*Journal of Chromatography*, 567 (1991) 389–404
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5876

# Development of analytical methods for some penicillins in bovine milk by ion-paired chromatography and confirmation by thermospray mass spectrometry

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(First received July 31st, 1990; revised manuscript received February 4th, 1991)

ABSTRACT

Analytical methods for the determination of cloxacillin, ampicillin/hetacillin, and amoxicillin in bovine milk were developed. The methods involved ultrafiltration of milk diluted with methanol, acetonitrile, and water on a 10 000-dalton cut-off filter. Separation of penicillins from other milk components was accomplished by ion-paired chromatography using a microbore column. The penicillins were detected using ultraviolet photodiode array (UV-PDA) detection and confirmed by thermospray liquid chromatography–mass spectrometry (LC–MS). The thermospray spectra of these compounds exhibited $[M+H]^+$ and $[M+Na]^+$ ions along with several fragment ions. The limits of detection for these antibiotics were estimated to be 50 to 100 ppb for LC with UV-PDA detection and 100–200 ppb for thermospray LC–MS detection.

## INTRODUCTION

Penicillins are $\beta$-lactam antibiotics, some of which exhibit antimicrobial activity against both gram-positive and gram-negative organisms [1]. Penicillins contain bulky side-chains attached to a 6-aminopenicillanic acid nucleus (Fig. 1).

The detection of penicillins in milk continues to be carried out by bioassay techniques. Methods involving fluorescence [2] and thin-layer chromatography [3] have been reported for analysis of cloxacillin in milk. The analysis of cloxacillin, ampicilllin/hetacillin, and amoxicillin in bovine milk by liquid chromatography (LC) with ultraviolet (UV) detection and thermospray liquid chromatography–mass spectrometry (LC–MS) has not previously been reported. Methods for their determination and confirmation are required to establish withdrawal periods for antibiotics from animals undergoing treatment and to check for antibiotic residues in bovine milk.

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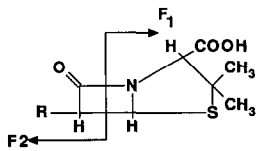


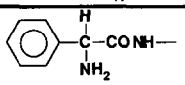
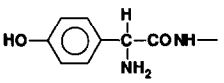
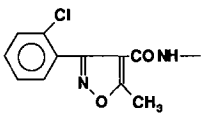

| | R | $[F_1+H]^+$ | $[F_2+H]^+$ | $[M+H+H_2O-CO_2]^+$ | $[M+H]^+$ |
|---|---|---|---|---|---|
| Ampicillin |  | 160 | 191 | 324 | 350 |
| Amoxicillin |  | 160 | 207 | 340 | 366 |
| Cloxacillin |  | 160 | 277 | 410 | 436 |

Fig. 1. Structures of penicillins discussed in the paper and the major thermospray MS ions detected.

The methodologies introduced here involve detection and measurement of penicillins directly from milk ultrafiltrates by ion-paired LC using ultraviolet photodiode array (UV-PDA) detection. Thermospray LC–MS was chosen for the confirmation technique because it had been used previously to analyze non-volatile and thermally labile compounds [4,5], including several $\beta$-lactams [6–11]. The sensitivity and specificity of LC combined with UV-PDA and MS methods for analyzing parts per billion (ppb) levels of penicillin in milk samples were also examined.

## EXPERIMENTAL

### *Materials and reagents*

The solutions for LC were prepared using highest-purity-grade acetonitrile and methanol (American Burdick & Jackson, Muskegon, MI, U.S.A.). LC-grade water was obtained from a Model 1000 Hydro Ultrapure water system (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, sodium octasulfonate and sodium dodecanesulfonate, were obtained from Regis (Morton Grove, IL, U.S.A.).

Cloxacillin, ampicillin, hetacillin sodium salts and amoxicillin were supplied by Sigma (St. Louis, MO, U.S.A.). A 1 mg/ml stock solution was prepared in acetonitrile–methanol–water (40:20:40, v/v). The working solution (1 $\mu$g/ml) was prepared daily from the stock solution.

The microseparation system, Centricon-10, employing a molecular mass cut-

off filter of 10 000 daltons, was obtained from Amicon, Division of W. R. Grace (Danvers, MA, U.S.A.).

*Milk samples*

Milk from dosed bovines was provided by the Food and Drug Administration (Washington, DC, U.S.A.). The dosed bovine milk was collected at various times (8–56 h) after intramammary infusion or intramuscular administration of the various penicillins. The control milk was collected from a cow not treated with penicillins 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 pencillins for assay validations.

*Sample preparation procedure*

An 0.5-ml aliquot of milk was diluted with an equal volume of acetonitrile–methanol–water (40:20:40, v/v). The sample was vortex-mixed for 10–15 s, placed in the microseparation system, and centrifuged for approximatley 30 min at 2677 *g* with a 45° fixed-angle rotor. A 10–100 $\mu$l aliquot of colorless ultrafiltrate was injected into an LC system equipped with either a UV-PDA or an MS detector.

*Liquid chromatography with ultraviolet photodiode array detection*

The LC equipment consisted of a Waters Model W600 multi-solvent delivery system (Milford, MA, U.S.A.) with a Waters U6K injector and temperature control accessory set at 40°C for amoxicillin, 50°C for ampicillin, and 60°C for cloxacillin.

Table I shows the mobile phases used for analysis of the penicillins. The mobile phase flow-rates were adjusted between 0.2 and 0.45 ml/min to give 9–13 min retention times for the penicillins on a Brownlee Microbore Phenyl Spheri-5 column with 5-$\mu$m particles (220 mm × 2.1 mm I.D.) (Sci-Con, Winter Park, Fl, U.S.A.). The milk ultrafiltrates and standards were analyzed in the 200–340 nm wavelength range using a Waters Model 990 UV-PDA detector.

