# Detection and Semiquantitative Estimation of Penicillin G and Cloxacillin in Milk by Thin-Layer Chromatography

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Milk was deproteinized with 2 volumes of acetonitrile. Cleanup was accomplished by partitioning between buffers and organic solvents first at acid pH (2.2), then at neutral pH (7), and finally into methylene chloride from pH 2.2 buffer. An aliquot of the methylene chlorine extract was spotted for thin-layer chromatography on silica gel plates that were developed with chloroform–acetone–glacial acetic acid (10:9:1). Penicillins were visualized by spraying the plate with 1 N HCl and then with starch solution. The plates were partially dried and exposed to iodine vapor. Penicillins formed blue-black spots. About 0.02 $\mu$g of penicillins in up to 5 mL of milk (0.004 ppm) could be readily detected.

Bioassay procedures for detection of penicillin in milk were originally developed when the only penicillin in common use with farm animals was penicillin G. In recent years, several of the newer semisynthetic penicillins have become available for use in treatment of mastitis and other animal diseases. While bioassay procedures can distinguish resulting penicillin residues from other antibiotics by use of penicillinase, they cannot distinguish penicillins from one another.

Chromatographic methods provide a means of separation and identification of different penicillins. The use of high-performance liquid chromatography (HPLC) for determination of certain penicillins in milk has been described in a separate paper (Moats, 1983). Thin-layer chromatography (TLC) on silica gel plates has been used to separate penicillins from one another (McGilveray and Strickland, 1967; Hellberg, 1968) and from metabolites and decomposition products (Fooks and Mattok, 1969; Birner, 1970; Manni et al., 1973). Separation of penicillins on reversed-phase plates was described by Biagi et al. (1969). Applications of TLC to residue analysis include determination of ampicillin in meat (Rybinska, 1980) and milk (Rybinska, 1981) and ampicillin and penicillin G in animal tissue (Yoshimura et al., 1981), all on silica gel plates with bioautography, and determination of four $\beta$-lactam antibiotics in milk using reversed-phase plates with bioautography (Herbst, 1982).

The present paper describes a method for determination of penicillin G and cloxacillin in milk using TLC together with a sensitive and relatively specific chemical procedure for visualizing the penicillins that is simpler and faster than the bioautographic method.

## EXPERIMENTAL SECTION

**Reagents.** Methylene chloride, acetonitrile, and petroleum ether (30–60 °C) were residue analysis grade; all others were reagent grade. Penicillin standards were obtained from Sigma Chemical Co. Stock solutions of 1 mg/mL were prepared in water and appropriate dilutions used. Thin-layer plates were E. Merck silica gel 60 HPTLC plates, 10 × 10 cm or 10 × 20 cm. Milk was obtained from the Beltsville herd and local commercial sources.

**Equipment.** An automatic spotter for TLC (Kontes Glass Co.), chromatographic tanks, Buchler Rotary Evapomix, and a Vortex mixer were used. Glassware was cleaned in special detergents for critical cleaning and rinsed in 1% HCl to ensure freedom from detergent residues.

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**Extraction of Sample: Milk.** Twenty milliliters of milk was mixed with 40 mL of acetonitrile in a small flask with vigorous swirling. After the precipitated proteins settled, the supernatant liquid was decanted through a plug of glass wool in the stem of a funnel and 30 mL of the clear to slightly opalescent filtrate collected.

For determination of recovery, appropriate dilutions of penicillins in water were added to the milk prior to extraction.

**Cleanup.** The acetonitrile extract was transferred to a separatory funnel and 5 mL of 0.1 M, pH 2.2, phosphate buffer was added. The mixture was extracted with 20 mL and then 10 mL of methylene chloride. The combined methylene chloride extracts were collected in a clean separatory funnel and 40 mL of petroleum ether (30–60 °C) was added. The organic layer was washed twice with 2-mL portions of water, which were discarded. It was then extracted 3 times with 1-mL portions of 0.01 M, pH 7, phosphate buffer. The combined pH 7 extracts were collected in a clean separatory funnel and 2 mL of 0.2 M, pH 2.2, phosphate buffer was added. The buffer layer was extracted with two 3-mL portions of methylene chloride, which was collected in a 15-mL conical graduated centrifuge tube. The methylene chloride was concentrated to 1 mL in the Buchler Rotary Evapomix.

