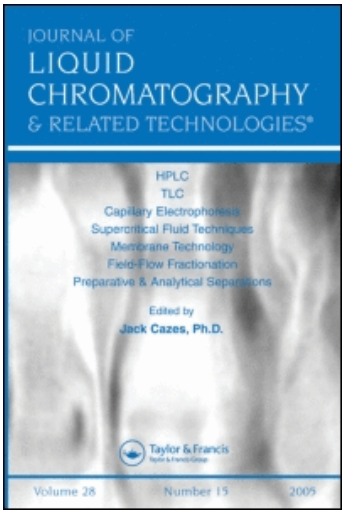

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High Performance Liquid Chromatographic Determination of Neomycin in Milk Using a Hisep Column

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF NEOMYCIN IN MILK USING A HISEP COLUMN

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ABSTRACT

A high performance liquid chromatographic (HPLC) method for the determination of neomycin in milk is described. Milk is passed directly through an amberlite CG-50 ion exchange resin column, and the neomycin which is retained on the column is derivatized with ortho-phthalaldehyde (OPA) reagent. The derivatized neomycin is eluted from the column with potassium borate buffer/methanol and analyzed by HPLC. A HISEP HPLC column with fluorometric detection was used. Recoveries ranged from 94 to 102% in samples fortified between 0.1 to 5ppm levels. The detection limit is 50ppb.

INTRODUCTION

Neomycin is a widely used broad spectrum aminoglycoside antibiotic that inhibits the growth of both gram positive and gram negative bacteria (1). It has a narrow therapeutic range, is potentially toxic like other aminoglycosides, and may cause ototoxicity and nephrotoxicity in case of overdosing (2,3). Antibiotic use for the treatment of

mastitis in cows may be a potential hazard to consumers due to persistence of residues in the milk (4). Although, neomycin is not approved for use in lactating cows, illegal use can result in the presence of residues of this antibiotic in milk. The Food and Drug Administration (FDA) has set a tolerance limit of 0.15 ppm of neomycin in milk (5).

Microbiological methods have been used for the detection of neomycin and other aminoglycosides in food products of animal origin (6,7-9). These methods, however, lack specificity (10). HPLC has become the analytical method of choice and has been successfully applied to the determination of aminoglycosides in biological fluids (11). The only HPLC method for the determination of neomycin in milk, reported by Shaikh et al, involves postcolumn derivatization with OPA reagent (4).

This paper describes the development of an analytical method for the determination of neomycin in milk and involves precolumn derivatization with OPA followed by HPLC analysis.

METHOD

Reagents and standards:

a.) Chemicals.- Neomycin sulfate, 2-mercaptoethanol and O-phthalaldehyde (Sigma Chemical Co., St. Louis, MO); sodium chloride, boric acid, potassium hydroxide, potassium tetraborate and HPLC grade methanol (Fisher Chemical Co., Fairlawn, NJ); ethylenediamine-tetraacetic acid, tripotassium salt, and amberlite CG-50 ion exchange resin (Aldrich Chemical Co., Milwaukee, WIS.)

b.) Mobile phase for LC.- Solvent A - ethylenediamine-tetraacetic acid, tripotassium salt (2.0 gm) was dissolved in one liter of distilled water: methanol, (300:700): Solvent B - methanol.

c.) Alkaline buffer.- Potassium tetraborate (76 gm) was dissolved in 400 ml distilled, pH adjusted to 11 with potassium hydroxide and final volume made to 500 ml with distilled water.

d.) Neomycin standard solutions.- Two standard solutions were prepared. Standard A: Neomycin sulfate (100 mg) was dissolved in 1000 ml distilled water. Standard B: 10 ml standard A was diluted to 100 ml with distilled water. Both the standards were stored in the refrigerator in polypropylene bottles. Milk samples were fortified using these standards.

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

f.) OPA reagent.- O-phthalaldehyde (100 mg) was dissolved in 1 ml methanol and 200 ul of 2-mercaptoethanol and 10 ml potassium borate buffer were added to it. This reagent was stored refrigerated in an amber glass capped vial. The reagent may be used for a week.

Apparatus:

a.) 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 200 ul was used for mixing the solvent. A FS-970 LC Fluorometer (Kratos Analytical Instruments, Westwood, NJ) was used as the detector.

b.) LC Column.- HISEP, 15cm X 4.6 mm, 5 um particle size (Supelco, Inc., Bellefonte, PA 16823-0048).

