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Simultaneous determination of seven penicillins in muscle, liver and kidney tissues from cattle and pigs by a multiresidue high-performance liquid chromatographic method

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Abstract

A high-performance liquid chromatographic (HPLC) method based on solid-phase extraction (SPE) was developed for determination of amoxicillin, penicillin G (benzylpenicillin), ampicillin, oxacillin, cloxacillin, nafcillin and dicloxacillin in muscle, liver and kidney tissues of pigs and cattle. The compounds were extracted in aqueous solution by precipitation of organic materials with a mixture of sulphuric acid and sodium tungstate. The extract was cleaned up by SPE on a divinylbenzene-co-*N*-vinylpyrrolidone polymeric sorbent. Further clean-up was performed by liquid–liquid partition with diethyl ether. The extract was derivatised with benzoic anhydride and 1,2,4-triazole mercury (II) reagent. Chromatography was performed by reversed-phase gradient HPLC on a C_{18} column with ultraviolet detection at 323 nm. The limits of detection estimated by a conservative model were in the range 8.9–11.1 μg/kg for amoxicillin, penicillin G, ampicillin, oxacillin, cloxacillin and nafcillin and 18.3–20.9 μg/kg for dicloxacillin. The mean recovery range was 66–77% for amoxicillin, 73–75% for penicillin G, 81–82% for ampicillin, 73–76% for oxacillin, 74–75% for cloxacillin, 66–72% for nafcillin and 58–65% for dicloxacillin.

Keywords: Penicillins

1. Introduction

In the European Union (EU), maximum residue limits (MRLs) have been established for amoxicillin (50 μg/kg), penicillin G (50 μg/kg), ampicillin (50 μg/kg), oxacillin (300 μg/kg), cloxacillin (300 μg/kg) and dicloxacillin (300 μg/kg) in muscle, liver and kidney [1]. Furthermore a provisional MRL of 30 μg/kg has been established for nafcillin. Few chromatographic procedures have been described for simultaneous determination of two or more penicillins in tissues [2–5]. Blanchflower et al. [4] reported a multiresidue liquid chromatographic method using mass spectrometric detection for detection of some monobasic penicillins in muscle and kidney tissues. However, the limits of detection were close to the EU MRLs. Boison and Keng [5] have recently published a sensitive high-performance liquid chromatography (HPLC) method for determination of amoxicillin, ampicillin, penicillin G and cloxacillin

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in bovine muscle tissues. No validation was reported for kidney and liver, which are generally more difficult to extract and clean up than muscle. To date, no single sensitive multiresidue HPLC method has been reported for the simultaneous determination of the EU listed amphoteric and monobasic penicillins in different tissue types.

This paper describes a sensitive multiresidue method for the determination of amoxicillin, penicillin G, ampicillin, oxacillin, nafcillin, cloxacillin and dicloxacillin in muscle, liver and kidney tissues from pigs and cattle.

2. Experimental

2.1. Reagents

Amoxicillin, penicillin G potassium salt, penicillin V potassium salt, ampicillin sodium salt, oxacillin sodium salt, cloxacillin sodium salt, nafcillin sodium salt and dicloxacillin sodium salt were purchased from Sigma (St. Louis, MO, USA). Penase (penicillinase) was obtained from Difco (Detroit, MI, USA). 1,2,4-Triazole was obtained from Merck–Schuchardt (Hohenbrunn, Germany). Acetonitrile and methanol of chromatography gradient grade, diethyl ether, mercury (II) chloride, sodium tungstate, benzoic anhydride and sodium thiosulphate were obtained from Merck (Darmstadt, Germany). Dimethylchlorosilane (DMCS) was purchased from Fluka (Buchs, Switzerland). Water was purified through a Millipore Milli-Q Plus system (Bedford, MA, USA).

Separate stock solutions of each penicillin were prepared at a concentration of 1000 μg/ml by dissolving the pure substances in water. These were stable for at least one month when stored at 5±2°C. A mixed standard solution containing 2 μg/ml amoxicillin, penicillin G, ampicillin, oxacillin, cloxacillin, nafcillin and dicloxacillin was prepared by diluting combined aliquots of the stock solutions with water. A separate internal control solution of penicillin V was prepared in the same manner. The 2 μg/ml standard solutions were stable for at least two weeks when stored at 5±2°C.

Derivatization reagent I (0.2 *M* benzoic anhydride) was prepared by dissolving 1.13 g benzoic anhydride in acetonitrile followed by dilution to 25 ml. Derivatization reagent II [2 *M* 1,2,4-triazole containing 2.6 m*M* mercury (II) chloride] was prepared by dissolving 6.905 g 1,2,4-triazole in 30 ml water and adding 5 ml 26 m*M* aqueous stock solution of mercury chloride. The pH was adjusted to 9.0±0.05 with 5 *M* sodium hydroxide. The mixture was diluted to 50 ml and equilibrated for at least 1 h. The mixture was then centrifuged for 10 min at 1500 *g* and the clear supernatant was used. The 26 m*M* mercury solution was stable for at least one month when stored at 20–25°C. The derivatization reagents I and II were prepared 1–4 h before use.

Phosphate buffer, pH 9.0 (25 m*M*) was prepared by dissolving 0.34 g KH_2PO_4 in water followed by adjustment of the pH with sodium hydroxide and dilution to 100 ml. Phosphate buffer pH 2.6 (25 m*M*) was prepared by dissolving 2.72 g KH_2PO_4 in water followed by adjustment of the pH by means of phosphoric acid and dilution to 100 ml. Phosphate buffer pH 6.5 (0.1 *M*), for mobile phase was prepared by dissolving 9.94 g Na_2HPO_4, 17.94 g $NaH_2PO^-_4H_2O$ and 4.96 g $Na_2S_2O_3$ in water followed by dilution to 2000 ml.

