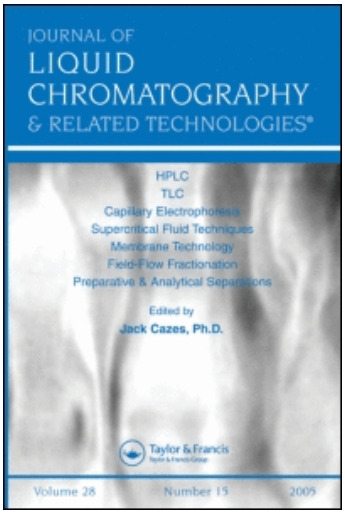

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AN HPLC METHOD FOR THE DETERMINATION OF PENICILLIN G RESIDUES IN VEAL CALF LIVER TISSUES

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ABSTRACT

A method developed in our laboratory and used for several years for the routine determination of penicillin G residues in animal tissues by liquid chromatography failed when it was used in a recent study for the determination of penicillin G residues in liver tissues of 2- to 5-week-old veal calves. The method was, therefore, modified as follows to permit the determination of penicillin G residues in liver tissues from very young calves. Penicillin G was extracted from calf liver tissue with acetonitrile instead of water. The acetonitrile extract was evaporated to near dryness, and the resulting residue was dissolved in 30 mL of 2% sodium chloride and cleaned up on a t-C_{18} Sep-Pak cartridge. The retained penicillin was then eluted with 1 mL of 60% acetonitrile/35%

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water/5% 0.2 *M* phosphate buffer solution, derivatized with 1 mL of 1,2,4-triazole/mercuric chloride solution at 65°C for 30 min, and analyzed by reverse-phase liquid chromatography with ultraviolet detection at 325 nm. The limits of detection and quantitation for this method are 1.5 and 5 ng/g, respectively, for penicillin G.

INTRODUCTION

In 1991, a sensitive analytical method for the determination of penicillin G residues in animal tissues was developed by scientists at the Health of Animals Laboratory and subsequently implemented successfully in several other laboratories. This method, which extracts penicillin G and the internal standard, penicillin V, into water after deproteinization with phosphotungstic acid, has been used successfully for several years for the determination of penicillin G residues in kidneys, muscle, liver, plasma, and urine of food animals (1).

In a recent study with scientists at the College of Veterinary Medicine, North Carolina State University, to determine the tissue depletion profile when very young calves (2–5 weeks old) were fed penicillin-contaminated milk from either treated cows or replacer milk formulations, it was discovered that this method was not able to extract penicillin G and the internal standard, penicillin V, added to control liver tissues from these calves. The method did, however, work well for both the muscle and kidney tissues from the same animals. After several unsuccessful attempts to modify the aqueous extraction procedure to permit the determination of penicillin G in these liver tissues, it was decided to significantly modify the extraction procedure. This was done by using acetonitrile instead of water for extraction and deproteinization.

This paper describes the modifications made to the previously published method to develop an alternative method for the accurate determination of penicillin G residues in liver tissues of these very young veal calves as part of a tissue depletion study.

EXPERIMENTAL

Apparatus

Solid-phase extraction cartridges, t-C_{18} Sep-Pak Vac (3 mL, 500 mg capacity, 17% carbon loadin), were obtained from Waters Chromatography (Mississauga, ON, Canada). Filter papers, GF/B, 5.5 cm, were obtained from Whatman Inc. (Clifton, NJ, USA). The solvent evaporator, a Zymark Turbo LV, was obtained from Zymark Ltd. (Mississauga, ON, Canada).

HPLC System

The system consisted of a Waters Chromatography 501 pump, a 712 WISP autoinjector, a Waters 486 programmable wavelength detector set at 325 nm and a sensitivity of 0.003 absorbance units full scale, and a Kipp and Zonen strip chart recorder. Components in the tissue extract were separated isocratically at room temperature on an Inertsil C_8 column, 5 μm, 4.6 × 150 mm (GL Sciences, Tokyo, Japan), at a mobile phase flow rate of 1.2 mL/min.