**Thin-Layer Chromatography.** An aliquot equivalent to 2–5 mL of the original sample, depending on sensitivity desired, was spotted on the TLC plates by using an automatic spotter (Kontes Glass Co.). Appropriate dilutions of penicillin standards in water were also spotted. The plates were developed with chloroform–acetone–glacial acetic acid (10:9:1 v/v/v) to within 1 cm of the top. The plates were removed from the chromatographic tank, dried with a stream of warm air, and sprayed until moist with 1 N HCl. After 10 min, the plates were dried and sprayed until moist with 0.5% soluble starch. The plates were then partially dried in a stream of warm air and placed for about 30 s in a closed chromatographic tank with iodine crystals on the bottom.

## RESULTS AND DISCUSSION

Penicillins have a carboxyl group with a p$K_a$ of about 2.7 (Hou and Poole, 1971). The acid forms of nonamphoteric penicillins are soluble in organic solvents, which provides the basis for isolation methods. However, some penicillins are rapidly degraded under strongly acid conditions (Hou and Poole, 1971) so these must be avoided in the isolation procedure.

It is desirable to deproteinize biological materials prior to extraction with organic solvents to prevent formation of emulsions. It is also desirable to remove lipids that would be coextracted with organic solvents and interfere

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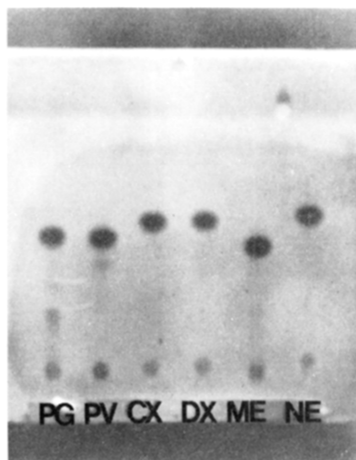


**Figure 1.** Penicillin standards (1 μg) on silica gel 60. Solvent system: chloroform–acetone–glacial acetic acid (10:9:1). PG = penicillin G; PV = penicillin V; CX = cloxacillin; DX = dicloxacillin; ME = methicillin; NE = nafcillin.

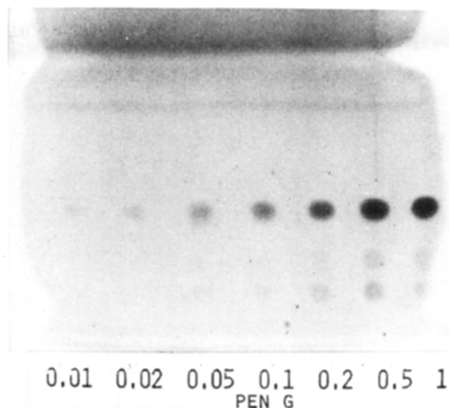


**Figure 2.** Detection of penicillin G standards on silica gel 60—0.01–1 μg spotted. Solvent system: chloroform–acetone–glacial acetic acid (10:9:1).

with the analysis. Since strongly acid deproteinizing agents cannot be used, tungstic acid, which gave a pH of about 2.0, was tested. This effectively precipitated proteins and fats from milk but recoveries of penicillin were erratic. Poor recoveries apparently resulted from coprecipitation of penicillin with milk proteins since penicillin added to the filtrate was quantitatively recovered. Acetonitrile proved satisfactory for precipitating proteins and, since it is a poor solvent for fats, most of the lipid present was coprecipitated with the proteins. For recovery of penicillins from milk, a ratio of acetonitrile to milk of 2:1 was optimal. A 1:1 ratio was ineffective in precipitating proteins, and recoveries of penicillin were poor when a 3:1 ratio was used. At the higher proportion of acetonitrile, penicillin apparently coprecipitated with the protein and lipid, which formed a sticky mass at the bottom of the flask.