HPLC eluent A was prepared by diluting 100 ml acetonitrile to 1000 ml with phosphate buffer, pH 6.5. HPLC eluent B was prepared by diluting 240 ml acetonitrile and 60 ml methanol to 1000 ml with phosphate buffer, pH 6.5. HPLC eluent C was prepared by diluting 300 ml acetonitrile and 200 ml methanol to 1000 ml with phosphate buffer, pH 6.5.

2.2. Materials

Solid phase extraction (SPE) cartridges, Oasis HLB (divinylbenzene-co-*N*-vinylpyrrolidone polymeric sorbent) 60 mg, were obtained from Waters (Milford, MA, USA). Glass fibre filters GF/B were obtained from Whatman (Maidstone, UK). Disposable Acrodisc LC 13 PVDF, 13 mm×0.45 μm, syringe filters were obtained from Gelman Sciences (Ann Arbor, MI, USA). Centrifuge tubes of 15 ml and 50 ml capacity, volumetric flasks and vials were made of polypropylene.

Glass fibre filters were silanized with 10% DMCS in toluene.

2.3. Instrumentation

The instruments used were a mini-food processor (Krups, type 708A), an Ultra Turrax homogeniser

model T25 with a 17 mm rotor (IKA, Staufen, Germany), a Sigma centrifuge Model 2-15 (Osterode, Germany), a VF2 test tube shaker (IKA), a vacuum manifold for SPE cartridges (Waters), a pH meter PHM 240 (Radiometer, Copenhagen, Denmark) and a temperature-controlled heating block with a manifold for nitrogen flow (Mikrolab Aarhus, Aarhus, Denmark).

The liquid chromatography system consisted of a Waters pump gradient system 600, a Waters 996 photodiode-array detector and a Waters 717 autosampler. 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 chromatographic system and acquisition of data were controlled by Waters Millennium 32 software.

The injection volume was 150 μl and the mobile phase flow-rate was set at 1.0 ml/min. The gradient was initiated with 80% eluent A and 20% eluent B followed by linear increase to 100% eluent B over 30 min. The gradient was then increased to 100% C over 19 min and held constant for 1 min. The system was returned to 80% eluent A and 20% eluent B over 2 min and conditioned for 10 min before the next injection. The column temperature was kept at 38–40°C using a circulator waterbath. Ultraviolet (UV) detection was performed at 323 nm.

2.4. Preparation of samples

Sample materials of muscle, kidney and liver from pigs and cattle were obtained from slaughterhouses in Denmark. Each tissue was homogenized in a food processor and a 2.5-g sample was transferred to a 50-ml polypropylene centrifuge tube. The sample was spiked with 200 μl internal control solution containing 2 μg/ml penicillin V and mixed with 20 ml water for 1 min using an Ultra-Turrax homogeniser. Organic materials were removed by addition of 2.0 ml 0.68 *M* sulphuric acid, immediately followed by 2.45 ml 0.68 *M* sodium tungstate solution. The mixture was then shaken vigorously for 1 min and allowed to stand for 5 min. The pH of the mixture was then checked. The mixture was discarded if the pH was outside the range 4.4–4.6 and the precipitation process was repeated with an increased or reduced volume of sodium tungstate solution. The mixture was centrifuged at 1500 *g* for 10 min and the supernatant was decanted into a 100-ml polypropylene volumetric flask. The extraction was repeated with 20 ml water, 2.0 ml 0.68 *M* sulphuric acid and 2.95 ml 0.68 *M* sodium tungstate solution. The supernatants were combined and diluted to 100 ml with water. A 50-ml volume of extract was transferred to a polypropylene beaker and the pH was adjusted to 8.5–8.9 with 5 *M* and 0.5 *M* sodium hydroxide solution. To avoid loss of amoxicillin, pH fluctuations were kept below pH 10.0 during the adjustment process. The extract was vacuum-filtered through a glass fibre filter.

An Oasis HLB SPE cartridge was washed with 2 ml methanol followed by 2 ml water. The filtrate was pulled through the cartridge at a flow-rate of 1–2 ml/min. The column was washed with 2 ml 25 m*M* phosphate buffer, pH 9.0 and dried by suction for 1 min. The penicillins were eluted with 4×1.0 ml acetonitrile. The eluate was collected in a 15-ml polypropylene tube.

A 150-μl volume of phosphate buffer, pH 9.0 was added to the eluate and the solution was evaporated to 50–80 μl at 45–50°C under a stream of nitrogen. A 400-μl volume of phosphate buffer, pH 9.0 and 75 μl derivatization reagent I were added to the concentrate. The mixture was vortex-mixed for 30 s and allowed to react at 20–24°C for 10 min. The solution was transferred to a 50-ml polypropylene tube containing 20 ml diethyl ether. The sample tube was rinsed with 500 μl water which was transferred to the tube containing ether. A 5.0-ml volume of phosphate buffer, pH 2.6 was added and the mixture was immediately shaken for 60 s. After 2–3 min standing, the diethyl ether phase was transferred to a 50-ml polypropylene tube containing 500 μl of 25 m*M* phosphate buffer, pH 9.0. The mixture was shaken immediately. A polypropylene syringe with needle was used in the transfer process. The extraction was repeated immediately with another 20-ml volume of diethyl ether. The combined diethyl ether extract was evaporated to 450–500 μl at 45–50°C under a stream of nitrogen. The solution was transferred to a weighed 15-ml polypropylene tube and diluted to a mass of 500 mg with 25 m*M* phosphate buffer, pH 9.0. A 75-μl volume of derivatization reagent I was added and the solution was vortex-mixed for 30 s and allowed to react at 20–24°C for 10 min. A 450-μl volume of derivatization reagent II was then added. The tube was closed, vortex-mixed for 60 s and allowed to react in a water

bath at 55±2°C for 30 min. The solution was then cooled in cold water and filtered through a 0.45 μm PVDF filter.

