Simultaneous Determination of Amoxicillin and Ampicillin in Bovine Milk by HPLC with Fluorescence Detection

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A sensitive liquid chromatographic analytical method using fluorescence detection was developed for the simultaneous determination of amoxicillin and ampicillin residues in raw and processed bovine milk. Aliquots of raw or processed milk (5 mL) were diluted to 40 mL with 0.01 M KH_2PO_4 (pH 4.5) buffer, and the soluble proteins were precipitated with the addition of sodium tungstate and sulfuric acid followed by centrifugation. The drug residues were concentrated by passing the supernatant through a C_{18} solid phase extraction cartridge. Amoxicillin and ampicillin were eluted from the cartridge and reacted with salicylaldehyde to form fluorescent derivatives, which were then analyzed with liquid chromatography and fluorescence detection. Average recoveries of spiked amoxicillin and ampicillin at 5, 10, and 20 ng/mL were >80%, with coefficients of variation (CV) <5%. The limit of detection (LOD) and limit of quantitation (LOQ) for amoxicillin were 1.1 and 2.4 ng/mL, respectively. The LOD and LOQ for ampicillin were 1.0 and 1.7 ng/mL, respectively.

Keywords: *Amoxicillin; ampicillin; antibiotics; milk; HPLC*

INTRODUCTION

Amoxicillin and ampicillin are two of the widely used β-lactam antibiotics. It is mandatory that every tanker load of milk be tested for β-lactam antibiotics residues in United States according to U.S. Food and Drug Administration (1991). Typically, microbiological inhibition and other rapid screening tests have been used for the detection of β-lactam antibiotics in milk (Messer *et al.*, 1982; Charm and Chi, 1982). False positive results have been reported due to the lack of specificity of these screening tests (Senyk *et al.*, 1990), which requires additional assays for the quantitative and confirmatory determination of specific β-lactam antibiotics in the positive milk samples after testing by screening methods.

Several HPLC methods have been reported for the analytical determination of various β-lactam antibiotics in milk. An LC-UV method reported by Boison *et al.* (1994) was able to detect penicillin G at 3 ng/mL in milk after derivatization with 1,2,4-triazole−mercuric chloride solution. Moats (1994) used an automated LC system for sample cleanup, and a different LC-UV system for the analysis of ampicillin and amoxicillin in milk, with a detection limit of 2−5 ng/mL. Straub *et al.* (1994) used perfusive-particle LC combined with ultrasonic nebulization electrospray mass spectrometry for determination of six β-lactam antibiotics residues in milk, with a detection limit of 3−5 ng/mL. Two more recent and creative methods for analyses of β-lactam antibiotics residues in milk were developed by Harik-Khan and Moats (1995) and Zomer *et al.* (1995). Harik-Khan and Moats used an automated LC for sample cleanup, screening kits for β-lactam activity testing, and an analytical LC-UV system for quantitative determination. The method reported by Zomer *et al.* used an LC for the separation of β-lactams and microbial receptor assay (Charm II) for identification and quantitation of β-lactams in milk.

It was reported by Jusko (1971) that a highly fluorescent derivative formed when ampicillin was heated in the presence of acid and formaldehyde. The reaction was further studied and a reaction pathway for the formation of the fluorescent derivative of ampicillin was proposed (Lebelle *et al.*, 1979; Uno *et al.*, 1981). Recently, utilizing the derivatization reactions of ampicillin and amoxicillin with formaldehyde, HPLC fluorescence methods were developed for the analysis of trace levels of ampicillin in serum (Lal *et al.*, 1994) and the determination of amoxicillin residues in fish tissues (Ang *et al.*, 1996).

Initial studies in our laboratory pertaining to modifying and applying the method of Ang *et al.* (1996) for the simultaneous analysis of amoxicillin and ampicillin in bovine milk were problematic due to co-eluting interfering HPLC peaks seen in the milk samples. Therefore, new fluorescent derivatives were developed by the reaction of amoxicillin and ampicillin with salicylaldehyde, and a straightforward and highly sensitive LC method was developed for the simultaneous determination of amoxicillin and ampicillin residues in raw and processed bovine milk.

