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Determination of ampicillin residues in milk by ion-pair reversed phase high performance liquid chromatography after precolumn derivatization[1]

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Abstract

A high performance liquid chromatography method has been developed for the determination of ampicillin residues in milk. The method involves extraction of ampicillin from milk with trichloroacetic acid solution followed by concentration on a conditioned C_{18} solid phase extraction column, acetylation with acetic anhydride in aqueous solution (pH 8.0) at ambient temperature for 3 min followed by reaction with 2 M 1, 2, 4-triazole and 10^{-2} M mercury (II) chloride solution (pH 9.0) at 65°C for 10 min. The resulting product is eluted on a C_{18} column with a mobile phase containing phosphate buffer (pH 6.5; 0.1 M), the ion-pairing agent tetrabutylammonium hydrogenosulphate, acetonitrile and methanol. The detection limit of the method is 3 ng ml^{-1} in milk.

Keywords: Ampicillin; Ion-pair reversed-phase liquid chromatography; Milk; Pre-column derivatization; Residues

1. Introduction

Ampicillin is a semi-synthetic penicillin with an amino group in the side-chain. It can be used for treating infections of milking cows. However, ampicillin residues in milk can cause allergic reactions in people with sensitivity to penicillin and also cause problems to the milk processing industry, in the manufacture of yoghurt, cheese and other milk products. For these reasons, several methods have been described for the assay of this antibiotic in the range of residue concentrations in milk (1–100 $\mu g\ l^{-1}$):

- ultraviolet (UV) detection methods at 220–230 nm which lack both sensitivity and selectivity [1,2] or include a lengthy and cumbersome extraction procedure [3];
- UV methods with precolumn derivatization reactions which permit a detection of the mercuric mercaptide of penicilloic acids at higher wavelengths (325–328 nm) and provide better selectivity with regard to co-extractives from milk [4].

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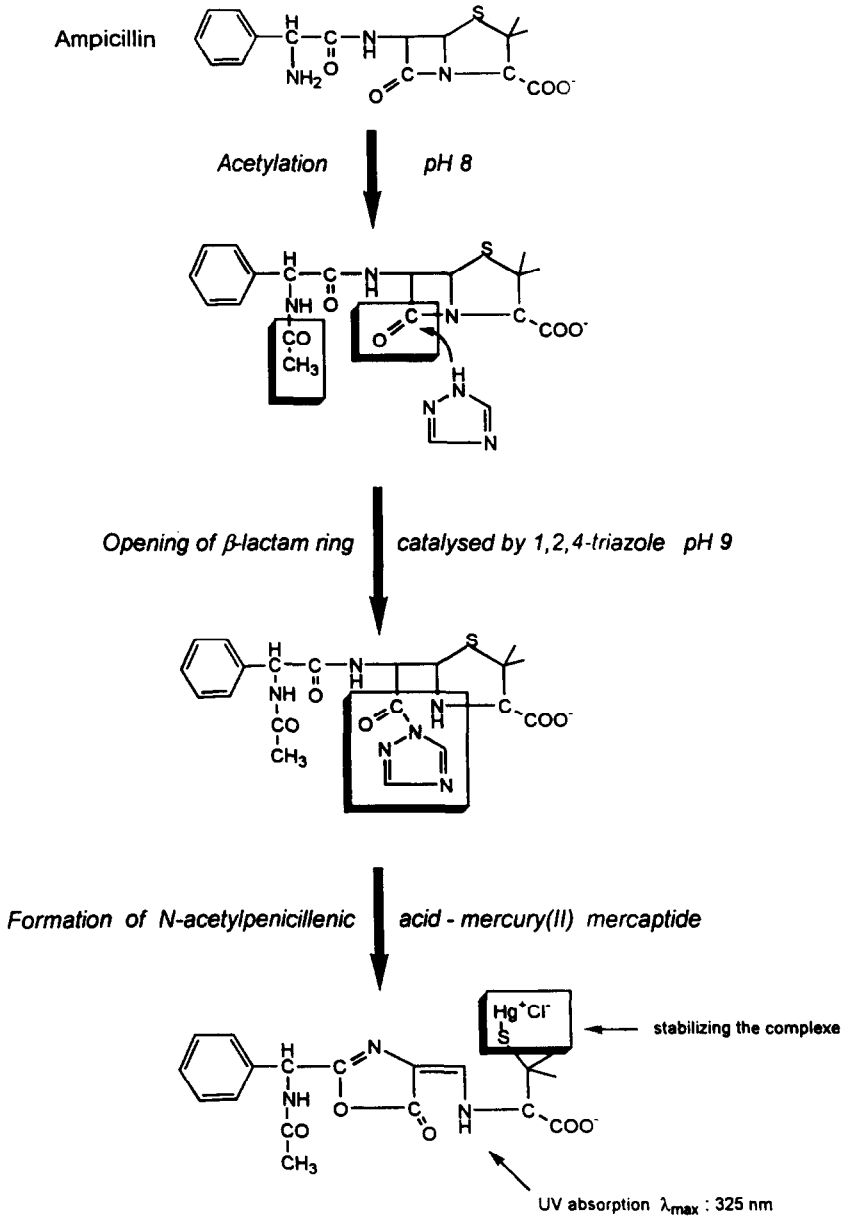

