferson, AR 72079-9502, USA

Received 5 December 1996; revised 14 February 1997; accepted 24 February 1997

Abstract

A rapid and sensitive HPLC method was developed for the determination of ampicillin residues in muscle tissues of beef, pork, chicken and catfish. Muscle tissues were blended with a food processor into paste. A 5-g aliquot of the blended tissues was homogenized with 14 ml of 0.01 *M* phosphate buffer (pH 4.5) using a tissue homogenizer. Proteins were precipitated with the addition of 1 ml trichloroacetic acid (75%, w/v) followed by centrifugation. After filtration, 1 ml of the supernatant was reacted with formaldehyde under acidic and heating conditions. The ampicillin fluorescent derivative was then analyzed by reverse phase HPLC with fluorescence detection. Recoveries of spiked ampicillin at 5, 10 and 20 ng/g were >85%, with coefficients of variation <5%. The limit of detection and limit of quantitation for ampicillin in the tissues were 0.6 ng/g and 1.5 ng/g, respectively. The method is also applicable to the analysis of ampicillin residue in dry milk powder. © 1997 Elsevier Science B.V.

Keywords: Ampicillin

1. Introduction

Ampicillin is a widely used β-lactam antibiotic. Improper use of antibiotics in livestock may result in undesirable residues in milk and animal tissues. The regulatory tolerance level of ampicillin residue set by the US Food and Drug Administration is 10 ng/g in uncooked edible tissues of swine and cattle [1]. In the USA, ampicillin has not been approved for use on fish, therefore no tolerance level of residue is established for fish tissues.

Microbiological methods have been used for the screening and testing of antibiotic residues in milk and tissues [2,3]. These methods are relatively non specific and lack the sensitivity for testing residues in animal tissues. Several high pressure liquid chromatographic methods (HPLC) with different sample preparation procedures have been developed for the analysis of antibiotic residues in milk and tissues [4].

Nagata and Saeki [5] reported a HPLC method with UV detection for determination of ampicillin in fish tissues. In their procedure, fish tissues were extracted with methanol and cleaned up by a Florisil cartridge. The detection limit of the method was 30 ng/g of ampicillin in fish tissues. The HPLC method developed by Boison et al. [6] was able to detect

*Corresponding author.

0378-4347/97/$17.00 Published by Elsevier Science B.V.
PII S0378-4347(97)00171-0

penicillin G residues in animal tissues at 5 ng/g. The animal tissues were extracted with water and cleaned up with a C_{18} cartridge. After derivatization with 1,2,4-triazole-mercuric chloride, penicillin G residues were analyzed by reverse phase HPLC with UV detection. The HPLC method with UV detection developed by Moats [7] could detect penicillin G, penicillin V and cloxacillin in beef and pork tissues at 5 ng/g. Several other HPLC methods have been developed for the analysis of β-lactam antibiotic residues in milk [8–10]. All these methods suffered from either tedious extensive sample preparation and clean-up or relatively low sensitivity. Our objective was to develop an analytical method for the quantitative determination of ampicillin residues in animal muscle tissues at the regulatory tolerance level of 10 ng/g.

The present paper describes a simple, rapid and highly sensitive method for the determination of ampicillin residues in animal muscle tissues by HPLC with fluorescence detection.

2. Experimental

2.1. Materials

Ampicillin [D-(−)-α-aminobenzylpenicillin trihydrate] reference standard was obtained from Sigma (St. Louis, MO, USA). Trichloroacetic acid (ACS reagent grade) and formaldehyde (37%, w/v) water solutions were obtained from Aldrich (Milwaukee, WI, USA). All solvents were analytical or HPLC grade and supplied by J.T. Baker (Phillipsburg, NJ, USA). All other chemicals were ACS reagent grade.

Beef, pork, chicken and catfish samples were purchased from a local market and stored at −70°C until used.

2.2. Sample preparation, extraction and deproteinization

Beef, pork, chicken or catfish muscle tissue was blended with a food processor into a homogenous paste.