Non-matrix calibration standards were prepared by transferring 20, 50, 100, 150 and 200 μl mixed standard containing 2 μg/ml of the individual penicillins to 15-ml propylene tubes. A 200-μl volume of 2 μg/ml penicillin V solution was added to each tube. The solution was diluted to a mass of 500 mg with 25 m*M* phosphate buffer, pH 9.0 and a 75 μl of derivatization reagent I was added. The mixture was vortex-mixed for 30 s and allowed to react at 20–24°C for 10 min. A 450-μl volume of derivatization reagent II was added, followed by vortex-mixing for 60 s and reaction at 55±2°C for 30 min. The solutions were cooled down in cold water. Derivatization of calibration standards was done in parallel with test samples.

2.5. Calculation

Linear regression with line forced through the origin was used for calculation of concentrations in samples.

2.6. Penase treatment of samples

The pH of 50-ml volumes of sample extract from precipitation of organic material was adjusted to 6.8–7.2. A 500-μl volume of penase solution containing 10 000 000 units/ml was added and the solutions were incubated at 25°C for 1, 2 and 24 h.

2.7. Ruggedness

The optimum pH for sample extraction on the polymeric sorbent was determined by analysis of muscle and liver tissues spiked to a level of 160 μg/kg with the individual penicillins. The pH of the extract obtained after precipitation of organic material was adjusted to selected values before SPE.

The capacity of the Oasis was checked by connecting to cartridges in series and applying 25% surplus volumes of sample extracts of muscle, kidney and liver tissues spiked to a level of 160 μg/kg with the individual penicillins. The cartridge load thus corresponded to extract from 3.1 g tissue. The amounts of penicillins adsorbed to each cartridge were determined following the procedure.

The optimal pH range for diethyl ether extraction was determined on muscle spiked to a level of 80 μg/kg with the individual penicillins.

The specificity of the method against a range of veterinary drugs was tested on tissue samples spiked to a concentration of 500 μg/kg with each of these compounds.

2.8. Limits of detection and quantification

The limits of detection (LODs) were determined on tissues from 10 different pigs and 10 different cattle. To obtain realistic LODs, the samples were spiked prior to extraction with penicillins to a peak height on chromatograms corresponding to ca. three times the short term baseline variation. The samples were thus spiked with amoxicillin, penicillin G, ampicillin, oxacillin, cloxacillin and nafcillin, each to a level of 6 μg/kg, and with dicloxacillin to a level of 12 μg/kg. The detection limits were determined as the mean results plus three times the standard deviation (SD) of the 20 measurements followed by correction for the mean recovery.

2.9. Precision and accuracy

The repeatability standard deviation (i.e., variability of independent analytical results obtained by the same operator, using the same apparatus under the same conditions on the same test sample and in a short interval of time), intra-laboratory reproducibility standard deviation (i.e., variability of independent analytical results obtained on the same test sample in the same laboratory by different operators under different conditions) and recovery of the method were determined on homogenised samples spiked with the individual penicillins to levels of 40 and 200 μg/kg. The spiked samples were stirred thoroughly with a glass spatula and equilibrated at 7±2°C for at least 1 h before extraction. The samples were analysed in duplicate on each of six different days equally divided between two analysts. Calculation of repeatability was done in accordance with ISO standard 5725-2, 1994 [6]. The intra-laboratory reproducibility was calculated by the same principle used for determination of reproducibility [6].

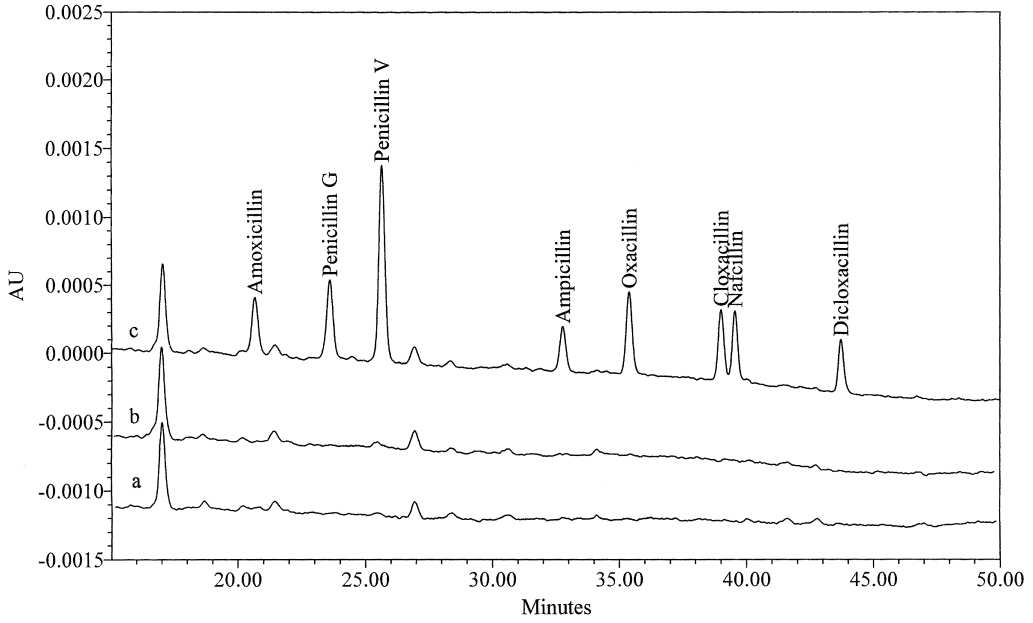

Fig. 1. Chromatograms of a control cattle muscle (a), a control pig muscle (b) and a pig muscle spiked to a level of 40 μg/kg with each of the penicillins (c). Penicillin V was used as internal control.