The linearity of the LC method was determined by analyzing solvent standards of 5, 20, 70, 200 and 500 ng in duplicate for the penicillins (note that ampicillin was not determined in duplicate). The recovery for the extraction method was determined by analyzing from four to seven spikes of each penicillin in bovine milk at the 100–1000 ppb level. The UV-PDA peak area at 220 nm for the spiked bovine milk extract was ratioed to the peak area for the identical spiking level for a solvent standard to determine the percentage recovery reported in Table II.

*Liquid chromatography with thermospray mass spectrometry*

Confirmation by thermospray LC–MS required the use fo different LC conditions from those used for UV-PDA, for compatibility with the mass spectrometer. The mobile phases (Table I) were introduced at flow-rates of 0.8–1.2 ml/min on a Brownlee Phenyl Spheri-5 analytical cartridge with 5-$\mu$m particles (220 mm

TABLE I

LC–UV AND THERMOSPRAY LC–MS MOBILE PHASES FOR DETERMINATION OF SOME PENICILLINS IN MILK

| Penicillin | Mobile phase (LC–UV) | Mobile phase (LC–MS) |
|---|---|---|
| Amoxicillin | 15% Acetonitrile<br>5% Methanol<br>2 m*M* Octanesulfonate<br>2 m*M* Dodecanesulfonate<br>0.4% Triethylamine<br>0.4% Phosphoric acid (85%)<br>79.2% Water | 1.5% Isopropanol<br>5% Acetic acid in 0.2 *M* ammonium acetate<br>93.5% Water |
| Ampicillin/hetacillin | 20% Acetonitrile<br>5 m*M* Dodecanesulfonate<br>0.4% Phosphoric acid (85%)<br>0.4% Triethylamine<br>79.2% Water | 10% Isopropanol<br>2% Acetic acid in 0.2 *M* ammonium acetate<br>88% Water |
| Cloxacillin | 27.5% Acetonitrile<br>2.5 m*M* Dodecanesulfonate<br>1.5 m*M* Octanesulfonate<br>0.1% Phosphoric acid (85%)<br>72.4% Water | 15% Isopropanol<br>2% Acetic acid in 0.2 *M* ammonium acetate<br>83% Water |

× 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 vaporizer at 320 and 120°C, respectively. A Finnigan MAT 4800 quadrupole mass spectrometer was operated in the pulsed positive-ion/negative ion detection mode under full scan conditions (150–550 daltons in 2 s) for initial acquisition of the spectra for penicillins. MS confirmation of penicillins in milk was performed in the positive-ion mode monitoring *m/z* 160, 277, 410, and 436 for cloxacillin, *m/z* 324 and 350 for ampicillin, or *m/z* 207 and 366 for amoxicillin (Fig. 1), with each ion being monitored for 200 ms.

## RESULTS AND DISCUSSION

Methodology was developed for the detection of penicillins directly from milk ultrafiltrates by ion-paired LC using UV-PDA detection. Various elution systems consisting of two ion-pair reagents (octane- and dodecanesulfonate) on a microbore phenyl column were investigated for separation of the individual penicillins from milk components. The best separation for each penicillin was achieved with the mobile phases shown in Table I. Under these LC conditions the pencillins are retained, permitting their separation from most bovine milk interferences, and providing a clean analytical window for their detection (Figs. 2–4).

Fig. 2A shows the chromatogram generated from the maximum absorption wavelength for all peaks found in the 210–340 nm range for 6 ng of cloxacillin standard (0–15 min run time) and for bovine milk spiked with 100 ppb of this analyte (15–29 min run time). The UV-PDA spectra (top) are shown for the major peaks in the chromatogram. Thge UV-PDA spectra for the cloxacillin standard (No. 1) and for cloxacillin spiked bovine milk (No. 12) show similar end absorption. Contour plots (Fig. 2B) for the cloxacillin standard (peak at 12.40 min) and for the spiked bovine milk (peak at 27.58 min sample injected at 15 min into the analysis) are symmetrical. The symmetry of the contour plot indicates the absence of UV-PDA-absorbing contaminants at the retention window for cloxacillin.

A representative chromatogram of 20 ng of amoxicillin standard and of bovine milk spiked with a concentration of 500 ppb of this analyte are shown in Fig. 3A.

