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Determination of penicillin G in bovine plasma by high-performance liquid chromatography after pre-column derivatization

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ABSTRACT

A simple, selective, and sensitive liquid chromatographic method with ultraviolet detection was developed for the analysis of penicillin G in bovine plasma. The assay utilizes a simple extraction of penicillin G from plasma (with a known amount of penicillin V added as internal standard) with water, dilute sulphuric acid and sodium tungstate solutions, followed by concentration on a conditioned C_{18} solid-phase extraction column. After elution with 500 μl of elution solution, the penicillins are derivatized with 500 μl of 1,2,4-triazole–mercuric chloride solution at 65°C for 30 min. The penicillin–mercury mercaptide complexes are separated by reversed-phase liquid chromatography on a C_{18} column. The method, which has a detection limit of 5 ng/ml (ppb) in bovine plasma, was used to quantitatively measure the concentrations of penicillin G in plasma of steers at a series of intervals after the intramuscular administration of a commercial formulation of procaine penicillin G.

INTRODUCTION

Previous studies on the disposition of procaine penicillin G in cattle have used standard, semi-quantitative, microbiological inhibition assays to measure the concentrations of penicillin G in bovine plasma [1–6]. There are no reports in the literature of a simple, sensitive liquid chromatographic method for measuring the concentrations of penicillin G in bovine plasma. Such a method is required to conduct pharmacokinetic studies in cattle and to evaluate other assay techniques [7]. We recently developed a simple, sensitive high-performance liquid chromatographic (HPLC) method for the determination of penicillin G residues in edible tissues of food-producing animals [8]. We now report a modification of this method for the determination of penicillin G in bovine plasma. To demonstrate the suitability of our method, a pharmacokinetic study was conducted in feedlot steers.

EXPERIMENTAL

Reagents

Ethacilin (300 000 I.U./ml of procaine penicillin G) was purchased from Rogar/STB (London, Canada). Penicillin V potassium salt and penicillin G socium salt were obtained from Sigma (St. Louis, MO, USA). 1,2,4-Triazole was obtained from Aldrich (Milwaukee, WI, USA), mercuric chloride from BDH (Toronto, Canada), sodium

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tungstate from Fisher Scientific (Nepean, Canada) and sodium thiosulphate was purchased from Anachemia Science (Winnipeg, Canada). Water was obtained from a Barnstead RO/Nanopure ultrafiltration unit. All other reagents were of reagent grade.

Materials

C_{18} solid-phase cartridges (J. T. Baker, 100 mg, 1 ml capacity) were purchased from John's Scientific (Toronto, Canada), and glass fiber filters, GF/B, were purchased from Whatman (Hillsboro, OR, USA).

Instrumentation

The liquid chromatography system consisted of a Waters 501 pump, a 712 autosampler, and a 994 photodiode-array detector (Waters Chromatography Division, Mississauga, Canada). Reversed-phase liquid chromatography was accomplished on a Waters Nova-Pak C_{18} column (4 μm, 150 mm × 3.9 mm I.D.). The operation of the pump, autosampler and detector and the acquisition of data were controlled by a Waters 820 chromatography work station.

Mobile phase flow through the column was set at 1 ml/min and the analytes were detected at 325 nm at a sensitivity setting of 0.005 a.u.f.s.

Preparation of derivatization reagent, mobile phase and elution solutions

The preparation of the derivatization reagent (2 *M* 1,2,4-triazole containing 0.01 *M* mercuric chloride), mobile phase (0.1 *M* phosphate buffer, pH 6.5, containing 0.0157 *M* thiosulphate–acetonitrile, 75:25, v/v) and elution solution (acetonitrile—0.2 *M* phosphate buffer, pH 6.5–water, 60:5:35, v/v) are all described in detail elsewhere [8].

