Determination by HPLC of chlortetracycline in pig faeces

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An HPLC assay used to determine chlortetracycline (CTC) in pig faeces is reported. Prodigy ODS3 (4.6 × 150 mm) was used for the stationary phase, whereas the mobile phase comprised oxalic acid, sodium oxalate and sodium decane sulfonate (66%)—each of 4 mM, and 34% acetonitrile. The mobile phase was pumped at a flow rate of 1 mL/min. Detection of CTC was by ultraviolet absorbance at 370 nm, and a 20 µL injection volume was used. Recovery from faeces was >90%, and coefficients of variability between runs were <10%. The lowest limit of quantification was 3.5 mg/kg, with an accuracy of <7% error. There was no interference from endogenous materials in the pig faeces, or commonly used antibiotics, and the method is suitable for use in drug disposition studies.

Keywords: chlortetracycline, HPLC, pig faeces

Introduction

Chlortetracycline (CTC) is a broad-spectrum antimicrobial agent used in animal husbandry for both prophylaxis and treatment of respiratory and alimentary tract infections. It is commonly administered for these reasons as an in-feed antibiotic in the pig industry.

Currently, there is much debate over the use of antimicrobial agents in livestock production, and their potential to select for antimicrobial resistance.1 The possibility of resistant organisms of animal origin becoming directly pathogenic to man, or transferring their resistance genes to pathogens of medical importance, is of particular concern. CTC is predominately excreted in urine and faeces and, due to enterohepatic re-circulation, elimination is prolonged. Pig intestinal flora are therefore exposed to fairly low concentrations of CTC over extended periods; conditions that exert a strong selective pressure for the development of resistance.2 The presence of antibiotics in livestock faeces is also of concern, with regard to their fate and their effect on the environment when they enter the soil.3 It is therefore important to have specific methods by which to assay these veterinary antibiotics, to assist and support studies investigating both the pressure that these agents apply in selecting resistant isolates, and their impact on the environment.

Although HPLC methods have been reported for the assay of CTC, these have been mainly for very low residue levels in animal tissues,4 following the setting of mandatory maximum residue limits by regulatory bodies in the EU. In this study, we report the development and validation of a simple reversed phase HPLC method, previously used to assay CTC in beef and pork tissues,5 and adapted for the assay of relatively high concentrations of CTC in pig faeces.

Materials and methods

HPLC

The HPLC method was based on that reported by Moats,5 with adaptations for CTC extraction from pig faeces. The HPLC apparatus consisted of a Concept series II pump (Science Marketing International, Gloucester, UK), a Model 200 ultraviolet (UV) detector (Thermo Finnigan, San Jose, CA, USA), a Trilab 2000 integrator (Trivector, Sandy, UK) and a Gina 50 T autosampler (Dionex, Macclesfield, UK). The stationary phase used was a Prodigy 5 µM ODS3 4.6 × 150 mm HPLC column (Phenomenex, Macclesfield, UK). The mobile phase consisted of 4 mM oxalic acid dihydrate, 4 mM sodium oxalate and 4 mM sodium decane sulfonate—66% (Sigma), with 34% acetonitrile. This was pumped at a flow rate of 1 mL/min (typical operating pressure ∼1200 psi). Detection of CTC was by UV absorbance at 370 nm, and an injection volume of 20 µL was used. Column durability was good, with no loss of performance following in excess of 300 injections.

Sample preparation and extraction method

Chlortetracycline hydrochloride (Sigma, Poole, UK) stock solutions were made up fresh on the day of use in 0.01 M orthophosphoric acid

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(AnaR Grade; BDH, Poole, UK), and protected from the light. Antibiotic-free pig faeces were provided by Bristol University, Division of Animal Health and Husbandry, Langford, UK. Samples of faeces (100 mg) and water samples (100 µL), spiked with known concentrations of CTC, were used for assay validation; aqueous samples were used as calibrators and internal controls. Samples were placed in glass homogenizers (Jencons Scientific, Leighton Buzzard, UK), to which acetonitrile 990 µL (HiperSolv grade; BDH) was added, and homogenized manually for 2 min. Samples were allowed to stand at 4°C for 30 min before the addition of 1.1 mL of 0.1 M H₃PO₄, and mixed.

The clear supernatant was taken off and 20 µL injected into the flow of the mobile phase for the HPLC assay.

**Assay validation**

Recovery of CTC from faeces was determined by preparing a set of faeces and aqueous samples (n = 6) spiked with CTC (3.5, 7.3, 100 and 400 mg/kg). Percentage recovery from faeces at each concentration was calculated as height of faeces peak/height of aqueous peak ×100. Reproducibility of extraction and assay was determined from the repeat assay of faecal and aqueous samples spiked with CTC to a concentration of 3.5, 7.3, 100 and 400 mg/kg (n = 6), and from six different pig faeces spiked to CTC 7.3 mg/kg. The percentage coefficient of variation (%CV) was calculated as height of faeces peak/height of aqueous peak ×100.