Blended muscle samples (5.0 g each) were weighed into 50-ml polyethylene centrifuge tubes. The muscle samples were fortified with ampicillin standard solution (250 ng/ml in water) at levels of 5, 10, and 20 ng/g. The fortified samples were allowed to equilibrated for 30 min before extraction. The sample was homogenized with 14 ml 0.01 *M* sodium phosphate buffer (pH 4.5) using a tissue homogenizer (Model Ultra-turrax T25, Ika-Labortechnik, Janke and Hunkel, Staufen, Germany) at 10 000 rpm for 2 min. Then 1 ml of trichloroacetic acid (75%, w/v) water solution was added into the tube, which was capped tightly and shaken vigorously for 30 s. The tubes were then centrifuged (3500 *g*) for 10 min. The supernatant was filtered through a #42 Whatman filter paper (Whatman, Clifton, NJ, USA) into another tube.

2.3. Derivatization reaction

A 1-ml aliquot of the filtrate was pipetted into a 15-ml graduated glass centrifuge tube. Then 0.2 ml of trichloroacetic acid (20%, w/v) water solution and 0.2 ml of formaldehyde (7%, w/v) water solution were added sequentially into the centrifuge tube, which was then vortexed for 20 s. The tube was capped loosely and heated in a water bath (100°C) for 30 min. After cooling down to room temperature, the contents in the graduated centrifuge tube were adjusted to 2 ml with 20% acetonitrile in water, mixed well then filtered through a 0.45-μm nylon microfilter (Whatman, Clifton, NJ, USA) into a sample vial and was ready for liquid chromatographic analysis.

2.4. Liquid chromatographic analysis

The liquid chromatographic system consisted of a 600E pump and pump controller, a 474 scanning fluorescence detector (Waters, Milford, MA, USA), with the excitation wavelength set at 346 nm and the emission wavelength set at 422 nm, a Model 717 autosampler (Waters) and a Prodigy 5 μm, ODS-3, 250 mm×4.6 mm I.D. HPLC column (Phenomenex, Torrance, CA, USA). HPLC data were acquired and processed using a PC and Millennium 2010 Chromatogram Manager software (Version 2.1, Waters). The injection volume was 100 μl. After injection, the sample was eluted with a mobile phase of acetonitrile–0.02 *M* KH_2PO_4 buffer with pH 3.5 (25:75, v/v) and a flow-rate of 1 ml/min. Peak area

of the ampicillin fluorescent derivative was integrated and used for quantitative analysis.

2.5. Calibration

Ampicillin stock solution (1 mg/ml, in water), which was prepared weekly and stored in refrigerator, was diluted with water to prepare calibration standard solutions of different concentrations. One ml aliquots of the standard solutions, 0.25, 0.5, 1.25, 2.5, 5 and 12.5 ng/ml were pipetted into six 15-ml graduated glass centrifuge tubes, respectively. These concentrations of ampicillin standard are equivalent to concentrations of 1, 2, 5, 10, 20 and 50 ng/g of ampicillin in the muscle tissue samples due to a dilution factor of 4 during the tissue sample extraction. A 0.2 ml-aliquot of trichloroacetic acid (20%, w/v, in water) solution and a 0.2-ml aliquot of formaldehyde (7%, w/v, in water) solution were added to each tube sequentially. The tubes were vortexed for 20 s and then heated in a water bath (100°C) for 30 min. After cooling to room temperature, the contents in each tube were brought to 2 ml with 20% acetonitrile in water. A 100-μl aliquot of each standard was injected into the HPLC (section 2.4) for analysis. The peak areas were used for construction of the calibration curve.

3. Results and discussion

This analytical method for the determination of ampicillin residues in animal tissues was developed following the criteria of the US FDA's general guideline [11]. The recovery and precision of the method were evaluated by analyzing samples fortified with ampicillin at levels of 5, 10 and 20 ng/g with $n \geq 5$ samples within each level. The recoveries, within-day variations and day-to-day variations were calculated and evaluated.

