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Quantification of penicillin-G and procaine in equine urine and plasma using high-performance liquid chromatography

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Abstract

A rapid and sensitive method for the extraction and quantification of penicillin-G and procaine in horse urine and plasma samples has been successfully developed. The method involves the use of solid-phase extraction (SPE) for penicillin-G, liquid–liquid extraction (LLE) for procaine, and high-performance liquid chromatography (HPLC) for the quantification of penicillin-G and procaine. The new method described here has been successfully applied in the pharmacokinetic studies of procaine, penicillin-G and procaine–penicillin-G administrations in the horse.

Keywords: Penicillin-G; Procaine

1. Introduction

Within the past 10 years, a number of sensitive methods for the detection of penicillin-G in milk [1], plasma [2–5], and tissue samples [6,7] have been published. These screening techniques include microbiological assay [8–13], immunoassay [14–16], and competitive binding [17–20]. None of these methods was adaptable to large scale sample extraction and quantification of penicillin-G in a reproducible and reliable manner. In this study, we have successfully developed a simple, cost effective and sensitive method for the extraction and quantification of penicillin-G in horse urine and plasma samples. The method we have developed allowed the study of pharmacokinetic profiles of penicillin-G and procaine in procaine–penicillin-G administration in the horse.

2. Experimental

2.1. Materials

Procaine–penicillin-G (PPG) was obtained from Phoenix Pharmaceuticals (St. Joseph, MO, USA) and was intramuscularly (i.m.) administered at 22 000 IU/kg of body mass. The potassium salts of penicillin-G and penicillin-V, procaine hydrochloride and

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tetracaine hydrochloride were purchased from Sigma (St. Louis, MO, USA). Each of these standard solutions contained 1 mg/ml prepared in distilled water and stored at 4°C until used. Standards for 1,2,4-triazole, mercuric chloride and triethylamine were also obtained from Sigma (St. Louis, MO, USA). A 2 *M* solution of 1,2,4-triazole was prepared with mercuric chloride (0.001 *M*) as the derivatizing reagent. Sodium thiosulphate was obtained from Baker and Adamson (Morristown, NJ, USA) and was prepared as a 0.0157 *M* solution in distilled water. Potassium hydroxide was purchased from Mallinckrodt (Paris, KY, USA). Potassium phosphate monobasic, ammonium hydroxide, hydrochloric acid, 85% *O*-phosphoric acid, distilled water (HPLC grade) and all organic solvents (HPLC grade) were commercially obtained from Fisher Scientific (Norcross, GA, USA). Potassium phosphate monobasic (0.1 *M*) was prepared at pH 7.0 and adjusted to pH 10 with KOH. Sodium thiosulphate buffer (0.0157 *M*) was added to potassium phosphate monobasic (0.1 *M*) and adjusted to pH 4.5 using phosphoric acid. Triethylamine solution was prepared at 0.0165 *M*. Chemsep C_{18} solid-phase extraction (SPE) cartridges (3 ml/500 mg) were purchased from Chemical Separations (King of Prussia, PA, USA). Sodium fluoride solution (10 mg/ml; Sigma) was added to the blood collection tube prior to blood collection to inhibit plasma esterase activity.

2.2. Apparatus

A Thermolyne Speci-Mix (Thermolyne, Dubuque, IA, USA), IEC HN-S II centrifuge (International Equipment, Needham, MA, USA), Turbo Vap LV evaporator and Benchmate II Automated SPE system (Zymark, Hopkinton, MA, USA) were used in the extraction process. A high-performance liquid chromatography (HPLC) system with diode array detection (DAD) automated with a 1090 liquid chromatograph and ChemStation software (Hewlett-Packard, San Fernando, CA, USA) was used for analysis. For analyte separation, a HPLC column, 150×2.1 mm Zorbax SB-C_{18} (MAC-MOD Analytical, Chadds Ford, PA, USA) was used with an inserted 4×2.1 mm C_{18} guard column (Hewlett-Packard) to prolong the life of the column.