Fig. 1. Ampicillin derivatization—postulated mechanism for the 1,2,4-triazole base-catalysed reaction of aminopenicillins with mercuric chloride [4,7].

This paper describes a method for the determination of ampicillin residues in milk which combines a modified extraction procedure based on the methods described by Terada and Sakabe [2] for ampicillin in milk and Boison and co-workers [5,6] for penicillin-G in animal tissue and milk and a precolumn derivatization reaction (Fig. 1) previously described by Haginaka and Wakaï [4] for ampicillin in serum and urine and by Bundgaard and Ilver [7].

The method is able to detect 3 $\mu g\ l^{-1}$ and to quantify down to 10 $\mu g\ l^{-1}$ of ampicillin in milk.

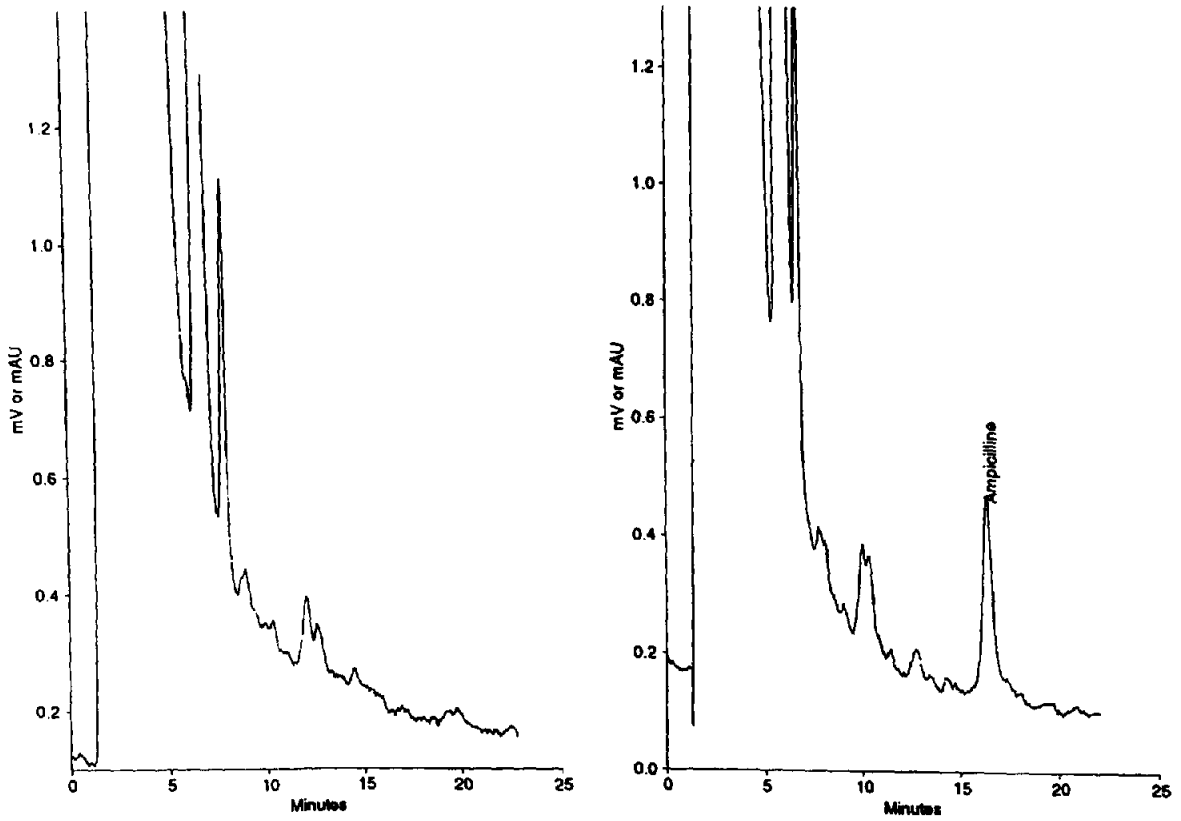

Fig. 2. Chromatogram obtained with (a) blank milk sample and (b) milk sample fortified with ampicillin to 10 $\mu g\ l^{-1}$.

2. Experimental

2.1. Apparatus

The following apparatus was used. Refrigerated centrifuge—model GR 4.11 (Jouan). Solid phase extraction cartridges—3 cm^3 (500 mg) Bond Elut C18 (Varian). Vacuum manifold—model VAC-ELUT (Touzart et Matignon). Liquid chromatograph—SP8800 Pump (Spectra Physics); manual injection loop R7125 (Rheodyne); Nova-Pak C18 column (4 μm; 3.9 mm × 150 mm); Kratos 783 variable wavelength UV detector (Kratos Analytical); data acquisition was controlled by a SP4290 integrator and a Winner386 station (Spectra Physics).

2.2. Materials and reagents

2.2.1. Standard

Sodium ampicillin with 92.4% as acid-base (Sigma Chemical Company).

2.2.2. Water

Demineralized water obtained with a Milli-Q ultrafiltration unit (Millipore).

2.2.3. Phosphate buffer (pH 6.5; 0.1 M) containing 0.0157 M thiosulfate and 0.02 M tetrabutylammonium hydrogenosulfate (TBAHS)

Weigh 4.969 g of anhydrous monobasic sodium phosphate, 10.139 g of diabasic sodium phosphate

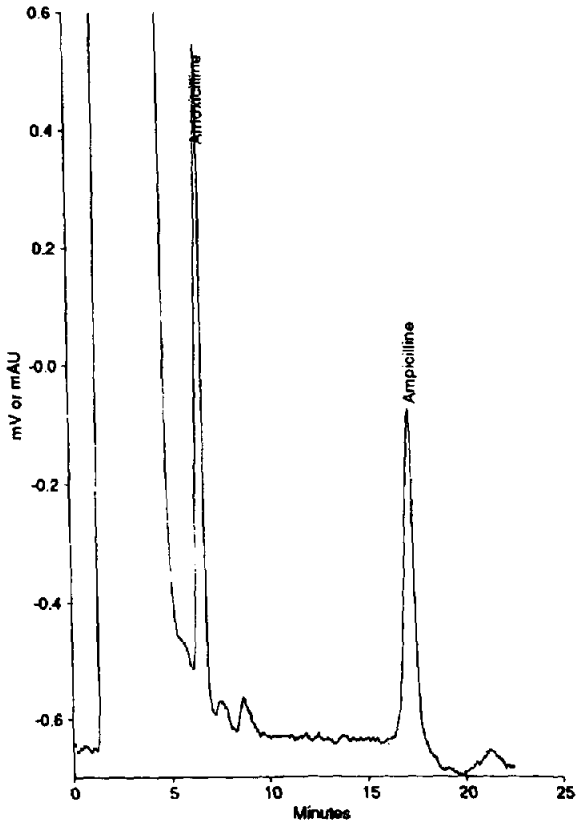

Fig. 3. Chromatogram obtained with a standard solution containing 50 $\mu g\ l^{-1}$ amoxicillin and 50 $\mu g\ l^{-1}$ ampicillin.

dihydrate, 3.894 g of sodium thiosulfate pentahydrate and 6.791 g of tetrabutylammonium hydrogenosulfate. Dissolve in 800 ml of water by stirring with a magnetic spinbar and dilute to volume in a 1 l volumetric flask. Mix thoroughly and filter through a 0.45 μm unit (Millipore) under vacuum.