3.1. Sample preparation and extraction

Ampicillin in the fortified animal muscle tissues stored in freezer at −70°C was stable for at least 7 days. Animal muscle tissues were blended for 5 min using a food processor in order to obtain a homogenized paste. 5 g of the tissues were extracted with 14 ml of 0.01 *M* phosphate buffer (pH 4.5). The proteins were precipitated by mixing with 1 ml of 75% trichloroacetic acid followed by centrifugation. The total volume of the sample was 20 ml at this point. A 1-ml aliquot of the filtered supernatant was used for the derivatization reaction, which represented one twentieth of the sample of 5 g. No further clean-up or concentration of the extract was needed. The procedure for sample preparation and extraction was simple, rapid and quantitative.

3.2. Derivatization reaction

A 1-ml extract was reacted with formaldehyde under acid and heating conditions. The reaction conditions were adopted from our previous report of the analytical method for amoxicillin that is similar in structure to ampicillin [12]. The structure of the fluorescent derivative of ampicillin is depicted in Fig. 1 according to Uno et al. [13]. The maximum excitation wavelength and maximum emission wavelength of the fluorescent derivative of ampicillin were 346 nm and 422 nm according to Jusko [14]. These parameters were used for the HPLC-fluorescence analysis of the ampicillin derivative. The

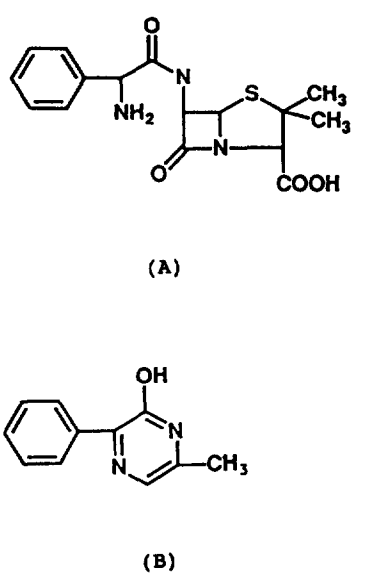

Fig. 1. Structures of (A) ampicillin and (B) ampicillin fluorescent derivative.

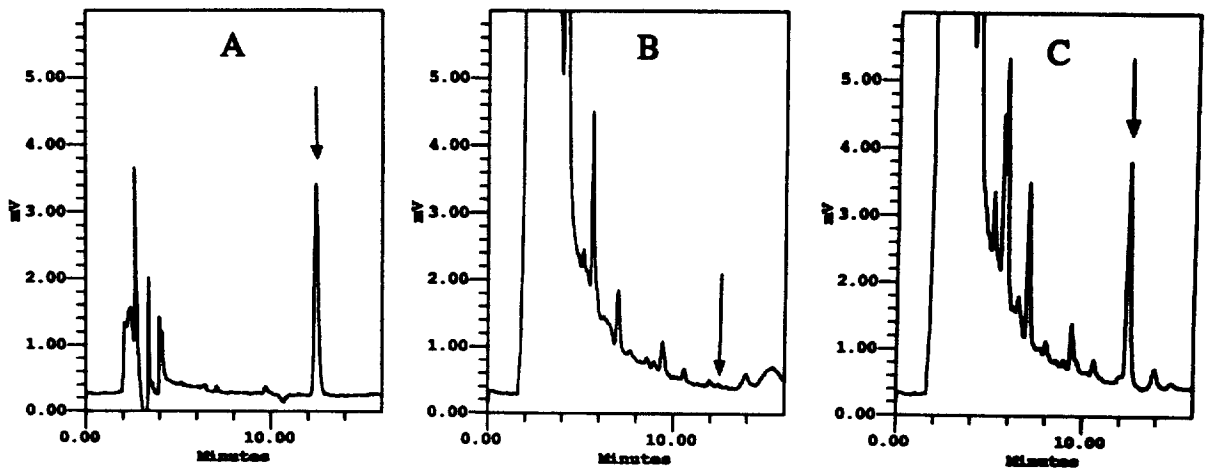

Fig. 2. Chromatograms of (A) standard ampicillin derivative, (B) beef muscle tissue blank and (C) beef muscle tissue fortified with ampicillin at 5 ng/g. Arrows indicate ampicillin peaks.

fluorescent derivative of ampicillin was stable in dark at room temperature within 48 h.