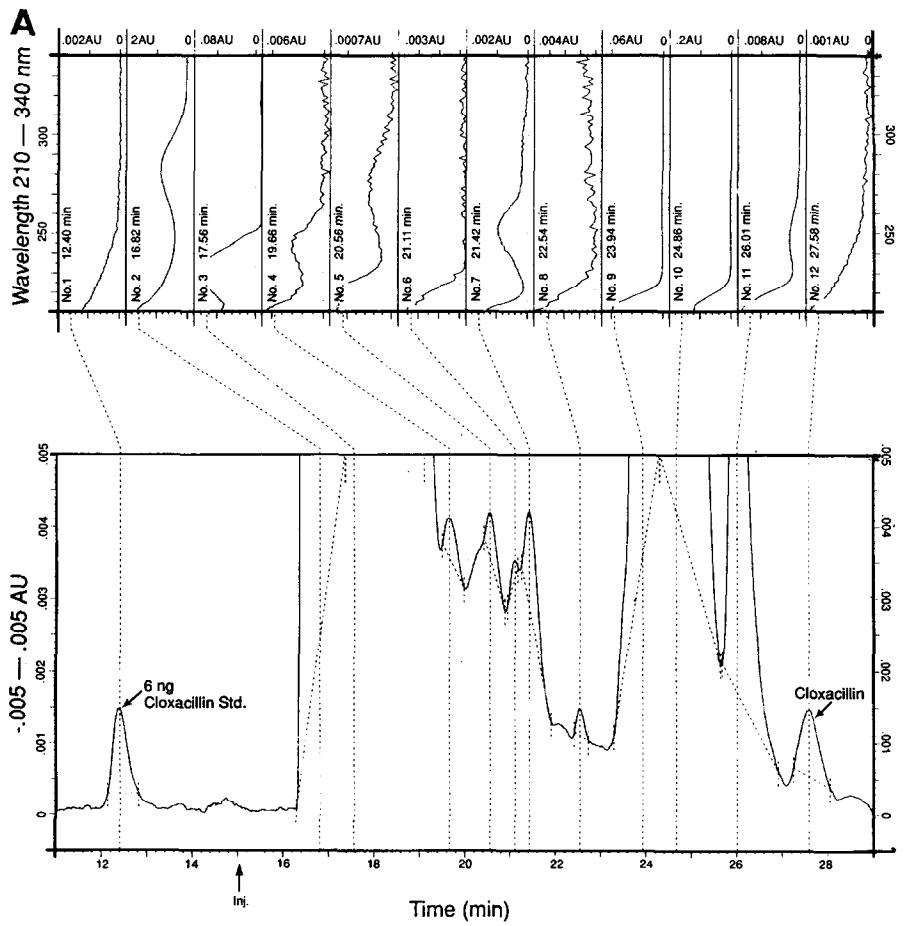



Fig. 2.

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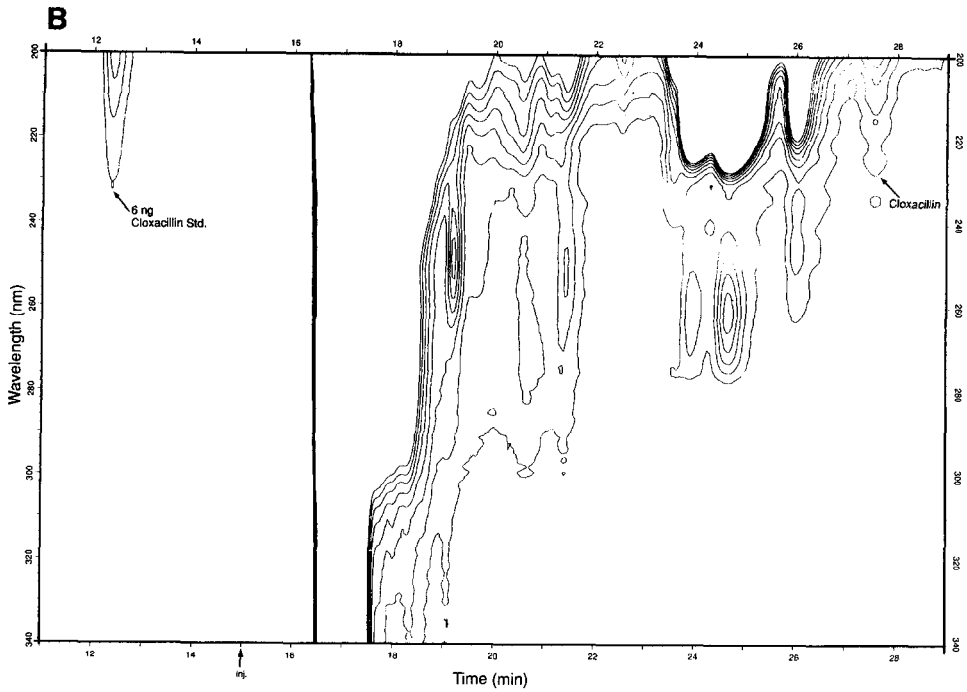


Fig. 2. LC–UV-PDA chromatograms for cloxacillin standard, spiked bovine milk, and milk taken after intramammary administration of this antibiotic. (A) LC–UV-PDA chromatogram acquired at the maximum wavelength for all detected peaks (bottom) in the 210–340 nm range and UV-PDA spectra for each major LC peak (top). Note that UV-PDA spectra Nos. 1 and 12 represent cloxacillin from the standard (6 ng) and a spiked bovine milk (100 ppb) after a 60-$\mu$l injection. (B) UV-PDA absorbance contour plot of a cloxacillin standard (6 ng) and spiked bovine milk (100 ppb).

UV-PDA spectra of the standard and of the principal peaks in the chromatogram are also shown. Although baseline resolution is not achieved for the separation of amoxicillin from milk components, no contaminants were detected in the UV-PDA spectrum (Nos. 1 and 6, ultrafiltrate). Also, the absorption contour plot at the retention time for amoxicillin (Fig. 3B) demonstrates that the milk matrix did not interfere with its detection. Using this method, analysis of milk obtained 24 h after intramuscular administration of amoxicillin revealed low or non-detectable quantities of the antibiotics. No amoxicillin was detected in bovine milk 24 h after intramuscular administration.

A chromatogram, monitoring UV absorbance at 220 nm, of 20 ng of ampicillin standard (0–10 min running time) and of bovine milk spiked at a concentration of 500 ppb ampicillin (10–20 min running time) is shown in Fig. 4A. The contours for the ampicillin standard (peak at 8.3 min) and for the spiked bovine milk (peak at 18.6 min, sample injected at 10 min into the analysis) (Fig. 4B) are