MATERIALS AND METHODS

Chemicals and Milk Samples. Salicylaldehyde (ACS grade) and 1-pentanesulfonic acid, sodium salt (ACS grade), were obtained from Aldrich Chemical Co. (Milwaukee, WI). Sodium tungstate (ACS grade) was obtained from Sigma Chemical Co. (St. Louis, MO). Amoxicillin and ampicillin reference standards were obtained from U.S. Pharmacopeial Convention, Inc. (Rockford, IL). All solvents were HPLC grade and supplied by J. T. Baker, Inc. (Phillipsburg, NJ). All other chemicals were ACS grade.

Raw nonprocessed milk samples were obtained from a processing plant (Little Rock, AR) and from a local farm.

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S0021-8561(96)00739-X

Homogenized processed whole milk was purchased from a local market. The milk samples were stored at −70 °C until analyzed.

Apparatus. The C_{18} solid phase extraction cartridges (3 cc, 500 mg) and the vacuum manifold were obtained from Waters (Milford, MA). The HPLC system consisted of a 600E pump and pump controller, a 470 scanning fluorescence detector (Waters), with the excitation wavelength set at 354 nm and the emission wavelength set at 445 nm, a model 7125 injector with a 100 μL sample loop (Rheodyne, Cotati, CA), and a Prodigy 5 μm, ODS-3, 4.6 mm × 250 mm HPLC column (Phenomenex Co., Torrance, CA). HPLC data were acquired and processed using a PC and Millennium 2010 Chromatogram Manager software (Version 2.1, Waters). After injection, the sample was eluted with a mobile phase (I) of acetonitrile: 0.02 M KH_2PO_4 buffer, pH 5.5 (32:68, v/v) maintained at 1 mL/min for 12 min. After 12 min the mobile phase was changed to mobile phase (II) 50:50 acetonitrile:buffer, the flow rate was increased to 1.5 mL/min, and maintained for 10 min. The HPLC system was equilibrated with the mobile phase (I) at 1.5 mL/min for 8 min prior to the next injection.

Standard Solutions. Amoxicillin and ampicillin standard stock solutions (1 mg/mL in water) were prepared monthly and stored at 4 °C. Intermediate standard solutions of 5 μg/mL were prepared weekly by dilution of stock solutions with water and stored refrigerated. Working standard solutions of different concentrations were prepared daily by diluting the intermediate standard solutions with water.

Identification of Fluorescent Derivatives. Amoxicillin or ampicillin standard stock solution (1 mL) was mixed with 200 μL of trichloroacetic acid:water solution (30%) and 20 μL of salicylaldehyde in a 15-mL centrifuge tube that was capped loosely. The mixture was heated in a 100 °C water bath for 45 min. The fluorescent derivatives of amoxicillin and ampicillin were purified with a semipreparative C_{18} reverse phase HPLC system. The fluorescent characteristics of the purified derivatives dissolved in 50:50 acetonitrile:water were determined with an LS 50B luminescence spectrometer (Perkin Elmer, Beaconsfield, Buckinghamshire, U.K.). The structures of the derivatives were confirmed by electron ionization mass spectrometry and ^{1}H-NMR. MS analysis was performed with a Finnigan MAT (Finnigan MAT Corp., San Jose, CA) 4023 quadrupole mass spectrometer. ^{1}H-NMR spectra were recorded on a Bruker AM500 spectrometer (Bruker Instruments, Inc., Billerica, MA) operating at 500.13 MHz. Detailed instrumental procedures have been described (Ang *et al.*, 1996).

Optimization of Derivative Reaction Conditions. In order to optimize the derivative reaction conditions used in the analytical procedure, we investigated the effects of the reaction time, the water bath temperature, the concentration of the 200 μL of trichloroacetic acid used, and the amount of salicylaldehyde added on the formation of the fluorescent derivatives of both amoxicillin and ampicillin. To optimize the reaction time, eight centrifuge tubes each containing 1 mL of standard solution (100 ng of amoxicillin and 100 ng of ampicillin) were each mixed with 200 μL of 30% (w/v) trichloroacetic acid aqueous solution and 20 μL of salicylaldehyde. The eight tubes were heated in a 100 °C water bath and then individually removed from the water bath after 10, 20, 25, 30, 35, 40, 50, and 60 min reaction time. The fluorescent derivatives formed in each tube were quantitatively determined by HPLC with fluorescence detection. The effects of heating temperature (with heating time, 30 min; salicylaldehyde, 20 μL; 30% trichloroacetic acid, 200 μL), the effects of different amounts of salicylaldehyde (with heating time, 30 min; temperature, 100 °C; 30% trichloroacetic acid, 200 μL), and the effects of the concentration of the 200 μL trichloroacetic acid (with heating time, 30 min; temperature, 100 °C; salicylaldehyde, 20 μL) were determined in a similar manner.

