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Metabolomics reveals novel biomarkers of illegal 5-nitromimidazole treatment in pigs. Further evidence of drug toxicity uncovered

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ABSTRACT

The aim of the study was to investigate the potential of a metabolomics platform to distinguish between pigs treated with ronidazole, dimetridazole and metronidazole and non-medicated animals (controls), at two withdrawal periods (day 0 and 5). Livers from each animal were biochemically profiled using UHPLC–QTof-MS in ESI+ mode of acquisition. Several Orthogonal Partial Least Squares-Discriminant Analysis models were generated from the acquired mass spectrometry data. The models classified the two groups control and treated animals. A total of 42 ions of interest explained the variation in ESI+. It was possible to find the identity of 3 of the ions and to positively classify 4 of the ionic features, which can be used as potential biomarkers of illicit 5-nitroimidazole abuse. Further evidence of the toxic mechanisms of 5-nitroimidazole drugs has been revealed, which may be of substantial importance as metronidazole is widely used in human medicine.

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1. Introduction

The drugs (1-methyl-5-nitro-1H-imidazol-2-yl)methyl carbamate (ronidazole), 1,2-dimethyl-5-nitro-1H-imidazole (dimetridazole) and 2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethanol (metronidazole) are members of the 5-nitroimidazole family and used in veterinary medicine for the treatment and prevention of anaerobic microorganism and protozoa infections. Previous studies have shown that the 5-nitroimidazoles have caused mutations in bacterial strains and in mammalian cell lines promoting the incidence of malignant tumours in laboratory animals (FAO/WHO, 1989).

As suspected carcinogens and mutagens their use for food-producing animals, as veterinary medicines or feed additives, has been forbidden within the European Union for decades. Thus, under council regulation CR 2377/90, ronidazole, dimetridazole and metronidazole were banned as veterinary medicines for livestock in 1993 (CR 3426/93), in 1995 (CR 1798/95) and in 1998 (CR 613/98) respectively. Subsequently ronidazole was banned as a feed additive in 1998 (CD 98/19 EC) and dimetridazole in 2001 (CR 2205/2001). In 2010, repealing CR 2377/90, the 5-nitroimidazoles under study were included on the list of prohibited substances under Commission Regulation 37/2010.

The use of 5-nitroimidazoles as veterinary medicines for food-producing animals were also banned in other countries such as the USA (AMDUCA, 1994), Canada (Canada Gazette, 2003) and in Australia (APVMA, 2007). Despite their ban within the EU, the administration of 5-nitroimidazole to livestock is still being undertaken as non-compliant samples have been found in recent years (EFSA, 2013)

Current analytical methods for the detection of 5-nitroimidazole residues are based on the detection and quantification of the parent compound and hydroxylated metabolites (Cronly, Behan, Foley, Malone, & Regan, 2009). Trace amounts of these residues are present in tissues of certain animal species such as poultry and pig after 2–3 days post treatment (Mallinson, Henry, & Rowe, 1992), making detection of illegal use very challenging. Furthermore, they form reactive metabolites that bind covalently with proteins (Miwa, West, Walsh, Wolf, & Lu, 1982) and these covalently-bound metabolites are not detectable using current analytical methods thus their depletion period has not been established. However their bioavailability during digestion of treated meat may involve a health risk for consumers. Therefore, development of a method which can detect the fraudulent abuse of these

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drugs at longer periods following cessation of treatment is required to enable authorities to prevent contaminated food products polluting the food chain.

Metabolomic-based analysis of animal tissues and biological fluids, using NMR and LC–MS combined with chemometrics has been demonstrated to be a powerful tool for identifying unique metabolic profiles associated with drug treatment, pollutant exposure and disease. (Graham et al., 2012, 2013; O'Kane, Chevallier, Graham, Elliott, & Mooney, 2013). Xenobiotics cause a downstream of responses in exposed organs by the induction of biological stresses that alter the metabolic profile of tissues, thereby generating specific metabolic signatures (Kang, Lee, Joo, & Kim, 2005). Biomarker detection of chemical exposure is a growing area of interest in food safety surveillance, as biomarker identification has some important advantages over classical targeted analytical techniques. After xenobiotic exposure, resulting biomarkers may be easier to detect than the causative agent itself in terms of tissue availability, sample preparation and sensitivity issues (O'Kane et al., 2013). In the present study, a metabolomics platform was developed to detect the illegal use of a range of 5-nitroimidazole drugs; ronidazole, dimetridazole and metronidazole in pigs following two withdrawal periods (day 0 and 5). Liver extracts from control animals and treated animals were analysed using high resolution mass spectrometry and the recorded metabolomic profiles were used to build multivariate statistical models capable of characterising both control and treated animals.