3.3. HPLC analysis

The retention time of the ampicillin fluorescent derivative was 12.5 min. Fig. 2 shows the chromatograms of ampicillin standard, beef muscle blank and beef muscle fortified with ampicillin at 5 ng/g. Fig. 3 Fig. 4 Fig. 5 illustrate the chromatograms of catfish muscle blank, catfish muscle fortified with ampicillin, chicken muscle blank, chicken muscle fortified with ampicillin, pork muscle blank, and pork muscle fortified with ampicillin, respectively. The ampicillin

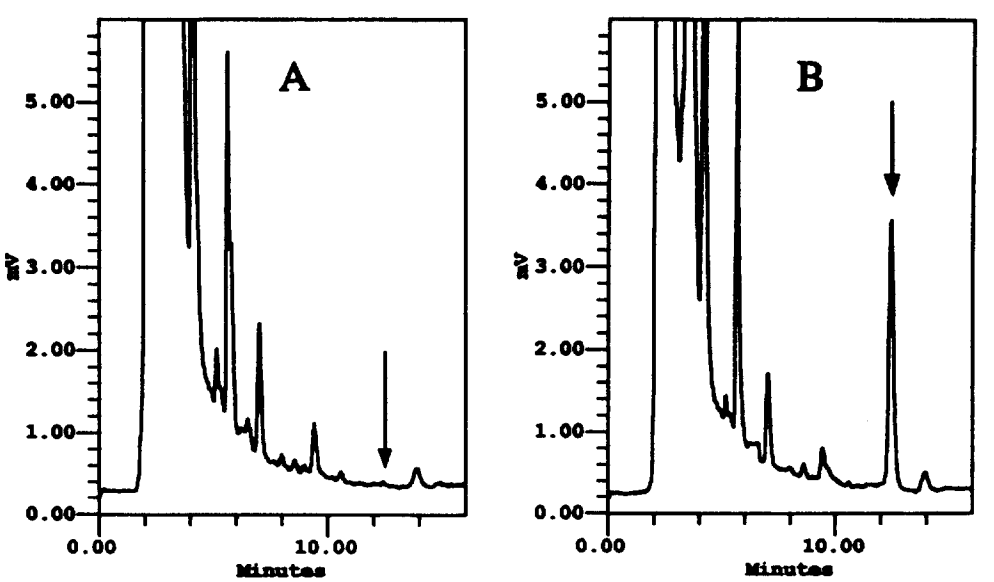

Fig. 3. Chromatograms of (A) catfish muscle blank and (B) catfish muscle tissue fortified with ampicillin at 5 ng/g.

