Determination of Some Monobasic Penicillins in Milk by Ion-Pair Liquid Chromatography

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An ion-pair liquid chromatographic method for the determination of four commonly used monobasic penicillins (penicillin G, penicillin V, oxacillin, and cloxacillin) in milk has been developed. According to this method, which provides quantitation down to 3–4 ppb, milk is acidified at pH 3 and extracted with dichloromethane. The extracted penicillins are partitioned into a phosphate buffer, pH 7, and following addition of ammonium sulfate, the extracts are purified by treatment with diethyl ether and repartitioned into acetonitrile. The acetonitrile extracts are concentrated into phosphate buffer, pH 7, and after addition of tetrabutylammonium hydrogen sulfate, the formed penicillin ion pairs are extracted into chloroform. Liquid chromatography was performed on a reversed-phase C_{18}, 5-μm, column using an aqueous acetonitrile, pH 6, mobile phase containing 5 mM tetrabutylammonium hydrogen sulfate. Overall recoveries were found to be better than 82.9%, whereas overall relative standard deviation for all penicillins ranged between 3.8% and 6.6%.

INTRODUCTION

Penicillin G (Pc-G), penicillin V (Pc-V), oxacillin (Oxc), and cloxacillin (Clxc) are extensively used in veterinary practice for treatment and prevention of bovine mastitis. This use has led to the need for methods to monitor residual levels of penicillins in milk, since traces of these compounds have been incriminated in allergic reactions in humans (Becker, 1976; Dewdney and Edwards, 1984; Burgat, 1984). The presence of penicillin residues in milk might also be responsible for starter culture inhibition in the manufacture of fermented dairy products such as cheese, yogurt, buttermilk, or sour cream (Sozzi and Smiley, 1980; Friend and Shahani, 1983; Orberg and Sandine, 1985; Shahani and Whalen, 1986).

Penicillins in milk have traditionally been regulated by bioassays (Katz, 1986; International Dairy Federation, 1987). These methods, which are based on testing for inhibition of microbial growth, are very sensitive and, therefore, useful for initial screening of residues, but their precision appears to be variable and their specificity questionable. Furthermore, the occurrence in milk of natural inhibitory substances (Busta, 1966; Carlsson and Bjorck, 1987) affects the accuracy of these assays.

Several physicochemical methods including thin-layer chromatography (Moats, 1983a), thin-layer chromatography and bioautography (Bossuyt et al., 1976; Herbst, 1982), electrophoresis and bioautography (Billon and Tao, 1979; Pietrangeli et al., 1986), and gas–liquid chromatography (Hamann et al., 1975; Meetschen and Petz, 1990) have also been reported for the determination of penicillins in milk. Most of them provide increased specificity and sensitivity but cannot be considered sufficiently simple, fast, or precise.

Considerable progress has been made recently on penicillin residues by the use of liquid chromatography (LC). However, current LC methods for the determination of penicillins in milk have, for the most part, dealt with single-component analysis using reversed-phase columns (Tyczkowska et al., 1989; Wiese and Martin, 1989; Moats, 1990a,b). Methods involving multiple analysis of penicillin residues in milk have also been described. However, some of them (Munns et al., 1985; Terada and Sakabe, 1985) lack the sensitivity required to monitor the ultralow levels (<20 ppb) of penicillins expected in milk, whereas Moats's (1983b) method, although adequately sensitive, is based on gradient chromatography.

Since multiresidue procedures are desirable for surveillance purposes, an ion-pair LC method capable of determining traces of Pc-G, Pc-V, Oxc, and Clxc in milk was developed. In this method separation of penicillins can be effected under isocratic conditions at a mobile-phase pH that favors the chemical stability of the analytes.

