Journal of Chromatography, 490 (1989) 101–113
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4660

DEVELOPMENT OF AN ANALYTICAL METHOD FOR PENICILLIN G IN BOVINE MILK BY LIQUID CHROMATOGRAPHY WITH ULTRAVIOLET–VISIBLE DETECTION AND CONFIRMATION BY MASS SPECTROMETRIC DETECTION

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(First received September 28th, 1988; revised manuscript received January 3rd, 1989)

SUMMARY

A liquid chromatographic (LC) method with ultraviolet–visible photodiode array (UV-VIS PDA) detection was developed to measure penicillin G in bovine milk. A liquid chromatographic–mass spectrometric (LC–MS) procedure was divised to confirm the LC method. The method involved diluting milk with a drug-releasing solvent consisting of acetonitrile–methanol–water and ultrafiltration through a 10 000 dalton cutoff filter. Penicillin G was separated from other components in the ultrafiltrate by ion-paired LC using a reversed-phase microbore column eluted with a 25% acetonitrile solution. The LC method was confirmed by thermospray LC–MS. The detection limit for penicillin G determination in milk was estimated to be 10 ppb for LC with UV-VIS PDA and 100 ppb for LC–MS.

0378-4347/89/$03.50

INTRODUCTION

Penicillin G belongs to the β-lactam group of antibiotics. Its structure (Fig. 1) incorporates the 6-aminopenicillanic acid nucleus. Penicillin G is administered in salt form with counter ions of procaine (Fig. 1), potassium, or sodium.

Bioassays are the most commonly employed methods for determining penicillin G concentrations in milk [1]. However, bioassay procedures provide neither the sensitivity nor specificity required for residue determinations. Methods involving fluorescence [1] and thin-layer chromatography [2] were reported for analysis of penicillin G in milk. However, these procedures are not acceptable for current regulatory purposes.

The methodology reported here involves determining penicillin G directly from milk ultrafiltrate by ion-paired liquid chromatography (LC). The method uses a microbore reversed-phase column to reach higher mass sensitivities and increased specificity for β-lactams when dealing with complex matrices [3,4]. An ultraviolet–visible photodiode array (UV–VIS PDA) detector was employed in developing the LC method to improve specificity and minimize liquid chromatographic–mass spectrometric (LC–MS) usage for confirmation of penicillin G. Thermospray LC–MS has been used to analyze non-volatile and thermally labile compounds [5–7], including several β-lactams [8–10]. The accuracy, sensitivity, and specificity of the LC and LC–MS methods for analyzing low concentrations of penicillin G in milk samples will be presented.

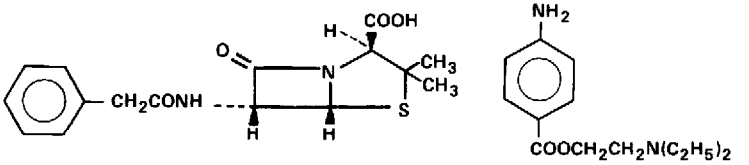

Fig. 1. Structure for penicillin G procaine

EXPERIMENTAL

Materials and reagents

The LC solutions were made from acetonitrile and methanol, highest purity solvent grade (American Burdick & Jackson, Muskegon, MI, U.S.A.). LC-grade water was obtained from the Model 1000 Hydro ultrapure water system obtained from Hydro Services and Supplies (Research Triangle Park, NC, U.S.A.). Phosphoric acid and triethylamine were LC grade (Fisher Scientific, Raleigh, NC, U.S.A.). The ion-pairing reagents, octanesulfonate (S8) and dodecanesulfonate (S12), were obtained from Regis (Morton Grove, IL, U.S.A.).

Penicillin G procaine was supplied by Sigma (St. Louis, MO, U.S.A.). A 1 mg/ml (calculated as penicilline G base) stock solution was prepared in ace-

tonitrile–methanol–water (40:20:40). The working solution (1 μg/ml) was prepared daily from the stock solution.

The microseparation system, Centricon-10, employing a molecular mass cutoff filter of 10 000 daltons, was obtained from Amicon, Division of W.R. Grace and Co. (Danvers, MA, U.S.A.).

