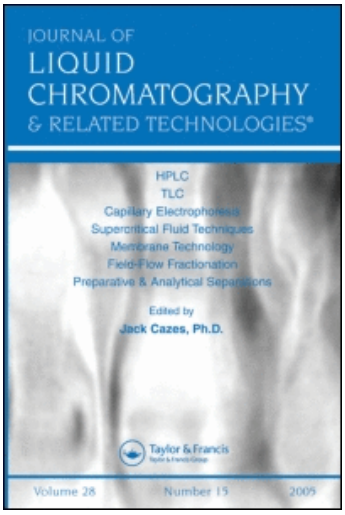

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

High Performance Liquid Chromatographic Determination of Gentamycin in Animal Tissue

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To cite this Article Agarwal, Vipin K.(1989) 'High Performance Liquid Chromatographic Determination of Gentamycin in Animal Tissue', Journal of Liquid Chromatography & Related Technologies, 12: 4, 613 — 628
To link to this Article: DOI: 10.1080/01483918908051762
URL: http://dx.doi.org/10.1080/01483918908051762

JOURNAL OF LIQUID CHROMATOGRAPHY, 12(4), 613-628 (1989)

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF GENTAMYCIN IN ANIMAL TISSUE

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ABSTRACT

A liquid chromatographic method for the determination of gentamycin in animal tissue is described. Gentamycin is quantitatively extracted from animal tissue with potassium phosphate buffer and the extract is deproteinated. The deproteinated extract is acidified to pH 6.4 - 6.5 with sulfuric acid and is purified by ion exchange gel chromatography (CM-sephadex). After elution from the ion exchange column with alkaline buffer, the eluent is further purified using a silica Sep-pak cartridge and derivatized on the cartridge with O-phthalaldehyde. The derivatized gentamycin is eluted from the Sep-pak cartridge with ethanol, and analyzed by liquid chromatography with fluorometric detection. Recoveries ranged from 86 to 108% in samples fortified with gentamycin at 0.5 to 10 ppm levels. The detection limit was 0.2 ppm.

INTRODUCTION

Antibiotics are widely used to treat a variety of serious infections in livestock and subtherapeutically

to prevent disease and to promote growth (1,2). Gentamycin, streptomycin, dihydrostreptomycin, and neomycin are the aminoglycoside antibiotics which have been approved for veterinary use in food producing animals only for oral administration (3). Gentamycin, which has a broad spectrum of activity against gram positive and gram negative bacteria, consists of three major components C1, Ca and C2. All of these resemble each other in chemical nature and have similar antibacterial activities (4).

Microbiological methods have traditionally been used to detect residues of aminoglycoside antibiotics including gentamycin in food products of animal origin (5,6). These methods have been found to be inaccurate because of their lack of specificity and the ability to distinguish among different aminoglycosides (7). A number of chemical and physical methods have also been reported for the determination of gentamycin, including thin layer chromatography (8,9), gas chromatography (10), and liquid chromatography (11-19). Liquid chromatography (LC) has become the analytical method of choice because of its specificity, sensitivity, and precision. LC has been successfully applied to the determination of gentamycin in biological fluids i.e., plasma and serum (11-19). Its application, however, for the determination of residues of gentamycin in tissues has not been reported.

This paper reports the development of an analytical method for the determination of gentamycin in animal tissue using liquid chromatography with fluorometric detection..

METHOD

Reagents and standards:

a.) Chemicals.- Gentamycin sulfate (Sigma Chemical Co., St. Louis, MO); potassium phosphate monobasic and dibasic, boric acid, potassium hydroxide, sodium hydroxide, acetic acid, sodium sulfate and HPLC grade methanol (Fisher Chemical Co., Fairlawn, NJ); 2-mercaptoethanol, O-phthalaldehyde (OPA) and 1-heptanesulfonic acid sodium salt (Sigma Chemical Co., St. Louis, MO); CM-sephadex, C-25 (Pharmacia Inc., Piscataway, N.J.)

b.) Mobile phase for LC.- Solvent A - Heptanesulfonic acid, sodium salt (5.0 gm) was dissolved in one liter of acetic acid: water: methanol, (10:290:700); Solvent B - methanol.