Table 2
Precision data for milk samples fortified to 10 $\mu g\ l^{-1}$

	Day 1	Day 2	Day 3	Day 4
	8.4	9.6	8.4	10.3
	8.6	8.0	8.8	10.4
	7.4	9.0	9.1	10.8
	8.1	8.2	8.3	9.6
Mean	8.1	8.7	8.7	10.3
SD	0.5	0.7	0.4	0.5
RSD (%)	6.3	8.2	4.2	4.5
Recovery (%)	44.3	47.5	47.3	56.0

2.2.4. Mobile phase

Measure 700 ml of 0.1 M phosphate buffer containing 0.0157 M thiosulfate and 0.02 M TBAHS into a 1 l volumetric flask. Add 120 ml of methanol (LC grade) and dilute to volume with acetonitrile (LC grade).

2.2.5. Elution solution

Measure 60 ml of 0.1 M phosphate buffer into a 100 ml volumetric flask and dilute to volume with acetonitrile.

2.2.6. Acetic anhydride solution 0.2 M

Measure 500 μl of acetic anhydride and transfer to a 25 ml volumetric flask (use only dry glassware with no trace of water). Then make up to 25 ml with acetonitrile.

2.2.7. Derivatizing reagent—2 M 1,2,4-triazole containing 0.01 M mercuric chloride

Weigh 13.78 g of 1,2,4-triazole (Merck) into a 100 ml beaker, add 60 ml of water and stir with a magnetic spinbar to dissolve. Add 10 ml of 0.1 M

Table 1
Recovery and accuracy of recovery of ampicillin from fortified milk samples

Fortification ($\mu g\ l^{-1}$)	Mean concentration found $\pm$SD ($\mu g\ l^{-1}$)	Recovery (%)	Mean concentration corrected by R[a] (54.5%) ($\mu g\ l^{-1}$)	RSD (%)	Accuracy (%)	Number of replicates
10	5.7 ± 0.3	56.6 ± 3.1	10.4 ± 0.6	5.5	104	4
20	11.1 ± 1.2	55.6 ± 5.9	20.4 ± 2.2	10.6	102	4
40	20.2 ± 2.4	50.6 ± 6.1	37.1 ± 4.5	12.1	93	4
80	44.3 ± 2.9	55.4 ± 3.6	81.2 ± 5.3	6.5	101	4

[a] R is the mean recovery determined for the method taking into account all the values corresponding to the four levels of concentration tested

Table 3
Precision data for milk samples fortified to 40 $\mu g\ l^{-1}$

	Day 1	Day 2	Day 3	Day 4
	38.1	35.0	37.7	38.8
	40.1	35.4	35.3	32.3
	33.8	34.0	–	41.0
	40.3	36.5	37.6	32.3
Mean	38.1	35.2	36.9	36.1
SD	3.0	1.0	1.3	4.5
RSD (%)	7.9	2.9	3.6	12.4
Recovery (%)	51.9	48.0	50.2	49.2

mercuric chloride solution, mix, and adjust to pH 9.0 ± 0.5 with 4 M NaOH. Transfer to a 100 ml volumetric flask and dilute to volume with water.

2.3. Preparation of standard solutions

2.3.1. Stock standard solution (SSS)—ampicillin (500 mg l^{-1})

Weigh 54.8 mg of standard sodium ampicillin and dissolve in 4 ml of methanol. Dilute to volume with water in a 100 ml volumetric flask. Prepare fresh stock standard solution every 2 months and keep frozen at -20°C.