Reagents

All solvents used in the study, including acetonitrile and methanol, were of liquid chromatography (LC) grade. Sodium chloride, sodium phosphate dibasic, sodium phosphate monobasic, and anhydrous sodium thiosulfate, all analytical grade reagents, were obtained from Fisher Scientific (Pittsburgh, PA, USA). 1,2,4-Triazole was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). The analytical standards, sodium penicillin G and potassium penicillin V, were both USP reference standards obtained from Sigma Chemical Co. (St. Louis, MO, USA). Water was obtained from a Barnstead reverse osmosis/nanopure ultrafiltration system.

The preparation of the derivatizing reagent (2 *M* 1,2,4-triazole containing 10^{-3} *M* mercuric chloride) and the elution solution has already been described (1). [CAUTION: Mercuric chloride is highly toxic and all efforts must be made to avoid contact. See Material Safety Data Sheets for more information.] The mobile phase was made by mixing 250 mL of acetonitrile with 750 mL of a 0.05 *M* phosphate buffer containing 0.0157 *M* thiosulfate, filtered through a 0.22-μm nylon filter and degassed.

Preparation of Standard Solutions

Stock solutions (100 μg/mL) were prepared by dissolving 0.0107 g of sodium penicillin G and 0.0111 g of potassium penicillin V into individual 100-mL volumetric flasks and diluting to volume with water. Two-milliliter aliquots were transferred into 4-mL vials and stored at −76ºC. (One stock standard solution was removed from storage each day, allowed to thaw at room temperature, and used for the day's analysis.)

Stock solutions should be prepared every 6 months. From this stock solution, a working standard solution of 2 μg/mL was prepared daily by appropriate dilution with water and used for the preparation of calibration solutions.

Sample Pretreatment

Five grams of partially thawed, finely chopped, control (drug-free) liver tissue was accurately weighed into each of six 50 mL disposable polypropylene centrifuge tubes. The control tissues were then fortified with 0, 12.5, 25, 50, 100, and 200 µL of a 2 µg/mL penicillin G working standard solution to prepare tissue calibration standards containing 0, 5, 10, 20, 40, and 80 ng/g of penicillin G, respectively. A 5.0-g amount of partially thawed, finely chopped, test liver tissue was also weighed into 50-mL disposable polypropylene centrifuge tubes. (Note: It is important to chop the liver tissue into fine, small pieces to facilitate the extraction of the penicillins from tissue with acetonitrile by mechanical agitation.)

Next 150 µL of a 2 µg/mL penicillin V working standard solution was added to each test liver tissue and to the control liver tissues to provide a constant amount, 60 ng/g penicillin V, as internal standard. Then 10 mL of acetonitrile was added to each centrifuge tube and allowed to sit for 10 min. The contents in each centrifuge tube were mixed on a Vortex at high speed for 20–30 s, agitated on a mechanical shaker for 5 min, and centrifuged at 2–5°C for 10 min at 3200*g*. (Note: It is critical to centrifuge at these reduced temperatures to avoid poor recoveries.) The supernatant was transferred, after centrifugation, into a 15-mL polypropylene centrifuge tube and the acetonitrile in the supernatant was evaporated to approximately 500 µL using prepurified nitrogen (inlet nitrogen pressure at 5–10 psi) with the Zymark Turbo LV evaporator unit with the water bath temperature set at 60°C. The 15-mL centrifuge tube was rinsed with three 10-mL aliquots of 2% sodium chloride and the rinses were vacuum filtered through a GF/B (glass fiber) filter paper into a 125-mL sidearm flask. The filtered liver extracts are now ready for solid-phase extraction cleanup.

Cleanup of Tissue Extracts on t-C_{18} Sep-Pak Cartridges and Derivatization for LC Analysis

A t-C_{18} Sep-Pak cartridge (3 mL, 500 mg capacity) equipped with a 20-mL solvent reservoir was conditioned with 5 mL of methanol, followed by 5 mL of water, and 5 mL of 2% sodium chloride. (Note: It is very important not to allow the cartridge to run dry at this stage.) The conditioned t-C_{18} cartridge was loaded with the liver tissue extract at a flow rate of about 3 mL/min.

