Journal of Chromatography, 626 (1992) 271–276
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 653

Short Communication

Development of an assay for penicillin G in chinook salmon muscle tissue

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(First received May 21st, 1992; revised manuscript received September 24th, 1992)

ABSTRACT

A high-performance liquid chromatographic method was developed to assay penicillin G (pen-G) in chinook salmon muscle tissue. Solid-phase extraction techniques were used to recover pen-G and the internal standard, phenoxymethylpenicillin, from the salmon tissue samples. Pen-G was analyzed using a 25 cm × 4.6 mm I.D. Ultrasphere OS (C_8) column and a mobile phase of acetonitrile–0.02 *M* phosphate buffer, pH 6.75 (43:200) with ultraviolet detection at 214 nm. A linear calibration curve ($r^2 = 0.9994$) was obtained for pen-G over the range 0.05–3.0 ppm. Recoveries of pen-G added to tissues at 0.1, 1.0 and 2.0 ppm were 63.9%, 64.2% and 65.4%, respectively, in salmon muscle tissue, and intra-assay variabilities of 4.89% at 1.0 ppm and 2.79% at 2.0 ppm were optimized for salmon muscle tissue. The detection limit of pen-G was 0.05 ppm in salmon muscle tissue, using a signal-to-noise ratio of 5:1.

INTRODUCTION

The use of antibiotics to treat diseases in salmon raised in the aquaculture industry has aroused concern due to the potential presence of antibiotic residues in salmon tissue at the time of marketing. Penicillin G (pen-G) is used to treat *Renibacterium salmoninarum*, which causes bacterial kidney disease (BKD), and *mycobacterium spp.*, which causes fish tuberculosis and mycobacteriosis in salmon [1]. Penicillin is one of the few food contaminants associated with Type I or immunoglobulin E-mediated food allergies [2]. In order to ensure that antibiotic levels in salmon are within acceptable levels, accurate methods are required which will provide specific and sensitive quantitation.

Microbiological methods have been developed for pen-G analysis in various animal tissues [3]. Sen-

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0021-9673/92/$05.00

sitivities for penicillin detection by thin-layer bioautography using the swab test on premise and microbial inhibition test may vary depending on the growth media and test organism [4]. Generally, chromatographic methods provide greater selectivity and sensitivity. High-performance liquid chromatographic (HPLC) methods can be useful for confirming antibiotic residue test results obtained by direct screening methods. A comparison between HPLC and bioassay methods for the analysis of procaine pen-G in swine tissues indicated that the HPLC method was more sensitive, on average, in detecting pen-G than the corresponding bioassay [5]. This difference may be attributed to the presence of naturally occurring inhibitory substances in the sample matrix which can interfere with the adventitious growth of the test organism [6]. There have been several methods published for the HPLC analysis of pen-G in other animal tissues including beef, pork, and dairy cow's milk [7–12]. While many of these methods used liquid–liquid separation techniques for sample preparation, the use of a solid-phase extraction procedure frequently offers increased speed and selectivity. To date, there are no published methods for the quantitative analysis of pen-G by HPLC in salmon tissue. In the present study, a procedure using solid phase extraction and analysis of pen-G by HPLC in chinook salmon muscle tissue is reported.

EXPERIMENTAL

Materials

Pen-G potassium salt and phenoxymethylpenicillin (pen-V) potassium salt were obtained from Sigma (St. Louis, MO, USA). HPLC-grade acetonitrile, disodium hydrogenorthophosphate heptahydrate and analytical-grade phosphoric acid (85%) were obtained from BDH (Toronto, Canada). Purified water was produced using a Milli-Q water purification system (Millipore Canada, Mississauga, Canada).

Apparatus

The HPLC system consisted of a Beckman Model 110A pump (Beckman Instruments, San Ramon, CA, USA), a Shimadzu SIL-9A autoinjector set to deliver 20-μl samples, a Shimadzu SPD-6AV variable-wavelength UV detector set at 214 nm, and a Shimadzu C-R3 integrator (Shimadzu Instruments, Columbia, MD, USA). The system was fitted with a Brownlee Labs. MPLC NewGuard column containing a 1.5 cm × 3.2 mm I.D. C_8 cartridge (Applied Biosystems, Santa Clara, CA, USA). A 25 cm × 4.6 mm I.D. Beckman Ultrasphere octylsilane (C_8) column with particle size of 5 μm was used. The mobile phase consisted of acetonitrile–0.02 *M* phosphate buffer, pH 6.75 (43:200) which was delivered isocratically at a flow-rate of 1.0 ml/min. The mobile phase was filtered prior to use using a Millipore HPLC solvent filtration system through a FP Vericel 47-mm, 0.45-μm membrane filter (Gelman Sciences, Ann Arbor, MI, USA).