The cleanup procedure used is a slight modification of that described for preparing samples for analysis by high-performance liquid chromatography (Moats, 1983). Since it was desirable to have samples in a volatile solvent to facilitate spotting for TLC, the sample extracts were repartitioned into methylene chloride from pH 2.2 buffer as a final cleanup step. Other approaches to cleanup such as column chromatography were either ineffective or gave poor recoveries of penicillins. The cleanup procedure used was satisfactory for recovery of a variety of nonamphoteric penicillins but did not work with the more polar carbenicillin or with amphoteric compounds such as ampicillin. Of the nonamphoteric penicillins, only penicillin G and cloxacillin are commonly used in veterinary practice and these are therefore the only ones likely to be found in milk.

The solvent system used for TLC on silica gel plates in the present study was chloroform–acetone–acetic acid (10:9:1 v/v/v) as described by Manni et al. (1973). Development of plates was rapid, requiring only 20 min. The EM silica gel 60 HPTLC plates were the most satisfactory of a number of precoated TLC plates tested. Penicillins were separated from interference in sample extracts, but separation of penicillins from one another was not completely satisfactory (Figure 1). Similar results have been reported by other authors using silica gel plates (McGilveray and Strickland, 1967; Hellberg, 1968). However, separation of the two penicillins of major interest was satisfactory.

Visualization of penicillin G on the plates was based on a method described by Thomas and Broadbridge (1970) for visualization of penicillins and penicilloic acids on a starch–agarose electrophoresis medium. They observed that penicilloic acids showed a strong affinity for iodine vapor, producing an intense blue-black spot. This method was capable of detecting as little as 0.01 μg of penicilloic acids. When penicillins were treated in situ with acid, they also could be detected at this level of sensitivity. Although Thomas and Broadbridge considered that penicillins were converted to the corresponding penicilloic acids by acid treatment, other investigators have reported that penicillins actually rearrange to the corresponding penillic acids in strong acid (Hou and Poole, 1971).

To adapt this method to thin-layer plates, the plates were sprayed until moist with 1 N HCL, allowed to stand 10 min, and exposed to iodine vapor in a closed tank. Penicillins were visible as brown spots, which faded as soon as the plate was exposed to the air. Spraying the plates with starch prior to exposing them to iodine vapor stabilized and intensified the reaction with iodine, forming intense blue-black spots. The best results were obtained when the plates were slightly moist when exposed to iodine vapor. If they were too moist or too dry, sensitivity decreased and spots faded more rapidly. Under optimum conditions, the color reaction faded slowly and spots remained visible for several days. The limit of sensitivity with penicillin G standards is about 0.01 μg (Figure 2). The color intensity produced with other penicillins was essentially the same as with penicillin G (Figure 1). The presence of other substances in sample extracts that produce slight darkening with this reaction may interfere with detection of very small amounts of penicillin. This color reaction is at least 10 times as sensitive as any other reaction for detecting penicillins on TLC plates. The acid–ferricyanide method of McGilveray and Strickland (1967) is sensitive to about 0.2 μg of penicillin but was not sufficiently specific to be used with tissue extracts. The iodine–sodium azide method is also reported to be sensitive to about 0.2 μg of penicillin (Hellberg, 1968).

With the iodine–sodium azide method (Hellberg, 1968) for detection of penicillin, iodine is consumed, leaving a white spot against a blue background rather than an intense blue-black spot as in the present case. The reason for this difference is not clear.

Recovery of penicillin G and cloxacillin added to milk at 0.01 μg/mL is shown in Figure 3. Aliquots equivalent to 2 and 5 mL of milk were spotted. The milk blank was essentially free of interferences in the area of the penicillin spots. Recoveries appear quantitative and spots are readily visible. Even with an aliquot equivalent to 5 mL of original milk, nonspecific darkening is slight. Since 0.02 μg or less of either penicillin in 5 mL of milk is readily detectable,

