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Development and validation of a liquid chromatography tandem mass spectrometry assay for the measurement of faecal metronidazole

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ABSTRACT

Background: Metronidazole is an oral antibiotic which is widely used in the treatment of patients with *Clostridium difficile* associated disease.

Methods: This article describes the validation of a LC-MS/MS assay for the measurement of metronidazole in human faecal samples.

Results: Matrix matched and aqueous standards showed no significant difference in performance for the routine calibration of the assay. D^4 deuterated metronidazole internal standard eluted with a different retention time to the undeuterated metronidazole on chromatography, hence zidovudine was used as an internal standard. Ion suppression was noted for both metronidazole and zidovudine due to unidentified compounds present in the faecal matrix and this was improved by extracting a smaller quantity of faeces and diluting the extract prior to analysis. Measurement uncertainty was 13% at 28,400 ng/ml, 7.2% at 3300 ng/ml, 3.9% at 320 ng/ml, 13.6% at 109 ng/ml and 30.9% at 20 ng/ml. The assay was shown to be linear on dilution and the sensitivity of the assay was superior to HPLC assays using UV detection. The limit of detection was 5 ng/ml, the limit of quantitation was 66 ng/ml and the upper limit of the working range was 30,000 ng/ml. Patient samples were stable at −20 °C for 12 months and extracted faecal samples were stable on storage for 1 week at 4 °C. There were no specific requirements for patient preparation or time of sample collection relative to taking metronidazole.

Conclusions: Metronidazole can be quantified in faecal samples using LC-MS/MS which opens up opportunities for further research in this area.

1. Introduction

Metronidazole is a nitroimidazole antibiotic originally introduced in 1959 [1] to treat trichomonas vaginalis infections but currently widely used for treatment of, and prophylaxis against, anaerobic bacterial infections such as those due to *Clostridium difficile*. Orally administered metronidazole is well absorbed by the gut mucosa. Once absorbed, metronidazole is metabolised (with a half-life of between 6 and 12 h) by the hepatic cytochrome P-450 enzymes to form two major metabolites, an acid metabolite and a hydroxy metabolite, which are excreted within the bile back into the gut and subsequently reabsorbed. While the activity of the acid metabolite is negligible, the activity of the hydroxy metabolite is approximately 65% of the activity of the parent drug. In healthy humans the efficient enterohepatic recirculation results in negligible concentrations of metronidazole in faeces [2]. Faecal concentrations of metronidazole in patients with colonic disease reflect leakage of the drug from the colonic vasculature through the inflamed mucosa into the lumen. The faecal concentration of metronidazole in patients with inflammatory bowel disease corresponds with the degree of disease activity [3].

Metronidazole is widely used in the treatment of patients with *Clostridium difficile* associated disease (CDAD). However, the median response time to treatment is 4–5 days. The reason for this long response time and the reasons why it is much longer in some patients are unclear. Up to 20% of patients will undergo a further episode of CDAD. There is little data on the relationship between faecal metronidazole concentrations and response time to treatment or the development of relapse. There is a need for a reliable assay for measurement of faecal metronidazole to support further research in this area.

There are several published chromatographic techniques for quantification of metronidazole in plasma, serum, saliva and gastric fluid, typically using HPLC with UV detection or LC-MS/MS [4–13]. There are few studies describing measurement of metronidazole in human faeces. Two published reports which describe measurement of faecal metronidazole in patients with Crohn's disease and colitis used reversed phase HPLC assays with UV detection although there are no details provided regarding the calibration and validation of these assays [2,3].

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Measurement of metronidazole using mass spectrometry might be expected to offer superior sensitivity and specificity compared to existing HPLC assays.

There is limited information in the literature describing the measurement of pharmacologically active compounds in faeces using tandem mass spectrometry, the calibration of LC-MS/MS assays for measurements of analytes in faecal material and the ion suppression effects which are seen secondary to the faecal matrix. This article describes the validation of a mass spectrometric assay for the measurement of faecal metronidazole.

2. Materials and methods

2.1. Reagents

Methanol Fluka Analytical LC-MS Chromasolv grade, ethylacetate and ammonium acetate were HiPerSolv Chromanorm reagents for HPLC purchased from VWR, Biosolve ULC/MS grade formic acid was used. $NaHCO_3$ was obtained from the Chaddlewood Co-operative and a solution with a concentration of 100 mg/ml was used as an extraction buffer. 0.2 μm hydrophilic Syringe Filters (Minisart PES 16534) were obtained from Sartorius, 5 ml glass vials with screw top lids were obtained from Chromacol.