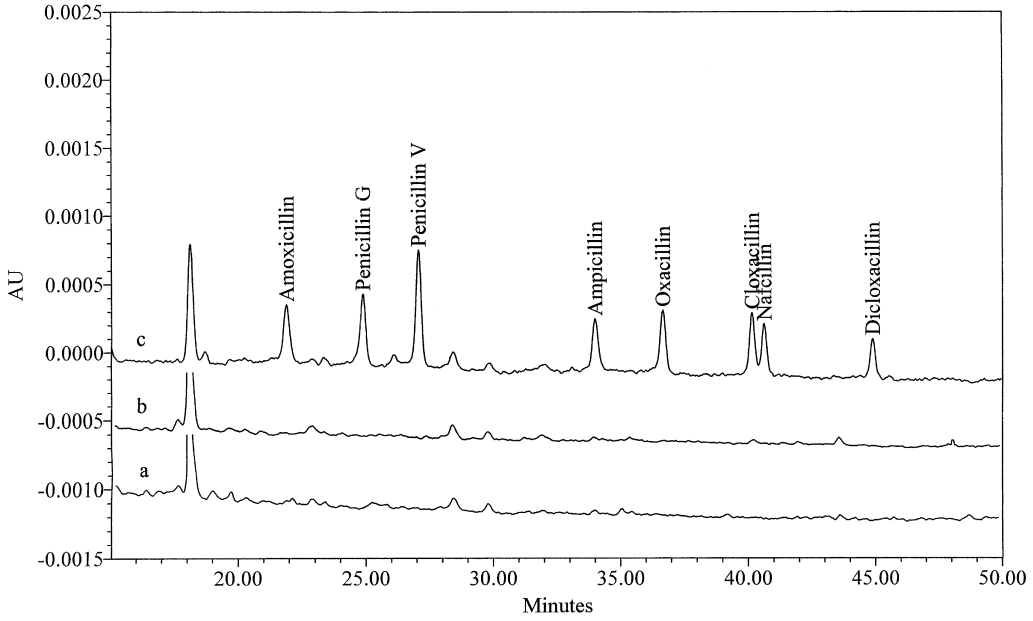

Fig. 2. Chromatograms of a control cattle kidney (a), a control pig kidney (b) and a pig kidney spiked to a level of 40 μg/kg with each of the penicillins (c). Penicillin V was used as internal control.

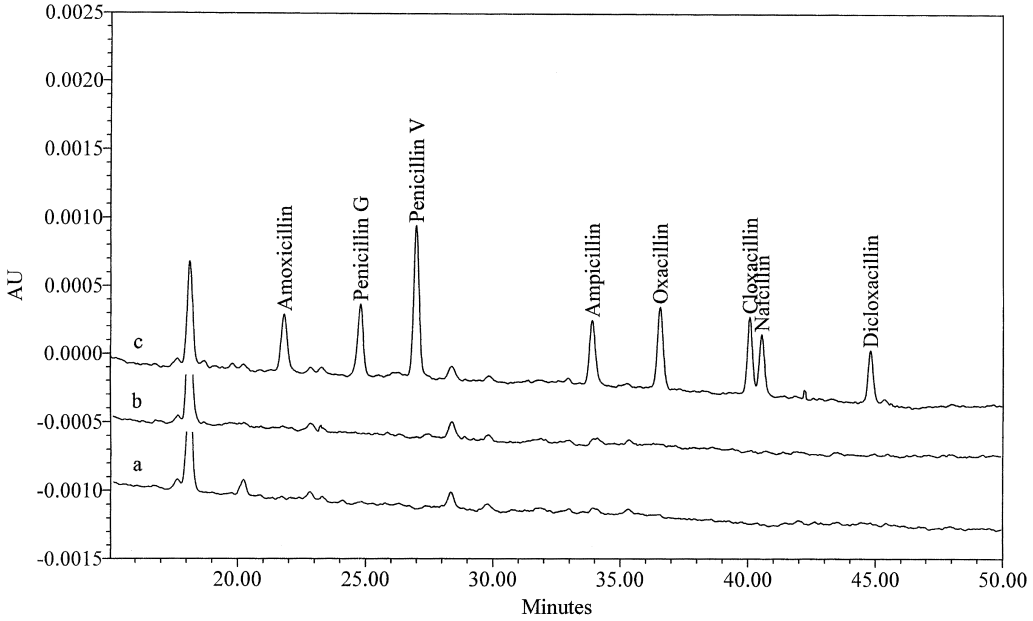

Fig. 3. Chromatograms of a control cattle liver (a), a control pig liver (b) and a pig liver spiked to a level of 40 μg/kg with each of the penicillins (c). Penicillin V was used as internal control.