Milk samples

Dosed bovine milk was provided by the Food and Drug Administration (Washington, DC, U.S.A.). The dosed bovine milk was collected at various periods (8–96 h) after inter-mammary infusion of penicillin G procaine. The control milk was collected from a cow not treated with penicillin G at the North Carolina School of Veterinary Medicine (Raleigh, NC, U.S.A.). The control milk was used for blank analysis or was spiked with penicillin G procaine for assay validation.

Sample preparation procedure

A 0.5-ml aliquot of milk was diluted with an equal volume of a solution consisting of acetonitrile–methanol–water (40:20:40). The sample was vortex-mixed for 10–15 s, placed in the microseparation system and centrifuged for approximately 30 min at 2677 *g* with 45° fixed-angle rotors. A 10–60 μl aliquot of colorless ultrafiltrate was injected into an LC system equipped with either a UV–VIS PDA or MS detector.

LC with UV–VIS PDA detection

The LC equipment consisted of a Waters Model W600 multisolvent delivery system with a Waters U6K injector and temperature control accessory set at 40°C (Waters Chromatography Division, Milford, MA, U.S.A.). The liquid chromatograph was coupled to a Model 990 UV–VIS PDA detector (Waters Chromatography Division).

The LC separations were performed using a mobile phase consisting of 25% acetonitrile (v/v) in water containing 0.0025 *M* octanesulfonate, 0.0025 *M* dodecanesulfonate, 0.5% (v/v) 85% phosphoric acid, and 0.5% (v/v) triethylamine. The mobile phase flow-rate was adjusted between 0.3 and 0.5 ml/min to give a 6–7 min retention time for penicillin G on a Brownlee Microbore Phenyl Spheri-5 analytical cartridge, 220 mm×2.1 mm I.D., 5-μm particles (Sci-Con, Winter Park, FL, U.S.A.). The milk ultrafiltrates and standards were analyzed in the wavelength range 200–360 nm by the UV–VIS PDA detector.

Liquid chromatography–mass spectrometry

Thermospray spectra were acquired using a mobile phase of isopropanol–0.2 *M* ammonium acetate–acetic acid (12.5:85.5:2) at a 1 ml/min flow-rate at 40°C, on a Brownlee Phenyl Spheri-5 analytical cartridge, 220 mm×4.6 mm

I.D. (Sci-Con). The thermospray interface (Finnigan MAT, San Jose, CA, U.S.A.) was operated with the temperatures of the source and vaporizor at 270 and 105 °C, respectively. A Finnigan MAT 4800 quadrupole mass spectrometer was operated in the pulsed positive-ion/negative-ion detection mode under full scan conditions for initial acquisition of the spectra for penicillin G procaine. For confirmation of penicillin G in milk, the instrument was operated in the positive-ion mode, monitoring m/z 309 and 335 each for 0.5 s.

The quantity of penicillin G, determined by LC–UV–VIS PDA or LC–MS, was calculated as follows:

$$\text{ppb}_{\text{penicillin G}} = \frac{\text{penicillin G (ng)} \times 2}{\text{injection volume (ml)}}$$

Usually the injection volume ranged between 0.010 and 0.060 ml. The multiplication by 2 in the equation accounts for the dilution of milk from the releasing solvent.

RESULTS AND DISCUSSION

Previous work in determining β-lactam antibiotics in serum and tissue extracts showed the usefulness of the microseparation system for sample cleanup [3,11–13]. These 10 000 dalton molecular mass cutoff filters were used successfully for simplifying the sample preparation procedure for milk. Penicillin G, like other β-lactam antibiotics, exhibits significant binding to matrix components in serum [13] and an even higher degree of binding to milk components. A major concern in developing an extraction procedure for penicillin G using ultrafiltration is the binding of analyte to milk components. Evaluating various drug releasing solvents (Fig. 2) shows the importance of protein binding on sample recoveries. For example, results from our laboratories showed that the absence of a releasing solvent limited the recovery to 50%, whereas the optimal releasing solvent increased the recovery to 83%. Based on recovery of penicillin G using various ratios of solvents (Fig. 2), acetonitrile–methanol–water (40:20:40) was chosen for the drug-releasing solvent.