Sample Preparation and Extraction. A 5-mL sample of milk in a 50-mL polyethylene centrifuge tube was diluted to 40 mL with 0.01 M KH_2PO_4 buffer (pH 4.5) and shaken vigorously for 20 s. 1 mL of 10% sodium tungstate solution and 1 mL of 0.5 M H_2SO_4 solution were added to the tube, which was then shaken for 20 s. The mixture was centrifuged at 3500 g for 20 min and supernatant filtered through a #42 Whatman filter paper (Whatman Inc., Clifton, NJ) into another clean tube. Prior to solid phase extraction, 1 mL of 3% (w/v) sodium 1-pentanesulfonate solution was added to the filtrate.

Solid Phase Extraction. The 3 cc (500 mg) C_{18} solid phase extraction cartridges were mounted onto a vacuum-operated solid phase extraction manifold. The cartridges were conditioned by applying 15 mL of methanol followed by 5 mL of water. The sample filtrate was then loaded onto the cartridge at an approximate flow rate of 1 mL/min. When all the filtrate had eluted through the cartridge, the cartridge was washed with 4 mL of water. The amoxicillin and ampicillin adsorbed on the cartridge were eluted with 1.6 mL of 60% methanol in water. The eluate was collected in a 50-mL round bottom flask and evaporated with a vacuum rotary evaporator set at 50 °C to about 700 μL to remove methanol. The concentrated extract was transferred from the flask to a 15-mL graduated centrifuge tube. The flask was rinsed with 500 μL of water, which was also transferred to the centrifuge tube.

Derivative Reaction. A 20-μL aliquot of salicylaldehyde and 200 μL of 30% trichloroacetic acid were added to the concentrate in centrifuge tube, which was then vortexed for 10 s. The centrifuge tube was capped loosely and heated in a boiling water bath (100 °C) for 45 min. After cooling to room temperature, the contents in the graduated centrifuge tube were brought to 2 mL with 50% acetonitrile in water, and a 100-μL aliquot was injected into the HPLC for analysis.

Calibration and Determination. To establish calibration curves, a series of standard solutions at concentrations of 10, 20, 50, 100, and 150 ng/mL of both amoxicillin and ampicillin was prepared. One milliliter of each standard solution was reacted with salicylaldehyde and trichloroacetic acid as described above. After dilution to 2 mL, a 100-μL aliquot was injected into the HPLC. For daily analysis of milk samples, 50 ng/mL standard solutions were reacted and used as daily calibration standards for quantitative determination of amoxicillin and ampicillin in milk samples.

Spiking of Milk Samples and Recovery. A standard solution containing 100 ng/mL each of both amoxicillin and ampicillin was used to spike milk samples at the levels of 5, 10, and 20 ng/mL. After the spiked milk samples were analyzed by the procedure described above, percentage recoveries were calculated using the daily calibration standards as external standards for quantitation. Five replicates of spiked milk samples at each level of 5, 10, and 20 ppb were analyzed to determine the recoveries and variation of the analytical procedure within the work day (within-day recoveries and variation). Spiked milk samples were also analyzed on different days to determine the day-to-day variation of the analytical procedure.

RESULTS AND DISCUSSION

Derivatization Reaction. Highly fluorescent derivatives were formed when amoxicillin or ampicillin was reacted with salicylaldehyde under acid conditions at elevated temperature. The reactions were consistent and quantitative. The fluorescent derivatives were stable in the dark at room temperature for up to 48 h.

The chemical structures of amoxicillin and ampicillin and their fluorescent salicylaldehyde derivatives are shown in Figure 1. The structures of the fluorescent derivatives were proposed according to the reaction pathway for the reaction of ampicillin with formaldehyde as suggested by Uno *et al.* (1981). Positive confirmation was obtained by ^{1}H-NMR (Table 1) and electron ionization MS (Table 2) analyses of these derivatives. The apparent molecular ions observed for the amoxicillin derivative (*m*/*z* 294) and ampicillin derivative (*m*/*z* 278) and their fragment ions (Table 2) are consistent with the structures presented in Figure 1. Additional ^{1}H-NMR analysis (Table 1) positively confirmed the chemical structures. Proton assignments were made via chemical shifts, homonuclear decoupling, and nuclear overhauser effect (NDE) experiments.