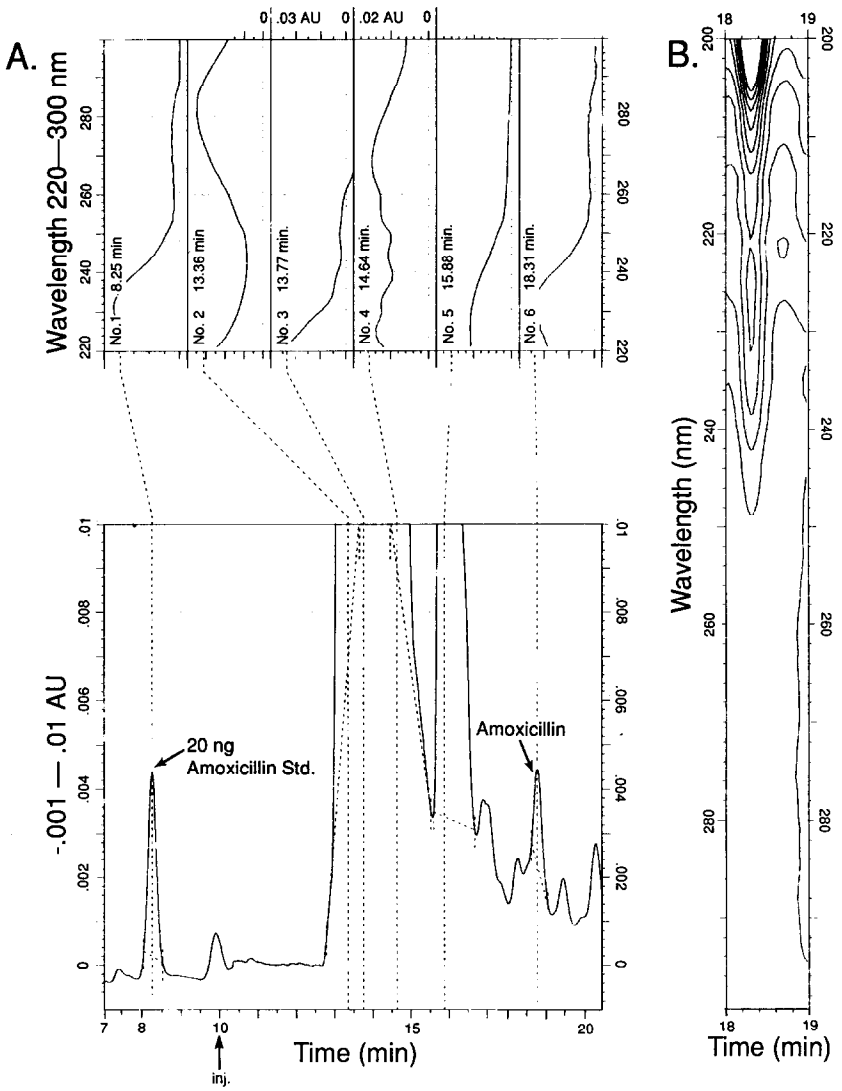


Fig. 3. LC–UV-PDA chromatograms for the amoxicillin standard (20 ng) and a bovine milk sample spiked with 500 ppb amoxicillin. (A) LC–UV-PDA chromatograms acquired at the maximum wavelength for all detected peaks (bottom) in the 220–300 nm range and UV-PDA spectra for each major LC peak (top). Note that UV-PDA spectra Nos. 1 and 6 belong to amoxicillin from the standard and spiked bovine milk, respectively. Injection volume was 40 μl. (B) A portion of the UV-PDA absorbance contour plot for amoxicillin in spiked bovine milk.

symmetrical, indicating the absence of contaminating absorbing compounds. No ampicillin was detected in bovine milk 24 h after intramuscular administration.

The analysis of hetacillin is complicated by its hydrolysis to ampicillin *in vivo* [12]. An *in vitro* experiment demonstrated that the majority of hetacillin was

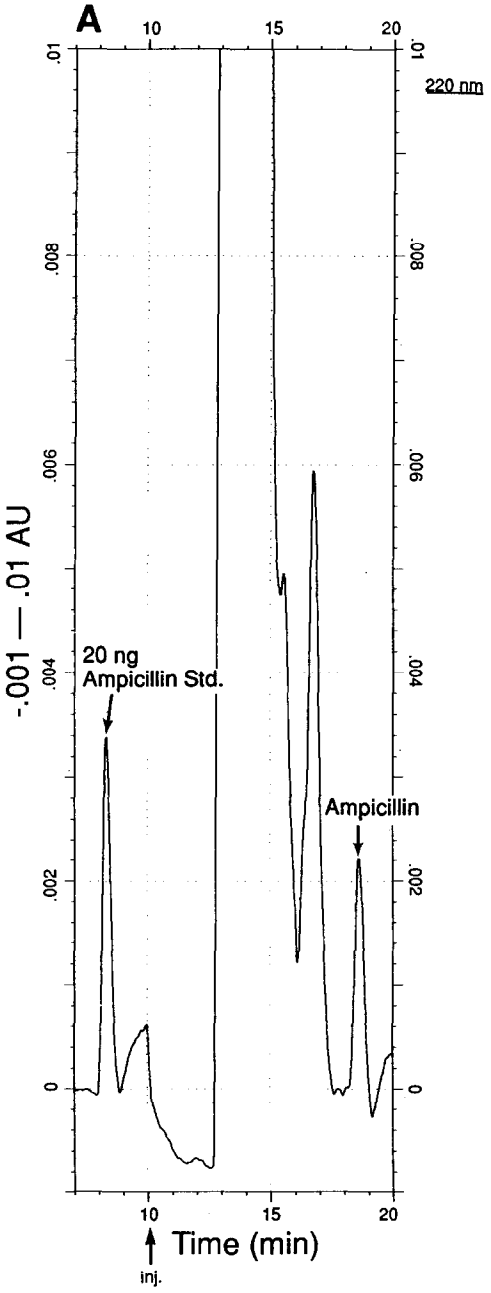
A
10
15
20
.01
.008
.006
.004
.002
0
220 nm
-.001 — .01 AU
20 ng
Ampicillin Std.
Ampicillin
Time (min)
inj.

Fig. 4.