Based on earlier studies with β-lactams, the Brownlee Microbore Phenyl Spheri-5 cartridge was determined to be optimum for LC separation [3,11–13]. Various elution systems consisting of two ion-pairing reagents (octanesulfonate and dodecanesulfonate) were investigated for the separation of penicillin G from milk components. The best separation of penicillin G from other matrix components was obtained with a 1:1 mixture of octanesulfonate and dodecanesulfonate ion-pairing reagents. A calibration using the UV–VIS PDA detector response at 210 nm was generated by measuring five calibration points over the 10–500 ng range in duplicate. The calibration was linear with a correlation coefficient of 0.9984.

LC–UV–VIS profiles of 12.5 ng (500 ppb) of penicillin G standard (0–10

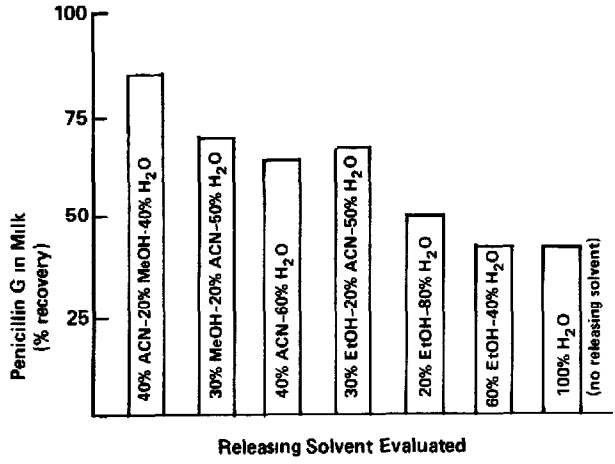

Fig. 2 Recovery of penicillin G from milk using various drug-releasing solvents for the ultrafiltration extraction. The solvents evaluated include acetonitrile (ACN), methanol (MeOH), ethanol (EtOH), and water (H_2O).

min run time) and control milk spiked with 12.5 ng of penicillin G (10–20 min run time) are shown in Fig. 3A. The LC profile shows the maximum wavelength absorbance of all peaks in the 210–360 nm range. The analysis of control milk revealed a clean analytical window for the detection of penicillin G. The LC peaks of penicillin G in aqueous solution and in spiked control milk were symmetric. The UV–VIS absorption spectra for penicillin G standard (No. 2) and spiked control milk (No. 9) have no distinct absorption maxima, and therefore are only of limited use in validating the presence of penicillin G. Spectra No. 1 and No. 8 are from procaine (UV_{max} 295 nm). The UV–VIS spectra for the other major LC peaks are presented, demonstrating that some potential interferences can be distinguished from penicillin G based on the UV–VIS absorption curves. The contour diagram of penicillin G analysis by LC–UV–VIS PDA over the wavelength range 200–330 nm is presented in Fig. 3B. The contours for penicillin G standard (retention time 6.58 min, injected at 0 min) and spiked control milk (retention time 16.91 min, injected at 10 min) were virtually identical, which indicated the absence of UV–VIS-absorbing interfering compounds.

LC–UV–VIS chromatograms (4.2–20 min run time) of penicillin G standard and dosed bovine milk are shown in Fig. 4A. The chromatograms were computer-generated at the maximum wavelengths for all peaks in the 210–360 nm range together with their respective UV–VIS spectra. Penicillin G was detected in the dosed bovine milk sample based upon identical LC retention time to the standard. The UV–VIS spectrum for procaine standard was acquired (spectrum No. 1), but procaine concentration in the milk sample appeared to be very low, as evidenced by a small peak before penicillin G (too weak for gen-

eration of a UV–VIS spectrum). Also, the UV–VIS spectra of the other major LC peaks are presented for comparison to penicillin G to help distinguish potential interferences.

The contour diagrams for the standard and dosed bovine milk samples over the range 200–360 nm is shown in Fig. 4B. The contours for 20 ng (800 ppb) of penicillin G (retention time 6.34 min, injected at 0 min) and 104.5 ng (4.18 ppm) of penicillin G for dosed milk (retention time 16.35 min, injected at 10 min) were symmetric and very similar.