2.3. Drug administration

A dose of 22 000 IU/kg of PPG was administered by the i.m. route to six healthy mares weighing 550±50 kg and ranging in age from 3 to 10 years.

2.4. Sample collection

Urine samples were continually collected via an in-dwelling Foley catheter and storage bag prior to drug administration and at 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 16, 20, 24, 28, 32 and 36 h post-aqueous PPG administration. The catheters were removed after 36 h and single sample voids were collected at 48 and 72 h. Urine samples were divided in two equal volumes immediately after collection. One portion was frozen at −20°C and designated as non-pH adjusted urine (≅pH 8.0). The remaining portion was adjusted to pH 5 (±0.15) with HCl or NH_4OH immediately after collection and designated as pH-adjusted urine and stored at −20°C until analysis. For long-term storage, both sets of urine were stored in a 400-ml plastic stock container and in a 50-ml plastic centrifuge tube for short-term storage. The urine sample in short-term storage was used in the analysis of the samples, while the long-term storage sampler was stored at −70°C during the analysis.

Blood samples were collected at 0 h as the control before drug administration and at 0.25, 0.5, 0.75, 1, 2, 3, 4, 6, 8, 10, 12, 16, 20, 24, 28, 32, 36, 48 and 72 h post-drug administration. Approximately 20 ml of blood was drawn from the neck vein via an indwelling catheter placed into the jugular vein and into 3×10 ml vacutainer tubes containing NaF (10 mg/ml) to inhibit plasma esterase activity [21,22]. The blood samples were then centrifuged at 2500 *g* for 15 min to obtain plasma (≅12 ml). Four test tubes each containing a 3-ml aliquot of plasma were immediately frozen at −20°C until analysis.

2.5. Pretreatment and analysis for penicillin-G

2.5.1. Sample extraction and clean-up

Urine samples frozen in 50-ml centrifuge tubes were thawed and a 150-μl aliquot was used for analysis. Prior to extraction, penicillin-V (100 μg) was added to the sample as the internal standard and the pH of the mixture was adjusted to 7.0 by adding

3 ml phosphate buffer (0.1 *M*, pH 7.0). For the extraction of plasma, a 2-ml aliquot was used with 50 μg of penicillin-V added as the internal standard. The pH of the plasma sample was adjusted to 7.0 by adding 3 ml phosphate buffer (0.1 *M*, pH 7.0) and the plasma–buffer mixture was filtered using 0.45-μm syringe filter (Whatman, Clifton, NJ, USA). Aqueous solutions of penicillin-G and penicillin-V were stored at 4°C after they had been prepared. Urine and plasma samples were extracted using a Benchmate II automated SPE system. The automated Benchmate was programmed to extract penicillin-G and penicillin-V using a list of reagents and Chemsep C_{18} 3 ml/500 mg SPE columns. The Benchmate was programmed to condition the column with 3 ml of methanol, 3 ml of distilled water, and 3 ml of phosphate buffer (0.1 *M*, pH 7) at a flow-rate of 0.20 ml/s. A 150-μl urine or 2.0-ml plasma sample preparation was applied onto the SPE column and filtered to waste at a flow-rate of 0.02 ml/s. The column was then rinsed with 5 ml of distilled water at a flow-rate of 0.05 ml/s, and dried under air pressure at 10 bar for 120 s. The drug analytes were eluted from the column with 5 ml methanol at a flow-rate of 0.02 ml/s. The methanol eluent was evaporated over air (5–10 bar pressure) in a Turbo Vap LV evaporator at 75°C for 45–60 min. The dried extracts were then capped and stored at 4°C until analysis.

2.5.2. Derivatization of analytes prior to analysis

The dried extracts of urine and plasma were reconstituted in 300 μl of distilled water. The penicillin-G and penicillin-V were derivatized by adding 150 μl (200 μl to plasma) of 1,2,4-triazole (2 *M*) prepared in 0.001 *M* mercuric chloride. The derivatization was completed by heating the sample mixture at 65°C for 15 min. The derivatized samples were then cooled, vortexed and aliquots placed into HPLC glass injection vials. The plasma samples were filtered using a 0.1-μm syringe filter (Corning) prior to HPLC analysis. Filtration of the urine samples was not necessary.