Fortification of milk samples: Milk samples were fortified using standards A and B. A 50 ul, 100 ul, and 250 ul aliquots of standard A were transferred to 15 ml polypropylene tubes. Appropriate aliquots of milk were added to these polypropylene tubes to give a total volume of 5 ml, resulting in milk samples fortified at 1, 2, and 5 ppm levels of neomycin. Similarly, 50 ul, 100 ul, and 250 ul of standard B were transferred to polypropylene tubes and made up to 5 ml with milk to obtain milk samples fortified at 0.1, 0.2, and 0.5 ppm levels.

Preparation of standards for calibration curve: Standard solutions A and B were used to prepare solutions of neomycin in water ranging from 0.1 ppm to 5 ppm levels.

Preparation of amberlite CG 50 resin column: A slurry was made by shaking amberlite CG 50 resin with the alkaline buffer and equilibrated for about two hours. A column was prepared by placing glass wool in the bottom of a Pasteur pipet and filled to a 3.5 cm height with the resin slurry. The column was washed with distilled water until the eluent was neutral.

Sample preparation: A 40 ml portion of milk was mixed with 2 gm sodium chloride, shaken well to dissolve the sodium chloride, and fortified with an appropriate amount of standard neomycin. Milk (5 ml), or the standard, was passed through the amberlite CG 50 resin column. For milk samples fortified at 0.2 and 0.1 ppm levels, 10 ml milk was passed through the column. The column was then washed with 8 ml of distilled water. OPA reagent (0.6 ml) was loaded on to the column and passed through. After two minutes the derivatized neomycin was eluted with 3 ml

of alkaline buffer/methanol (20:80). The eluent was collected in a graduated vial and final volume made to 4 ml with methanol. The sample vial was covered with aluminum foil, stored in freezer (-8° to -10° C), and analyzed by HPLC after fifteen minutes.

Liquid Chromatography:

The following operating conditions were used. A linear gradient starting with 0% solvent B at zero time to 60% solvent B in 15 minutes on curve 4 and maintaining a flow rate of 1.7 ml/minute was used. Curve 4 was the concave curve on LDC gradient master which holds the initial solvent gradient for about 6 minutes. The peaks were quantitated based on peak heights. An excitation wavelength of 340nm with a KV 418 emission filter was used. The detector was set at 0.5 uA range and the sensitivity was reduced if the concentration of neomycin was greater than 2ppm. The injection volume was from 10 ul to 20 ul.

RESULTS

Figure 1, a representative chromatogram of the OPA derivative of standard neomycin, shows two peaks with retention times of 8.2 and 19.8 minutes. The calibration curve for the OPA derivative of neomycin standard was obtained by plotting the peak heights of the sum of the two peaks (peak 1 and 2, Figure 1) versus the neomycin concentration in the 0.1 to 5 ppm range. To obtain the calibration curve, all the standards were passed through the amberlite CG 50 resin column, derivatized on the column, and eluted with alkaline buffer/methanol. The curve was linear with a correlation coefficient of 0.989.

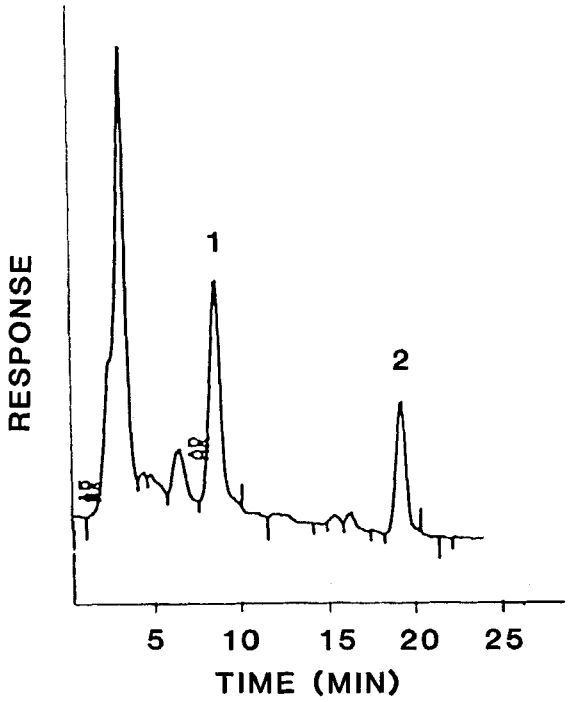

Figure 1. Chromatogram of the OPA derivative of standard neomycin at 2 ppm level.