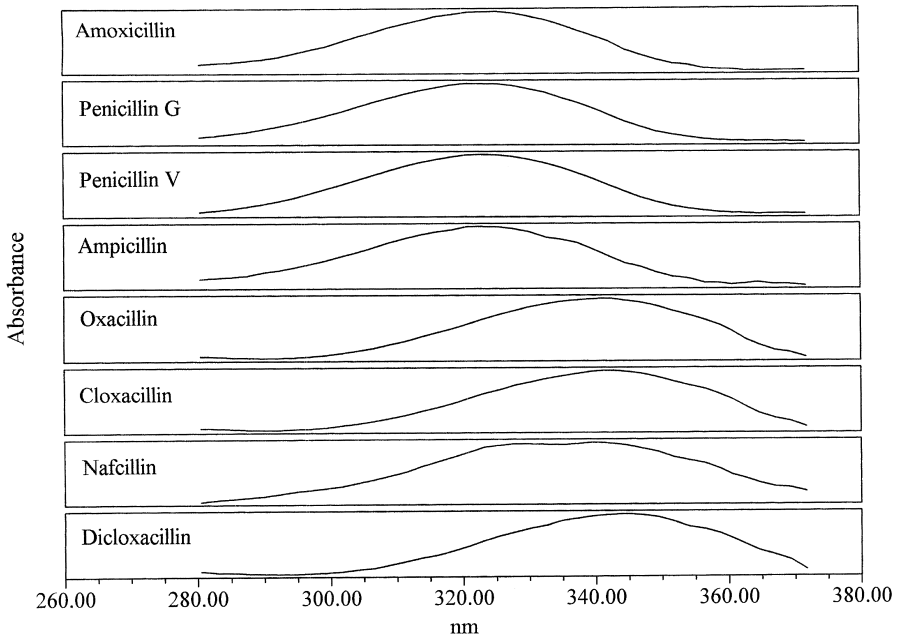

Fig. 4. UV absorbance spectra of derivatized penicillins.

3. Results and discussion

The sample extract was derivatised before HPLC. The NH_2 group in amoxicillin and ampicillin was derivatised with benzoic anhydride to facilitate extraction with diethyl ether and to increase retention on C_{18} columns. The wavelength of detection was moved to the higher UV-region by complexing penicillins with the 1,2,4-triazole mercury (II) reagent using a previously described procedure [7]. Benzoic anhydride was added to sample extracts before and after diethyl ether extraction. The second addition of benzoic anhydride was performed to obtain closely similar reaction conditions in sample and standard solutions for the 1,2,4-triazole mercury derivatisation of penicillins. The extraction procedure was optimised to give maximum recovery and insignificant interference.

Typical chromatograms of blank control muscle and muscle tissue spiked to a level of 40 μg/kg with amoxicillin, penicillin G, ampicillin, oxacillin, cloxacillin, nafcillin and dicloxacillin are shown in Fig. 1. Corresponding chromatograms for liver and kidney are shown in Figs. 2 and 3. The methanol concentration in the mobile phases and the column temperature were important parameters in separation of cloxacillin and nafcillin peaks. A circulator waterbath was used to obtain a well defined column temperature. The resolution was stable during the entire validation study (more than 200 injected sample extracts).

The LODs were ca. 10 μg/kg for all penicillins except dicloxacillin, which could be detected down to ca. 20 μg/kg (Tables 1–3). The LODs were thus a factor of 3–30 below the EU MRLs [1].

The UV spectra of the different derivatized penicillins are shown in Fig. 4. It was also possible to verify the authenticity of the penicillin peaks by a prior penase treatment of sample extract with 100 000 IU/ml at 25°C. The necessary reaction time

Table 1
Limits of detection[a]

	Mean±SD (μg/kg)		LOD (μg/kg)
	Pigs	Cattle	Overall
Amoxicillin	3.62±0.90	4.24±1.09	10.5
Penicillin G	3.69±0.78	3.85±0.71	9.6
Ampicillin	4.06±0.72	4.84±1.17	9.8
Oxacillin	3.12±0.55	4.00±0.73	9.2
Cloxacillin	3.90±0.97	4.36±1.25	10.8
Nafcillin	3.92±0.74	3.68±0.98	10.1
Dicloxacillin	6.97±1.26	7.36±1.26	18.3

[a] Muscle tissues from pigs (n=10) and cattle (n=10) were spiked to a level resulting in a signal equal to ca. three times the short term baseline variation (6 μg/kg for all penicillins except dicloxacillin which was spiked to level 12 μg/kg). The LOD was calculated from the mean and the SD obtained on the combined set of data from pigs and cattle. The LODs were corrected for recovery.

Table 2
Limits of detection[a]

	Mean±SD (μg/kg)		LOD (μg/kg)
	Pigs	Cattle	Overall
Amoxicillin	4.75±1.11	4.55±1.03	10.1
Penicillin G	5.03±0.97	4.02±0.71	9.9
Ampicillin	4.43±0.91	6.62±1.25	11.1
Oxacillin	4.91±1.47	3.88±0.62	11.0
Cloxacillin	5.64±1.25	5.75±1.22	9.8
Nafcillin	4.57±1.01	5.04±0.82	9.5
Dicloxacillin	7.58±1.50	5.70±0.96	20.4

[a] Kidney tissues from pigs (n=10) and cattle (n=10) were spiked to a level resulting in a signal equal to ca. three times the short term baseline variation (6 μg/kg for all penicillins except dicloxacillin which was spiked to level 12 μg/kg). The LOD was calculated from the mean and the SD obtained on the combined set of data from pigs and cattle. The LODs were corrected for recovery.