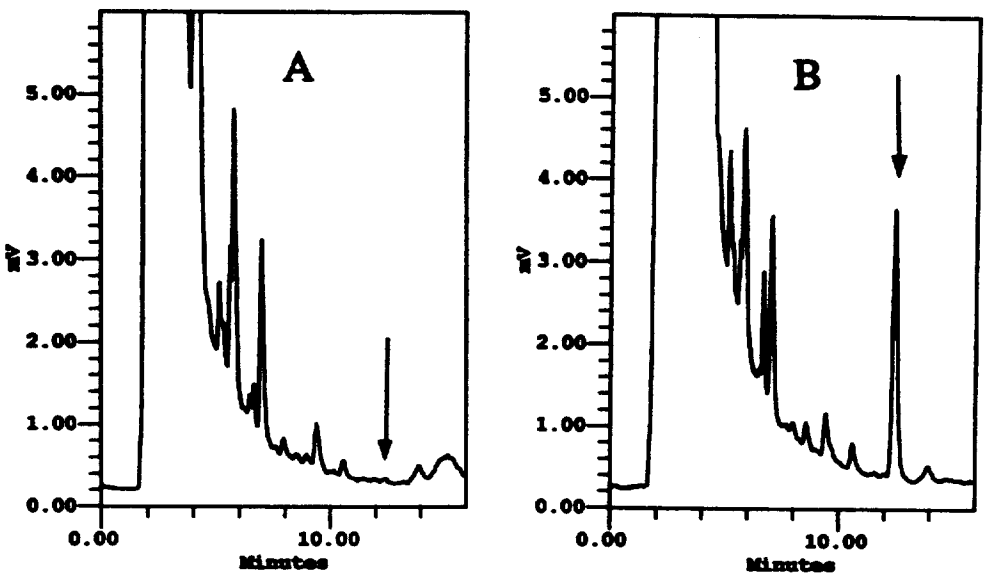

Fig. 4. Chromatograms of (A) chicken muscle blank and (B) chicken muscle tissue fortified with ampicillin at 5 ng/g.

fluorescent derivative was well resolved from interfering peaks in the muscle tissue samples. A pH of 3.5 was necessary for the phosphate buffer of the HPLC mobile phase to separate the ampicillin fluorescent derivative from interfering peaks. There were interfering peaks that co-eluted with the ampicillin fluorescence derivative when the pH value of the phosphate buffer of the HPLC mobile phase was higher than pH 4.0.

The linearity of the calibration curve for ampicillin

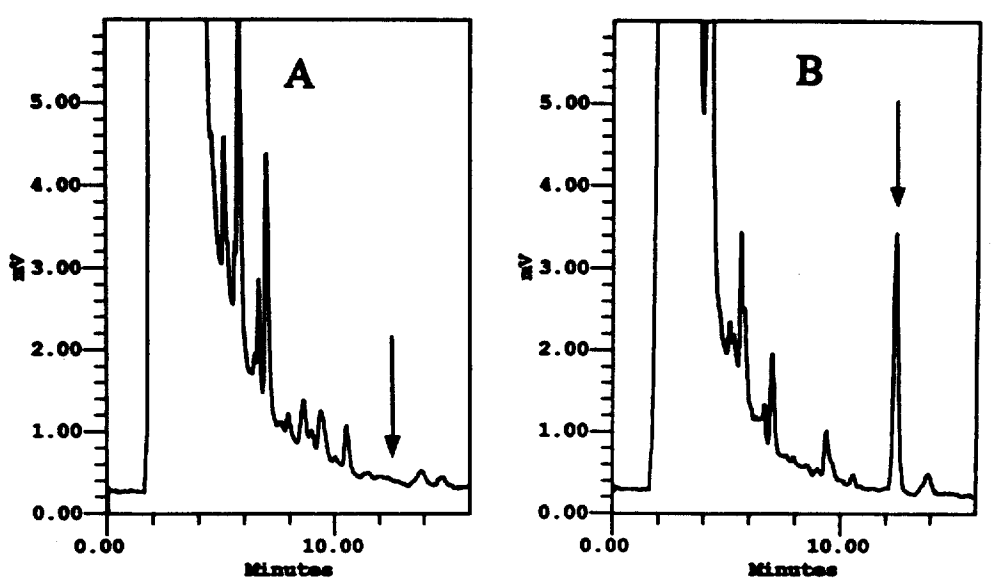

Fig. 5. Chromatograms of (A) pork muscle blank and (B) pork muscle tissue fortified with ampicillin at 5 ng/g.

Table 1
Recoveries of ampicillin from fortified beef muscle tissue samples (within-day)

Fortification level of ampicillin (ng/g)	Recovery (%)		n	C.V. (%)
	Range	Mean		
5	86.9–95.9	91.4	5	3.6
10	85.5–91.0	89.0	5	2.5
20	90.9–97.6	95.4	5	2.8

was good (r=0.9999) for a range of concentrations equivalent to 1 ng/g to 50 ng/g of ampicillin in the muscle tissue samples. The linear regression equation was $y=a_1x-a_0$, where y is the ampicillin content in tissue samples (ng/g) and x is the peak area count (mV). The coefficients a_1 and a_0 were 0.077 and 0.029, respectively. The confident interval (α=0.05) of the intercept (a_0) of the regression equation was calculated to be −0.006 to 0.064 which includes the point of 0. Therefore, for practical reasons an one-point calibration standard (10 ng/g) could be used for routine analyses.