The spectrophotofluorometric excitation and emission spectra of the derivatives of amoxicillin and ampicillin

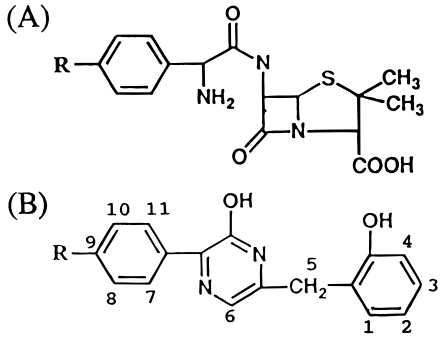

Figure 1. (A) Chemical structure of amoxillin (R = OH) and ampicillin (R = H). (B) Chemical structure of amoxicillin salicylaldehyde fluorescent derivative (R = OH) and ampicillin salicylaldehyde fluorescent derivative (R = H).

Table 1. 500 MHz Proton Spectral Parameters for the Fluorescent Derivatives of Amoxicillin and Ampicillin Formed after Reaction with Salicylaldehyde

derivative of amoxicillin[a]		derivative of ampicillin[b]	
proton	chemical shift, ppm[c]	proton	chemical shift, ppm
1	7.14	1, 6	7.17
2	6.74	2	6.76
3, 6	7.07	3	7.09
4	6.81	4	6.83
5	3.73	5	3.78
7, 11	8.18	7, 11	8.25
8, 10	6.75	8, 9, 10	7.36−7.41

[a] Coupling constants are $J_{1,2} = 7.5$ Hz, $J_{1,3} = 1.7$ Hz, $J_{2,3} = 7.7$ Hz, $J_{2,4} = 1.1$ Hz, $J_{3,4} = 8.2$ Hz, and $J_{7,8} = 9.0$ Hz. [b] Coupling constants are $J_{1,2} = 7.5$ Hz, $J_{1,3} = 1.7$ Hz, $J_{2,3} = 7.5$ Hz, $J_{2,4} = 1.3$ Hz, $J_{3,4} = 8.2$ Hz, $J_{7,8} = 8.2$ Hz, and $J_{7,9} = 1.9$ Hz. [c] In DMSO-d_6 after assigning the residual solvent peak to 2.49 ppm.

Table 2. Chromatographic and Mass Spectrometric Data of the Fluorescent Derivatives of Amoxicillin and Ampicillin Formed after Reaction with Salicylaldehyde

derivative of	HPLC retention time[a]	fluorescence[b]		EI mass spectra[c]
		λ(ex)	λ(em)	
amoxicillin	10 min	354 nm	445 nm	77 (18), 91 (15), 120 (17), 133 (16), 146 (18), 160 (58), 249 (10), 277 (11), 294 (100), 295 (20)
ampicillin	21 min	347 nm	425 nm	77 (23), 89 (15), 91 (12), 104 (19), 116 (10), 117 (12), 130 (17), 144 (45), 146 (22), 147 (11), 233 (10), 261 (18), 278 (100), 279 (21)

[a] HPLC retention time using the conditions described in Materials and Methods. [b] Fluorescence excitation and emission maxima of each derivative determined as described in Materials and Methods. [c] Electron ionization mass spectral data. Only ion fragments with relative intensities of at least 10% are listed. The number in parentheses is the relative intensity observed.

are depicted in Figure 2. The maximum excitation wavelength was 354 nm for the derivative of amoxicillin and 347 nm for the derivative of ampicillin. The maximum emission wavelength was 445 nm for the derivative of amoxicillin and 425 nm for the derivative of ampicillin. These results are very similar to those of the fluorescent formaldehyde derivatives of amoxicillin and ampicillin reported by Ang *et al.* (1996) and Jusko (1971). This suggests that the salicylaldehyde derivatives and the formaldehyde derivatives of amoxicillin and ampicillin may have similar fluorophores.