Evacuation of the cartridge was continued for another 3 min after which the solvent reservoir and adaptor were removed and the retained penicillins were eluted with 1 mL of elution solution (60% acetonitrile/35% water/5% 0.2 *M* phosphate buffer) into labeled Kimax 12 × 75 mm borosilicate glass tubes. The

cleaned-up liver tissue extracts were then derivatized to form the mercaptides, as previously described (1) and analyzed by reverse-phase HPLC.

Liquid Chromatographic Analysis

Peak heights of the peak responses of the extracts from the calibration and test samples were measured. Using regression analysis, a calibration curve of response ratio (peak height of penicillin G/peak height of penicillin V) versus concentration of penicillin G added to control tissue was constructed. From the measured response ratio in the test extract, the concentration of penicillin G residue in the test extract was calculated from the calibration curve data.

Experiments to Optimize the Extraction of Penicillins from Calf Liver Tissue

To optimize the volume of acetonitrile required to maximally recover penicillin G from this matrix, control liver tissues were fortified at 40 ng/g and extracted with one of the following compositions: one 10-mL portion of acetonitrile; two 5-mL portions of acetonitrile; or one 10-mL portion of acetonitrile followed by one 5-mL portion of acetonitrile. The acetonitrile extracts were evaporated to dryness and cleaned up on C_{18} cartridges as described, and the penicillins were analyzed by liquid chromatography to determine the extent of recovery from the tissue matrix. In addition, the effect of mechanical agitation with or without prehomogenization of the finely chopped liver tissues on the recovery of the penicillins from liver tissues using acetonitrile was investigated. The results of these experiments are presented in Table 1.

Experiments to Define Operational Characteristics of the Analytical Method

Limit of Detection (LOD), Limit of Quantitation (LOQ), Recovery, Accuracy, and Precision

Control liver tissues, obtained from the animals that served as controls in the experiment, were analyzed to establish the specificity of the assay for penicillins G and V (the internal standard). Six sets of matrix calibration standards were analyzed over 2 days to determine the LOD, the LOQ for the analytical method, and the mean recoveries for penicillins G and V from liver tissue (Tab. 2). To determine the interassay precision and accuracy of the method, one set of a

Table 1. Optimization of Experimental Parameters for the Recovery of Penicillins G and V from Calf Liver Tissues Using Acetonitrile[a]

Experiment Conducted	Total Volume (mL) of ACN	% Pen G Recovered	% Pen V Recovered
1. Extract fortified tissue with 2 5-mL portions of ACN	10	68	68
2. Extract fortified tissue with 1 10-mL portion of ACN	10	74	70
3. Extract fortified tissue with 1 10-mL portion of ACN followed by 1 5-mL portion of ACN	15	71	68
4. Effect of tissue homogenization before mechanical agitation on recovery		46	35
5. Effect of mechanical agitation without prior tissue homogenization on recovery		72	71

[a]ACN, acetonitrile; Pen, penicillin.

matrix calibration standard and one set of control liver tissues fortified at 15, 30, and 75 ng/g were also prepared and analyzed on each of 4 consecutive days (Tab. 3). The intraassay precision and accuracy of the analytical method were determined by preparing one set of matrix calibration standards covering the range of 5 to 80 ng/g liver tissue (5, 10, 20, 40, and 80 ng/g) and analyzing them together with four replicates of control liver tissues fortified at 15, 30, and 75 ng/g (Tab. 4).