2.3.2. Intermediate standard solution (ISS)—ampicillin (40 mg l^{-1})

Pipet 4 ml of ampicillin SSS into a 50 ml volumetric flask and dilute to volume with water.

2.3.3. Working standard solutions (WSS)—ampicillin (500, 1000, 2000 and 4000 $\mu g\ l^{-1}$)

WSS500: Pipet 250 μl of ampicillin ISS into a 20 ml volumetric flask and dilute to volume with water.

WSS1000: Pipet 500 μl of ampicillin ISS into a 20 ml volumetric flask and dilute to volume with water.

WSS2000: Pipet 1 ml of ampicillin ISS into a 20 ml volumetric flask and dilute to volume with water.

WSS4000: Pipet 2 ml of ampicillin ISS into a 20 ml volumetric flask and dilute to volume with water.

2.4. Preparation of ampicillin-fortified milk samples

To construct a calibration curve, take 5 ml of blank milk into each of four centrifuge tubes and fortify with 100 μl portions of ampicillin working standard solutions WSS500, WSS1000, WSS2000 and WSS4000 to obtain samples fortified to 10, 20, 40 and 80 $\mu g\ l^{-1}$, respectively. Homogenize each sample by vortexing for 20 s.

2.5. Extraction, clean-up and analytical procedure

2.5.1. Acidic extraction

Add 0.5 ml of 30% trichloroacetic acid solution and mix thoroughly by vortexing for 10 s. Cap the tubes and centrifuge at 3300 g for 30 min (θ:0°C). Make a hole in the solid cream plug and transfer the supernatant to a clean centrifuge tube taking care to avoid cream pieces. Add 100 μl of 4 M sodium hydroxide solution to the supernatant to pH 5.2, stir by vortexing for 10 s. Add 1 ml of 20% sodium chloride solution to the supernatant and homogenize by vortexing for 10 s.

2.5.2. C_{18} column clean-up

Mount a 50 ml solvent reservoir on the C_{18}

Table 4
Precision data—within-day and between-day variations

Fortification ($\mu g\ l^{-1}$)	Mean concentration found ($\mu g\ l^{-1}$)	Recovery (%)	Within-day variation ($\mu g\ l^{-1}$)	Between-day variation ($\mu g\ l^{-1}$)	RSDr[a] (%)	RSDR[b] (%)
10	9.0 ± 1.0	49.8 ± 4.7	1.5	3.0	6.0	11.7
40	36.5 ± 2.8	49.8 ± 3.9	3.2	3.3	7.2	7.5

[a]RSDr is the relative standard deviation for repeatability (within-day variations).
[b]RSDR is the relative standard deviation for reproducibility (between-day variations).

cartridge with an adapter and place on a solid phase extraction vacuum manifold. Wash the C_{18} cartridge with 20 ml of methanol followed by 20 ml of water and 10 ml of 2% sodium chloride solution and discard the washes. Do not allow the cartridge to run dry at this stage. Pour the supernatant solution homogenized with 1 ml of 20% sodium chloride solution into the reservoir and pull the sample through the C_{18} cartridge by vacuum at a flow rate of 3 ml min^{-1}. Wash the column with 1 ml of 2% sodium chloride followed by 1 ml of water. Discard the washes and remove the adapter and the reservoir from the cartridge. Place a clean 5 ml glass centrifuge tube under the C_{18} cartridge and elute the sample immediately with 1 ml of the elution solution.

2.5.3. Precolumn derivatization

Add 20 μl 2 M sodium hydroxide solution to the eluate in the 5 ml centrifuge tube to pH 8 and stir by vortexing for 10 s. Add 10 μl of 0.2 M acetic anhydride solution, mix, and allow reaction for about 3 min to acylate the amino group of the ampicillin side-chain. Add 0.5 ml of derivatizing reagent, cap, stir by vortexing and allow reaction for about 10 min in a 65°C water bath. Remove the centrifuge tube from the water bath and quickly cool to room temperature by immersing the tubes in a beaker of water.

2.5.4. LC determination

Inject a 200 μl sample into the chromatograph operated in an isocratic mode using a mobile phase (18% acetonitrile–12% methanol–70% phosphate buffer pH 6.5; 0.1 M) at a flow rate of 0.8 ml min^{-1}. Measure the peak areas of ampicillin detected at 325 nm.