Recovery and reproducibility

The mean percentage of recovery (n = 6) of CTC from faecal samples spiked to a concentration of 3.5, 7.3, 100 or 400 mg/kg CTC were: 100.0%, 100.0%, 95.8% and 90.1%, respectively. This extraction procedure was reproducible: %CVs were 9.1, 6.2, 5.1 and 5.4 for faecal samples, and 10.8, 9.2, 8.0 and 9.3 for aqueous samples. Samples from six different pigs used to assess variations in extraction recovery had a %CV of 5.8 and a mean percentage recovery of 90.7.

**Linearity and accuracy**

Linearity (linear regression) of faeces and aqueous samples in the range 0–400 mg/kg was good, r² = 0.9993 (y = 0.0327x), 1.0000 (y = 0.0338x), respectively. Accuracy was good across a wide range of CTC concentrations, with percentage errors using aqueous calibrators of –7.0% for a 3.5 mg/kg sample, –3.7% for a 25 mg/kg sample, +1.0% for a 150 mg/kg sample and –5.3% for a 300 mg/kg sample.

**Sensitivity and specificity**

CTC retention time was ~6 min (Figure 1). The lowest limit of quantification was 3.5 mg/kg, and was calculated as a peak height at least three times the baseline noise and a %CV of <10 for six replicates. The lowest limit of detection was ~1.0 mg/kg and was calculated as a peak height greater than three times the baseline noise but with a %CV of >10 for six replicates. CTC levels in faeces from three pigs given in-feed CTC at 15 mg/kg of pig, daily for 2 days, were 41.0, 54.2 and 59.4 mg/kg (Figure 1). There was no interference from endogenous material in either the CTC-free faeces or from the assay of aqueous neomycin, lincomycin, tylosin (Sigma), avilamycin (Eli Lilly, Liverpool, UK), ciprofloxacin or enrofloxacin (Bayer AG, Wuppertal, Germany) (data not shown).

**Results**

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Stability

CTC extracted from faeces and aqueous samples spiked to a concentration of 5, 25, 150 and 400 mg/kg were found to be stable at +5°C for at least 24 h; %CV (n = 11) over 24 h for the faecal samples were 6.3, 7.2, 5.9 and 7.4; aqueous samples 8.7, 7.7, 7.5 and 6.6, respectively. Over a period of 3 h there was no significant degradation of CTC in 0.01 M H3PO4, or in faeces at room temperature; the slope of the linear regression line was –0.00006, 0.00003 with a change in peak heights of 1.2% for a 1.0 mg/L sample in 0.01 M H3PO4 and 3.2% for a 7.3 mg/kg sample in faeces.

Discussion

The extent to which CTC is bound to intestinal contents and, consequently, the selective pressure to which enteric bacteria are exposed, are likely to vary as digested food travels along the gut. Environmental conditions are known to alter along the length of the gut and also to differ with diet.6,7 Both water content and pH have been shown to affect the binding and release of antibiotics in the intestinal contents.8 CTC additionally forms chelates with cations, undergoes reversible epimerization under different pH conditions, and is metabolized to a number of metabolites, some with a degree of antimicrobial activity.9 Although such an approach potentially could underestimate the selective pressure exerted by CTC, in general, the degree of metabolism of CTC is relatively low and the major effect is likely to be from parent CTC.10 For these reasons we chose to use a direct assay method (HPLC) to determine the total CTC levels in the collected pig faeces, rather than an indirect one such as bioassay.

In this study, we have been able to develop simple methods to detect CTC in pig faeces, using an adaptation of Moats’ extraction procedure and reverse phase HPLC method. The extracted solutions were stable at +5°C for at least 24 h, permitting the use of a chilled autosampler (data not shown) for the assay of large batches of samples. The extraction process reproducibly extracted >90% of CTC from faeces, with a <10% error in accuracy. To increase the sensitivity of the assay, solid phase extraction or other concentration methods would need to be developed, but this would greatly increase the cost and procedure time of the assay. However, the detection limit of our assay was at a clinically relevant concentration, and it is not clear whether there would be any advantage in being able to detect CTC at levels below this.

In conclusion, we have developed a simple method for the assay of CTC in faecal material that is suitable for drug disposition and environmental impact studies. The extraction method developed allowed small quantities of faeces to be processed in a convenient and cost-effective way, using aqueous calibrators and standard laboratory HPLC equipment.

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References