MATERIALS AND METHODS

Chemicals. Penicillin G, oxacillin, and cloxacillin, as their sodium salts, and penicillin V, as its potassium salt, were purchased from Sigma (St. Louis, MO). Tetrabutylammonium hydrogen sulfate (TBA–HSO_4) and LC grade acetonitrile were obtained from Merck-Schuchardt (Munchen, FRG), while penicillinase was obtained from Leo Pharmaceutical Products (Denmark). All other chemicals used were of analytical reagent grade and obtained from Merck-Schuchardt. Deionized water was distilled before use.

Stock solutions of Pc-G, Pc-V, Oxc, and Clxc, ca. 1 mg/mL, were prepared in water and were stable for 1 month if stored at −25 °C. Aliquots of these solutions were further diluted with mobile phase to give working solutions containing each penicillin in the range 0.020–3 μg/mL. Working solutions were prepared daily.

Aqueous 0.5 M TBA–HSO_4 solution, pH 7, was prepared by dissolving the appropriate amount of TBA reagent in 1 M disodium hydrogen phosphate solution. A 0.05 M phosphate buffer, pH 7, was also prepared by adding the appropriate volume of 0.05 M sodium dihydrogen phosphate solution to a volume of a 0.05 M disodium hydrogen phosphate solution.

Instrumentation. LC separations were carried out on a Gilson (Villiers-le-Bel, France) system consisting of a Model 802 manometric module, a Model 302 piston pump, a Model HM/HPLC dual-beam variable-wavelength UV–vis spectrophotometer set at 210 nm, and a Model N1 variable-span recorder. A Hplc-technology (Macclesfield, U.K.) Model TC 831 column oven, set at 40 °C, permitted temperature regulation. Injections were made on a reversed-phase, 25 × 0.46 cm, column packed with

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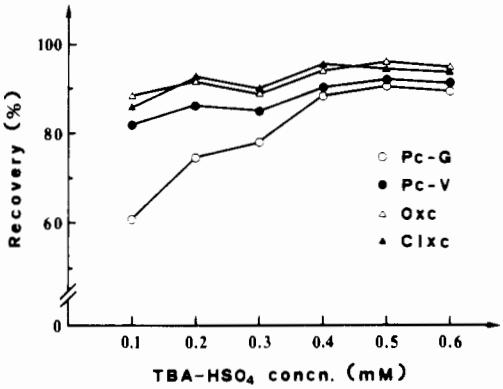

Figure 1. Effect of TBA-ion concentration on extraction of aqueous penicillin solutions by chloroform.

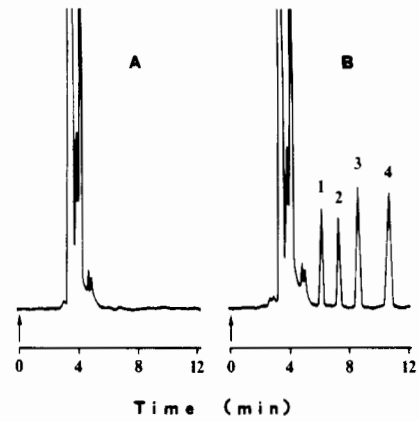

Figure 2. Typical chromatograms of a blank milk sample (A) and a sample (B) spiked with 30 ppb of Pc-G (1), Pc-V (2), Oxc (3), and Clxc (4). Conditions: mobile phase, 5 mM TBA-HSO_4 in acetonitrile–0.02 M phosphate buffer (38:62 v/v), pH 6; column, Nucleosil C_{18} (5 μm); column temperature, 40 °C; flow rate, 1 mL/min; wavelength, 210 nm; detector sensitivity, 0.020 aufs; chart speed, 0.5 cm/min; injection volume, 100 μL.

Nucleosil C_{18}, 5 μm, (Macherey-Nagel-Duren, FRG), through a Rheodyne 7125 sample injector equipped with a 100-μL loop.

LC Procedure. The mobile phase used was a mixture of acetonitrile and 0.02 M phosphate buffer (380:620 v/v), containing 5 mM TBA-HSO_4. The phosphate buffer was prepared by mixing 470 mL of 0.02 M disodium hydrogen phosphate solution with 150 mL of 0.02 M phosphoric acid solution and adjusting the pH at 6 with dilute phosphoric acid after addition of TBA reagent. The mobile phase was degassed using helium and delivered at a rate of 1 mL/min. A 100-μL aliquot of the final milk extract was injected onto the column. Recordings were made at a chart speed of 0.5 cm/min and a detector sensitivity of 0.020 AUFS.