Accuracy data for penicillin G determination in spiked control milk samples are presented on Table I. The mean recovery of penicillin G from spiked control milk within the 100–1000 ppb range was 81.9%. The UV–VIS PDA detection limit was estimated to be 0.3 ng (10 ppb) using an injection volume of 60 μl and based on a 3:1 signal-to-noise ratio at 210 nm.

Thermospray LC–MS proved very specific and sensitive for the detection of penicillin G. The thermospray spectrum for penicillin G exhibited a $[M+H]^+$

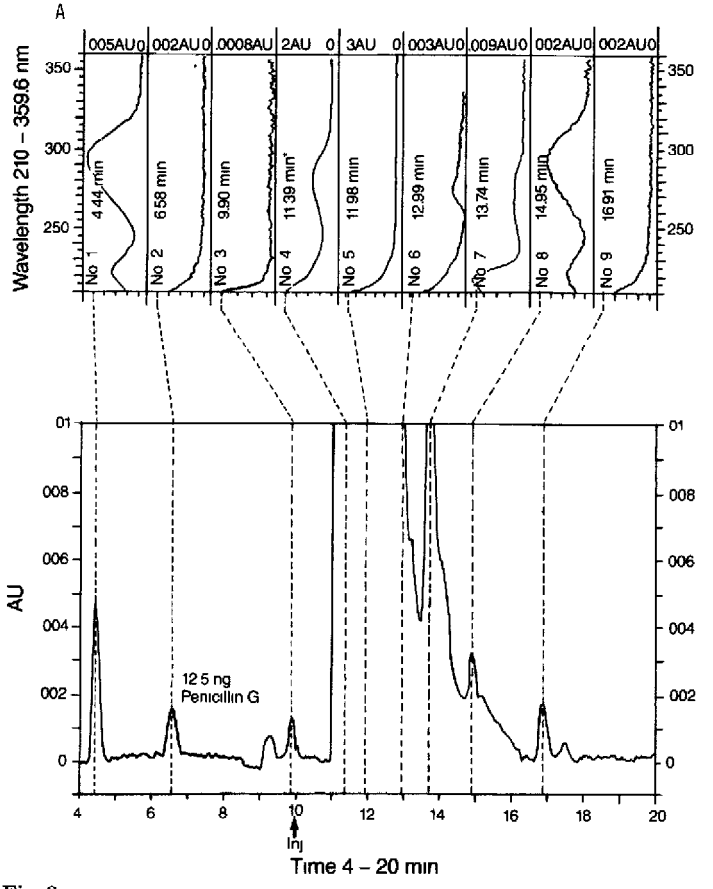

Fig. 3.