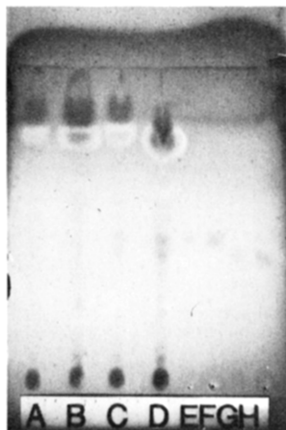


**Figure 3.** Recovery of penicillin G and cloxacillin from spiked milk samples. (A) Control, 2 mL equiv spotted; (B) control, 5 mL equiv spotted; (C) 0.01 ppm of penicillin G, cloxacillin, 2 mL equiv spotted; (D) 0.01 ppm of penicillin G, cloxacillin, 5 mL equiv spotted; (E) cloxacillin, 0.02 μg of standard; (F) cloxacillin, 0.05 μg of standard; (G) penicillin G, 0.02 μg of standard; (H) penicillin G, 0.05 μg of standard. EM silica gel 60 HPTLC plate. Developing solvent: chloroform–acetone–glacial acetic acid (10:9:1 v/v/v).

the sensitivity limit of the procedure is about 0.004 ppm. This is near or below the reported limits of reliable detection of these compounds by other procedures that are, for penicillin G, 0.005 ppm by HPLC (Moats, 1983), 0.0012 ppm by bioassay (Vilim et al., 1979) and by bioassay and TLC (Herbst, 1982), 0.006 ppm by the Charm test (MacCaulay and Packard, 1981; Charm and Chi, 1982), 0.015 ppm by bioassay and Delvotest P (MacCaulay and Packard, 1981), and 0.005 ppm for unspecific β-lactam antibiotics by the AOAC *Bacillus stearothermophilus* disc assay (Peeler et al., 1982). Bioassay procedures are less sensitive for cloxacillin with reported detection limits of 0.02 ppm in milk (Herbst, 1982; Vilim et al., 1979) as compared with 0.004 ppm by TLC and 0.002 ppm by HPLC (Moats, 1983). As compared with the TLC procedures of Herbst (1982), the present method is much faster (3 h) does not require maintenance of bacterial cultures and is more sensitive for detection of cloxacillin. More cleanup is required and other β-lactam antibiotics are not detected.

The use of reversed-phase plates for TLC analysis of pencillins was also investigated since these have been reported (Herbst, 1982; Biagi et al., 1969) to give better separations of penicillins than has been achieved on silica gel. Silica gel plates dipped in a solution of DD-200 silicone oil and commercial $C_{18}$ reversed-phase plates from several manufacturers were tested. Good separations of penicillins could be obtained on the plates using a solvent system of 10:9:1 (v/v/v) water–acetone–glacial acetic acid. Color development was somewhat less intense and less consistent on the reversed-phase plates, and longer exposure to acid was required for satisfactory color development with acid-resistant penicillins such as cloxacillin. A more serious drawback was that only a small amount of sample extract could be spotted without serious interference with development of chromatograms. The use of reversed-phase plates would require better cleanup than was achieved in the present procedure.

The procedure described using silica gel plates is suitable for separation and semiquantitative estimation of penicillin G and cloxacillin in milk on the assumption that related penicillins with similar $R_f$ values are not likely to be present. The absence of spots with the appropriate $R_f$ values is a reliable negative test. Identification can be further confirmed by HPLC. A semiquantitative estimate of the amount present can be made by visual comparison with standards spotted on the same plates. Attempts to quantitate with a densitometer were unpromising since color intensity was not linear with concentration and the color intensity of both spots and background faded fairly rapidly. This method can be used for confirmation of other test procedures for cloxacillin and penicillin G or for direct screening of milk samples since the time required (3–4 h) is generally less than that for bioassay procedures. The method is less suitable for quantitation than HPLC but is equally sensitive and does not require expensive equipment.

The procedure was tested with milk from individual cows from the Beltsville herd and with milk from local commercial sources, all from the Holstein breed of cows. However, it is not anticipated that milk from other breeds would present special problems. Application of these procedures for the determination of penicillins in tissues, blood serum, and colostrum is also under study and will be reported separately.

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**Registry No.** Penicillin G, 61-33-6; cloxacillin, 61-72-3.

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