An unfortified milk sample was processed through the complete cleanup and derivatization procedure and examined by HPLC (Figure 2). No interferring peaks were present in the chromatogram. The fortified milk samples were then carried through the complete cleanup and derivatization procedure and examined by HPLC. Figure 3 shows a representative chromatogram of a milk sample fortified at 1 ppm level of neomycin. The two peaks of the OPA derivative of neomycin (labeled as 1

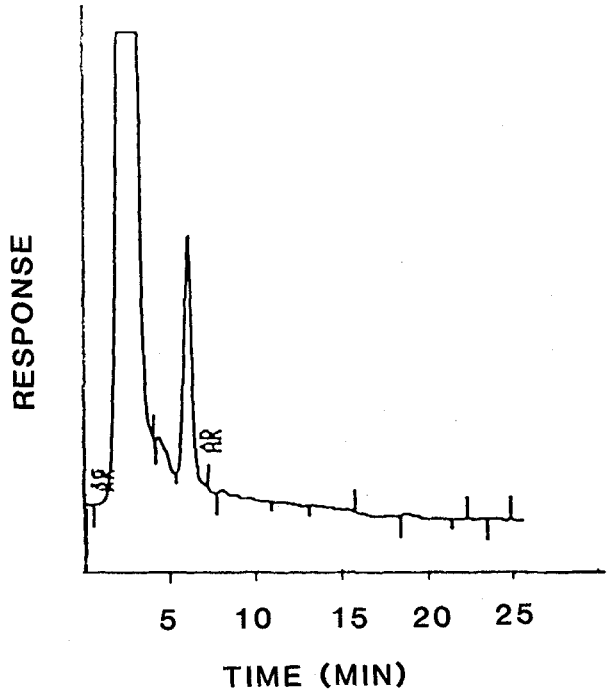

Figure 2. Chromatogram of unfortified milk sample.

and 2) are well resolved. Figure 4 shows a chromatogram of a milk sample fortified at 0.1 ppm level and both the peaks of OPA derivative of neomycin are also well resolved. The recoveries of neomycin from milk samples were calculated based on the peak heights of sum of both peaks (peak 1 and 2) and ranged from 94 to 102% in samples fortified between 0.1 to 5 ppm levels (Table 1).

Forty samples of raw milk from individual producers in connecticut were tested for neomycin using this method. None of the samples contained a detectable amount of neomycin (DL=50 ppb).

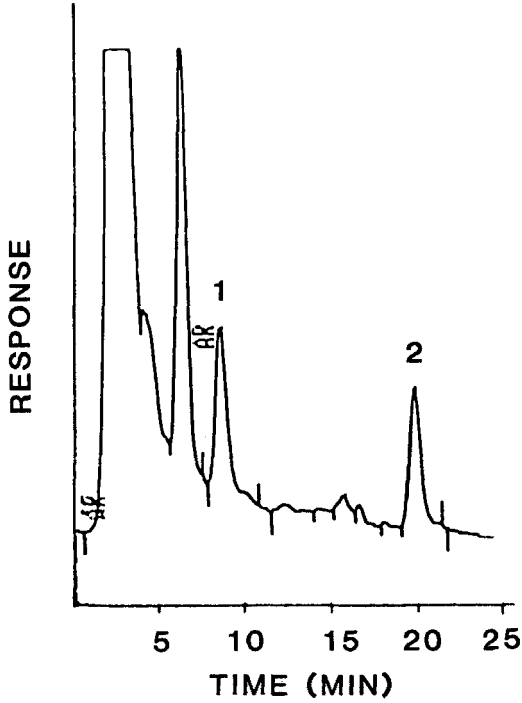

Figure 3. Chromatogram of milk sample fortified at 2ppm level with neomycin.