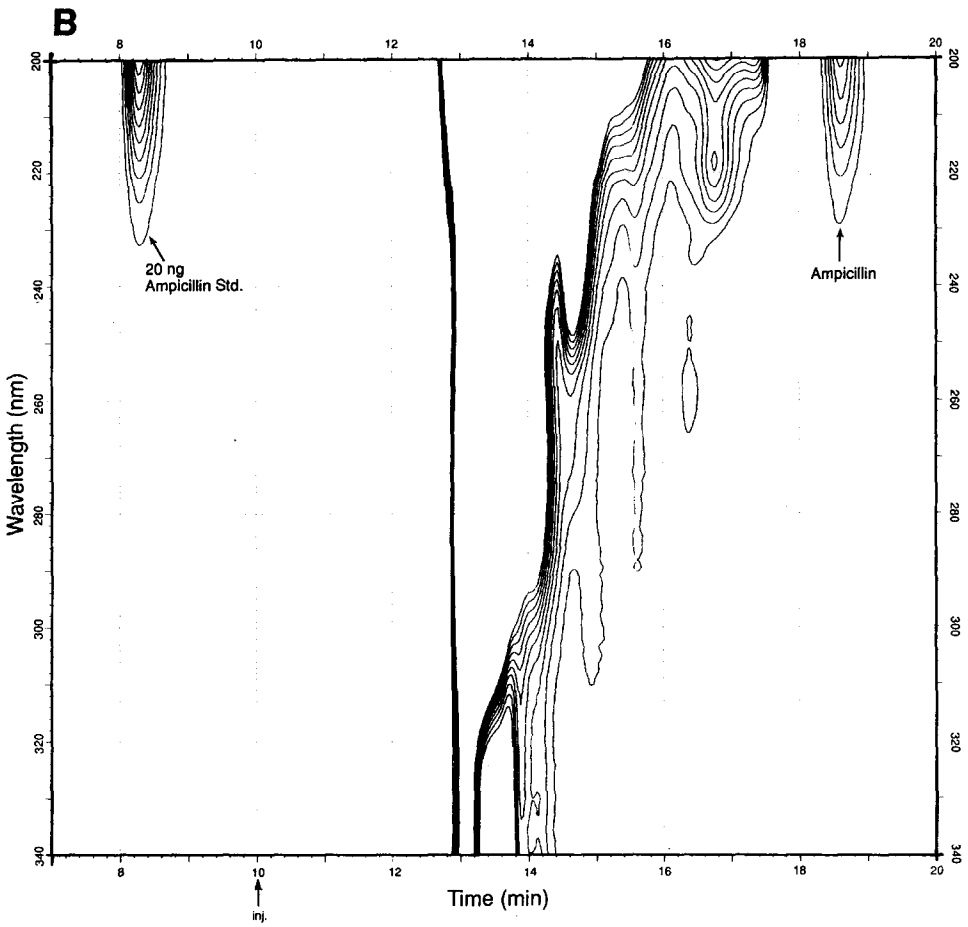


Fig. 4. LC–UV-PDA chromatograms for ampicillin standard (20 ng) and bovine milk sample spiked with 500 ppb ampicillin. (A) LC–UV-PDA chromatograms acquired at 220 nm on a UV-PDA for an ampicillin standard (0–10 min, sample injected at 0 min, not shown) and a bovine milk sample spiked with ampicillin (10–20 min, sample injected at 10 min). Injection volume was 30 μl. (B) UV-PDA absorbance contour plots of ampicillin standard and spiked bovine milk.

converted to ampicillin in less than 4 h at room temperature. Elevated temperatures (*e.g.*, 37°C) would increase the rate of conversion to ampicillin. The fast conversion of hetacillin to ampicillin and the need to monitor milk beyond 24-h time periods dictate the use of analytical methods for measuring ampicillin. Therefore, a measured value of ampicillin in a milk sample could result from the administration of hetacillin or ampicillin or a combination of both antibiotics.

Linearity of the LC–UV-PDA method was determined by injecting penicillin standards from 5 to 500 ng. The UV-PDA method was linear over the concentration range for all penicillins. The resulting linear correlation coefficients ($r$) were

TABLE II

STATISTICAL SUMMARY OF LC–UV ANALYSES OF SOME PENICILLINS IN BOVINE MILK

| Compound | Concentration spiked (ppb) | *n* | Concentration found (ppb) | | Coefficient of variation (%) | Recovery (%) |
|---|---|---|---|---|---|---|
| | | | Range | Mean ± S.D. | | |
| Ampicillin | 1000 | 5 | 820–920 | 872 ± 3.6 | 4.2 | 87.2 |
| | 250 | 5 | 178–206 | 193 ± 11.2 | 5.8 | 77.3 |
| | 100 | 5 | 56–74 | 66 ± 6.5 | 9.8 | 66.0 |
| Cloxacillin | 1000 | 5 | 810–920 | 882 ± 4.4 | 5.0 | 88.2 |
| | 250 | 5 | 238–241 | 240 ± 3.8 | 1.6 | 95.9 |
| | 100 | 5 | 74–86 | 79 ± 7.3 | 9.3 | 79.2 |
| Amoxicillin | 500 | 7 | 324–467 | 384 ± 5.4 | 14.4 | 77.0 |
| | 200 | 4 | 158–169 | 163 ± 5.0 | 3.0 | 81.5 |

greater than 0.995 for all of these antibiotics. The linear correlation coefficient for ampicillin was 0.9986 ($n = 5$), for cloxacillin 0.9955 ($n = 10$), and for amoxicillin 0.9999 ($n = 9$).

Statistical summaries of accuracy and precision for the LC–UV-PDA analysis of penicillins in spiked bovine milk samples are presented in Table II. The mean recoveries of ampicillin and cloxacillin from spiked control milk in the 100–1000 ppb range were 76.8 and 87.7%, respectively. The mean recovery of amoxicillin in the 200–500 ppb range was 79.2%.

Thermospray LC–MS proved to be very specific for the detection and confirmation of penicillins. New analytical LC conditions were developed due to the incompatibility of the ion-paired LC non-volatile buffer with thermospray MS. The new condition substituted ammonium acetate buffer for the sulfonates and made use of standard 4.6 mm I.D. LC columns to achieve flow-rates more compatible with thermospray. The thermospray mass spectrum for cloxacillin exhibited an $[M + H]^+$ ion ($m/z$ 436) and $[M + Na]^+$ adduct ions, and fragment ions at $m/z$ 410, 277, and 160 (Fig. 5A). The peak at $m/z$ 410, $[M - 26]^+$, is postulated to be a thermal degradation product of cloxacillin involving opening of the β-lactam ring followed by hydration and loss of carbon dioxide [7]. The ions at $m/z$ 277 and 160 are protonated fragment ion resulting from the opening and cleavage of the β-lactam ring. The ion at $m/z$ 160 is common to all of the penicillin atibiotics measured.