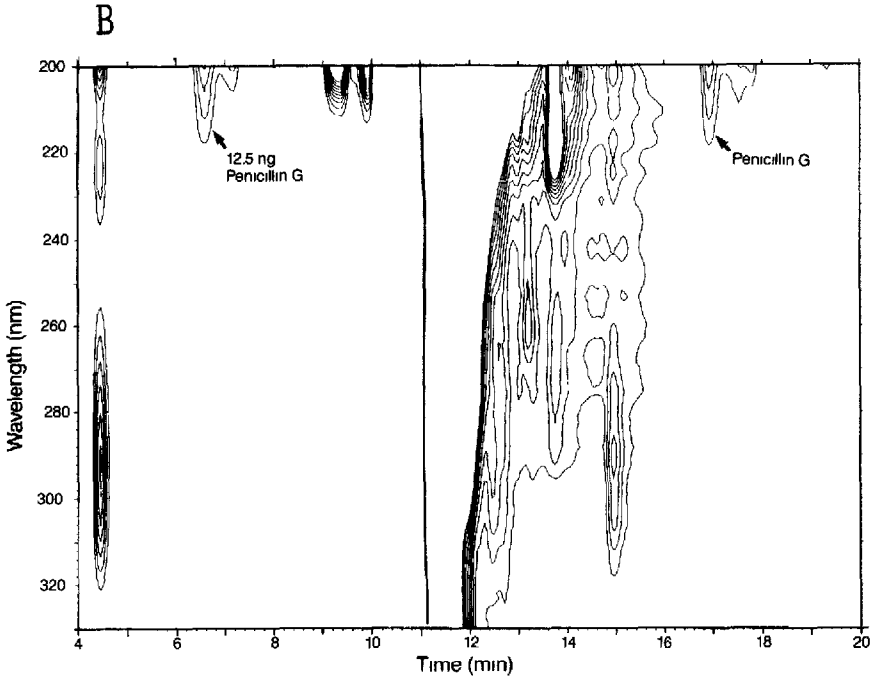

Fig. 3. LC-UV-VIS chromatograms for penicillin G standard (12.5 ng) and penicillin G spiked into control milk (12.5 ng). (A) LC-UV-VIS chromatogram [at 0.01 absorbance units (AU)] acquired at the maximum wavelength for all detected peaks and the UV-VIS spectra (210-360 nm) for each major LC peak. Note that UV-VIS spectra No. 2 and No. 9 are penicillin G in standard and milk, respectively. (B) Contour plot of LC-UV-VIS chromatogram of penicillin G standard and spiked milk sample.

ion (m/z 335), several adduct ions ($[M+NH_4]^+$ and $[M+Na]^+$), and a few fragment ions at m/z 309, 176, and 160 (Fig. 5a) The peak at m/z 309 is postulated to be a thermal degradation product of penicillin G involving opening of the β-lactam ring followed by hydration and loss of carbon dioxide [9]. The ions at m/z 176 and 160 are protonated fragment ions from the opening and cleavage of the penicillin ring. The ion at m/z 160 should be representative for all penicillin antibiotics. The negative-ion spectrum of penicillin G (Fig. 5b) exhibited an $[M-H]^-$ ion (m/z 333) and several fragment ions at m/z 307, 251, 174, and 158. The m/z 307 ion is believed to result from the same thermal degradation as discussed for the ion at m/z 309 in the positive-ion mode. Likewise, the ions at m/z 174 and 158 are the fragment ions (minus hydrogen) from ring opening and cleavage of the molecule. The negative-ion mode of operation was about ten times less sensitive for penicillin G compared to the positive-ion detection mode. The thermospray LC–MS spectrum of procaine exhibits an intense $[M+H]^+$ ion (m/z 237). Procaine is easily separated from

penicillin G and could be used to confirm the presence of penicillin G procaine versus other salts of penicillin G. Procaine was at least two orders of magnitude more sensitive in the positive-ion detection mode compared to the negative-ion detection mode.

Thermospray LC–MS was capable of determining the quantity of penicillin G present in a sample at levels comparable to the LC–UV–VIS PDA method. Multiple-ion detection (monitoring the positive ions of m/z 309 and 335) was employed to achieve suitable sensitivity for penicillin G. The low-mass ions representative of penicillin G (m/z 160 and 176) were not monitored due to significant background ion current from the milk. Fig. 6 shows excellent signal-to-noise ratios for the analysis of three standards of penicillin G used for calibration. The estimated detection limit by LC–MS was 3 ng (100 ppb) (signal-to-noise ratio of 2:1 for the $[M+H]^+$ ion at m/z 335). Calibration curves for penicillin G, using either m/z 309 or m/z 335 ions, were linear over the range

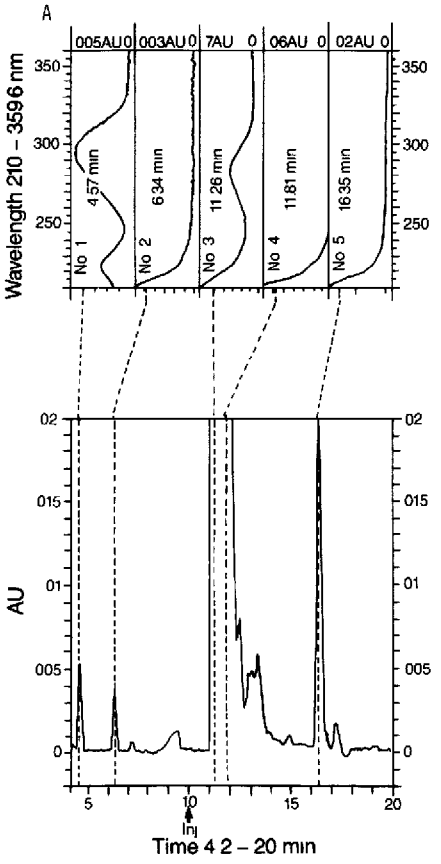

Fig. 4.

