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 50T 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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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 \(\times 100\). 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 the standard deviation/mean \(\times 100\). Linearity, the correlation between drug concentration and peak height, was determined by assaying a series of faeces and aqueous samples spiked with known concentrations of CTC \((0, 3.5, 25, 150, 300\) mg/L). The accuracy of the measure of CTC in faecal samples was calculated using a standard curve of prepared aqueous calibrators \((0, 3.5, 25, 150, 300\) mg/L), and a separately prepared internal control \((75\) mg/L). A calibration curve was plotted of target concentration versus peak height, and the CTC concentration of assayed faecal samples was calculated by simple linear regression analysis. Percentage error as a measure of accuracy was calculated as \([\text{measured concentration} – \text{target concentration}] / \text{target concentration}] \times 100\).

Specificity was also determined by the assay of six commonly used veterinary antibiotics on the CTC HPLC set-up. To determine whether there were any endogenous compounds present that could interfere with the CTC peak, faeces from six pigs given antibiotic-free feed were assayed. As a guide to the concentration range likely to be found in faecal samples, faeces were collected from pigs that had consumed feed daily containing \(15\) mg/kg CTC per weight of pig (the recommended therapeutic dose); after 2 days the CTC was extracted and assayed as described. The stability of the CTC was assessed in \(0.01\) M H\(_3\)PO\(_4\) and in aqueous neomycin, lincomycin, tylosin (Sigma), avilamycin (Eli Lilly, Liverpool, UK), ciprofloxacin or enrofloxacin (Bayer AG, Wuppertal, Germany) (data not shown).

**Results**

**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 \(90.7\).
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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 Moats8 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