In order to obtain a high fluorescent response, the derivatization reaction conditions were optimized and the results of these experiments are shown in Figure 3. The optimum combination of the reaction conditions

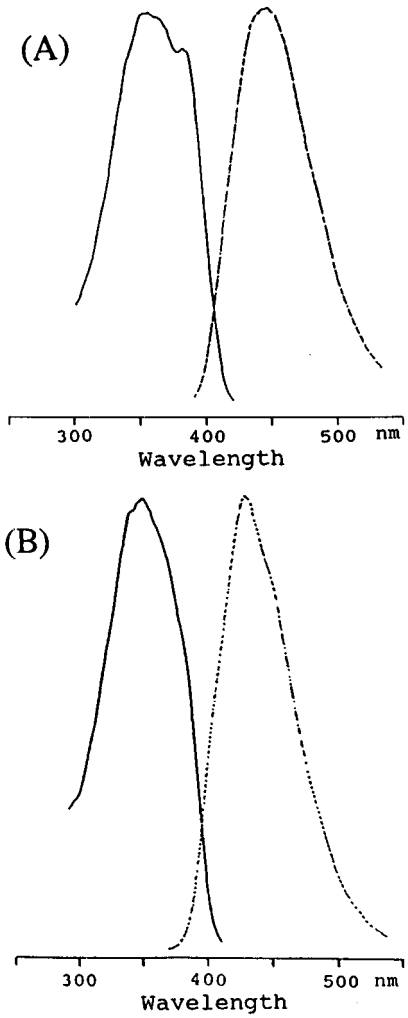

Figure 2. (A) Spectrofluorometric excitation (354 nm) and emission (445 nm) spectra for the fluorescent derivative of amoxicillin. (B) Spectrofluorometric excitation (347 nm) and emission (425 nm) spectra for the salicylaldehyde fluorescent derivative of ampicillin.

consisted of 1 mL of standard solution mixed with 200 μL of 30% trichloroacetic acid and 20 μL of salicylaldehyde and then heated at 100 °C for 45 min.

Sample Extraction and Derivatization. A 5-mL aliquot of milk sample was diluted to 40 mL with 0.01 M KH_2PO_4 to obtain a complete extraction of amoxicillin and ampicillin. The milk proteins were precipitated by adding 1 mL of 10% sodium tungstate aqueous solution and 1 mL of 0.5 M sulfuric acid solution. These reagents had been successfully used to denature and precipitate fish proteins by Luo *et al.* (1996) and animal tissue proteins by Boison *et al.* (1991). After centrifugation and filtration, a clear milk extract was obtained. Sodium pentanesulfonate was added to the extractprior to solid phase extraction to increase the retention of amoxicillin and ampicillin on the C_{18} cartridge and therefore to enhance their recoveries. When applying the extract onto the C_{18} cartridge, the flow rate was adjusted to 1 mL/min or lower. Higher flow rate (e.g., 1.5 mL/min) resulted in a lower recovery. After solid phase extraction, the C_{18} cartridge was eluted with 60% methanol in water. The methanol in the eluate was evaporated with a rotary evaporator since its presence seemed to adversely effect the derivatization reaction. At the completion of the reaction, the reaction mixture was diluted to 2 mL with 50% aqueous acetonitrile to dissolve any excess unreacted salicylaldehyde.