DISCUSSION

The only HPLC method for the determination of neomycin residues in milk as reported by Shaikh et al, requires defatting and deproteination of milk as sample pretreatment steps before HPLC analysis (4). In the proposed method both steps, defatting and deproteination, were eliminated. It was possible to avoid the deproteination step primarily because of the use of a HISEP column. This column has been shown to work effectively for serum and plasma samples without deproteinating these

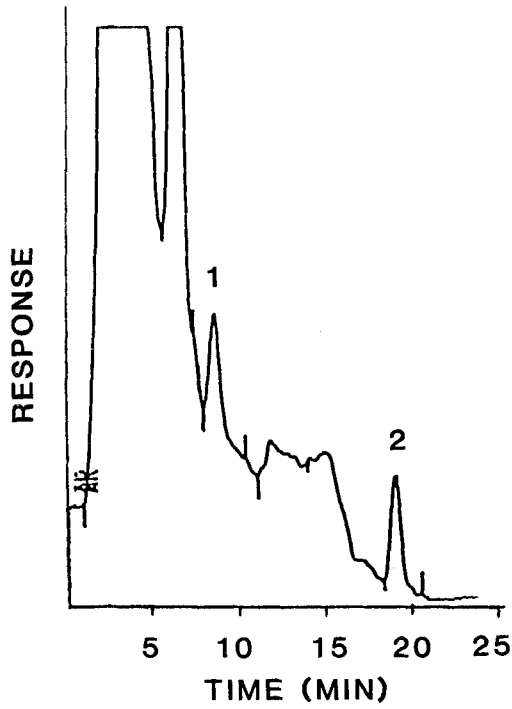

Figure 4. Chromatogram of milk sample fortified at 0.1 ppm level with neomycin.

samples. This HISEP column is made of silica based material containing hydrophobic regions shielded by hydrophilic network (12). This hydrophilic network shields the large protein molecules from contact with the inner hydrophobic regions, and thus protein molecules are passed unretained on the column. Drugs and other smaller metabolites, however, penetrate the hydrophilic network and are retained on the column. These compounds can then be eluted by adjusting the mobile phase.

Table I

Recovery of neomycin from milk fortified with neomycin between 0.1 to 5 ppm levels.

Fortification level ppm	Recovery %
0.1	94
0.1	109
0.1	103
Average + SD	102 ± 6.2
0.2	93
0.2	87
0.2	103
Average + SD	94.3 ± 6.7
0.5	101
0.5	96
0.5	95
Average + SD	97.3 ± 2.7
1.0	102
1.0	98
1.0	93
Average + SD	97.7 ± 3.6
2.0	104
2.0	99
2.0	92
Average + SD	98.3 ± 5.0
5.0	98
5.0	99
5.0	93
Average + SD	96.7 ± 2.5

In preliminary experiments milk samples were defatted by centrifugation at 4°C. When milk samples were used without defatting no change in recoveries was observed and also the chromatograms showed no interferring peaks. Therefore the defatting step was eliminated. The use of sodium chloride was necessary as poor recoveries of neomycin were obtained in the absence of sodium chloride. The concentration of sodium

chloride also affected the recoveries of neomycin and 5gm sodium chloride in 100ml milk gave the maximum recoveries (>95%).

Precolumn derivatization of primary amines with OPA has not gained much success due to the unstable nature of the OPA derivatives. In this report, the derivatization conditions were optimized to obtain OPA derivative of neomycin which were stable at -8° to -10°C for at least four hours. Derivatization with OPA reagent was done on the amberlite CG 50 resin column for two minutes. After elution of the derivatized neomycin from the column, it was also necessary to leave the derivatized neomycin for about 15 minutes in the freezer (-8° to -10°C) in order to get complete derivatization. The peak heights were found to increase during the first fifteen minutes following the elution from the column and remained constant for atleast four hours when stored at -8° to -10°C.

The use of amberlite CG 50 ion exchange resin was chosen for two reasons. First, neomycin was selectively retained on the column when milk was passed through the column and other interferences present in the milk were not retained on the column. Second, the derivatization with OPA reagent was carried out effectively and conveniently on the column. The OPA derivatized neomycin was then easily eluted with alkaline buffer/methanol.

The retention time of peak 1 (Figure 4) was close to other eluting peak present in the milk sample and therefore at lower fortification levels (less than 0.5 ppm), this peak was sometimes not resolved to the base line. In such cases it is recommended to reduce the flow rate of the mobile phase to 1.5 ml/minute in order to resolve peak 1 better if necessary. Since an increse in the peak heights of OPA derivative of neomycin was observed up to fifteen minutes, it was also important to

inject the sample into the LC only after storing for that initial period of fifteen minutes in order to obtain quantitative results.

In conclusion, this proposed method for the analysis of neomycin in milk is simple, sensitive and quantitative. This method allows the detection of neomycin in milk below the tolerance level of 0.15 ppm as specified by the FDA. The use of a HISEP column for milk analysis without deproteination has successfully been demonstrated for the first time. The use of HISEP column for the analysis of other drug residues in milk may also become important.

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