Table 2. Recovery of Penicillin G and Peniciilin V from Control Calf Liver Tissue Fortified with Penicillin G and a Constant Amount of Penicillin V as Internal Standard

Drug Added (ng/g)	Pen G Peak Response for		Mean Method Recoveries (%)
	External Standard	Fortified Liver Sample	
Pen G			
5.0	6.4 ± 0.2 (n = 6)	4.8 ± 0.8 (n = 6)	75
10.0	10.8 ± 0.3 (n= 6)	9.2 ± 1.3 (n = 6)	85
20.0	21.3 ± 0.2 (n = 6)	14.1 ± 4.2 (n = 6)	66
40.0	42.0 ± 0.5 (n = 6)	31.4 ± 2.6 (n = 6)	75
80.0	87.0 ± 0.8 (n = 6)	63.2 ± 7.1 (n = 6)	73
Pen V, 60.0			
(Internal standard)	24.1 ± 0.4 (n = 6)	17.0 ± 2.7 (n = 16)	71

Table 3. Inter-assay Precision and Accuracy of the Analytical Method

[Pen G] (ng/g)	[Pen G] (ng/g) Determined in Fortified Control Liver on				[Pen G] Determined (ng/g)	Accuracy (%)
	Day 1	Day 2	Day 3	Day 4		
15.0	16.2	16.5	18.3	16.0	16.8±1.0[a]	+12
30.0	28.3	31.1	30.4	28.8	29.7±1.3	−1
75.0	77.0	78.5	85.5	72.9	78.5±5.2	+5

[a]Mean ± S.D.

To verify the accuracy of the method for measuring the concentration of penicillin G residues in actual liver tissues, control liver tissues were fortified by one analyst, coded, and given to another analyst, who was not involved in the sample preparation for analysis using the developed analytical methodology. The results of the analysis of the verification experiments are also shown in Table 4.

Application of the Developed Analytical Method

Thirteen Holstein calves, ranging in age from 2 to 5 weeks, were housed in individual hutches on a North Carolina State University dairy farm (Dairy Educational Unit, Lake Wheeler). Calves were allowed a 5-day acclimation period and were bucket-fed antibiotic-free milk replacer at 12% body weight (BW) once daily; water was provided ad libitum. Procaine penicillin G was added to antibiotic-free replacer milk at 0.68 mg/kg BW (equivalent to calves consuming fluid milk containing penicillin G at 3.33 ppm at 12% BW). Calves were weighed before feeding on day 1. The 12 oldest calves, 3 to 5 weeks of age, were fed the milk replacer with procaine penicillin G once and then transported

Table 4. Intra-assay Precision and Accuracy of the Analytical Method

[Pen G] Added to Control Liver (ng/g)	[Pen G (ng/g)] Experimentally Determined in Replicate				[Pen G] Determined (ng/g)	Accuracy (%)
	1	2	3	4		
15.0	18.0	18.0	16.0	16.0	17.0 ± 1.2[a]	+13
30.0	30.0	29.5	29.0	28.8	29.3 ± 0.5	−2
75.0	86.0	75.5	73.0	79.0	78.4 ± 5.6	+ 5

[a]Mean ± S.D.

to an abattoir for slaughter. Three calves were slaughtered at each of the following times: 4, 6.5, 9.5, and 13 h after drinking the penicillin G-contaminated milk replacer. The youngest calf (2 weeks old) served as the control animal. It was fed milk replacer without added procaine penicillin G and slaughtered 3 h later.

One whole kidney, a liver lobe, and muscle (semimembranosus and semitendinosus) were collected, immediately stored in ice, transported to the laboratory, split into 2 aliquots of approximately 0.5 kg each, and stored at −79°C until assayed. Muscle and kidney tissues were analyzed with the previously published method (1), while the liver samples were analyzed with the alternative method described in this manuscript.

RESULTS AND DISCUSSION

Figure 1a shows a chromatogram of a control (drug-free) liver tissue sample obtained from a 12-week-old Holstein calf from Brandon, Manitoba, Canada, used in a previous study, fortified with penicillin G at 40 ng/g and analyzed following the previously described method (1). Figure 1b is a chromatogram of a control liver tissue sample obtained from the 2-week-old Holstein calf used as a control calf in this study, fortified with penicillin G at 40 ng/g, and analyzed according to the previously described method.

Figure 1c is a chromatogram of an incurred liver tissue extract, obtained from one of the 12 experimental calves used in this study that was fed penicillin G-contaminated milk replacer once with 3.33 ppm of penicillin G at 12% BW and slaughtered 4 h later, which was analyzed following the previously described procedure. Because the previously described method worked successfully when it was applied to the 12-week-old calf liver tissues (Fig. 1a), it is quite apparent from both Figures 1b and c that the same method was unable to extract penicillins G and V from either fortified control liver tissue or incurred liver tissue samples from these very young calves.

