necessary to more completely characterize the light stability of B_6 vitamers in food systems under various conditions.

Registry No. PN, 65-23-6; PL, 66-72-8; PM, 85-87-0.

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Determination of Penicillin G, Penicillin V, and Cloxacillin in Milk by Reversed-Phase High-Performance Liquid Chromatography

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A method is described for determination of three commonly used monobasic penicillins, penicillin G, penicillin V, and cloxacillin, in milk by high-performance liquid chromatography. Penicillins were extracted from milk with 2:1 acetonitrile. Cleanup was accomplished by partitioning between buffers and organic solvents, first at acid (pH 2.2) and then at neutral (pH 7.0) pHs. For HPLC, a reversed-phase C_{18} column was used. The solvent system was (A) 0.01 M H_3PO_4 and (B) acetonitrile. A solvent gradient of 80 A:20 B–40 A:60 B in 20 min, flow rate 1 mL/min, was used to elute all penicillins studied within 20 min. Recoveries of penicillins from spiked samples were 88–105%. Limits of detection were about 0.002 ppm for cloxacillin and 0.005 ppm for penicillin G and V. The procedure should be satisfactory for most monobasic penicillins but did not work with the more polar carbenicillin and amphoteric compounds such as ampicillin.

Methods have been described for determination of penicillins in pharmaceutical preparations by high-performance liquid chromatography (HPLC) using both reversed-phase (LaBelle et al., 1979; Larsen and Bundgaard, 1978; Tsuji et al., 1979; White et al., 1975) and anion-exchange columns (Blaha et al., 1975; Tsuji and Robertson, 1975) and also in blood serum using reversed-phase HPLC (Thijssen, 1980; Vree et al., 1978).

Thijssen (1980) and Vree et al. (1978) used perchloric acid to precipitate proteins in the determination of respectively isoxazoyl penicillins and ampicillin and amoxicillin in blood serum by HPLC. Although precipitation of proteins with strong acid appears satisfactory for acid-stable penicillins, it is unsuitable for some penicillins which are rapidly degraded under acid conditions (Hou and Poole, 1971).

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The application of HPLC methods to determination of penicillin residues in milk and animal tissue has not previously been reported. Detection of penicillin at the residue levels required by regulatory agencies requires isolation from more complex substrates and much higher sensitivity than is required for clinical applications. This paper describes a method for determination in milk of three penicillins commonly used in veterinary practice.

MATERIALS AND METHODS

Chemicals. Acetonitrile was UV grade and residue analysis grade, petroleum ether (30–60 °C) and methylene chloride were residue analysis grades, and other chemicals used were reagent grades. Penicillins were obtained from Sigma Chemical Co.

Equipment. A Buchler Rotary Evapomix and vortex mixer were used. All glassware was cleaned in special detergents designed for critical cleaning and rinsed in 1% hydrochloric acid and distilled water before use.

HPLC Apparatus. A Varian Model 5000 liquid chromatograph was used with a Varian UV-50 detector set at

220 nm. A Varian MCH-10 (10-μm particle size) 30 cm × 4.6 mm C_{18} reversed-phase column was used. Samples were injected with a Valco automatic loop injector with a 200-μL loop.

HPLC Procedure. A flow rate of 1 mL/min was used. Solvents were (A) 0.01 M H_3PO_4 and (B) acetonitrile. A solvent program of 80 A:20 B–40 A:60 B in 20 min was used. A 200-μL aliquot of the sample extract in water was injected onto the column. Quantitation was based on peak height which was linear up to 6 μg of penicillin injected.

Extraction of Sample: Milk. A total of 20 mL 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 Procedure. The filtrate (30 mL) was transferred to a separatory funnel, 5 mL of pH 2.2, 0.2 M phosphate buffer and 20 mL of methylene chloride were added and shaken vigorously, and the methylene chloride (lower layer) was drawn off into a clean separatory funnel. The water layer was extracted with an additional 10 mL of methylene chloride which was combined with the first extraction. Then 40 mL of petroleum ether (30–60 °C) was added to the methylene chloride extracts and the organic layer was washed twice with 1-mL portions of distilled water which were discarded. The organic layer was then extracted 3 times with 1-mL portions of 0.01 M, pH 7, phosphate buffer, collecting the combined pH 7 extracts in a 15-mL conical graduated centrifuge tube. Two milliliters of petroleum ether was added to the tubes and the contents were mixed for 10 s on a Vortex mixer and centrifuged for 1 min at low speed, and the petroleum ether was carefully removed with a pipet and discarded. The residual petroleum ether was stripped off at reduced pressure in a Buchler Rotary Evapomix. Then 3 mL of saturated ammonium sulfate and 2 mL of acetonitrile were added to the tubes. The contents were vortexed in a vortex mixer for 10 s and centrifuged for 1 min at low speed, and the acetonitrile layer was carefully transferred to a clean centrifuge tube. The aqueous layer was extracted with an additional 2 mL of acetonitrile. Then 0.8 mL of water was added to the acetonitrile extracts, and the acetonitrile was evaporated under reduced pressure in the Buchler Rotary Evapomix. The volume of the remaining water layer was adjusted to 1 mL and an aliquot used for higher performance liquid chromatography.