3. Results and discussion

The method has been applied to the analysis of ampicillin residues in raw milk, skimmed milk and lyophilized skimmed milk and the operator was able to prepare 8–10 samples for LC analysis in the same day.

The method has been tested (for raw milk) in terms of specificity, linearity, recovery, accuracy, limit of detection and precision (within- and between-day variations).

Concerning the specificity of the method, the chromatograms corresponding to the extracts of blank milk reveal no peak interfering with ampicillin (Fig. 2a and b). Amoxicillin is detected with a retention time of about 6 min and does not interfere with ampicillin detection (Fig. 3). Other penicillins, such as penicillin-G, oxacillin and cloxacillin, were not detected by this method.

The linearity of the response has been verified for samples of milk fortified from 10 to 80 ng ml^{-1}. The regression equation of the calibration curve was $Y = 1.0065X - 0.6393$ with a correlation coefficient of $r = 0.9916$.

To determine procedure recoveries, the UV responses for ampicillin in fortified samples subjected to extraction, clean-up and LC analysis were compared with those for equivalent external ampicillin standards. The mean recovery of the method ($54.5 \pm 4.9\%$) was measured taking into account all the values corresponding to the four concentrations. Results in Table 1 show that recovery is linear over the range of concentrations studied and the standard deviation of recovery is within the correct limit ($< 5\%$).

Accuracy has been measured for milk samples fortified to 10 $\mu g\ l^{-1}$, 20 $\mu g\ l^{-1}$, 40 $\mu g\ l^{-1}$ and 80 $\mu g\ l^{-1}$ with four replicates for each concentration and considering the calibration curve used for linearity testing. Results are shown in Table 1.

The limit of detection was defined as $LD = 3C/SB$ where C is the concentration of the samples fortified at the lowest concentration tested (10 $\mu g\ l^{-1}$) and SB is the signal-to-noise ratio of the response obtained with the same fortified samples. LD has been measured to 3 ng ml^{-1}. The precision of the method was assessed for within-day and between-day variations. Precision data were determined using the same operator and the same material for milk fortified at 10 $\mu g\ l^{-1}$ and 40 $\mu g\ l^{-1}$. The operator carried out four trials each day for each concentration and during four days. Results are shown in Table 2 and Table 3. They correspond to the raw values corrected by the mean recovery of the method ($54.5 \pm 4.9\%$). Only one of the 16 values for the 40 $\mu g\ l^{-1}$ samples was doubtful and was discarded. Over the

four days of routine use of the methodology, the mean recoveries ranged from 42% to 59% at the 10$\mu g\ l^{-1}$ level of concentration and from 44% to 56% at the 40 $\mu g\ l^{-1}$ level of concentration. Thus, the range of recoveries in routine use (Table 4) has been estimated to be $49.8 \pm 4.7\%$ at the 10 $\mu g\ l^{-1}$ level and $49.8 \pm 3.9\%$ at the 40 $\mu g\ l^{-1}$ level.

The mean RSDR (Table 4) was 11.7% for 10 $\mu g\ l^{-1}$ samples and 7.5% for 40 $\mu g\ l^{-1}$ samples. These were within the limit fixed by Directive 93/256/EEC concerning reference analysis methods for residue analysis: RSDr = 16% and RSDR = 32% for 10 $\mu g\ l^{-1}$; RSDr = 13% and RSDR = 26% for 40 $\mu g\ l^{-1}$.

4. Conclusion

An analytical method for selective determination of ampicillin in milk is presented. Based upon results obtained by other research groups on benzylpenicillin, a neutral side-chain penicillin, it permits the quantification of ampicillin, an amphoteric penicillin, at a residue level down to 10 $\mu g\ l^{-1}$ in milk. Although fortified milk samples have been used for the study and the validation, the method has also been applied with success to contaminated milk. It is suitable for use in raw milk as well as skimmed milk or lyophilized skimmed milk. With slight modification of the procedure, it could also be applicable for meat.

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