The negative-ion spectrum of cloxacillin (Fig. 5B) exhibited an $[M - H]^-$ ion ($m/z$ 434) and several fragment ions at $m/z$ 408 and 275. The ion at $m/z$ 408 results from the previously mentioned thermal degradation discussed for the ion at $m/z$ 410 in the positive-ion detection mode. Likewise, the ion at $m/z$ 275 is the fragment ion resulting from ring opening and cleavage of the β-lactam ring. The negative-ion detection was several times less sensitive for cloxacillin than the positive-ion detection mode.

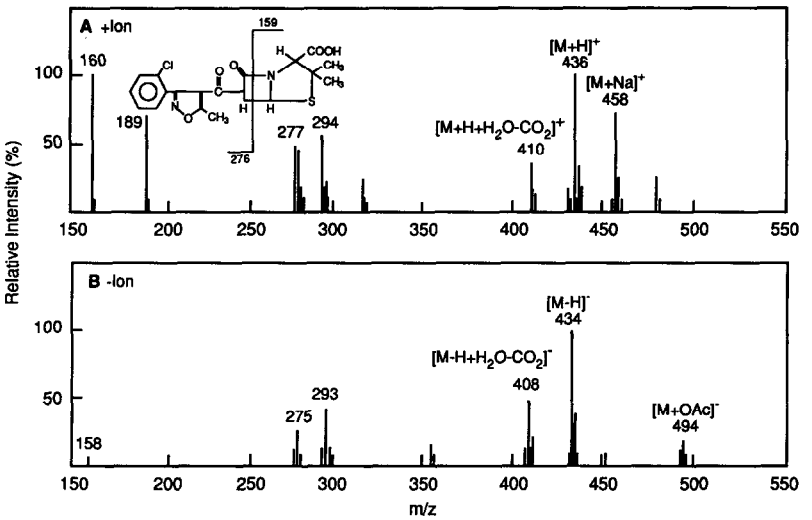


Fig. 5. Thermospray LC–MS spectra of cloxacillin acquired using (A) positive-ion and (B) negative-ion detection.

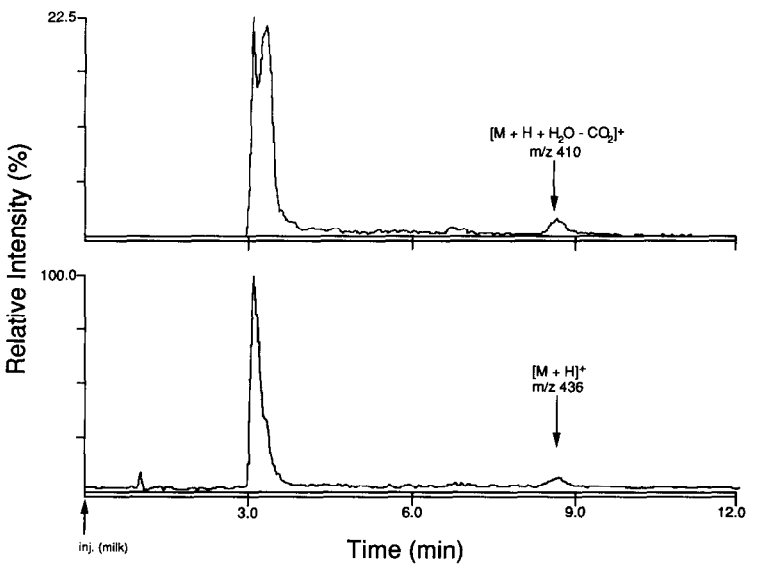


Fig. 6. Thermospray LC–MS chromatograms monitoring the $[M + H + H_2O - CO_2]^+$ ion at $m/z$ 410 and $[M + H]^+$ ion at $m/z$ 436 for a bovine milk sample spiked with 500 ppb cloxacillin. Injection volume was 50 $\mu$l.

Multiple-ion detection, monitoring the positive ions $m/z$ 410 and 436, was employed to achieve suitable sensitivity for the detection of cloxacillin. The low mass ions representative of cloxacillin at $m/z$ 160 and 277 were monitored only for the high concentration of the antibiotic in bovine milk. Furthermore the ions at $m/z$ 160 and 277 maximize several seconds after the $[M + H]^+$ ion. This delay is believed to result from the thermal decomposition of the penicillin on the source walls followed by vaporization into the thermospray aerosol to form the ions at $m/z$ 160 and 277. This process also accounts for the slight peak tailing observed for these ions. This phenomena was observed for all the penicillins investigated. For concentrations below 1 ppm of cloxacillin, the low-mass ions were not monitored owing to a significant ion current in each mass channel. The confirmation was based upon the presence of the $m/z$ 410 and 436 ions (Fig. 6) and the ratio of their areas. The ratio of the peak area for $m/z$ 410 to $m/z$ 436 was 0.7; and peak ratios within 25% of this value was required for the confirmation of cloxacillin.

While thermospray LC–MS was primarily used for confirmation, measurement of penicillin concentrations in milk and meat could be performed. A calibration curve generated using the ion at $m/z$ 436 was linear over the range 100–500 ng of cloxacillin with a correlation coefficient of 0.9996 ($n = 8$). The cloxacillin concentrations obtained from analyzing bovine milk collected 8–56 h after intramammary administration were determined by LC–MS. The measured values compared closely to those obtained for cloxacillin by LC–UV-PDA (Table III). The level of cloxacillin in milk quickly decreased from 56 ppm at 8 h to 6 ppm at 32 h and was below the detection limit of 100 ppb at 48 h. The mean difference in measured values between LC–MS and LC–UV was 13%.