Figure 1d is the chromatogram of an incurred liver tissue sample from the same animal shown in Figure 1c when it was analyzed using the alternative method described in this paper. This liver tissue was determined to contain penicillin G at a concentration of 195 ng/g, a value that could not have been determined using the previously described method.

When we first made this observation (Fig. 1b), it was hypothesized that the very young age of the calves involved in this particular study may have contributed to the failure to extract the penicillins from their liver tissues. It was thought that, perhaps, the livers from these very young veal calves were so metabolically active that the penicillins were being rapidly degraded or metabolized as soon as they were added to control liver tissue. This speculation was, however, abandoned when it was demonstrated that by using the modified extraction pro-

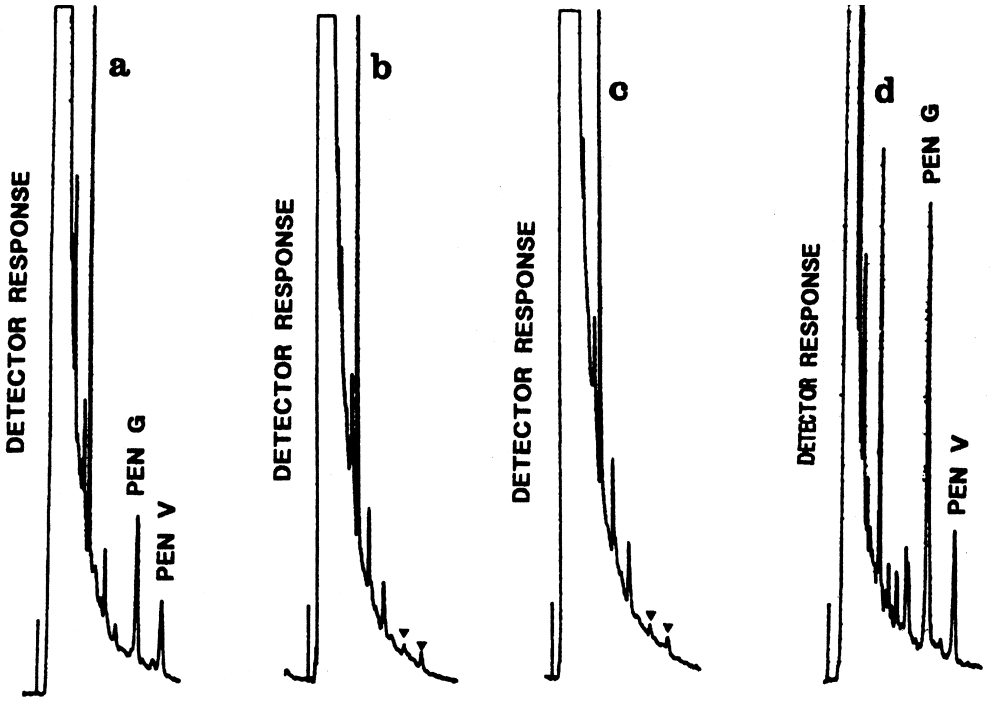

Figure 1. a) LC-ultraviolet(UV) chromatogram of a control (drug-free) liver tissue from a 12-week-old Holstein calf from Brandon, Manitoba, Canada, used in a previous study, fortified with penicillin (PEN) G at 40 ng/g, and the internal standard penicillin G and analyzed using the previously published procedure. b) LC-UV chromatogram of a control (drug-free) liver tissue obtained from a 2-week-old Holstein calf used as a control in the North Carolina State University experiment, fortified with penicillin G at 40 ng/g with penicillin V as internal standard and analyzed following the previously published procedure. c) LC-UV chromatogram of a 3-week-old Holstein calf fed penicillin G-contaminated milk replacer (equivalent to 3.33 ppm of penicillin G) once at the rate of 12% body weight, slaughtered 4 h later, and analyzed using the previously published procedure. d) LC-UV chromatogram of the liver tissue from the same animal in Figure 1c that was processed and analyzed according to the modified analytical procedure described in this manuscript. Chromatographic conditions for the detection and determination of the liver extracts are described in detail in the text.

cedure described in this paper (Fig. 1d) the penicillins could actually be efficiently extracted. This was an indication to us that the penicillins were not actually being rapidly metabolized or degraded as we had previously thought.