Preparation of standard solutions and reagents

All pen-G solutions were prepared immediately before use. A 500 μg/ml stock solution was prepared by dissolving 5 mg of pen-G in 10.0 ml of 0.02 *M* phosphate buffer, pH 6.75. Varying volumes of stock solution were diluted with the phosphate buffer to give concentrations of 0.5, 1.0, 4.0, 10, 20, and 30 μg/ml.

The internal standard, pen-V, was used for the calibration curve samples and for the analysis of spiked fish samples. It was also used for the recovery study as an external standard. A 1.0 mg/ml stock solution was prepared by dissolving 10.0 mg of pen-V in 10.0 ml of 0.02 *M* phosphate buffer, pH 6.75. The working solution was prepared by diluting 0.16 ml of pen-V stock solution to a final volume of 10.0 ml with the phosphate buffer, resulting in a final concentration of 16.0 μg/ml. To each 5-g muscle tissue sample, 0.5 ml of internal standard working solution was added, resulting in a final concentration of 1.6 μg/g tissue.

Extraction procedure

In a 50-ml polypropylene centrifuge tube, 15 ml of acetonitrile and 0.5 ml of the internal standard solution were added to 5.0 g of finely chopped salmon muscle tissue. Each sample was homogenized three times for 15-s intervals at medium speed using a Brinkman Polytron Model PT 10/35 homogenizer (Brinkman Instruments, Rexdale, Canada). The samples were centrifuged for 5 min at 1000 *g* at 4°C (GH 3.7 bucket rotor, *g* avg) in a Beckman Model GPR centrifuge (Beckman Instruments). The supernatant was transferred to a 50-ml culture tube and

stored in the dark at 4°C. An additional aliquot (15 ml) of acetonitrile was added to the salmon muscle residue and homogenized as before. The supernatants were combined and evaporated to dryness under nitrogen in a 40°C water bath. A 2-ml aliquot of acetonitrile was added to the residue and vortex-mixed for 20 s. A 20-ml volume of 0.02 *M* phosphate buffer, pH 6.75 was added and the sample was vortex-mixed for an additional 20 s. The resulting solution was passed through an activated Bakerbond SPE octadecyl (C_{18}) high-capacity disposable 6-ml column (J.T. Baker, Phillipsburg, NJ, USA). The C_{18} column was initially activated by passing through two 6-ml portions of 50% (v/v) acetonitrile, followed by two 6-ml portions of the phosphate buffer. The pen-G and internal standard were eluted from the cartridge with two 4-ml portions of acetonitrile. The combined eluates were evaporated to dryness under nitrogen in a 40°C water-bath. The samples were reconstituted with 1 ml of mobile phase and vortex-mixed for 15 s prior to analysis.

Calibration curve, assay precision and recovery

A calibration curve was prepared from salmon muscle tissue samples (5 g) by adding 0.5 ml of the internal standard (pen-V) solution (1.6 μg/g tissue) and 0.5 ml of the appropriate pen-G standard solutions to give final concentrations of 0.05, 0.1, 0.4, 1, 2, and 3 μg/g tissue. The calibration curve was constructed by plotting the peak-area ratios of pen-G to those of the internal standard against the known concentrations of pen-G.

The recovery study was performed by adding 0.5 ml of 1.0, 10.0, and 20.0 μg/ml pen-G solution to 5 g of salmon muscle tissue. Duplicate samples were prepared at each concentration. The samples were processed as described above, however, the internal standard solution was added just before the final evaporation step. The peak-area ratios of pen-G to the internal standard were compared against the peak-area ratios of identical amounts of unextracted standard solutions.

Intra-assay coefficient of variation

Determination of the intra-assay coefficient of variation was performed by injecting each of the 1.0- and 2.0-ppm salmon extracts five times. These extracts were prepared in the same manner as for the calibration curve.