2.5.3. Analysis of urine and plasma samples

Concentrations of penicillin-G and penicillin-V were determined using HPLC. The separation column was a Zorbax SB-C_{18}, 150×2.1 mm column with a guard column. Separation of penicillin-G and penicillin-V was achieved with a pre-mixture of 74% phosphate buffer (0.1 *M*) containing 0.0157 *M* potassium thiosulfate (pH 4.5) and 26% acetonitrile. flow-rate was at 0.350 ml/min, and the detection wavelength was fixed at 325 nm with a window of 4 nm. The reference wavelength was 450 nm with a window of 100 nm. Injection volume was 10 μl per sample and the retention times for penicillin-G and penicillin-V were ≅7.0 and 8.5 min, respectively.

2.6. Preparation and analysis of procaine

2.6.1. Sample pretreatment and clean-up

The method of analysis of procaine in urine and plasma was a modification of the method of Stevenson and co-workers [21,22]. A frozen aliquot of urine was thawed and a 5-ml aliquot used for sample extraction and analysis. Tetracaine (20 μg) was added to the sample and the pH adjusted to between 9.75 and 10.25 using 1–1.5 ml of NH_4OH (1.0 *N*). Dichloromethane (DCM; 5 ml) was added to the sample for extraction, the sample tubes were capped and mixed on a roto-rack for 10 min prior to centrifugation (2500 *g* for 10 min), and the aqueous (top) layer discarded. The organic (bottom) layer was transferred to a clean test tube and the extracts evaporated to dryness in a water bath at 65°C. Dried extracts of the samples were capped and stored at 4°C until analysis.

Plasma samples were thawed and to a 2-ml aliquot, tetracaine (5 μg) was added as the internal standard and the sample mixture was adjusted to pH 10.00 prior to extraction by adding 3 ml phosphate buffer (0.1 *M*, pH 10.00). DCM (5 ml) was added to the samples, mixed and centrifuged as described above for urine. The organic (bottom) layer was transferred to a clean test tube, evaporated to dryness in a water bath at 65°C, capped and stored at 4°C until analysis.

2.6.2. Sample analysis

Dried sample extracts were reconstituted in 200 μl of methanol. A 100-μl aliquot of the reconstituted extract was placed in an HPLC injection vial (2-ml glass vial with 125 μl polypropylene insert). The concentration of procaine and tetracaine in urine and plasma samples was determined using HPLC auto-

mated with a 1090 liquid chromatograph and ChemStation software. Detection of procaine and tetracaine was accomplished by UV detection using a deuterium lamp set at a fixed wavelength of 288 nm with a window of 4 nm. The reference wavelength was fixed at 450 nm with a window of 100 nm. Separation of procaine and tetracaine was achieved with a 150×2.1 mm Zorbax SB-C_{18} column with an inserted 4×2.1 mm C_{18} guard column. The guard column was changed approximately every three weeks or after 150 injections. The mobile phase comprised a pre-mixed acetonitrile–triethylamine (0.0165 *M*) (85:15) adjusted to pH 3.0 using 85% phosphoric acid. The flow-rate was at 0.35 ml/min and 10 μl of sample was injected onto the column. Retention time recorded for procaine and tetracaine was ≅1.45 and 1.85 min, respectively.

3. Results and discussion

3.1. Recoveries of procaine and penicillin-G from urine and plasma

Using the optimized procedure for the extraction and clean-up of the samples, recoveries for procaine and penicillin-G from both urine and plasma were determined. Urine and plasma samples were spiked with known concentrations of penicillin-G and procaine, extracted and analyzed. Recoveries of procaine and penicillin-G from both urine and plasma were calculated using peak area ratios of the extracted procaine to tetracaine (internal standard) vs. the peak ratio of unextracted procaine to tetracaine, and those of extracted penicillin-G to penicillin-V (internal standard) vs. unextracted penicillin-G to penicillin-V. The results are summarized in Table 1.