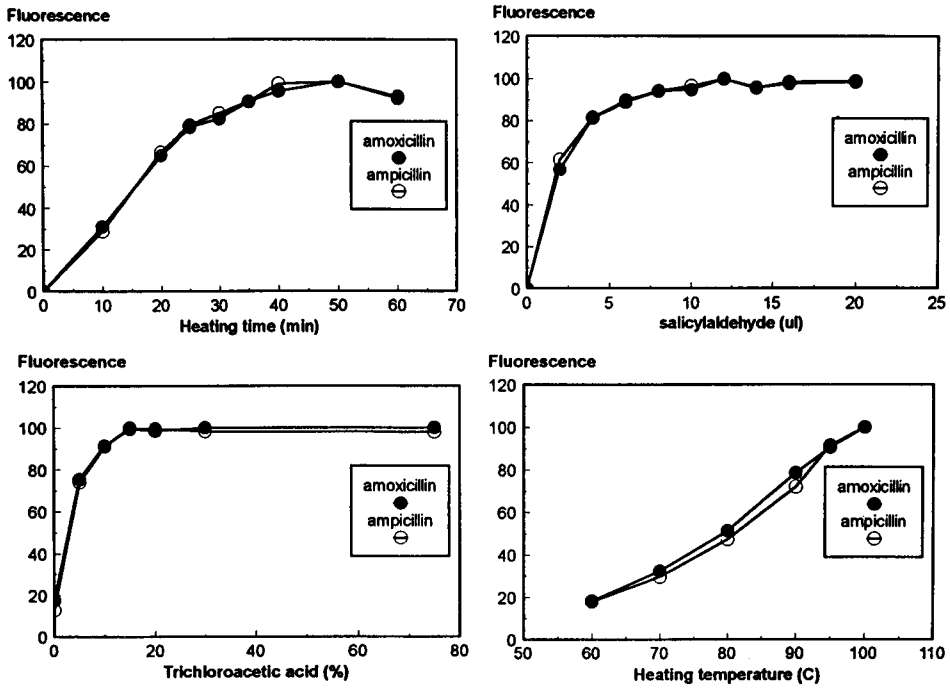

Figure 3. Effects of heating time (A), amount of salicylaldehyde (B), concentration of trichloroacetic acid (C), and heating temperature (D) on the formation of fluorescent derivatives of amoxicillin and ampicillin.

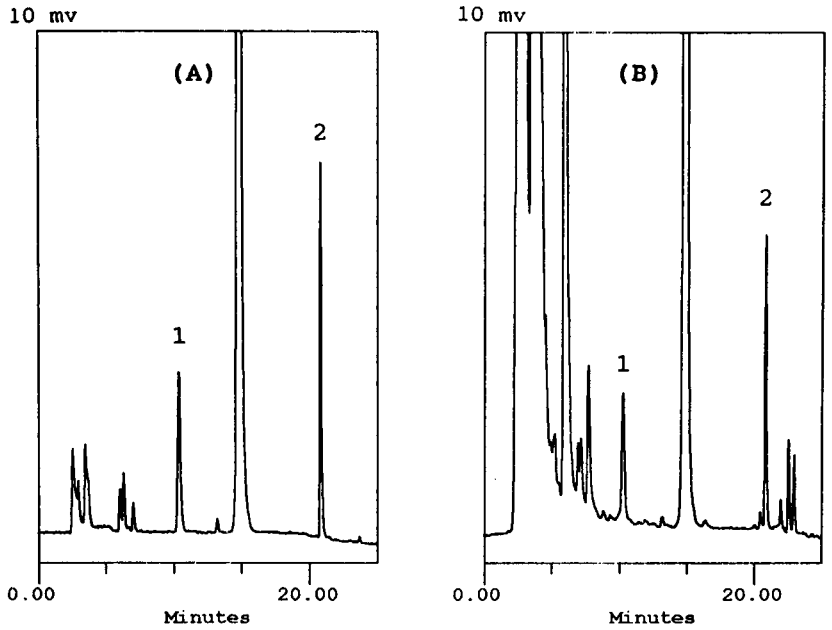

Figure 4. (A) Chromatogram of standard amoxicillin derivative (peak 1) and ampicillin derivative (peak 2). (B) Chromatogram of milk sample spiked with 10 ng/mL each of amoxicillin and ampicillin.

HPLC Separation and Determination. Figure 4A shows a chromatogram of a mixture of standard solution of amoxicillin (peak 1) and ampicillin (peak 2), and each peak represents an equivalent of 2.5 ng of each drug injected on the column. The off-scale peak at the retention time of 15 min is the unreacted residue of salicylaldehyde. A 100-μL aliquot was injected into the HPLC for separation and quantitative determination. The amoxicillin derivative was eluted at approximately 10 min by the mobile phase (I) (acetonitrile:buffer, 32:68) at flow rate of 1 mL/min. The mobile phase (I) was run for 12 min and then changed to mobile phase (II) (acetonitrile:buffer, 50:50) at a flow rate of 1.5 mL/min in order to elute the ampicillin derivative, which was relatively less polar. The ampicillin derivative was thus eluted at approximately 21 min. Figure 4B shows the chromatogram from the analysis of a raw milk sample spiked at 10 ng/mL with amoxicillin and ampicillin. The chromatograms of raw milk and processed milk samples were very similar.