Table 3
Limits of detection[a]

	Mean±SD (μg/kg)		LOD (μg/kg)
	Pigs	Cattle	Overall
Amoxicillin	5.27±1.52	5.55±1.15	10.4
Penicillin G	4.66±0.49	4.86±1.26	9.5
Ampicillin	4.73±1.07	6.40±1.32	10.7
Oxacillin	4.15±0.90	4.89±0.66	9.5
Cloxacillin	4.79±0.99	5.35±0.60	9.1
Nafcillin	4.57±0.91	5.16±0.48	8.9
Dicloxacillin	7.47±2.07	6.46±1.23	20.9

[a] Liver tissues from pigs (n=10) and cattle (n=10) were spiked to a level resulting in a signal equal to ca. three times the short term baseline variation (6 μg/kg for all penicillins except dicloxacillin which was spiked to level 12 μg/kg). The LOD was calculated from the mean and the SD obtained on the combined set of data from pigs and cattle. The LODs were corrected for recovery.

for complete breakdown of penicillins at a level of 160 μg/kg was measured to be less than 1 h for amoxicillin, penicillin G, penicillin V, ampicillin, oxacillin and nafcillin. Up to 10% of the cloxacillin and dicloxacillin was still intact after 2 h incubation. No residues were detected after 24 h incubation.

The results for precision and recovery are summarised in Tables 4–6. The relative repeatability standard deviation (RSD_r) was in all cases below 10% for levels 40 and 200 μg/kg. The mean recoveries were generally independent of the level in the range 40–200 μg/kg. The mean recoveries for muscle, kidney and liver tissues were 77, 66, 67% for amoxicillin, 75, 74, 73% for penicillin G, 82, 81, 82% for ampicillin, 76, 75, 73% for oxacillin, 74, 75, 74% for cloxacillin, 72, 70, 66% for nafcillin, and 65, 65, 58% for dicloxacillin. The mean recoveries for penicillin V were 90, 90 and 86%.

Table 4
The relative repeatability standard deviation (RSD_r), intra-laboratory reproducibility standard deviation ($RSD_{R,intra}$) and recovery on spiked muscle samples (one duplicate analysis at each level was conducted on each of six days)

Penicillin	Fortification level (μg/kg)	RSD_r (μg/kg)	$RSD_{R,intra}$ (μg/kg)	Recovery (mean±SD) (%)
Amoxicillin	40[a]	8.34	13.3	79.1±9.4
	40[b]	7.25	11.2	76.7±7.6
	200[a]	4.67	7.43	77.9±5.2
	200[b]	3.22	9.53	73.5±4.5
Penicillin G	40[a]	5.49	7.27	75.0±4.6
	40[b]	6.07	8.15	75.5±5.2
	200[a]	2.47	5.36	74.7±3.8
	200[b]	1.76	2.80	75.5±1.9
Penicillin V	160[a]	6.07	7.30	89.8±5.3
	160[b]	4.28	7.48	90.6±6.2
Ampicillin	40[a]	6.25	9.46	81.1±6.8
	40[b]	6.47	6.74	83.2±3.6
	200[a]	4.28	4.48	82.4±2.7
	200[a]	3.51	6.13	79.8±4.5
Oxacillin	40[a]	5.36	6.17	75.0±3.7
	40[b]	3.37	3.97	76.8±2.4
	200[a]	3.18	3.80	76.0±2.3
	200[b]	2.09	3.24	76.8±2.2
Cloxacillin	40[a]	4.69	5.44	74.6±3.2
	40[b]	5.38	6.29	75.1±3.6
	200[a]	3.08	3.47	74.0±2.0
	200[b]	1.97	2.73	72.9±1.7
Nafcillin	40[a]	4.69	8.02	73.6±5.4
	40[b]	6.55	7.22	73.8±4.1
	200[a]	6.69	7.86	70.7±4.4
	200[b]	3.16	7.40	72.0±5.1
Dicloxacillin	40[a]	8.03	12.7	65.2±7.4
	40[b]	8.98	10.4	67.4±5.6
	200[a]	1.82	5.38	66.1±3.5
	200[b]	3.88	5.58	63.2±3.1

[a] Pigs.
[b] Cattle.

Table 5
The relative repeatability standard deviation (RSD_r), intra-laboratory reproducibility standard deviation ($RSD_{R,intra}$) and recovery on spiked kidney samples (six duplicates at each level were conducted on different days)

Penicillin	Fortification level (μg/kg)	RSD_r (μg/kg)	$RSD_{R,intra}$ (μg/kg)	Recovery (mean±SD) (%)
Amoxicillin	40[a]	6.10	8.41	64.3±4.6
	40[b]	6.47	7.13	66.0±4.2
	200[a]	3.28	5.31	64.8±3.1
	200[b]	3.86	4.24	68.8±2.2
Penicillin G	40[a]	7.45	7.45	71.7±2.7
	40[b]	6.36	7.38	75.3±4.4
	200[a]	4.05	5.20	73.0±3.2
	200[b]	2.90	4.93	76.0±3.4
Penicillin V	160[a]	4.93	6.92	87.6±5.2
	160[b]	6.07	6.77	91.5±4.8
Ampicillin	40[a]	8.91	10.90	76.1±6.8
	40[b]	9.21	9.35	85.3±5.7
	200[a]	5.82	8.59	79.2±6.0
	200[b]	4.70	4.70	84.2±2.7
Oxacillin	40[a]	1.58	6.38	69.3±4.3
	40[b]	8.90	8.90	75.2±4.7
	200[a]	4.05	5.86	76.9±3.9
	200[b]	1.60	5.05	79.9±3.9
Cloxacillin	40[a]	3.42	5.66	73.5±4.4
	40[b]	4.01	7.11	74.5±4.9
	200[a]	4.23	5.91	75.3±3.8
	200[b]	2.75	5.06	75.9±3.5
Nafcillin	40[a]	5.61	10.83	65.2±6.5
	40[b]	8.42	8.42	70.8±3.9
	200[a]	4.27	7.02	69.4±4.4
	200[b]	1.98	4.88	72.7±3.4
Dicloxacillin	40[a]	7.05	10.44	61.9±5.6
	40[b]	7.12	7.12	65.8±2.3
	200[a]	5.07	8.06	65.3±4.7
	200[b]	7.29	9.13	66.4±5.0

[a] Pigs.
[b] Cattle.