RESULTS AND DISCUSSION

Treatment of milk with 2 volumes of acetonitrile was effective in precipitating proteins and lipids, and recovery of penicillins in the clear filtrate was essentially quantitative. The acetonitrile extraction avoids extremes of pH which might result in degradation of penicillins. With a higher proportion of acetonitrile to milk, the precipitated milk constituent formed a gummy mass at the bottom of the flask, and recoveries of penicillins were reduced. This also occurred if the milk was not vigorously stirred during addition of the acetonitrile. With the acetonitrile extraction, there was no loss of penicillin from binding to proteins as occurred when protein precipitants such as tungstic acid were used.

The penicillins were partitioned into methylene chloride at pH 2.2 and then reextracted into a small volume of pH 7 buffer. This effectively concentrated the penicillins without the necessity of evaporating large volumes of solvent. Petroleum ether was added to the organic phase to facilitate extraction into the pH 7 buffer. Three 1-mL extractions recovered >95% of the penicillins present. Injection of the sample into the HPLC column in pH 7 buffer sometimes caused peak broadening and apparent low recoveries. This was especially true if the pH 7 extract was concentrated. The buffer salts were eliminated by partitioning the sample into acetonitrile from half-saturated ammonium sulfate. Penicillins were nearly completely recovered in the acetonitrile layer from the first extraction and a second wash gave complete recovery. To obtain the final sample in water, water was added to the acetonitrile extract prior to evaporation to avoid possible losses from evaporating the sample to dryness. Efforts to achieve further cleanup by partitioning, column chromatography, and Sep-PAKS (Waters) were unsuccessful. Either no useful separations were obtained or recoveries of penicillins were poor and erratic.

Table I. Buffer Salts Tested for Reversed-Phase Separation of Penicillins in a Buffer–Acetonitrile System

0.01 M $(NH_4)_2SO_4$	0.01 M tetraethylammonium chloride
0.01 M NH_4Ac	0.01 M tetramethylammonium chloride
0.01 M $NH_4H_2PO_4$	0.02 M, pH 2.5, phosphate buffer
0.02 M NH_4Cl	0.02 M, pH 2.2, phosphate buffer
0.01 M, pH 7, phosphate buffer	0.01 M H_3PO_4

The sodium salts of penicillins were not retained to any extent from water on the reversed-phase column. However, retentions were satisfactory if ammonium or phosphate salts were present in the aqueous phase. Some salts tested in the aqueous phase are listed in Table I. It is well-known that the ammonium salts of penicillins are more soluble in organic solvents than the sodium salts (Hou and Poole, 1971). This probably accounts for the better retention of penicillin on the reversed-phase column in the presence of ammonium salts. The type of ammonium salt present had no effect on retention times nor did variation of pH in the range 4–7. Retention times in phosphate buffers were less than with ammonium salts at the same proportion of acetonitrile. Retention times in $NH_4H_2PO_4$ were similar to those with other ammonium salts. Retention times were substantially increased when tetramethyl- and tetraethylammonium chlorides were used in place of ammonium chloride. However, since retention times of interfering substances were also altered, the use of alkyl-substituted compounds offered no advantage. Retention times were also increased when acid buffers were used, and the best separations from interferences were obtained in phosphate buffers pH 2.5 or below. Dilute (0.01 M) H_3PO_4 proved generally satisfactory.

Columns other than the C_{18} reversed-phase column were also investigated including reversed-phase phenyl and C_8 columns and an anion-exchange column. The C_8 column (Alltech) gave a pattern essentially identical with that of the C_{18} column used but slightly larger retention times. The phenyl column (Waters) did not give as good separation of penicillins from minor interfering peaks. The anion-exchange column did not separate penicillins from interferences in sample extracts.