3.4. Recoveries and variations

Samples of beef, pork, chicken and catfish muscle tissues were fortified with ampicillin at 5, 10 or 20 ng/g. Five replicates of samples at each level were analyzed in one day to evaluate the within-day recoveries and variations. Also samples of beef, pork, chicken and catfish muscle tissues fortified with ampicillin at 10 ng/g were analyzed on different days (n=5) to evaluate day-to-day recoveries and variations of the analytical procedure. These results are summarized in Table 1 Table 2 Table 3 Table 4 Table 5. The average recoveries were higher than 85% and the relative standard deviations (C.V. values) were less than 5%. These results met the requirements described in the US FDA's general guideline [11].

Table 2
Recoveries of ampicillin from fortified pork muscle tissue samples (within-day)

Fortification level of ampicillin (ng/g)	Recovery (%)		n	C.V. (%)
	Range	Mean		
5	87.1–95.6	91.9	5	3.7
10	86.6–91.1	88.5	5	1.8
20	89.0–94.4	92.0	5	2.2

Table 3
Recoveries of ampicillin from fortified chicken muscle tissue samples (within-day)

Fortification level of ampicillin (ng/g)	Recovery (%)		n	C.V. (%)
	Range	Mean		
5	87.6–94.3	92.2	5	2.9
10	89.7–95.5	92.7	5	2.9
20	91.0–96.5	94.9	5	2.3

Table 4
Recoveries of ampicillin from fortified catfish muscle tissue samples (within-day)

Fortification level of ampicillin (ng/g)	Recovery (%)		n	C.V. (%)
	Range	Mean		
5	90.5–97.8	93.9	5	2.9
10	85.2–94.4	89.9	5	4.3
20	93.5–97.7	95.2	5	1.8

The same procedure was also applied to the analysis of ampicillin residue in dry milk powder. Two grams of dry milk powder was extracted with 17 ml of phosphate buffer and 1 ml of trichloroacetic acid with a dilution factor of 10. The recoveries of fortified ampicillin at 25 ng/g or 50 ng/g were higher than 80% with relative standard deviations less than 5%.

3.5. Limit of detection and limit of quantitation

More than thirty blank samples of beef, pork, chicken and catfish were analyzed. The backgrounds of all the blank samples were similar. No significant interfering peak was found at the retention time of

Table 5
Recoveries of ampicillin from fortified animal muscle tissue samples (day-to-day)

Muscle tissue	Recovery (%)		n	C.V. (%)
	Range	Mean		
Beef	84.8–91.1	87.8	5	2.8
Pork	85.3–94.2	89.6	5	3.6
Chicken	86.1–92.7	88.7	5	2.8
Catfish	89.1–96.8	91.4	5	3.6

Ampicillin fortification level was 10 ng/g for all muscle tissues.

the ampicillin fluorescent derivative. The limit of detection (LOD) and limit of quantitation (LOQ) were estimated according to the guidelines of the American Chemical Society [15]. The LOD was defined as the mean blank response plus three times the standard deviation of replicate analyses of blank samples. The LOQ was defined as the mean blank response plus ten times the standard deviation of replicate analyses of blank samples. Therefore, based on the analytical results of the blank samples, The LOD and LOQ of the analytical method for ampicillin residues were calculated as 0.6 ng/g and 1.5 ng/g of muscle tissues, respectively.