The calibration curves were linear in the range 0–400 ng/ml. The relative standard error of slope converting peak area (μV×s) to concentration units (ng/ml) was less than 1.0% for all compounds. The coefficients of determination (R^2) were better than 0.999.

The effect of sample pH on recovery from SPE is shown in Fig. 5. The largest pH sensitivity was observed in relation to amoxicillin. Generally, the optimal pH range for extraction was 8.5–9.0. The procedure describes a step for further clean-up by liquid–liquid extraction with diethyl ether. This was performed at pH 2.6 to obtain complete recovery of the penicillins (Fig. 6). However, as penicillin G is unstable at low pH, the dwell time under the acidic condition should not exceed 5 min [7]. A standing time of 2–3 min after shaking the diethyl ether–aqueous phase was found to be sufficient for maxi-

Table 6
The relative repeatability standard deviation (RSD_r), intra-laboratory reproducibility standard deviation ($RSD_{R,intra}$) and recovery on spiked liver samples (one duplicate analysis at each level was conducted on each of six days)

Penicillin	Fortification level (μg/kg)	RSD_r (μg/kg)	$RSD_{R,intra}$ (μg/kg)	Recovery (mean±SD) (%)
Amoxicillin	40[a]	8.45	8.86	65.9±4.3
	40[b]	7.31	7.31	70.4±4.0
	200[a]	3.39	3.40	64.8±1.7
	200[b]	3.00	3.63	68.1±2.0
Penicillin G	40[a]	4.82	8.44	72.9±5.6
	40[b]	3.88	4.80	70.4±2.8
	200[a]	3.78	6.43	72.1±4.2
	200[b]	2.97	3.85	76.2±2.5
Penicillin V	160[a]	5.33	5.80	85.8±3.8
	160[b]	5.08	8.46	86.6±6.6
Ampicillin	40[a]	5.17	8.35	83.0±6.2
	40[b]	6.40	8.57	84.0±6.1
	200[a]	3.30	7.98	80.1±6.1
	200[b]	3.87	7.21	80.0±5.4
Oxacillin	40[a]	3.80	3.80	70.0±2.0
	40[b]	4.73	6.35	74.7±4.0
	200[a]	4.52	6.68	71.4±4.2
	200[b]	2.69	5.03	76.7±3.6
Cloxacillin	40[a]	5.70	6.57	74.2±3.8
	40[b]	4.65	8.33	77.7±5.9
	200[a]	1.96	2.95	70.4±1.8
	200[b]	2.37	5.52	74.4±3.9
Nafcillin	40[a]	5.65	6.04	64.9±2.9
	40[b]	4.19	5.00	69.0±2.8
	200[a]	3.91	4.28	61.0±2.0
	200[b]	3.15	6.76	68.4±4.4
Dicloxacillin	40[a]	4.44	6.42	56.1±3.1
	40[b]	6.93	6.98	59.9±3.0
	200[a]	3.86	3.64	56.2±1.4
	200[b]	4.40	8.00	61.2±4.5

[a] Pigs.
[b] Cattle.

Table 7
Veterinary drugs included in the specificity test of the method

Oxytetracycline	Tylosin	Albendazole
Tetracycline	Spiramycin	Fenbendazole
Chlortetracycline	Streptomycin	Febantel
Sulphadiazine	Dihydrostreptomycin	Albendazole sulphoxide
Sulphathiazole	Ceftiofur	Mebendazole
Sulphamerazine	Cefoperazone	Fenbendazole sulphoxide
Sulphamethazine	Trimethoprim	Fenbendazole sulphone
Sulphadoxine	Dexamethazone	Oxybendazole
Sulphamethoxazole	Thiabendazole	
Methylsulphaphenazole	Levamisole	