The thermospray mass spectrum for ampicillin exhibited an $[M + H]^+$ ion ($m/z$ 350), a sodium adduct ion ($[M + Na]^+$), and fragment ions at $m/z$ 324, 191, and 160 (Fig. 7A). The ions at $m/z$ 324 ($[M - 26]^+$) and the ions at $m/z$ 207 and

TABLE III

COMPARISON OF MEASURED QUANTITIES OF CLOXACILLIN BY LC–UV AND THERMOSPRAY LC–MS

| Sample | LC–UV (ppm) | LC–MS (ppm) |
|---|---|---|
| Control | 0 | 0 |
| Dosed milk 8 h | 56 | 51 |
| Dosed milk 24 h | 8 | 7 |
| Dosed milk 32 h | 6 | 5 |
| Dosed milk 48 h | N.D.[a] | N.D. |
| Dosed milk 56 h | N.D. | N.D. |

[a] N.D. = not detected.

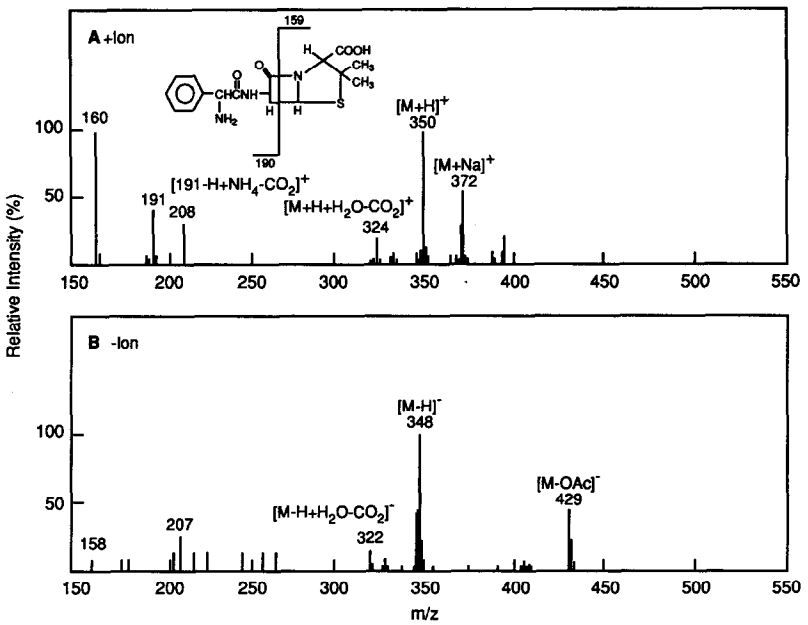


Fig. 7. Thermospray LC–MS spectra of ampicillin acquired using (A) positive-ion detection and (B) negative-ion detection.

160 are protonated fragment ions previously discussed for cloxacillin. The negative-ion spectrum for ampicillin (Fig. 7B) exhibited an $[M - 26]^-$ (*m/z* 322) ion. The negative-ion detection mode was several times less sensitive than the positive-ion detection mode. Multiple-ion detection, monitoring the positive ions *m/z* 324 and 350, was employed to achieve suitable sensitivity for determination of ampicillin in milk. Fig. 8 shows that these ions (*m/z* 324 and 350) comaximize in the milk sample and standard verifying the presence of ampicillin. The low mass ions *m/z* 160 and 191 were not monitored because of significant background ion current in these channels, presumably due to the milk matrix. Also, the $[M + Na]^+$ ion for ampicillin as well as for the other penicillins was not monitored due to the variability of the peak intensity, presumably due to variability in sodium concentration in samples or standards.

The thermospray spectrum for amoxicillin exhibited a $[M + H]^+$ ion (*m/z* 366), an adduct ion $[M + Na]^+$ (*m/z* 388), and fragment ions at *m/z* 340, 207, 224, and 160 (Fig. 9A). The ion at *m/z* 340, $[M - 26]^+$, was discussed previously for the other penicillins. The negative-ion spectrum for amoxicillin (Fig. 9B) exhibited an $[M - H]^-$ ion (*m/z* 364) and fragment ions at *m/z* 338 and 205. As was observed with the other penicillins, the negative-ion mode of operation was several times less sensitive for amoxicillin compared to the positive-ion detection mode. To improve thermospray LC–MS sensitivity, only the two most intense

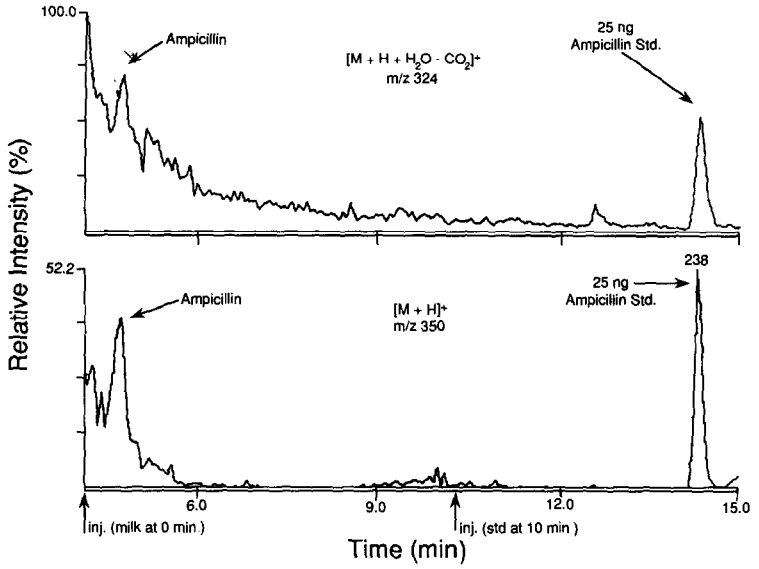


Fig. 8. Thermospray LC–MS ion chromatograms monitoring the $[M + H + H_2O - CO_2]^+$ ion ($m/z$ 324) and $[M + H]^+$ ion ($m/z$ 350) for a bovine milk sample spiked with 500 ppb ampicillin (injection volume was 100 $\mu$l) and for a 25-ng ampicillin standard.