Table 1
Recovery of penicillin-G and procaine in plasma and urine (n=5)

Sample	Concentration spiked (μg/ml)	Recovery (%)	C.V.[a] (%)
Penicillin-G			
Plasma	0.50	72.86	3.15
	5.00	74.44	2.93
	50.00	80.00	0.84
Urine	1.00	71.70	3.68
	50.00	75.24	1.56
	100.00	92.23	3.32
Procaine			
Plasma	0.25	79.94	11.94
	2.50	75.96	10.80
	25.00	94.09	4.03
Urine	0.20	70.87	10.18
	2.00	75.88	7.52
	10.00	88.83	2.10

[a] Coefficient of variation (%)=standard deviation of concentration detected/mean concentration detected×100.
% Recovery=concentration detected/concentration spiked×100.

3.2. Calibration curves for procaine and penicillin-G in urine and limits of detection

The linearity of the methods for procaine and penicillin-G was investigated over the concentration range of 0.025–150 μg/ml using spiked urine and plasma samples for HPLC–DAD analysis. Reliable detection limits for both penicillin-G and procaine were obtained with a signal-to-noise ratio of >3. Table 2 shows the calibration curves for penicillin-G and procaine in horse urine and plasma and the detection limits. As shown in Table 2, the calibration curves for procaine and penicillin-G were linear with a mean r^2 of 0.9985. The limit of detection (LOD) for penicillin-G was 0.05 μg/ml and 0.1 μg/ml in plasma and urine, respectively. The limit of detection for procaine in plasma and urine was 0.01 μg/ml.

3.3. Precision and accuracy of procaine and penicillin-G

The precision and accuracy of procaine and penicillin-G quantification were determined using five procaine and penicillin-G-free urine and plasma samples. The samples were spiked with varying concentrations of the analytes and performed intra- and inter-day assays. The results are summarized in Tables 3 and 4. The coefficient of variation (C.V.) for penicillin-G in plasma and urine for intra- and inter-day assays ranged from 1.28 to 5.49% whereas that for procaine was 0.78 to 9.14%. The relative difference for intra- and inter-day analyses ranged from 0.10 to 16.56% irrespective of the drug (procaine or penicillin-G) and the sample (urine or plasma). The accuracy for quantification of penicillin-G in plasma

Table 2
Calibration curves for penicillin-G and procaine in urine and plasma and limits of detection by HPLC

Sample	*n*	Calibration curve (μg/ml)	r^{2a}	CR[b] (μg/ml)	LOD[c] (μg/ml)
Penicillin-G					
Plasma	5	$y=0.0503x-0.1104$	0.9985	0.05–50	0.05±0.00740
Urine	6	$y=0.0221x+0.0192$	0.9989	0.10–150	0.10±0.0104
Procaine					
Plasma	5	$y=0.3441x-0.001$	0.9987	0.025–25	0.01±0.0015
Urine	7	$y=0.0688x+0.0294$	0.9996	0.10–10	0.01±0.0013

[a] Correlation coefficient.
[b] Concentration range.
[c] Limit of detection.

and urine was within an acceptable range, in that, the highest relative difference for intra-day assays was 4.80% in plasma and 8.14% in urine (Table 3), 7.37% in plasma and 9.85% in urine for inter-day assays (Table 4). For quantification of procaine, the highest relative difference was 12% in plasma and 6% in urine for intra-day assays (Table 3) whereas it was 15.84% in plasma and 16.56% in urine for inter-day analyses (Table 4). From these results, it can be noted that under all conditions, the relative difference was less than 20%. As long as the relative difference was below 20%, the accuracy of the method was demonstrated.