Milk Extraction. A 7-g sample was weighed into a 50-mL glass-stoppered centrifuge tube and acidified at pH 3 with 1 mL of 0.6 N sulfuric acid. A volume (30 mL) of dichloromethane was added, and the tube was shaken for 2 min. The suspension formed was centrifuged for 3 min at 6000*g*. A 25-mL aliquot of the bottom organic layer, containing the compounds of interest, was pipetted to a 50-mL evaporating flask, and a volume (2 mL) of 0.05 M phosphate buffer, pH 7, was added. The resulting mixture was rotary-evaporated under reduced pressure at 30 °C to a volume of ca. 6 mL, transferred to a 15-mL tube, vortexed, and centrifuged for 1 min at 2000*g*. The top aqueous layer separated was pipetted to another tube, while the remaining bottom layer was combined with flask washing by 2 mL of the phosphate buffer. The resulting mixture was submitted to the described procedure of vortexing, centrifuging, and transferring the top aqueous layer into the same tube.

Cleanup Procedure. A volume (1 mL) of saturated ammonium sulfate solution was added in the tube. Following addition of diethyl ether (3 mL), tube content was vortexed for 0.5 min and centrifuged for 1 min at 2000*g*. The upper organic layer was discarded, while traces of diethyl ether in the bottom aqueous layer were removed by gentle nitrogen stream. Tube content was then extracted twice with 2-mL portions of acetonitrile and, after centrifuging for 1 min at 2000*g*, the combined top layers were mixed into a 25-mL evaporating flask with 3 mL of 0.05 M phosphate buffer, pH 7, to be further rotary-evaporated at 35 °C to a volume of ca. 3 mL. Following addition of 0.5 mL of 0.5 M aqueous TBA-HSO_4 solution, adjusted at pH 7, and 5 mL of chloroform, the flask content was shaken vigorously for 2 min, transferred to a 10-mL tube, and centrifuged for 1 min at 2000*g*. A 4.5-mL aliquot from the separated bottom organic layer was pipetted to a clean centrifuge tube. The content of the tube was evaporated to dryness with nitrogen at 30 °C and redissolved in 1 mL of mobile-phase solution; a 100-μL aliquot was used for liquid chromatography.

Calibration curves were constructed by plotting peak heights vs concentration from 100-μL injections of each of the working solutions. The penicillin concentrations in the analyzed samples were calculated by reference to calibration curves and multiplication by the appropriate dilution factor.

Peak Confirmation. Confirmation of the presence of penicillins in the suspected samples was based on the disappearance of the recorded peaks after a penicillinase treatment. In this procedure a volume (100 μL) of an aqueous solution of penicillinase was added to the phosphate buffer, pH 7, prior to ammonium sulfate addition. The mixture was allowed to stand at room temperature for at least 15 min, submitted to the cleanup procedure, and injected onto the column. Although 200 units of penicillinase was adequate for hydrolyzing 600 ng of Pc-G and Pc-V, quantities as high as 2500 and 50 000 units were used to hydrolyze the corresponding quantity of Oxc and Clxc, respectively. Furthermore, addition of 200 units of penicillinase into the final milk extract 5 min before sample injection was effective in hydrolyzing Pc-G and Pc-V without chromatographic problems.

RESULTS AND DISCUSSION

The extraction of monobasic penicillins with dichloromethane was most efficient when milk samples had been acidified at pH 3 or lower due to the suppressed ionization of the carboxylate moiety of these compounds. When the pH was lowered, however, a remarkable degradation of Pc-G occurred, a behavior that has been well studied in the past (Schwartz, 1965; Hou and Poole, 1971; Blaha et al., 1976). Therefore, milk samples had to be acidified at the upper pH value (pH 3) as a compromise between extraction efficiency and stability considerations.