In order to confirm the estimated LOQ, eight samples (two for each) of beef, pork, chicken and catfish muscle tissues were fortified with ampicillin at 2 ng/g and analyzed. The average recovery of ampicillin was 83% with relative standard deviation (C.V.) 5.2%, which indicated the analyses of ampicillin residues were quantitative at the fortified level of 2 ng/g of muscle tissues. Fig. 6 is the chromatogram of a typical tissue sample fortified with ampicillin at 2 ng/g. The ampicillin peak indicated by an arrow was determined to be 1.7 ng/g, which is very close to the estimated value of LOQ.

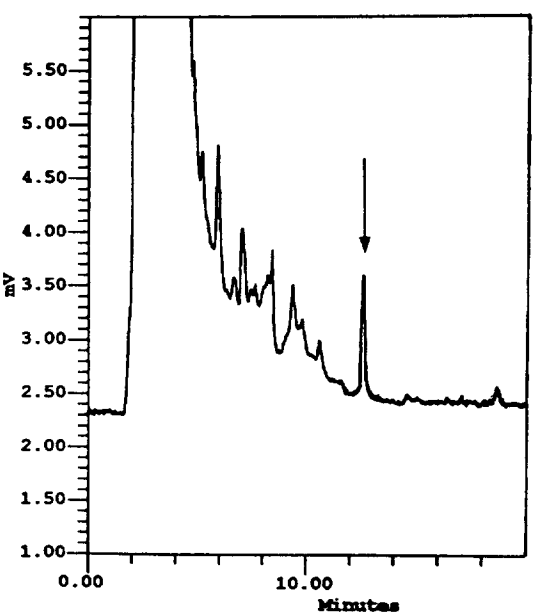

Fig. 6. Chromatogram of a typical animal muscle tissue sample fortified with ampicillin at 2 ng/g. The ampicillin peak indicated by an arrow was determined to be 1.7 ng/g.

3.6. Conclusion

A highly sensitive analytical method for the HPLC determination of ampicillin residues in animal muscle tissues has been developed. The sample preparation and extraction procedure was simple, rapid and quantitative. No extensive sample clean-up procedure was required. The method described is sensitive, precise and accurate. It meets the requirements of US FDA's general guideline for monitoring drug residues in animal tissues.

References

[1] Code of Federal Regulations, CFR 21.556.40, 1995.
[2] J.W. Messer, J.E. Leslie, G.A. Houghtby, J.T. Peeler, J.E. Barnett, J. Assoc. Off. Anal. Chem. 65 (1982) 1208.
[3] S.M. Plakas, A. DePaola, M.B. Moxey, J. Assoc. Off. Anal. Chem. 74 (1991) 910.
[4] M. Petz, in V.K. Agarwal (Editor), Antibiotic Drug Residues in Food Products of Animal Origin, Plenum Press, New York, NY, 1992, p. 147.
[5] T. Nagata, M. Saeki, J. Assoc. Off. Anal. Chem. 69 (1986) 448.
[6] J.O. Boison, C.D.C. Salisbury, W. Chan, J.D. MacNeil, J. Assoc. Off. Anal. Chem. 74 (1991) 497.
[7] W.A. Moats, J. Chromatogr. 593 (1992) 15.
[8] R.F. Straub, R.D. Voyksner, J. Chromatogr. 647 (1993) 167.
[9] W.A. Moats, J. AOAC Int. 77 (1994) 41.
[10] E. Verdon, P. Couedor, J. Pharm. and Biomed. Anal. 14 (1996) 1201.
[11] U.S. Food and Drug Administration, Memorandum: General Principles for Evaluating the Safety of Compounds Used in Food Producing Animals, FDA, Rockville, MD, USA, 1986.
[12] C.Y.W. Ang, W.H. Luo, E.B. Hansen, J.P. Freeman, H.C. Thompson, J. AOAC Int. 79 (1996) 389.
[13] T. Uno, M. Masada, Y. Kuroda, T. Nakagawa, Chem. Pharm. Bull. 29 (1981) 3651.
[14] W.J. Jusko, J. Pharm. Sci. 5 (1979) 728.
[15] American Chemical Society Committee on Environmental Improvement, Anal. Chem., 52 (1980) 2242.