It was, therefore, concluded that the failure could be directly traced to the ineffectiveness of the aqueous extraction procedure used in the previous method. We have not as yet been able to offer a reasonable explanation for the failure of the previous method to extract the penicillins from this particular tissue matrix, considering that the method still worked successfully for the muscle and kidney tissues from these same very young calves. It is also interesting to note that the acetonitrile extraction procedure also worked successfully on the muscle and kidney tissues from these very young veal calves and, in fact, gave identical analytical results as the aqueous extraction method.

If any analysts who have used the previous method in the past have made similar observations as we have just made, we would be most grateful if they could share this information with us and also assist us with an explanation for the failure of the aqueous extraction method to extract penicillins from liver tissues of these very young veal calves.

To make them useful for the study under consideration, the experimental parameters for the extraction procedure (i.e., the extraction efficiency and repeatability), the part of the previous method that was significantly modified, were optimized and validated. Table 1 shows that one 10-mL portion of acetonitrile was sufficient to maximally extract both penicillins from the liver tissue matrix. In addition, chopping the liver samples into very fine small pieces permitted the penicillins to be maximally extracted by using mechanical agitation with acetonitrile only, without the need for prehomogenization of the liver tissues. Not homogenizing the liver tissues before the mechanical agitation step represents a time savings of about 30 min in sample pretreatment time for a set of 12 tissues including calibration standards.

Table 2 shows that recoveries ranging from 66 to 85% for penicillin G and 71% for penicillin V can be obtained when this method is used to analyze penicillins G and V added to control livers from these young calves. While these are slightly lower than the recoveries (85–94%) achieved with the aqueous extraction method, both methods have the same LOD for penicillin G [signal/noice (S/N) = 3]. The slightly lower recovery may be because penicillins are known to undergo some degree of degradation in acetonitrile (2,3). A LOQ of 5 ppb (S/N = 10) was calculated for this method. Like the previous method, this LOQ makes the alternative method suitable for the determination and monitoring of penicillin G residues in livers of very young beef calves. A maximum residue limit of 50 ppb has been established for all matrices in beef cattle.

Table 3 shows that the interassay variability calculated for this method was less than 15% and was not significantly different from the intraassay variability shown in Table 4. Results of the verification experiment in which control calf

Table 5. Verification of the Accuracy of the Analytical Method

[Pen G] Added to Control Liver (ng/g)[a]	[Pen G] Experimentally Determined (ng/g)	Accuracy (%)
10.0	9.4	−6
10.0	10.0	0
15.0	16.4	+9
30.0	28.6	−5
40.0	37.5	−6
50.0	48.4	−3
75.0	80.4	+7

[a]These samples were prepared by one analyst, randomized, coded, and given to another analyzt who was not involved in the preparation of the samples to analyse using the alternate method.

liver samples were fortified, coded, and randomized by one analyst and presented to another analyst for analysis using the alternative method are presented in Table 5. The results demonstrate that this method can be used to accurately determine the unknown concentrations of penicillin G added to liver tissues from these very young beef calves.

The optimized and validated analytical method was then used to determine the concentrations of penicillin G residues in livers of 2 to 5-week-old calves fed penicillin-contaminated milk replacer and withdrawn from the milk for varying periods of time before slaughter.

CONCLUSION

An alternative analytical method with detection sensitivity equivalent to that of the previous method for the determination of penicillin G residues in food animal tissues has been developed for use under circumstances such as that in this study where the original method fails. The alternate method is selective, can be performed rapidly, and is suitable for use in a routine environment for regulatory monitoring of penicillin G residues.

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