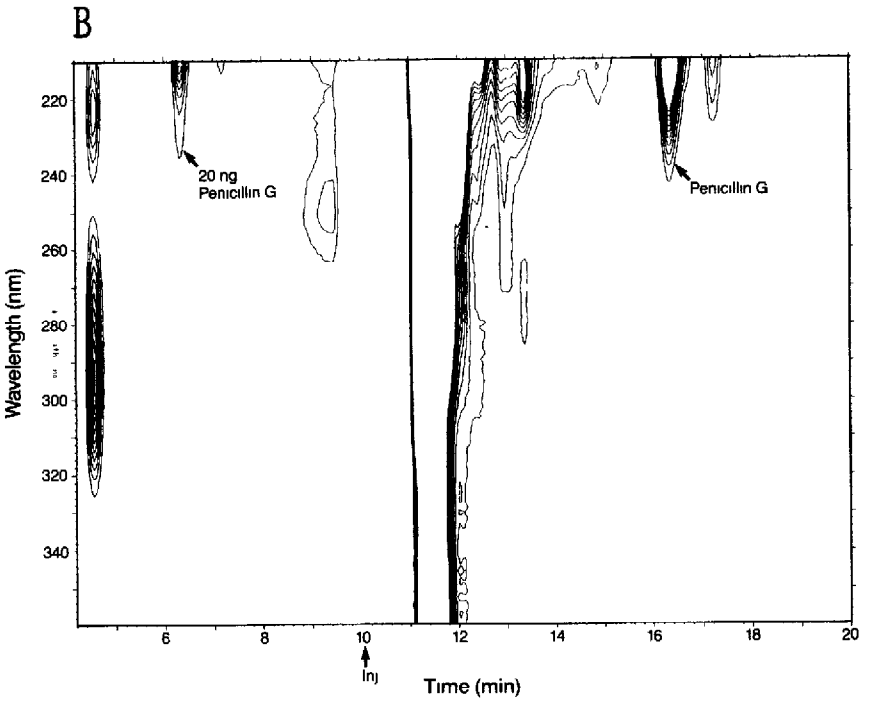

Fig. 4. LC-UV-VIS chromatograms for penicillin G standard (20 ng) and bovine milk samples. (A) LC-UV-VIS chromatogram [at 0.02 absorbance units (AU)] acquired at the maximum wavelength for all detected peaks and the UV-VIS spectra (210-360 nm) for each major LC peak. Note that UV-VIS spectra No. 2 and 5 are penicillin G from the standard and dosed milk, respectively. (B) Contour plot of penicillin G standard and dosed milk.

TABLE I

STATISTICAL SUMMARY FOR PENICILLIN G RECOVERING FROM SPIKED MILK SAMPLES DETERMINED BY LC-UV-VIS PDA DETECTION

Amount spiked (ppb)	n	Amount recovered (ppb)		Coefficient of variation (%)	Mean recovery (%)
		Range	Mean ± S.D.		
1000	5	700-850	762 ± 68.01	8.92	76.2
250	5	208-233	225.0 ± 10.51	4.67	90.0
100	5	73- 87	79.6 ± 5.16	6.50	79.6

20–500 ng (linear correlation coefficient based on the m/z 335 ion using five calibration points).

Thermospray LC–MS was used to confirm the LC separation methodology developed for penicillin G. The ion chromatogram for the $[M+H]^+$ (m/z 335) and $[M+H+H_2O-CO_2]^+$ (m/z 309) ions of penicillin G shows no interfer-