The extracted penicillins were partitioned into a phosphate buffer, pH 7, and after addition of ammonium sulfate solution, the mixture was partly purified by treatment with diethyl ether. Additional purification was effected by extracting the mixture with acetonitrile since penicillins were quantitatively recovered in the top acetonitrile layer as their ammonium salts (Hou and Poole, 1971; Moats, 1983b, 1984), whereas some interfering components were eliminated. However, further cleanup was quite indispensable since concentration of the acetonitrile extract into phosphate buffer, pH 7, followed by liquid chromatography resulted in recording of interfering peaks and baseline drift.

Further cleanup was effected by converting penicillin anions to ion pairs with TBA cations since the formed pairs are readily extracted into chloroform. This extraction was most efficient when the added TBA-HSO_4 aqueous solution had been adjusted at pH 7, where penicillins are completely ionized and ion-pair formation is at maximum. Extraction efficiency was also affected by the concentration of TBA-HSO_4 aqueous solution (Figure 1); best results were obtained at concentrations greater than 0.4 M. This ion-pair extraction of penicillins permitted evaporation of the resulting chloroform extract to dryness without any degradation of the contained compounds.

The effectiveness of the cleanup procedure permitted chromatographic analysis of milk samples under isocratic

Table I. Recovery Data for Penicillin Analysis in Milk

concn added,[a] ppb	mean concn found ± SD, ppb				mean rec, %			
	Pc-G	Pc-V	Oxc	Clxc	Pc-G	Pc-V	Oxc	Clxc
4.0	3.5 ± 0.4	4.6 ± 0.5	3.9 ± 0.2	4.1 ± 0.3	87.5	114.5	97.5	101.7
10.0	8.1 ± 0.5	9.3 ± 0.4	9.2 ± 0.5	9.0 ± 0.7	80.8	93.3	91.8	89.7
30.0[b]	23.6 ± 0.7	25.2 ± 1.4	26.3 ± 1.2	26.1 ± 1.0	78.6	84.1	87.6	86.9
50.0	39.7 ± 1.5	42.6 ± 2.1	45.9 ± 1.2	46.8 ± 1.4	79.5	85.2	91.8	93.6
71.4	59.8 ± 2.6	63.0 ± 3.2	64.3 ± 3.6	66.5 ± 2.1	83.7	88.3	90.1	93.2
100.0[b]	82.5 ± 2.2	90.5 ± 2.5	90.9 ± 2.4	89.6 ± 2.8	82.5	90.5	90.9	89.6

[a] Six replicates. [b] Seven replicates.

Table II. Precision Data for the Determination of Penicillins in Milk Samples Spiked at 30.0 ppb

day	mean concn found ± SD, ppb				rel SD, %			
	Pc-G	Pc-V	Oxc	Clxc	Pc-G	Pc-V	Oxc	Clxc
1[a]	23.6 ± 0.7	25.2 ± 1.4	26.3 ± 1.2	26.1 ± 1.0	3.1	5.6	4.6	3.8
2[b]	25.3 ± 0.4	24.7 ± 1.4	27.0 ± 1.1	25.6 ± 1.2	1.8	5.5	4.1	4.6
3[b]	25.3 ± 0.5	27.1 ± 0.9	27.0 ± 0.4	28.1 ± 0.5	1.9	3.3	1.4	1.7
			Variance Estimates					
within day					2.4	4.9	3.7	3.5
among days					4.0	4.4	0.7	4.7
overall					4.6	6.6	3.8	5.8

[a] Seven replicates. [b] Six replicates.

Table III. Concentrations of Pc-G in Milk following Intramuscular Injections of Procaine Penicillin G

hours after first injection[a]	concn of Pc-G[b] found, ppb	hours after last injection	concn of Pc-G found, ppb
12	19.9	12	20.9
24	24.8	24	4.8
36	24.8	36	<4
		48	<4

[a] Four injections of 3 000 000 units/12 h. [b] Values not corrected for recovery.