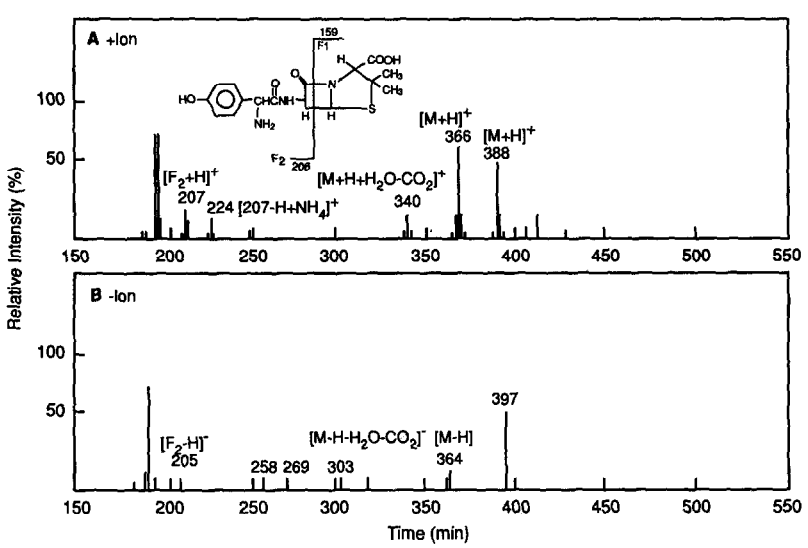


Fig. 9. Thermospray LC–MS spectra of amoxicillin acquired using (A) positive-ion detection and (B) negative-ion detection.

positive ions ($m/z$ 207 and 366) for amoxicillin were monitored. The $[M - 26]^+$ ion for amoxicillin ($m/z$ 340) was not monitored owing to its low relative abundance compared to the other penicillins.

The LC–MS confirmation of amoxicillin in bovine milk proved difficult because of the high polarity of this analyte, preventing baseline separation from the milk matrix (Fig. 10A). The addition of a liquid extraction step using 8% zinc sulfate in 80% methanol [1:1 (v/v) zinc sulfate solution and milk) prior to ultrafiltration precipitated many of the milk interferences. LC–MS detection limits near 200 ppb could be achieved with this additional clean-up step (Fig. 10B). No amoxicillin was found in bovine milk at 24 h or longer after intramuscular administration.

Comparison of the LC–UV and LC–MS detection limits showed both methods could detect all antibiotics down to 100 ppb. The UV-PDA detection limit

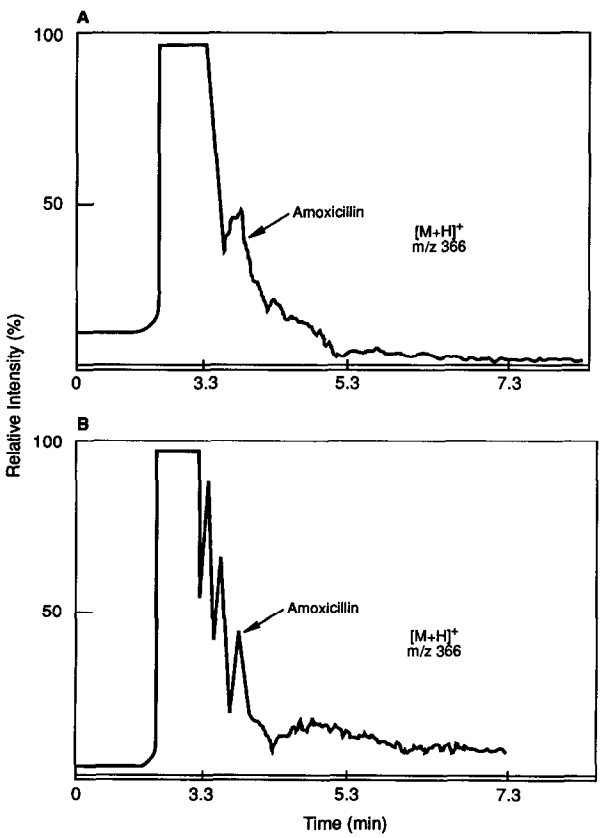


Fig. 10. Thermospray ion chromatogram for the $[M + H]^+$ ion ($m/z$ 366) of amoxicillin in spiked bovine milk. (A) Chromatogram obtained from milk with 500 ppb of amoxicillin without additional clean-up; (B) chromatogram obtained from milk with 500 ppb of amoxicillin using additional liquid extraction clean-up step.

based on a 3:1 signal-to-noise ratio at 210 nm was estimated to be 50 ppb for cloxacillin, 75 ppb for ampicillin, and 100 ppb for amoxicillin. The thermospray LC–MS detection limit based on a 3:1 signal-to-noise ratio was estimated to be 100 ppb for cloxacillin, 200 ppb for ampicillin, and 200 ppb for amoxicillin. Further work to improve the LC–MS separation of amoxicillin form the milk matrix or the incorporation of an additional clean-up step should further improve the detection limits for this antibiotic.

## CONCLUSIONS

Ion-paired LC separated penicillins from bovine milk components permitting detection by UV-PDA or MS. The need for volatile buffers in thermospray LC–MS required the development of different chromatographic conditions for LC–MS confirmation. Thermospray LC–MS using volatile ammonium buffers proved adequate for the separation of the penicillins from the milk matrix and for the confirmation of these antibiotics in milk.

The LC–UV-PDA methods, combined with thermospray LC–MS confirmation, could be used to determine the levels of cloxacillin, ampicillin/hetacillin, and amoxicillin in bovine milk at various periods after intramammary administration. The methods will prove useful for determining withdrawal periods for these antibiotics in cattle as well as for residue analysis. The presence of low ppm levels of penicillins in bovine milk indicate that withdrawal periods longer than 24 h are required to achieve acceptable tolerance limits of these antibiotics (less than 100 ppb in milk) after intramammary administration.

## ACKNOWLEDGEMENTS

This research was supported by the Food and Drug Administration (FDA) under Cooperative Agreement No. FD-U-000244. The authors wish to thank Greg Guyer of FDA for supplying bovine milk samples.

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