The fluorescence intensity of the ampicillin derivative was relatively stronger than that of the amoxicillin derivative. Therefore the fluorescence detector of HPLC was set at 354 nm for excitation wavelength and 445 nm for emission wavelength in order to obtain good sensitivity for both the ampicillin and the amoxicillin derivatives. The fluorescence detector showed good linearity for the amoxicillin derivative ($r = 0.9993$) and the ampicillin derivative ($r = 0.9990$) in the range of 0.5−7.5 ng injected on column of amoxicillin and ampicillin standard equivalents. The regression equations of the amoxicillin and ampicillin standard curves were as follows: amoxicillin injected (ng) = 0.0188 × peak area (mV·s^{-1})−0.026; ampicillin injected (ng) = 0.0189

Table 3. Recoveries (in %) of Amoxicillin and Ampicillin from Spiked Milk Samples (Within-Day)[a]

spike level of amoxicillin (amox.) and ampicillin (amp.)		raw milk I		raw milk II		processed milk	
		mean	CV	mean	CV	mean	CV
5 ng/mL	amox.	84.0	3.7	83.0	4.7	86.8	3.1
	amp.	83.4	3.7	84.8	3.1	85.4	2.0
10 ng/mL	amox.	82.0	1.8	81.6	3.3	83.2	4.5
	amp.	82.2	1.6	87.0	2.0	82.8	2.8
20 ng/mL	amox.	82.4	3.8	82.0	2.3	85.4	2.2
	amp.	82.4	3.2	82.7	2.1	83.3	2.6

[a] The number of replicates (n) = 5.

Table 4. Recoveries (in %) of Amoxicillin and Ampicillin from Spiked Raw Milk Samples (Day-to-Day)[a]

spike level of amoxicillin and ampicillin		spike recovery	
		mean	CV
5 ng/mL	amoxicillin	84.6	3.2
	ampicillin	83.4	3.8
10 ng/mL	amoxicillin	82.3	1.7
	ampicillin	82.0	2.5
20 ng/mL	amoxicillin	82.9	3.4
	ampicillin	81.6	3.1

[a] Number of replicates (n) = 5.

× peak area ($mV \cdot s^{-1}$) − 0.054. The amount of amoxicillin or ampicillin injected on column would be 2.5 ng for a milk sample containing 10 ng/mL of amoxicillin or ampicillin. The intercepts of both calibration curves were negligible. Therefore, for practical reasons a one-point calibration standard could be used for routine analysis of milk samples.

The limit of detection (LOD) and limit of quantitation (LOQ) were estimated according to the American Chemical Society (1980) guidelines. LOD was calculated as mean blank response plus three times the standard deviation of replicate analyses of blank samples. LOQ was calculated as mean blank response plus ten times the standard deviation of replicate analyses of blank samples. The LODs for amoxicillin and ampicillin in milk were 1.1 and 1.0 ng/mL, respectively. The LOQs for amoxicillin and ampicillin in milk were 2.4 and 1.7 ng/mL, respectively.

Spiked Recoveries of Amoxicillin and Ampicillin. The FDA's guidelines for the approval of an analytical method for residue analysis (1986) were followed for the design of the recovery experiment. The within-day recoveries and relative standard deviations (CVs) of spiked amoxicillin and ampicillin in raw milk I, raw milk II, and processed milk at the levels of 5, 10, and 20 ng/mL are summarized in Table 3. The recoveries of the three milk samples were similar and higher than 80% with CVs less than 5%. The day-to-day spike recovery experiments were performed only on raw milk I because of the similarity of the three milk samples in the within-day recoveries. The results of day-to-day recoveries are summarized in Table 4. These results indicated adequate accuracy and good precision of the analytical method.

Compared with other analytical methods for the analyses of β-lactam antibiotics as discussed in the section of introduction, this analytical method is superior in terms of sensitivity and selectivity. The disadvantage is that this method is only suitable for the analyses of amoxicillin and ampicillin. No fluorescent derivatives were formed when salicylaldehyde was reacted with other common β-lactam antibiotics such as penicillin G, cloxacillin, and cephapirin.

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Received for review September 27, 1996. Revised manuscript received January 21, 1997. Accepted January 22, 1997.[⊗]

JF960739L

[⊗] Abstract published in *Advance ACS Abstracts*, March 15, 1997.