Under isocratic conditions, retention times of individual penicillins varied greatly at a given proportion of acetonitrile. Therefore, for multiresidue analysis, a solvent program was essential to elute all penicillins in a reasonable length of time. A program of 0.01 M H_3PO_4–acetonitrile (80:20–40:60) in 20 min was satisfactory and eluted the three penicillins within 20 min. Solvent programming also

Table II. Recovery of Penicillins Added to Milk

	amount added, ppm	amount found, ppm 1	2	av	% recovery, mean ± SD
penicillin G	0.050	0.048	0.052	0.050	100.5 ± 3.0
	0.100	0.100	0.102	0.101	
penicillin V	0.050	0.052	0.050	0.051	102.7 ± 2.3
	0.100	0.105	0.102	0.103	
cloxacillin	0.050	0.043	0.049	0.046	93.5 ± 6.7
	0.100	0.102	0.088	0.095	

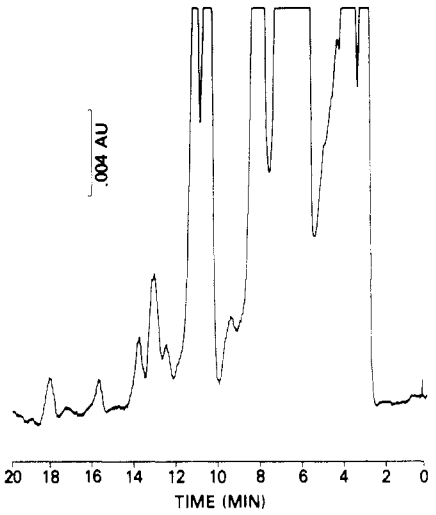

Figure 1. Milk blank, 2 mL equiv; solvent, 0.01 M H_3PO_4–acetonitrile (80:20–40:60 in 20 min); flow rate 1 mL/min, 0.02 AUFS at 220 nm; C_{18} 10-μm reversed-phase column.

has the advantage that late eluting peaks remain as sharp as earlier eluting ones. For the base line to remain reasonably flat with so steep a solvent gradient, the acetonitrile must have a low absorbance (0.02 AU or less) at 220 nm.

The injection volume of 200 μL was selected so that enough of the sample could be used to obtain the desired sensitivity with the procedure. With this relatively large injection volume, care must be used in selection of the solvent in which the sample is injected. Otherwise, peaks may be distorted and apparent recoveries may be low. In the present case, no problems were encountered when both samples and standards were injected in water. When samples and standards were injected in acetonitrile, apparent low recoveries resulted, and it is likely that some of the penicillin was carried through the column with the injection solvent front. Some distortion of peaks was sometimes noted when the sample buffer was pH 7 and the HPLC solvent was pH 2.5 or below.

Recoveries from milk samples spiked at 0.05 and 0.1 ppm with the three penicillins are shown in Table II and ranged from 88 to 105%. Figures 1 and 2 show chromatograms of a blank and a milk spiked with 0.01 ppm of each of the trial penicillins. The penicillins were separated from minor interfering peaks eluting after 14 min and gave readily identifiable peaks of the expected heights. The limit of detection is about 0.05 ppm for penicillin G and penicillin V and 0.002 ppm for cloxacillin. The total absence of any detectable peak at the retention times of the penicillins should be a reliable indication that the compounds are not present above very low levels (<0.002–0.005 ppm). These may be compared with levels of sensitivity reported for bioassay procedures which was low as 0.002 IU/mL (0.0012 ppm) for penicillin G in milk and 0.02 ppm for cloxacillin (Herbst, 1982; Vilim et al., 1979). However, Macauley and Packard (1981) found that of four bioassay

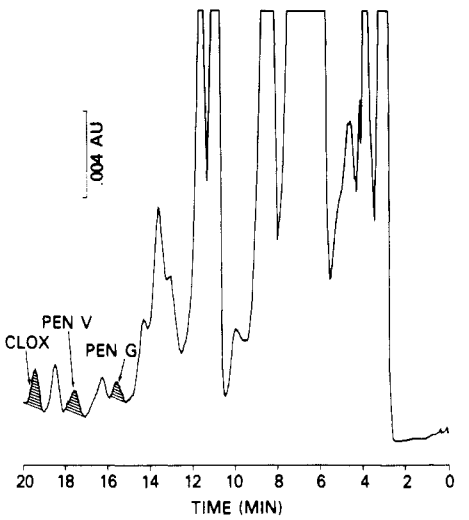

Figure 2. Milk, 2 mL equiv with 0.01 ppm of penicillin G, penicillin V, and cloxacillin; conditions as in Figure 1.

Table III. Milk Residue Study

		ppm at hours after last infusion 12	24	48	72
penicillin G:	treated quarter[a]	51.1	1.08	neg	neg
	control quarter	neg[c]	neg	neg	neg
cloxacillin:	treated quarter[b]	22.0	0.35	neg	neg
	control quarter	neg[d]	neg	neg	neg

[a] Three successive infusions of 100 000 units of procaine penicillin G. [b] Three successive infusions of 200 mg of sodium cloxacillin. [c] <0.005 ppm. [d] <0.002 ppm.

procedures tested, none would consistently detect penicillin G at levels less than 0.025 IU/mL (0.015 ppm) and one (Delvotest P) gave some false positives. The Charm test consistently detected penicillin G at 0.01 IU/mL but not at lower levels. Thus, the HPLC method is slightly less sensitive than the best reported for penicillin G but decidedly more sensitive for cloxacillin and is the only procedure capable of detecting cloxacillin at the tolerance level of 0.01 ppm in milk. Bioassays and the Charm test are not capable of distinguishing one penicillin from another.