2. Materials and methods

2.1. Animal experiments

Twenty-nine male pigs (crossbreed large white/landrace) of twelve weeks-old with an average 30 kg body weight, were randomly divided into four groups: three groups of six (treatment groups) and one group of eleven (control group), and housed separately. Animals were left in their pens for a fourteen day acclimatisation period. During this period a standard feed for grower pigs (Prime Grower Pellets, Thompsons, UK) and water ad libitum was supplied. Feed intake was monitored to calculate the consumption per animal per day. Batches of feed were mixed with the respective drugs. The target dosage was achieved by using the feed intake per animal per day and the average animal body weight. Dosages were based on previously described recommended therapeutic values (Debuf, Bishop, & Sciences, 1994; Foix, 1980; Schulze & Kielstein, 1987) to replicate current fraudulent activities. Following the acclimatisation period three groups pigs ($n = 6$) were each separately treated with either ronidazole (20 mg/kgbw/day) (RNZ) or dimetridazole (50 mg/kgbw/day) (DMZ) or metronidazole (25 mg/kgbw/day) (MNZ). The remaining group of 11 control animals were provided non-medicated feed for the duration of the trial. All groups were supplied with water ad libitum over the course of the experiment. Following 14 days of treatment with the respective drugs, all treated animals were returned to non-medicated feed for the duration of the withdrawal period. On day zero after cessation of treatment three animals per treated group and six animals from the control group were removed and sacrificed. The remaining animals were sacrificed on day 5 post-treatment cessation. Post-mortem liver samples were collected from all animals and stored at −80 °C until analysis. Animal experiments were conducted under approved institutional protocols and accordingly with guidelines established in the guide for the care and use of laboratory animals.

2.2. Sample preparation

The method used was based on the method previously employed by Graham et al. (2013) for the extraction of polar metabolites from post-mortem human brain tissue. Briefly, the left lobe of each liver was collected from partially thawed livers (each liver was partially thawed under constant temperature of 4 °C for 24 h). Consequently the left lobe was frozen, lyophilised and manually milled to a fine powder. In a 2 mL Eppendorf tube 50 mg of milled liver was extracted in 1 mL of ice cold methanol/water (4:1). The samples were mixed by rotation at 4 °C for 10 min using a roller mixer (SRT 9Roller Mixer, Stuart equipment, UK) and sonicated in an ice-water bath for 15 min (Ultrasonic cleaner USC 600 TH, VWR INTERNATIONAL, UK). Proteins were precipitated by centrifugation at 16,000g for 20 min at 4 °C and the supernatants collected. A pooled sample was prepared by combining 20 μL of each extract from each liver sample.

2.3. UHPLC–QTof-MS analysis

Sample and pooled supernatants were evaporated to dryness in a centrifugal vacuum concentrator (miVac Quatro Concentrator, Mason Technology, Dublin, Ireland) at 37 °C for 10 h. Following this, samples were reconstituted in 400 μL of Ultrapure water and filtered by centrifugation using 0.22 μm Constar Spin-X centrifuge tube filters (Corning Incorporated, Corning, NY 14831, USA) at 4 °C for 5 min at 10,000g. All solvents used were purchased from Sigma–Aldrich (Dorset, UK) and were LC–MS grade or equivalent.

Replicate injections ($n = 6$) of samples from each individual animal were randomly analysed using a Waters Acquity I-Class UHPLC System (Mildford, MA, USA) coupled to a Waters Xevo G2S QTof mass spectrometer (Manchester, UK) equipped with an electro spray ionisation source operating in positive mode and a lock spray interface for accurate real time mass correction. UHPLC separation was performed using an Acquity HSS T3 UHPLC column (2.1×100 mm, 1.8 μm, Waters, Milford, MA, USA) with an injection volume of 3 μL and a flow rate of 0.4 mL min^{-1} over 26 min. The sample manager and column temperature were maintained at 6 and 50 °C, respectively. The mobile phase consisted of A (Water + 0.1% formic acid) and B (methanol + 0.1% formic acid). The elution gradient started from an isocratic period of 2 min at 1% B followed by 4 consecutive linear gradients; 1–25% B over 4 min; 25–80% B over 4 min; 80–90% B over 2 min and 90–100% B over 9 min, then an isocratic period of 100% B over 2 min and returned to the initial conditions (1% B) over 1 min. The initial conditions were maintained for 2 min prior to the next injection. The capillary voltage was 1 kV and the sampling cone was set at 30 V. Source temperature was 120 °C and desolvation gas temperature was 450 °C. The cone gas flow was set to 25 L h^{-1} and the desolvation gas flow was 850 L h^{-1}. Mass spectral data were acquired in centroid mode using the MS^E function (low energy: 4 eV, high energy ramp from 25 to 35 eV) over the range m/z 50–1200, with a scan time of 0.1 s. A Leucine enkephalin lock mass calibrant was used throughout (5 μL min^{-1} infusion). A solution of 0.5 mM of sodium formate was employed for calibration. Data was collected over a range 50–1500 m/z. Prior to all analyses ten pooled conditioning samples were injected. For quality control, pooled samples were injected at intervals every ten samples throughout the entire experiment to determine the chromatographic reproducibility and peak intensities (Graham et al., 2013).

2.4. Data processing and multivariate data analysis

The raw data from the spectral analysis was processed using TransOmics software (Waters, UK). Peaks were automatically aligned and manually reviewed. Peak picking was made automatically for sensitivity with a peak width of 0.1 min. After that ion intensities were normalised to the total spectral intensity and the normalised data was imported to SIMCA-13.0.3 (Umetrics, Umea,

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