3.4. Analysis of procaine and penicillin-G in equine urine and plasma samples

Using the optimized method described above, the chromatograms for procaine and penicillin-G in urine and plasma samples were obtained as shown in Figs. 1 and 2. The chromatograms shown in Fig. 1 represent those for procaine in plasma and urine

Table 3
Intra-day assay precision and accuracy of the method ($n=5$)

Sample	Added (μg/ml)	Detected (μg/ml)	Detected−added[a]	C.V.[b] (%)	Relative difference[c] (%)
Penicillin-G					
Plasma	0.50	0.51±0.0109	+0.010	2.14	2.00
	5.00	4.89±0.2495	−0.110	5.10	2.22
	50.00	52.40±2.8760	+2.40	5.49	4.80
Urine	1.00	0.97±0.031	−0.03	3.20	3.00
	50.00	54.07±2.718	+4.07	5.03	8.14
	150.00	146.70±1.885	−3.30	1.28	2.20
Procaine					
Plasma	0.25	0.22±0.0201	−0.0300	9.14	12.00
	2.50	2.66±0.1047	+0.160	3.94	6.40
	25.00	23.34±1.229	−1.760	5.29	7.04
Urine	0.20	0.19±0.0056	−0.0100	2.80	5.00
	1.00	1.06±0.0321	+0.0600	3.03	6.00
	10.00	10.01±0.0985	+0.0100	0.98	0.10

[a] Concentration detected−concentration added.
[b] Coefficient of variation=standard deviation of concentration spiked/mean concentration detected×100.
[c] Relative difference (%)=(concentration added−concentration detected)/concentration added×100

Table 4
Inter-day assay precision and accuracy of the method (n=5)

Sample	Added (μg/ml)	Detected (μg/ml)	Detected−added[a]	C.V.[b] (%)	Relative difference[c] (%)
Penicillin-G					
Plasma	0.50	0.54±0.01440	+0.0368	2.66	7.36
	5.00	4.82±0.2569	+0.2680	5.33	5.37
	50.00	48.50±2.195	−2.4850	4.53	4.97
Urine	1.00	0.896±0.0142	−0.0985	1.58	9.85
	50.00	50.61±1.179	+0.6100	2.53	1.22
	150.00	147.41±3.818	−2.595	2.59	1.73
Procaine					
Plasma	0.25	0.21±0.0148	−0.0396	7.07	15.84
	2.50	2.82±0.0869	+0.3230	3.08	12.92
	25.00	25.39±1.1980	+0.3925	0.78	1.57
Urine	0.20	0.17±0.0038	−0.0331	2.25	16.56
	1.00	1.06±0.0234	+0.0623	3.02	6.23
	10.00	10.01±0.0981	+0.0110	0.98	0.11

[a] Concentration detected−concentration added.
[b] Coefficient of variation=standard deviation of concentration spiked/mean concentration detected×100.
[c] Relative difference (%)=(concentration added−concentration detected)/concentration added×100

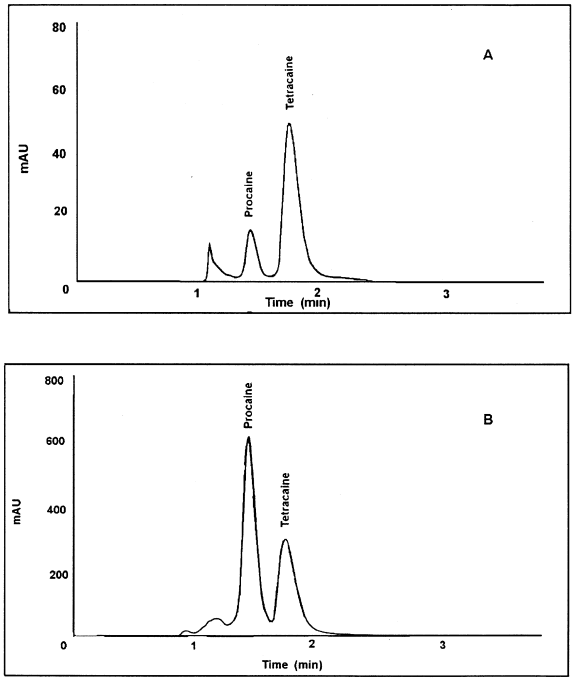

Fig. 1. HPLC chromatograms of procaine in horse samples. (A) Procaine in horse plasma collected 15 min after administration. (B) Procaine in horse urine collected 30 min after administration.