Preparation of plasma samples for analysis

The standard curve for penicillin G was constructed by spiking control drug-free plasma (2 ml) with known amounts of the 5 μg/ml penicillin G standard and 120 μl of a 5 μg/ml penicillin V standard to provide plasma concentrations in the 50–400 ng/ml range for penicillin G and 300 ng/ml for penicillin V. For plasma samples with penicillin G concentrations greater than 400 ng/ml, either 1-ml plasma samples were used, or penicillin concentrations were estimated from a calibration curve of response ratio (UV response for penicillin G/UV response for penicillin V) *versus* penicillin G concentrations from 500 to 2000 ng/ml, containing 900 ng/ml penicillin V. For test plasma (2 ml), 120 μl of the 5 μg/ml penicillin V standard was added. Water (30 ml), 2 ml of 0.17 *M* sulphuric acid and 2 ml of 5% sodium tungstate solution were added to standard and test samples, which were then vortex-mixed for 30 s. The samples were centrifuged at 2200 *g* for 10 min. The supernatant was vacuum-filtered through a GF/B filter into a 125-ml flask. A 10-ml volume of 20% sodium chloride solution was added to the filtrate in the 125-ml flask, and the solution was mixed thoroughly.

A 75-ml solvent reservoir was mounted onto the C_{18} cartridge with an adapter and these were placed on a solid-phase extraction vacuum manifold. The cartridge was washed with 5 ml of methanol, 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 filtrate from the 125-ml flask was poured into the reservoir and pulled through the cartridge at a flow-rate of approximately 3 ml/min. The cartridge was then washed with 5 ml of 2% sodium chloride and 5 ml of water, after which air was drawn through the cartridge for 5 min. The adapter and reservoir were removed from the cartridge, a clean 10-ml glass centrifuge tube was placed under the cartridge, and the penicillins were eluted with 500 μl of elution solution. (It is important that the penicillins be eluted from the cartridge immediately after the cartridge has been washed with sodium chloride and water.)

Derivatization reagent (500 μl) was added to the eluate in the centrifuge tube, and the tubes were capped and vortex-mixed for 20 s, then allowed to react in a 65°C water bath for 30 min. The centrifuge tube was removed from the bath and immersed in a beaker of water to cool the reaction mixture to room temperature. The contents of the tube were vortex-mixed, filtered

through a 0.45-μm. Acro filter disk into a 1-ml LC vial, and 50–100 μl aliquots were injected into the HPLC system.

Experiment to optimize the elution of penicillins G and V from Baker solid-phase extraction cartridges

Blank drug-free plasma samples (2 ml) fortified at different concentrations (25, 100 and 400 ng/ml) with penicillin G and 300 ng/ml penicillin V were extracted, loaded onto the C_{18} cartridges, eluted with four 250-μl portions of the elution solution and derivatized as previously described. Equivalent standard solutions of derivatized penicillins G and V in water were injected directly into the HPLC column and peak heights of peaks at the retention times for penicillin G and penicillin V were measured and compared with those from the processed samples in order to determine the amount of penicillins G or V recovered in the 250-μl fraction.

Precision, accuracy and recovery

Intra-assay precision of the method was determined by replicate analyses ($n = 5$) of control drug-free bovine plasma fortified with various concentrations of penicillin G. These samples were assayed on three consecutive days to determine day-to-day variation of the assay. The accuracy of the developed analytical method was evaluated by calculating the amounts of penicillin G found in the fortified samples from the calibration curve. Recovery was calculated over the range of concentrations studied by comparing the concentrations of penicillin G found in the fortified samples after extraction and HPLC analysis with external penicillin G standards.

Animal experiment

Two healthy, antibiotic-free finished steers were injected intramuscularly with procaine penicillin G (Ethacilin) once daily for five consecutive days. One steer, weighing 455 kg, was injected with a dose of 24 000 I.U./kg, and the other steer, weighing 440 kg, was injected with a dose of 66 000 I.U./kg body weight. The 24 000-I.U. dose was administered as a single intramuscular injection of 36.4 ml while the 66 000-I.U. dose was given in three equal-volume intramuscular injections of 32.3 ml. The injection sites were as follows: day 1, right lower hamstring area (semimembranosus and semitendinosus muscle); day 2, right lateral neck; day 3, left lateral neck; day 4, right upper hamstring area; and day 5, right gluteal muscle.