Table IV. Concentrations of Clxc in Milk following Three Successive Intramammary Infusions of 200 mg/48 h of Sodium Cloxacillin

quarter[a]	Clxc in milk, ppb, from each quarter at hours after treatment						
	12	24	36	48	60	72	84
LF[b]	28 900[c]	255.2	61.3	9.0	4.9	<3	<3
RF	<3	<3					
LR	<3	<3					
RR	7.2	<3	<3				

[a] LF, left front; RF, right front; LR, left rear; RR, right rear. [b] Treated quarter. [c] Values not corrected for recovery.

conditions. When a Nucleosil C_{18} 5-μm stationary phase and aqueous acetonitrile containing dilute phosphoric acid as eluent were used, the chromatograms recorded were free of interfering extraneous peaks. However, large differences in k' values, peak tailing, and degradation of Pc-G under these acidic conditions were consistently noted. Gradient elution has been successfully used under similar conditions (Moats, 1983b, 1984) to elute penicillins in a reasonable length of time eliminating peak tailing, but an isocratic LC system based on a mobile phase with a pH value compatible with the chemical stability of these compounds would be valuable. Recently Fletouris et al. (1991) proposed an aqueous acetonitrile, pH 6, mobile phase containing 5 mM TBA–HSO_4 for the isocratic separation of penicillins in aqueous solutions. When this system was used, Pc-G, Pc-V, Oxc, and Clxc were eluted in 5.9, 7.1, 8.3, and 10.3 min, respectively. No changes in retention times were noted with continual column use.

Regression analysis of the data obtained by running a series of working solutions showed the response to be linear for all compounds in the range examined [correlation coefficient (r), 0.9999 for Pc-G, 0.9997 for Pc-V, 0.9999 for Oxc, and 0.9999 for Clxc]. The absence of any interfering peaks in sample chromatograms (Figure 2) permitted determination down to 4 ppb for Pc-G and Pc-V and 3 ppb for Oxc and Clxc (peak to noise ratio, 3).

The recovery of the method was studied by spiking milk samples at six fortification levels with aqueous standard solutions and analyzing six replicates. The concentrations examined ranged from 4 to 100 ppb. Least-squares and regression analysis of the data presented in Table I showed that the relationship between "added" and "found" was adequately described, for all four compounds, by a linear regression ($y = -0.48 + 0.829x$, $r = 0.9995$ for Pc-G; $y = -0.32 + 0.893x$, $r = 0.9991$ for Pc-V; $y = -0.03 + 0.907x$, $r = 0.9998$ for Oxc; $y = 0.27 + 0.906x$, $r = 0.9993$ for Clxc). Therefore, the slopes (0.829 ± 0.033 for Pc-G; 0.893 ± 0.051 for Pc-V; 0.907 ± 0.020 for Oxc; and 0.906 ± 0.046 for Clxc) of these regression lines could be used as estimates of overall recovery for Pc-G (82.9 ± 3.3%), Pc-V (89.3 ± 5.1%), Oxc (90.7 ± 2%), and Clxc (90.6 ± 4.6%) determination in milk.

The precision of the method was also studied by assaying, on each of three different days, several milk samples spiked with penicillins at the 30 ppb level. To estimate the components of variance, the concentrations found (Table II) were subjected to "analysis of variance and expected mean squares for the one way-classification–unbalanced design" (Wernimont, 1987). The analysis of variance showed that within-day and among-days precisions, expressed as relative standard deviation (percent), were 2.3 and 3.9 for Pc-G, 4.9 and 4.3 for Pc-V, 3.7 and 0.6 for Oxc, and 3.5 and 4.6 for Clxc, respectively. It also suggested that overall precisions, which are in fact the overall uncertainties of single determinations, were 4.6% for Pc-G, 6.5% for Pc-V, 3.7% for Oxc, and 5.8% for Clxc.