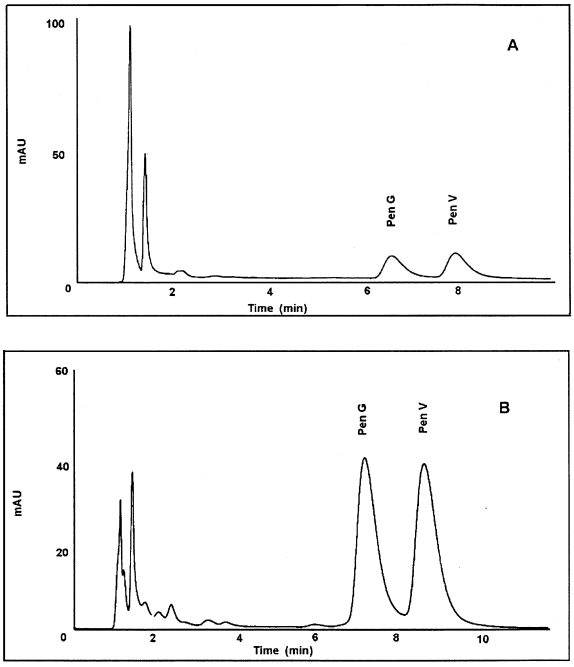

Fig. 2. HPLC chromatograms of penicillin-G in horse samples. (A) Penicillin-G in horse plasma collected 3 h after administration. (B) Penicillin-G in horse urine collected 30 min after administration.

samples collected at 15 min (panel A) and 30 min (panel B) after the administration of PPG and analyzed by HPLC–DAD following extraction. Fig. 2 shows the chromatograms for penicillin-G in plasma collected at 3 h (panel A) and that in urine at 30 min (panel B) post administration of PPG to horses.

The concentrations of procaine and penicillin-G in equine plasma and urine were analyzed using HPLC–DAD. Both procaine and penicillin-G were detected for long periods of time (72 h) following the administration of PPG. With the i.m. administration of procaine hydrochloride as a single drug formulation, procaine was eliminated so rapidly that it could only be detected and quantified for 4 to 6 h in plasma and 12 to 16 h in urine. However, with the i.m. administration of penicillin-G as a single drug formulation, the drug was detected and quantified up to 28 to 36 h in plasma and 48 h in urine. On the contrary, with the administration of PPG as a combined drug formulation, both procaine and penicillin-G were eliminated slower than during the administration of these drugs as individual drug formulations. With PPG administration, penicillin-G and procaine were quantified for 72 h in plasma and urine. The results of this study showed that both procaine and penicillin-G can be detected in urine and plasma for 72 h after the i.m. administration of PPG to horses.

SPE is a sensitive and powerful technique for chemical isolation and clean-up of penicillin-G from urine and plasma samples. The automatic SPE procedure developed for this study is adaptable to large scale sample extraction and clean-up procedures that resulted in cleaner sample extracts for HPLC analysis than those extracts by LLE. Pre-column derivatization of the penicillin-G extract improved its HPLC sensitivity and chromatographic behavior. Unlike methods previously reported with higher sensitivity by other investigators [5,23] than that of this method, the present method did not require deproteinization of plasma samples and thus, cost and time of sample pretreatment were reduced. The filtration step introduced for plasma samples combined with SPE greatly improved the recovery of penicillin-G from plasma.

Improved recovery of procaine extracted from plasma and urine was obtained at pH 10. For analysis, both procaine and penicillin-G were detected by HPLC at lower pH as the acid condition of the mobile phase improved the resolution of procaine from impurities and of penicillin-G from the internal standard penicillin-V.

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