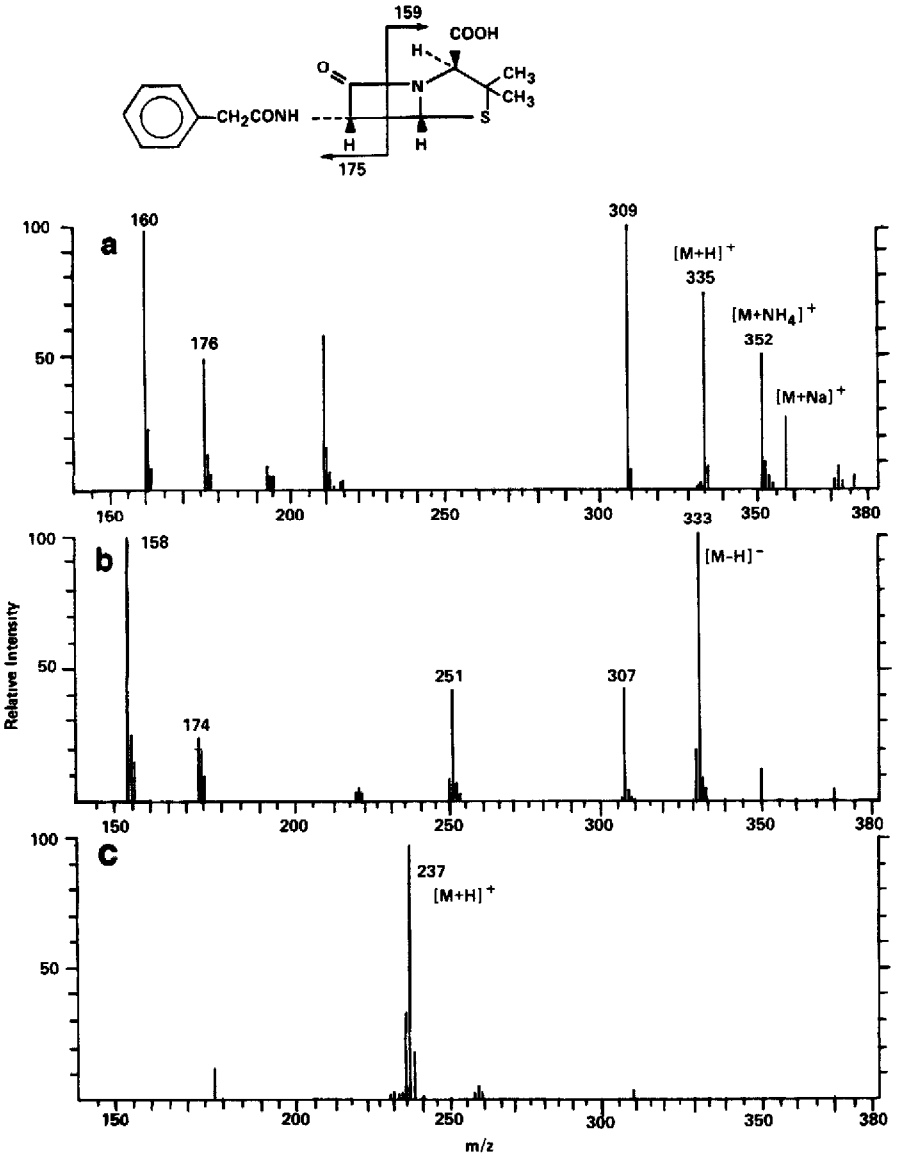

Fig. 5. Thermospray LC–MS spectra of penicillin G procaine. (a) Penicillin G positive-ion detection; (b) penicillin G negative-ion detection; (c) procaine positive-ion detection.

ence from the control milk at the retention time of the drug (not shown). The LC–MS profile of a dosed bovine milk sample showed a peak at the proper retention time as well as the comaximization of the ions at m/z 309 and 335, confirming the presence of penicillin G (Fig. 7). Comparing the measured quantity of the drug in milk by LC–UV–VIS PDA and LC–MS (Table II)