c.) Extraction buffer.- 0.1M potassium phosphate buffer (Dissolve 17.0 gm dibasic potassium phosphate in 1000 ml (0.1M) aqueous sodium sulfate solution) This buffer is buffer 4 in Table I. Other extraction buffers were prepared in a similar manner and the pH were adjusted with either phosphoric acid or potassium hydroxide as required.

d.) Alkaline buffer.- 10 mM sodium hydroxide in 0.2 M sodium sulfate solution.

e.) Gentamycin standard solutions.- Gentamycin sulfate (100 mg) was dissolved in 1000 ml distilled water and stored in the refrigerator in a polypropylene bottle. Working Standards: 1.0 ml, 2.5 ml, 5.0 ml, 10 ml, 25 ml and 50 ml of stock solutions were diluted to 100 ml with distilled water to provide concentrations of 1.0, 2.5, 5.0, 10, 25, and 50 μg/ml. Meat samples were fortified using these diluted standards.

f.) Potassium borate buffer.-Boric acid (3.1 gm) was dissolved in 100 ml distilled water and pH was adjusted to 10.5 with 50% potassium hydroxide solution. Final volume was made to 125 ml with distilled water.

g.) OPA reagent.- O-phthalaldehyde (100 mg) was dissolved in 1 ml methanol and 200 μl of 2-mercaptoethanol and 20 ml potassium borate buffer (f) were added to it to prepare the reagent. This reagent was stored refrigerated in an amber glass capped vial. The reagent may be used for a week.

Apparatus:

a.) Silica Sep-pak cartridges (Waters, Milford, MA)

b.) Centrifuge: International Centrifuge Model EXD (International Centrifuge Co., Boston, MA)

c.) Centrifuge Tube: 100 ml polypropylene tube.

d.) Vortex mixer. Bronwill vortex mixer or equivalent.

e.) Liquid chromatograph.- LDC/Milton Roy (Riviera

Beach, FL) ConstaMetric III pumps were used for solvent delivery. A Gradient Master (LDC/Milton Roy) was used to control gradient flow. A Lee mixer (The Lee Co., Westbrook, CT) with a mixing volume of 200μl was used for solvent mixing. An FS-970 LC Fluorometer (Kratos Analytical Instruments, Westwood, NJ) was used as the detector.

f.) LC Column.- Ultremex C18, 25cm X 4.6 mm, 5μm particle size (Phenomenex, Rancho Palos Verdes, California).

Extraction:

Ground beef tissue (5.0 gm) was placed in a 100 ml polypropylene tube, fortified with the appropriate amount of standard and mixed thoroughly. Potassium phosphate buffer (15 ml) was added to the tube, shaken on the vortex mixer for about 10 minutes and allowed to stand for about 10 minutes. The tube was again shaken on the vortex mixer and then centrifuged for about 20 minutes at 2000 rpm. The supernatant was withdrawn to another polypropylene tube and 10 ml fresh potassium phosphate buffer was added to the tube containing tissue pellet. The pellet with buffer was shaken on the vortex mixer and allowed to stand for about 5 minutes. After shaking again, the tube was centrifuged and the supernatant was withdrawn. The same procedure was repeated three more times using 10 ml buffer each time.

All the supernatants were combined in a 100 ml polypropylene tube.

Deproteination:

The polypropylene tube containing the extract was immersed in boiling water for about 5 minutes with occasional shaking, cooled to the room temperature, and then centrifuged for 10 minutes at 2000 rpm. The supernatant was filtered through a filter paper on a glass funnel into a 100 ml beaker. Potassium phosphate buffer (10 ml) was added to the centrifuge tube, shaken well on the vortex mixer, and centrifuged for about 10 minutes. The supernatant was filtered into the same 100 ml beaker. This step was repeated with an additional 10 ml buffer. The filter paper and the funnel were washed with an additional 2 ml buffer. The pH of the extract was adjusted to 6.4-6.5 with 1M sulfuric acid and the total volume was made to exactly 60 ml with distilled water. The extract was transferred to a 100 ml polypropylene tube. This solution can be stored in the refrigerator for 2-3 days for later use.