Table III shows the results of a milk residue study conducted by using single cows given standard treatments for mastitis consisting of three successive infusions of 100 000 units of procaine penicillin G or 200 mg of sodium cloxacillin, respectively. Untreated quarters were used as controls. No crossover from treated to untreated quarters was observed, and no detectable residues were present after 24 h in milk from the treated quarters. Well under 0.01 ppm of the penicillins should have given a discernible peak if any was present. The results are not inconsistent with those of comparable studies using bioassays although small amounts of residues have been found after 36 h in the milk of some treated cows (Rollins et al., 1970).

The method has been tested with milk from individual cows, mixed herd milk, and a large number of commercial milk samples purchased in the local area. It should be useful for identification and confirmation of residue presumptively identified as penicillin by other tests. It could also be used for directly screening milk samples for these particular penicillins since the time required (3 h) is somewhat less than for bioassay procedures. The method should be suitable for determination of most monobasic penicillins. However, more polar compounds such as carbenicillin and ampicillin were not recovered by this cleanup procedure. The adaptation of these methods to

determination of residues in blood and tissues is being studied.

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High-Pressure Liquid Chromatographic Method for the Analysis of 2-Amino-3-methylimidazo[4,5-*f*]quinoline, a Mutagen Formed during the Cooking of Food

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A quantitative method for the analysis of 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), a mutagen formed during the cooking of beef and fish, has been developed. A crude extract containing basic materials is purified by preparative TLC and reverse-phase HPLC. Quantitation is done on a cyanopropyl bonded-phase column run in the reverse-phase mode. Recovery is about 45% and the limit of detection is less than 25 pmol/50 g of beef. Control experiments with spiked extracts show a high degree of precision. The method has been used to show that there are relatively large differences in the formation of IQ between meat samples with a high or low fat content. The presence of other additives such as BHA, Celite, and casein decreases the formation of mutagen during cooking.

There is a great deal of current interest in the formation of mutagenic activity during the cooking of food (Weisburger and Horn, 1982). A number of mutagens have been isolated and their structures characterized (Sugimura, 1982). At least six of these mutagens have been reported to occur in fried beef (Barnes et al., 1983).

Although there is one report of an attempt at quantitation in the literature, analytical methods have not yet been developed to measure routinely the amount of mutagen in a complex foodstuff. Partially, this may be due to the fact that the compounds are primary heterocyclic amines, and hence do not always chromatograph well, and partly because food is such a complex material that extensive preliminary cleanup is required.

It is important that a quantitative analytical method be available, because any assessment of human risk from these mutagens and potential carcinogens necessarily requires an accurate measure of exposure. Also, studies of the chemistry involved in the formation of the mutagens will demand a technique for quantifying the product. Up to now, the only method available to serve this purpose has been a test for bacterial mutagenicity. Although reasonably precise, such a bioassay may be subject to inaccuracy because of its response to enhancers or inhibitors of mutagenesis (Pariza et al., 1982; Sugimura et al., 1980).

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In this paper, we describe a quantitative chemical method for 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), an extremely potent mutagen, which is formed during the frying of ground beef. This compound is interesting because of its structural similarity to the known colon, breast, and prostate carcinogen 3,2′-dimethyl-4-aminobiphenyl, as shown in Figure 1.

MATERIALS AND METHODS

Chemicals. All solvents used in extraction and chromatography were HPLC grade. Butylated hydroxyanisole (BHA) and casein were from Sigma (St. Louis, MO) and Celite 503 was from J. T. Baker (Phillipsburg, NJ). 2-Methylbenzimidazole and 5-aminoquinoline were from Aldrich (Miwaukee, WI). IQ standard was synthesized according to published procedures (Kasai et al., 1980) and cochromatographed with a reference sample kindly provided by Dr. T. Sugimura (Tokyo). The structure of the synthesized compound was confirmed by mass spectroscopy and ^{1}H NMR and found to be identical with that reported in the literature.

Meat. Ground beef with high or low fat content was purchased from a local supermarket. Water content was determined by weight difference before and after lyophilization. Fat content was determined by hexane extraction in a Soxhlet apparatus for 24 h. The low-fat meat contained 56.2% water and 10.6% fat on a wet weight basis or 24% fat on a dry weight basis. High-fat meat contained 52% water and 27.5 or 57.3% fat on a wet weight and dry weight basis, respectively.

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