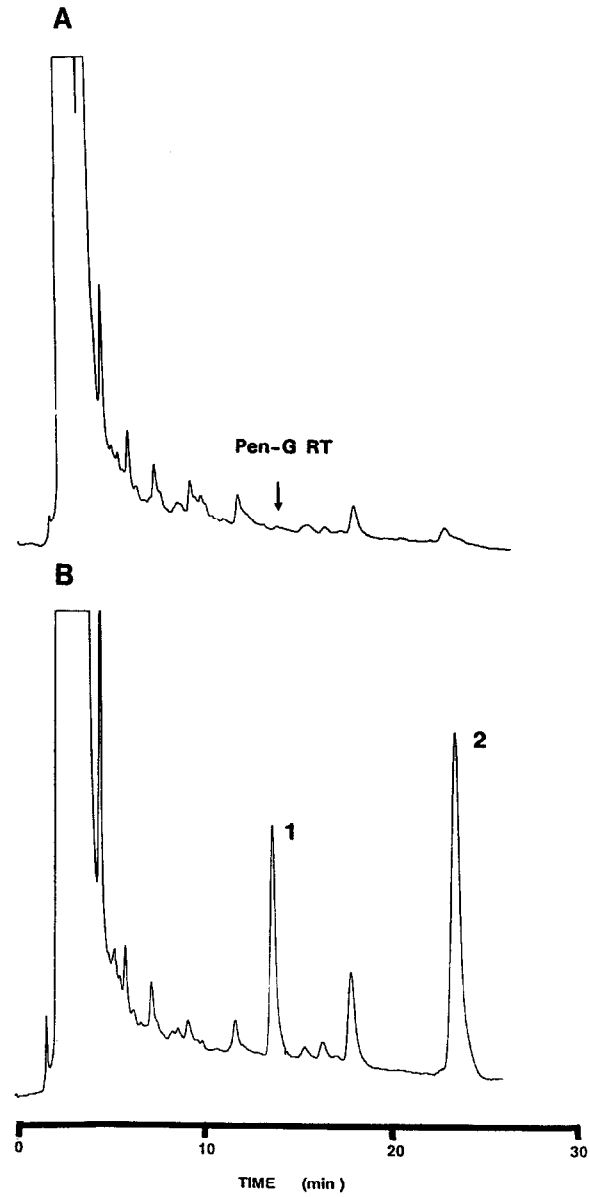

Fig. 1. Chromatographic conditions: column, Ultrasphere OS (C_8) 5-μm (25 cm × 4.6 mm); mobile phase, acetonitrile–0.02 *M* phosphate buffer, pH 6.75 (43–200); HPLC flow-rate 1.0 ml/min; ultraviolet detection wavelength, 214 nm; injection volume, 20 μl. Peaks: 1 = Penicillin G; 2 = Penicillin V (RT = retention time). (A) Representative chromatogram from a blank salmon muscle tissue extract. (B) Representative chromatogram of a salmon muscle tissue extract to which 1.0 ppm of pen-G and 1.6 ppm of the internal standard, pen-V, had been added.

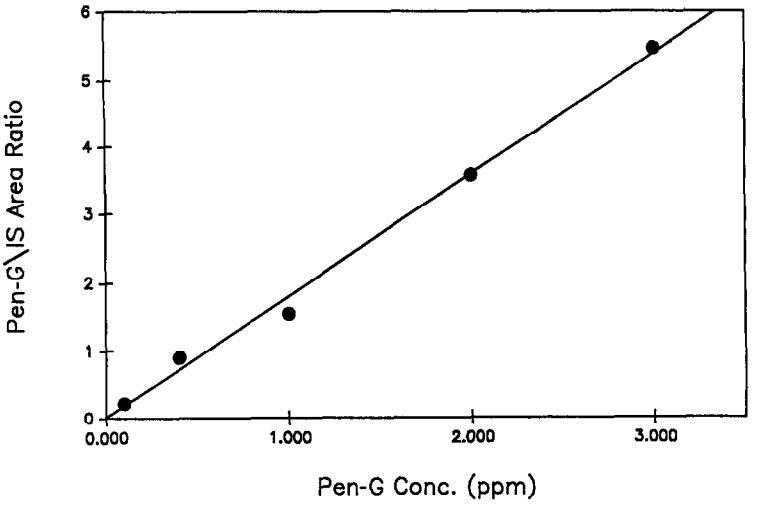

Fig. 2. Calibration curve for pen-G extracted from salmon muscle tissue over the concentration range 0.05–3.0 ppm. r^2 = 0.9994; y-intercept = 0.0262; slope = 0.0630. IS = internal standard.

RESULTS AND DISCUSSION

Chromatograms of a blank salmon extract and a salmon extract containing pen-G and the internal standard are shown in Fig. 1. The internal standard, pen-V, was selected for its appropriate elution and extraction properties. Other penicillin analogues were examined as possible internal standards. Amoxicillin, ampicillin, cloxacillin, dicloxacillin, oxacillin, methicillin, and nafcillin were evaluated but rejected due to unfavorable retention times or co-elution with endogenous substances. Fig. 2 shows a linear calibration curve obtained for pen-G extracted from salmon muscle tissue over the concentration range 0.05–3.0 ppm (y = 0.0262 + 0.0630x; r^2 = 0.9994).