Cleanup and derivatization with OPA:

Separation of gentamycin from the other compounds present in the tissue was done by modified ion exchange gel chromatography, originally described by Anhalt and Brown(12), followed by a silica Sep-pak cartridge cleanup. CM-sephadex (C-25) ion exchange resin was

equilibrated in 0.2M sodium sulfate solution for 24 hours. A column was prepared by placing glass wool in the bottom of a Fisher brand pasteur pipet and filled about 4 cm (approximate bed volume 1.5 ml) with CM-sephadex resin. The column was washed with 2 ml 0.2M sodium sulfate solution and then a 12 ml portion of the extract was passed through the column. Some pressure was applied to the top of the column with pipet bulb in order to maintain a flow of about 2-4 ml per minute. Pressure should not be released before removing the bulb from the pipet to avoid any disruption of the column bed due to air drawn into the column. The column was washed with 5.0 ml of 0.2M sodium sulfate solution. Gentamycin was eluted from the column with alkaline buffer (10 mM NaOH in 0.2M sodium sulfate solution). The initial 0.6 ml of eluent (void volume) was discarded and the next 2 ml of eluent was collected in a 5 ml syringe fitted with a silica Sep-pak cartridge. (Silica cartridge was conditioned with 5 ml distilled water before using.) The eluent was passed through the Sep-pak Cartridge. (For OPA derivatization of the standards the aqueous solution of standard gentamycin were loaded on the silica sep-pak.) The Sep-pak cartridge was washed with 5 ml distilled water, 5 ml ethanol and finally with 5 ml distilled water. The elution flow rate was maintained at about 5 ml/minute. The OPA reagent (2 ml) was loaded

into the syringe, passed through the silica cartridge at a rate of about 1 ml/minute, and allowed to stand for about one minute. The OPA derivatized gentamycin was then eluted with ethanol. The first 0.5 ml (void volume) of eluent was discarded and the next 3 ml of eluent was collected in a graduated sample vial. The total eluent was made to exactly 4 ml by adding water and 10-20 μl was injected into the HPLC.

Liquid Chromatography:

The following operating conditions were used.- A gradient elution starting with 20% solvent B at zero time to 60% solvent B in 15 minutes on curve 5 maintaining a flow rate of 1.6 ml/minute. (curve 5 was the most concave curve on LDC gradient master). The peaks were quantitated based on peak heights. An excitation wavelength of 340nm with a KV 418 filter as emmission filter was used. The detector was set at 0.5 A range and the sensitivity was reduced if the concentration of gentamycin was greater than 2ppm.

RESULTS AND DISCUSSION

Calibration plots for the OPA derivative of gentamycin standards were obtained by plotting the mean peak heights versus the antibiotic concentrations in the 2 to 50 ng range. The plots were linear, and in all cases correlation coefficients were found to be greater than 0.999.

Several different buffers were used for extraction to obtain complete recovery of gentamycin from the tissues. The results of the recovery of gentamycin from ground beef tissue fortified at the 2 ppm level are shown in Table I. Only alkaline buffers were found to extract gentamycin. An acidic buffer (monobasic potassium phosphate, pH 4.5, buffer 1 in table I) did not extract gentamycin from the tissue. Addition of sodium sulfate to the buffers improved recoveries. Recoveries above 92% were obtained using 0.1M potassium phosphate buffer in 0.1M sodium sulfate solution (pH 8.8, buffer no 4 in Table I). Therefore 0.1M potassium phosphate buffer (pH 8.8) in 0.1M sodium sulfate was used as extracting buffer.

Gentamycin is a complex of three major components. Under the HPLC conditions used, all three components are well separated. Figure 1 shows a representative chromatogram of the OPA derivative of standard gentamycin. The three components, C1, C1a and C2 have retention times of 10.2, 16.6, and 18.0 minutes. The third peak labelled as component C2 consists of C2 and a minor component C2a which is always present in the standard. C2a sometimes shows as a shoulder in the third peak (component C2) due to minor changes in the chromatographic conditions. An unfortified ground beef tissue sample was processed through the complete

Table I

Recovery of gentamycin from ground beef tissue fortified at 2 ppm level.