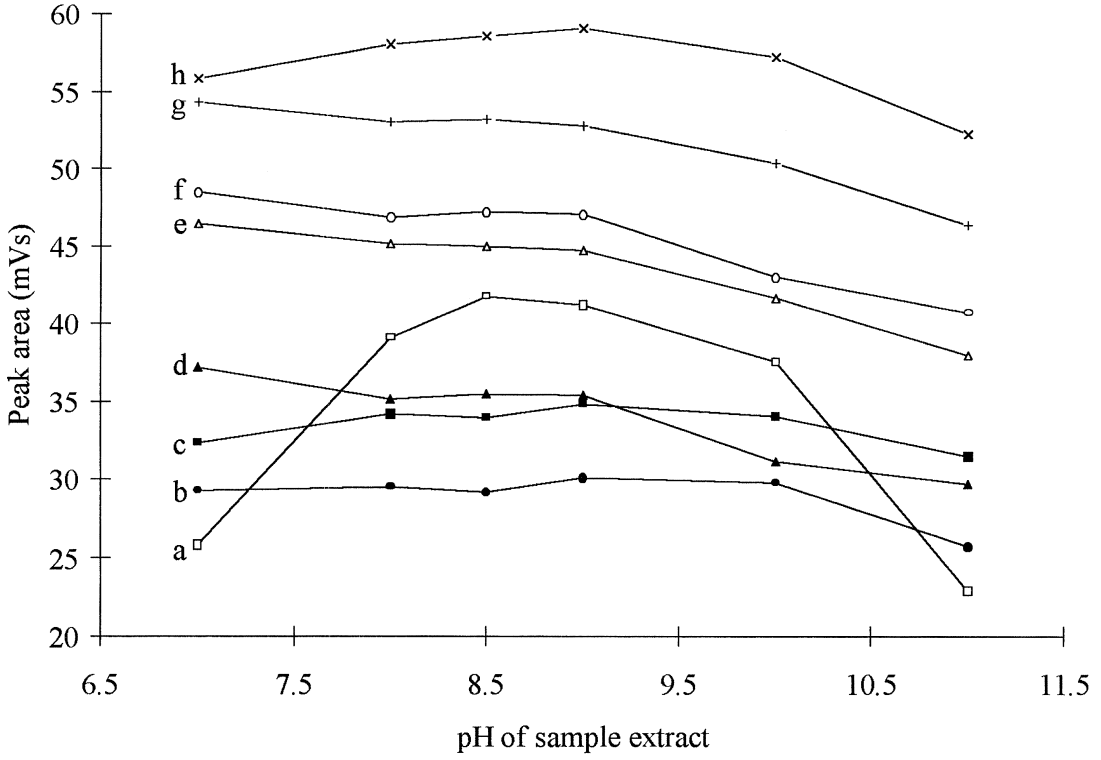

Fig. 5. Recovery of amoxicillin (a), ampicillin (b), penicillin V (c), dicloxacillin (d), cloxacillin (e), nafcillin (f), oxacillin (g) and penicillin G (h) from solid-phase extraction as a function of sample pH.

mal removal of interfering compounds. The capacity of the SPE cartridge was checked by applying 25% surplus volume of sample extract to two cartridges connected in series. Analytes were detected in the acetonitrile eluate from the first cartridge but not the second.

Uncontrolled adsorption effects of penicillins to glass surfaces were avoided by using polypropylene materials and glass silanized with 10% DMCS in toluene.

The stability at 5±2°C of final sample extracts of muscle, kidney and liver spiked to a level of 40 μg/kg was investigated over a period of seven days. No significant changes in response were observed for the individual penicillins in the different tissues.

The method was designed to use penicillin V as internal control compound, checking the overall performance on the individual sample. Application of penicillin V as internal standard in calculation of concentrations did not improve the precision of the results.

The specificity of the method was tested on tissues spiked to a level of 500 μg/kg with a broad range of veterinary drugs (Table 7). No analyte peaks were observed in chromatograms.

For the complete procedure, including the liquid/liquid extraction step, it was possible for a single trained analyst to obtain 6–8 sample extracts ready for HPLC within a working day of 8 h.

4. Conclusion

The HPLC method described in this paper provides a sensitive and reliable procedure for the quantitative analysis of amoxicillin, penicillin G, ampicillin, oxacillin, cloxacillin, nafcillin and dicloxacillin in muscle, kidney and liver tissues from

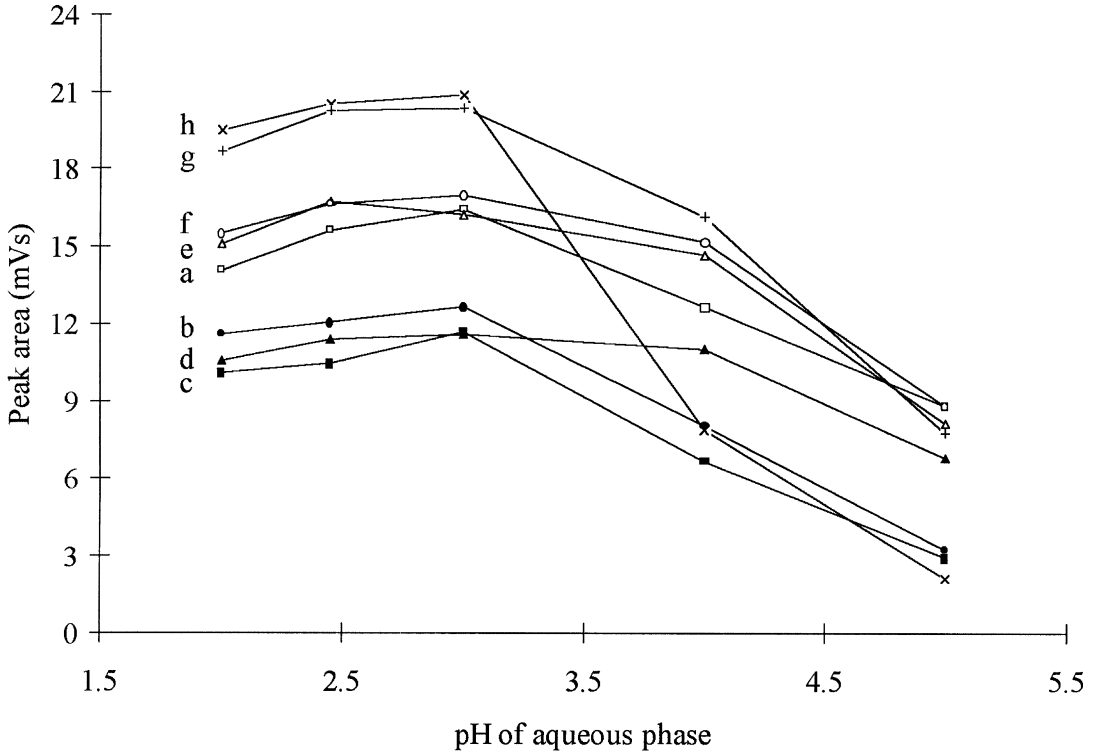

Fig. 6. pH dependence of extraction of amoxicillin (a), ampicillin (b), penicillin V (c), dicloxacillin (d), cloxacillin (e), nafcillin (f), oxacillin (g) and penicillin G (h) into diethyl ether phase.

pigs and cattle. The low detection limits and the high selectivity make the method suitable as a confirmation method in residue analyses.

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