Blood samples were collected from the left jugular vein immediately before and 1 h after each day's injection for the first four days. On the fifth day, blood samples were collected at 0 (control), 15, 30 and 60 min, 2, 4, 6, 8, 10, 12, 24, 36 and 48 h and at 3, 4, 5, 6, 7, 8 and 10 days following the injections. Blood was collected into heparin-

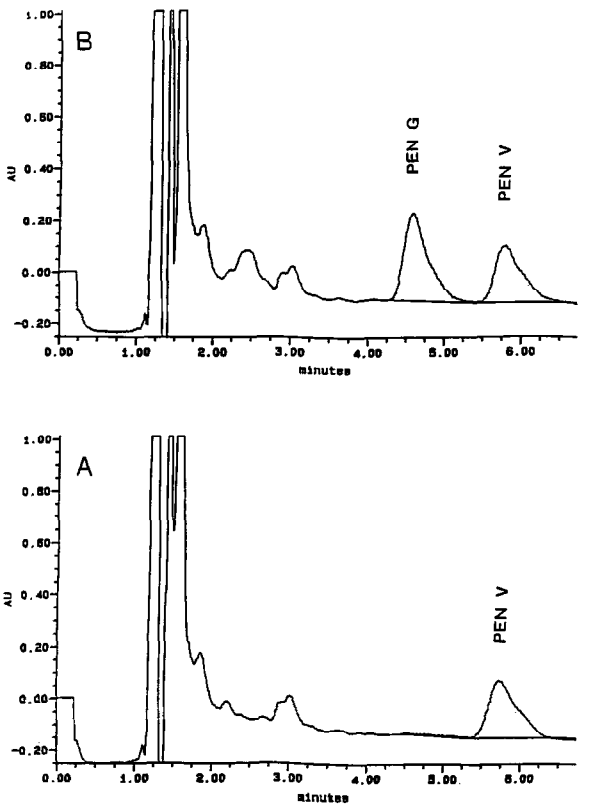

Fig. 1. Chromatograms of (A) a control drug-free plasma and (B) plasma containing 150 ng/ml penicillin G collected on the 7th day from a steer injected intramuscularly with 24 000 I.U./kg body weight procaine penicillin G once daily for five consecutive days. Each sample was fortified with penicillin V as internal standard at a concentration of 300 ng/ml and subjected to the assay procedure.

TABLE I

OPTIMIZATION OF THE ELUTION OF PENICILLINS G AND V FROM 100-mg CAPACITY BAKER C_{18} SOLID-PHASE EXTRACTION CARTRIDGES

Concentration level (ng/ml)	Drug	Amount added (ng)	Amount recovered[a] (ng)					Recovery (%)
			1st	2nd	3rd	4th	Total	
400	Pen. G	800	32	753	<2	N.D.[b]	785	98
	Pen. V	600	48	540	<2	N.D.	588	98
100	Pen. G	200	193	3	N.D.	N.D.	196	98
	Pen. V	600	580	10	N.D.	N.D.	590	98
25	Pen. G	50	46	N.D.	N.D.	N.D.	46	92
	Pen. V	600	588	7	N.D.	N.D.	596	99

[a] Amounts recovered in four 250-μl portions of the elution solution.
[b] Not detected.

TABLE II

INTER-ASSAY PRECISION FOR THE ANALYSIS OF PENICILLIN G IN BOVINE PLASMA

Theoretical concentration (ng/ml)	Observed concentration (mean ± S.D., n = 5) (ng/ml)			C.V. (%)
	Day 1	Day 2	Day 3	
20.0	19.3 ± 1.3	20.5 ± 0.8	18.7 ± 1.5	5
50.0	48.5 ± 2.8	45.8 ± 1.5	46.0 ± 4.0	3
75.0	74.3 ± 4.2	76.5 ± 5.0	74.7 ± 7.0	2
150.0	143.6 ± 9.3	147.3 ± 7.5	151.4 ± 6.5	3
300.0	298.6 ± 8.5	290.0 ± 9.8	295.5 ± 8.5	1

TABLE III

ESTIMATION OF THE INTRA-ASSAY PRECISION AND ACCURACY OF THE DEVELOPED ANALYTICAL METHODOLOGY USING FORTIFIED PLASMA OBTAINED FROM A DRUG-FREE HOLSTEIN STEER

Amount added (ng)	Amount found (mean ± S.D., n = 5) (ng)	C.V. (%)	Accuracy[a] (%)
20.0	19.3 ± 1.3	5	93
50.0	48.5 ± 2.8	4	96
75.0	74.3 ± 4.2	5	99
150.0	143.6 ± 9.3	6	95
300.0	298.6 ± 8.5	3	99

[a] Accuracy = (amount found × 100)/(amount added).