Characterization of the recorded chromatographic peaks was based mainly on the retention behavior of each penicillin. Confirmation, however, of the presence of penicillins in the suspected samples could be made possible by subjecting the samples, prior to chromatography, to treatment with penicillinase and comparing the chromatograms recorded. Such a confirmatory test using 200 units of penicillinase has been also proposed by Terada and Sakabe (1985) in the analysis of Pc-G, Pc-V, and ampicillin in milk. It was observed, however, that such a

Table V. Concentrations of Pc-G in Milk following Three Successive Intramammary Infusions of 375 000 Units/12 h of Benzathine Penicillin G

	Pc-G in milk, ppb, from each quarter at hours after treatment											
quarter[a]	12	24	48	72	96	120	168	192	240	264	288	312
RF[b]	44 600[c]	12 500	4100	1600	700	300	131.6	62.8	26.5	9.9	<4	<4
LF	<4	<4										
RR	<4	<4										
LR	14.1	<4	<4									

[a] RF, right front; LF, left front; RR, right rear; LR, left rear. [b] Treated quarter. [c] Values not corrected for recovery.

quantity was not effective in hydrolyzing Oxc and Clxc due to the greater stability of these compounds toward penicillinase. Pertinent experiments showed that use of 2500 and 50 000 units of this enzyme could effectively hydrolyze as much as 600 ng of Oxc and Clxc, respectively. The chromatograms obtained after this treatment were similar to that of the blank sample of Figure 2.

To validate the method with real samples, a milk residue study was conducted on three dairy cows (A, B, and C) treated with recommended doses of Pc-G and Clxc. Cow A was administered intramuscularly, in the neck region, four doses of procaine penicillin G (Ilcocillin P, Ciba-Geigy), 3 000 000 units each, at 12-h intervals. Cows B and C were administered standard treatments for mastitis consisting of three successive intramammary infusions into the same quarter of sodium cloxacillin (Orbenin L. A., Beecham Animal Health), 200 mg/48 h each, and benzathine penicillin G (Tardomyocel-L, Bayer), 375 000 units/12 h each, respectively. Control milk samples, taken from each cow before treatment, and all other samples collected during the trial at 12-h intervals were stored at −25 °C until analyzed. The analysis data of Table III showed that Pc-G could be detected in milk (cow A) 12 h after the first intramuscular injection (first milking). During treatment its concentration in milk remained relatively constant but thereafter decreased gradually to no detectable levels 36 h after the last injection. These findings lend support to those of comparable experiments (Schipper et al., 1965; Jacobs and Hanselaar, 1966) using bioassays.

As far as intramammary infusions are concerned, it was found that Clxc was present in milk from the treated quarter (cow B) for 60 h after the last infusion (Table IV), while Pc-G (Table V) persisted in milk from the treated quarter (cow C) for as long as 11 days after the last infusion. Crossover from treated to untreated quarters was also observed; both penicillins could be detected in milk from an untreated quarter 12 h after treatment. These findings cannot be considered inconsistent with those of comparable experiments using gas–liquid chromatography (Hamann et al., 1975) or bioassays (Rollins et al., 1970; Geleta et al., 1984; Egan and Meaney, 1985); a shorter elimination time for Pc-G in milk from treated quarter has been reported, but this inconsistency may be attributed to the different salts of the penicillin used. The results of the trial are, however, in direct contrast to those of another study (Moats, 1983b) using LC; drug crossover from treated to untreated quarters was not observed, whereas Pc-G and Clxc could not be detected in milk from treated quarters 24 h after treatment.

In conclusion, the results of the present study show that the proposed LC method is an efficient and reliable means of quantitating Pc-G, Pc-V, Oxc, and Clxc residues in milk. Therefore, the method should be useful for routine identification and confirmation of residues presumptively identified as penicillins by other tests. Since one analyst can easily process 12 samples in an 8-h working day, the method should be also suitable to serve as a directly screening sensitive test for Pc-G, Pc-V, Oxc, and Clxc contamination in milk.

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