The double-extraction procedure using acetonitrile was found to be the most efficient method for drug recoveries. The use of acetone for extraction resulted in chromatograms exhibiting fewer endogenous substances, however, the recovery of pen-G was substantially lower. Extractions using chloroform produced chromatograms exhibiting numerous interfering peaks and low recovery of pen-G. Protein precipitators, including perchloric acid, sodium tungstate–sulphuric acid, trichloroacetic acid, and zinc sulphate–barium hydroxide, caused significant degradation of pen-G. This effect was probably due to hydrolysis of the β-lactam ring, resulting in the formation of benzylpenicilloic acid and benzylpenilloic acid [12]. High-capacity solid-phase extraction cartridges containing 1000 mg of C_{18} sorbent resulted in a higher recovery of pen-G than regular C_{18} cartridges containing 500 mg. Over the concentration range 0.1–3.0 ppm, the mean recovery was found to be 64.5% (Table I). Using a signal-to-noise ratio of 5:1, the detection limit was found to be 0.05 ppm.

It is unlikely that the relatively low recovery rate of pen-G could be attributed to the degradation of this drug. The extraction efficiency of pen-G from

TABLE I

RECOVERY OF PENICILLIN G FROM CHINOOK SALMON MUSCLE TISSUE

Sample concentration (ppm)	Recovery[a] (%)
0.1	63.9
1.0	64.2
2.0	65.4
Mean	64.5

[a] Each value represents a single determination at each sample concentration.

TABLE II

INTRA-ASSAY VARIABILITY OF PENICILLIN G IN CHINOOK SALMON MUSCLE TISSUE

Injection No.	Sample concentration (ppm)	Pen G/internal standard peak-area ratio	Calculated concentration (ppm)
At 1.0 ppm			
1	1.0	0.396	0.91
2	1.0	0.406	1.04
3	1.0	0.402	1.00
4	1.0	0.406	0.99
5	1.0	0.402	1.01
Mean		0.402	0.99
Standard deviation		0.0041	0.0485
Coefficient of variation (%)		1.02	4.89
At 2.0 ppm			
1	2.0	0.690	2.11
2	2.0	0.652	1.99
3	2.0	0.678	2.07
4	2.0	0.669	2.04
5	2.0	0.646	1.97
Mean		0.667	2.04
Standard deviation		0.018	0.057
Coefficient of variation		2.69	2.79

beef tissue has been reported to be higher than that obtained in this study with chinook salmon [7]. The extraction and chromatographic assay were performed rapidly at an optimal pH which should minimize pen-G degradation [13]. An alternative explanation for the low recovery rate of pen-G may be the high cholesterol and lipid content and the presence of carotenoids in salmon muscle tissue. Pen-G may remain with the carotenoids or lipid components of the tissue and may not be recovered in the tissue extracts. Nettleton and Exler [14] have recently shown a higher content of total fat in cultivated coho salmon compared with either wild coho salmon or wild and cultivated rainbow trout. Carotenoids are the pigments responsible for the orange–red colour of salmon muscle tissue, with astaxanthin being the major substance involved [15]. The presence of astaxanthin may be an additional factor in the recovery of pen-G.

The results of the intra-assay coefficient of variation are shown in Table II. The coefficients were found to be 4.89% for the 1.0-ppm extract and 2.79% for the 2.0-ppm extract.

The use of a reversed-phase C_8 column gave optimal resolution of drug and internal standard as well as freedom from interference from endogenous substances. Moats [16] investigated the use of C_8, C_{18}, phenyl, and anion-exchange columns for the analysis of pen-G in milk. There were essentially no differences in the patterns between the C_8 and C_{18} columns. However, the resolution of pen-G from interfering peaks was lower than with the phenyl column. The anion-exchange column did not resolve pen-G from interfering substances.

For analysis of salmon muscle tissue, the C_8 column produced chromatograms with fewer endogenous substances, particularly in the area where pen-G and the internal standard eluted.

The analytical wavelength used was 214 nm and was found to give the greatest sensitivity. Other published reports used wavelengths ranging from 200 to 220 nm [5,7,9–12].

ACKNOWLEDGEMENTS

Funding was provided by the Science Council of

British Columbia, the Department of Fisheries and Oceans, Canada and the Natural Sciences and Engineering Research Council.

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