Extraction Solvent	pH	Recovery, % ± SD*
1. 0.2M pot. phosphate	4.5	no recovery
2. 0.2M pot. phosphate	8.0	71.3 ± 2.1
3. 0.2M pot. phosphate	8.8	79.2 ± 3.8
4. 0.1M pot. phosphate + 0.1M sod. sulfate	8.8	98.1 ± 4.1
5. 0.1M pot. phosphate + 0.2M sod. sulfate	8.8	92.6 ± 6.0
6. 0.1M pot. phosphate	10.0	91.7 ± 0.7
7. 0.1M pot. phosphate + 0.1M sod. sulfate	10.0	92.2 ± 3.0

* average of three trials.

pot. = potassium; sod. = sodium

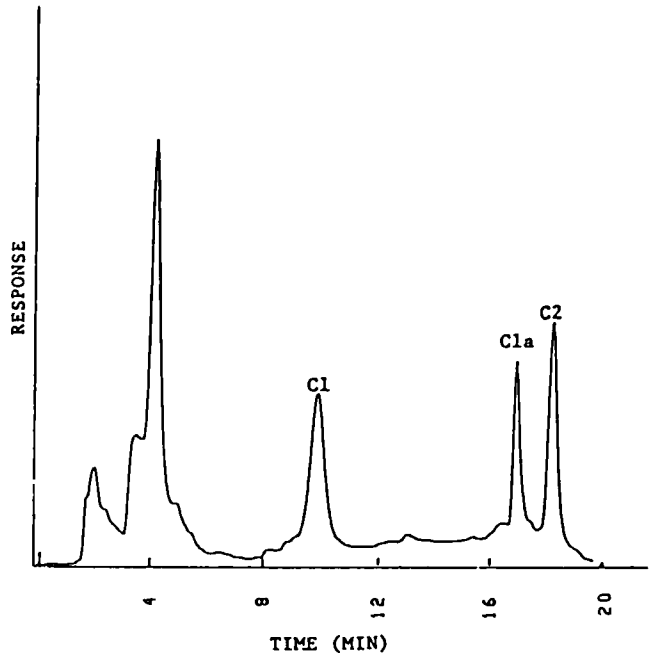

Figure 1. Chromatogram of the OPA derivative of standard gentamycin.

extraction, cleanup and derivatization procedure and examined by HPLC (Figure 2). There were no interfering compounds present in the sample which eluted at the retention time of C1a and C2. A broad peak, however, was present at the retention time of the C1 component. This made it impossible to quantitate the C1 component in the spiked sample. It was decided, therefore, to quantitate gentamycin based on the sum of the peak heights of C1a and C2.

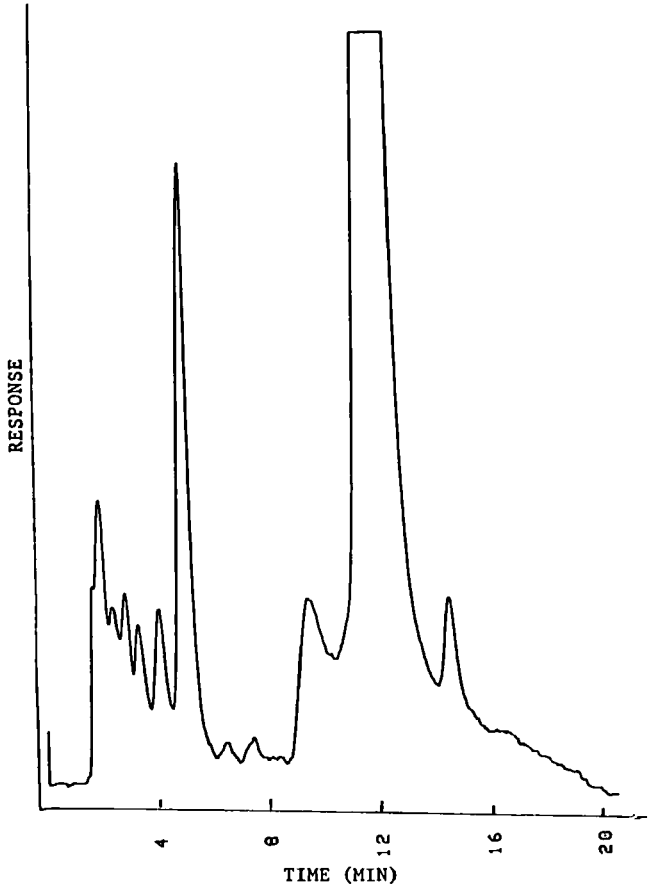

Figure 2. Chromatogram of beef tissue extract.