TABLE IV

RECOVERY OF PENICILLIN G FROM SPIKED PLASMA SAMPLES USING THE PRECOLUMN DERIVATIZATION HPLC METHOD

Concentration in standard (ng/ml)	Detector response (peak height) (mm)		Recovery (%)
	External standard	Spiked standard	
10.0	5.3	4.9	92
25.0	13.2	11.4	86
50.0	26.0	24.8	95
100.0	51.5	46.5	90
200.0	103.3	96.8	94
400.0	207.3	186.9	90

ized tubes and centrifuged at 4500 *g* for 10 min. The plasma was separated and stored at −20°C until analyzed.

RESULTS AND DISCUSSION

Typical chromatograms of extracted control and test plasma samples are shown in Fig. 1. The retention times for penicillins G and V were 4.6 and 5.8 min, respectively. There was no interference with the assay from endogenous compounds or other β-lactam antibiotics such as ampicillin, amoxicillin and cloxacillin.

The results of an experiment conducted to optimize the elution of penicillins G and V from the 100-mg Baker cartridges are shown in Table I. It can be seen that under these conditions, penicillin G and penicillin V are both quantitatively eluted from the solid-phase extraction cartridges with 500 μl of the elution solution.

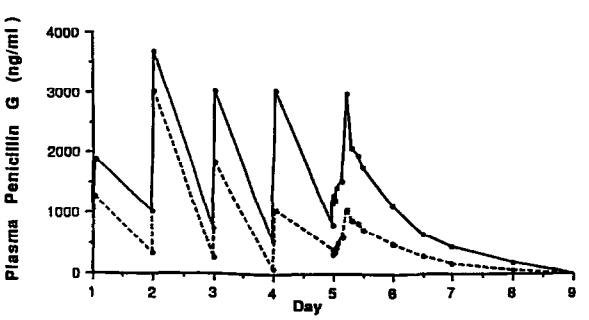

Fig. 2. Plasma concentration–time profile for the two steers injected intramuscularly with procaine penicillin G at 24 000 (---) or 66 000 (——) I.U./kg body weight per day for five consecutive days.

The results summarized in Tables II and III show that the coefficients of variation (C.V.) for both the inter- and intra-assay precision do not exceed 10%, and the method shows accuracies greater than 95% of the expected values over the range of concentrations studied. Table IV shows that more than 85% of penicillin G spiked into drug-free plasma was recovered with this method.

To demonstrate the pharmacokinetic capabilities of this method for bovine plasma, samples from two steers injected with procaine penicillin G were subjected to the assay procedure. Fig. 2 shows the plasma penicillin G concentration–time curves from two steers injected intramuscularly with doses of 66 000 or 24 000 I.U./kg body weight per day for five consecutive days.

On day 5, the maximum plasma concentration of penicillin G was reached at a time (T_{max}) 6 h after the administration of the drug in both animals. In addition, the maximum plasma concentrations (C_{max}) attained on day 5 following drug administration were 1.06 and 2.99 μg/ml for the 24 000 and 66 000 I.U./kg doses, respectively. There were no detectable penicillin G concentrations in plasma samples collected beyond nine days analysed with this method which has a detection limit of 0.005 μg/ml (5 ppb).

CONCLUSION

The HPLC method described in this paper provides a highly sensitive and reliable assay procedure for the analysis of penicillin G in bovine plasma. The extraction process is simple and rapid, and the pre-column derivatization step provides the sensitivity and reliability required for the quantitative analysis of penicillin G levels in bovine plasma following intramuscular administration of penicillin G. This method, which has a detection limit of 5 ppb in bovine plasma, is currently being used in our laboratory to measure pharmacokinetic parameters in the plasma of finished beef steers injected with a commercial formulation of a 1:1 mixture of procaine penicillin G and benzathine penicillin G.

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