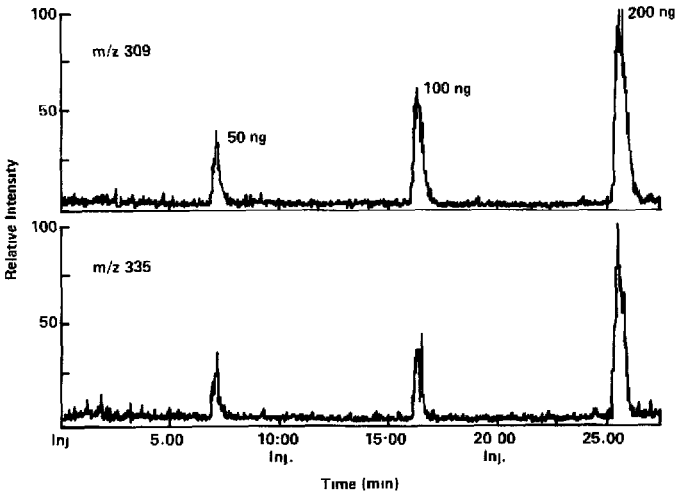

Fig. 6. Thermospray LC–MS chromatograms monitoring the $[M+H]^+$ ion (m/z 335) and $[M+H+H_2O-CO_2]^+$ ion (m/z 309) for 50, 100, and 200 ng of penicillin G injected.

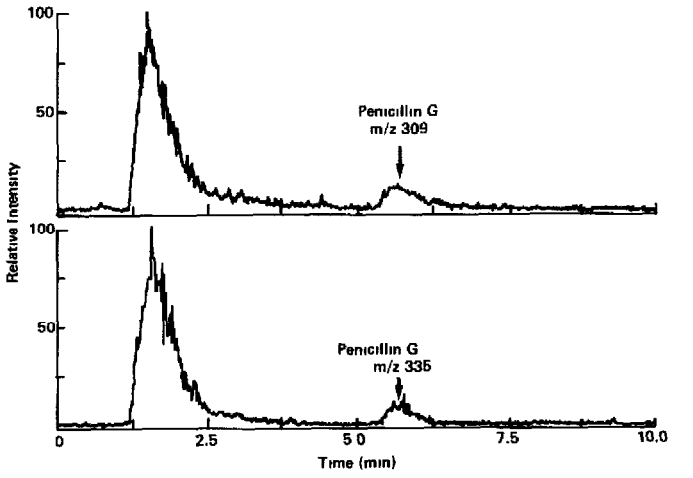

Fig. 7. Thermospray LC–MS chromatograms confirming the presence of penicillin G in dosed milk sample shown in Fig. 4. The $[M+H]^+$ ion (m/z 335) and $[M+H+H_2O-CO_2]^+$ ion (m/z 309) both maximize at the proper retention for penicillin G by LC–MS.

shows agreement between the two methods. The mean difference in measured values between LC–MS and LC–UV–VIS PDA was 32%, with the highest errors at the lower concentration levels.

LC–UV–VIS PDA and LC–MS were useful in showing the quantity of penicillin G in bovine milk at various times after dosing. The measurement of penicillin G at various times after dosing is important for determining the

TABLE II

COMPARISON OF LC-UV-VIS PDA AND LC-MS MEASURED VALUES OF PENICILLIN G IN MILK

Penicillin G was dosed through udder infusion.

Time after dose (h)	*n*	Quantity of penicillin G (ppm)	
		LC-UV-VIS PDA	LC-MS
12	1	12.0	14.8
24	2	0.6, 0.7	0.3, 0.4
26	2	10.4, 10.0	12.3, 12.5

TABLE III

MEASUREMENT OF PENICILLIN G BY LC-UV-VIS PDA AT VARIOUS TIMES AFTER DOSING CATTLE BY UDDER INFUSION

Time after dosing (h)	Quantity of penicillin G (ppm)
8	37.9
24	1.02
32	0.1
48	<0.01
72	<0.01

withdrawal period for the drug. During the withdrawal period bovine milk is contaminated with the drug and therefore cannot be consumed. The quantity of penicillin G in bovine milk, determined by LC-UV-VIS and confirmed by LC-MS, at various times after dosing is presented in Table III. The level of penicillin G in milk quickly decreases from 37.9 ppm at 8 h to 1.02 ppm at 24 h. Penicillin G concentration in milk continues to decrease until it is below detection limits of 10 ppb at 48 h. Clearly, penicillin G residue is found in milk at the low ppb level 32 h after dosing cattle, indicating a withdrawal period of greater than 32 h.

ACKNOWLEDGEMENTS

This research was supported by the Health and Human Services of the Food and Drug Administration under cooperative agreement No. FD-U-000244-01. The authors wish to thank Greg Guyer of FDA for supplying dosed bovine milk samples and for discussions about this research.

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