The ground beef samples were spiked with standard gentamycin at 0.2, 0.5, 1, 2, 5, and 10ppm levels. All these samples went through the entire purification and derivatization procedure and examined by LC. Figure 3 shows a representative chromatogram of a sample spiked at the 2ppm level. Gentamycin C1a and C2 are well

Table II

Recovery of gentamycin from ground beef tissue fortified with gentamycin, extracted with 0.1M potassium phosphate buffer in 0.1M sodium sulfate.

Fortification level ppm	Recovery %
0.2	88.2
0.2	69.4
0.2	91.5
Average ± SD	83.0 ± 9.8
0.5	97.8
0.5	93.3
0.5	105.2
Average ± SD	98.8 ± 4.8
1.0	93.3
1.0	85.9
1.0	97.0
Average ± SD	92.1 ± 4.5
2.0	97.4
2.0	102.6
2.0	95.9
Average ± SD	98.6 ± 3.0
5.0	95.3
5.0	101.2
5.0	103.1
Average ± SD	99.8 ± 3.2
10.0	107.6
10.0	102.7
10.0	93.3
Average ± SD	101.2 ± 5.9

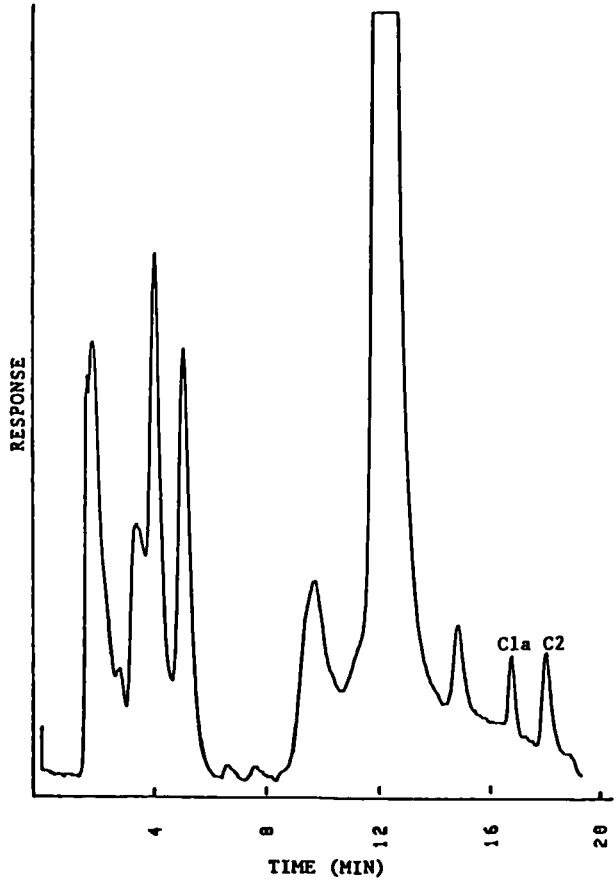

Figure 3. Chromatogram of beef tissue extract fortified at 2ppm level with gentamycin.

resolved. The recoveries, calculated based on the sum of the peak heights of C1a and C2, are shown in Table II and ranged from 85.9% to 107.6% in samples fortified between 0.5ppm to 10ppm levels. For samples which were fortified at levels lower than 0.5ppm it was necessary to use larger volumes of the extract for cleanup and

derivatization steps. For example, in samples fortified at 0.2ppm level, 25ml of the extract was used instead of the usual 12 ml. This improved the resolution of the peaks but quantitation remained difficult because of the increased base line noise. The recoveries ranged from 69.4% to 91.5% with a standard deviation of 9.8.

In conclusion, the method presented in this report can successfully be applied for the determination of gentamycin in animal tissues at a level as low as 0.2ppm. The method is accurate and reproducible.

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