2.2. Standards and controls

Metronidazole (Fluka) purchased from Sigma-Aldrich was used to prepare the calibrators for the assay by preparation of 50 ml of a 4 mg/ml stock solution in 10% MeOH (*aq*) which was then serially diluted to produce a range of standard solutions with concentrations 0.1 μg/ml, 0.5 μg/ml, 1 μg/ml, 5 μg/ml, 10 μg/ml, 50 μg/ml and 100 μg/ml. Patient faecal samples were used as positive control material and a faecal sample from an individual who had not taken any metronidazole was used as a negative control. One zidovudine capsule (250 mg) was obtained from Derriford Hospital Pharmacy, crushed using a pestle and mortar and 10 ml of 10% CH_3OH (*aq*) was added to give a stock solution of 25 g/L. The mixture was vortexed and centrifuged to remove particulate material. The supernatant was diluted in 10% CH_3OH (*aq*) to produce a working internal standard solution with a concentration of 10 μg/ml. Metronidazole–(ethylene-d^4) hydrochloride (Fluka) was purchased from Sigma-Aldrich

2.3. Sample preparation

50 mg faeces was accurately weighed into a 5 ml glass vial. To each vial, 100 μl zidovudine internal standard (10 μg/ml), 100 μl 10% CH_3OH (*aq*) and 1800 μl $NaHCO_3$ extraction buffer was added. Each vial was vortexed thoroughly to mix the sample. 2.5 ml ethylacetate was added and the vial was vortexed to mix. The vials were centrifuged at 2195 rcf for 10 min to separate the organic and aqueous layers and the organic layer was decanted into a second glass vial and dried down under nitrogen at 40 °C for 20 min using a Techne Dri-Block DB-3. The contents of the vial were reconstituted with 2 ml 10% CH_3OH (*aq*). Each extract was filtered using a Minisart syringe filter and diluted 1/10 with 10% CH_3OH (*aq*) prior to analysis. Samples were stored protected from light at 4 °C until analysis and presented for analysis in 2 ml glass vials. The measured metronidazole concentration in ng/ml could then be converted into a value of μg/g faeces using the accurate weight of sample which had been extracted.

2.4. LC-MS/MS

Chromatography was performed using a Waters Acquity UPLC BEH C18 1.7 μm 2.1 × 50 mm column and a Waters Acquity UPLC system with a binary solvent manager. Tandem mass spectrometry was performed by positive electrospray ionisation using a Waters TQD mass spectrometer. Chromatography was performed at 55 °C. The mobile phases used for the chromatography were; 2 mM ammonium acetate and 0.1% formic acid in water for the aqueous buffer (Buffer A) and 2 mM ammonium acetate and 0.1% formic acid in methanol for the organic buffer (Buffer B) at a flow rate of 0.4 ml/min. The column was equilibrated with 10% organic buffer B until the pressure stabilised (as defined by a delta pressure ripple under 10 psi/min) The mobile phase composition at baseline was 10% buffer B, changing with a linear gradient to 25% buffer B from 0.2 to 0.6 min and further increasing over a linear gradient to 100% buffer B over the period up to 1.8 min before reverting back to 10% buffer B at 2 min to enable the column to re-equilibrate for the next sample. The retention times for metronidazole, hydroxymetronidazole, d^4 metronidazole and zidovudine were 0.87–0.91, 0.65–0.66, 0.62 and 1.35–1.39 min respectively. The column effluent was diverted to the MS source between 0.4 and 1.6 min, but otherwise diverted to waste. The mass spectrometry conditions were desolvation gas flow 800 L/h, desolvation temperature 450 °C, source temperature 120 °C, capillary voltage 2.6 kV, cone voltage 25 V, extractor voltage 3 V, cone gas 200 L/h, collision gas (argon) 0.25 L/min. The following mass transitions were selected for metronidazole (172 > 128 (quant) and 172 > 82 (qual)), hydroxymetronidazole (188.2 > 126.2 (quant) and 188.2 > 123.2 (qual)), d^4-metronidazole (176 > 128 (quant) and 176 > 82 (qual)) and zidovudine (268.1 > 127.1 (quant) and 268.1 > 142 (qual)). System check criteria for acceptance were based on the retention times and peak areas taken from the 1 μg/ml standard for the quantitative transition for both metronidazole and zidovudine. Metronidazole results were not reported for samples where the ion ratio for the quantitative transition relative to the qualitative transition was outside the limits of tolerance. The acceptance range for ion ratios was determined from the standard solutions with a tolerance of ±20%. Typical quantitative/qualitative ion ratios for metronidazole were in the range 5–9. The low mass resolution was 517 on MS1 and 514 on MS2, high mass resolution 778 on MS1 and 854 on MS2. Dwell times were 0.05 s for each mass transition channel monitored in the MRM on an automatic delay setting for switching frequency. The number of scans over each peak was evaluated.

2.5. Validation

The method validation procedure was based on that proposed by Honour. [14].

2.6. Ion suppression

Ion suppression was evaluated by injecting a faecal extract which was negative for metronidazole onto the UPLC column whilst continuously infusing a 100 ng/ml solution of metronidazole or zidovudine (at a flow rate of 20 μl/min) into the mass spectrometer source. The faecal extract was prepared from 0.5 g sample extracted into 800 μl $NaHCO_3$ extraction buffer and 200 μl 10% MeOH (*aq*) as described above. This concentration gave counts in the range 5×10^4–8×10^6 which were in the upper half of the working range.

2.7. Calibration

Faecal samples spiked with metronidazole, faecal extracts spiked with metronidazole and aqueous standards were compared for suitability for calibration of the assay. 100 μl (of 1 μg/ml, 100 ng/ml or 10 ng/ml) of metronidazole in 10% CH_3OH (*aq*) were added to 0.5 g faeces, 800 μl extraction buffer and 100 μl 1 μg/ml zidovudine internal standard were added and the samples extracted according to the sample preparation procedure described above. 800 μl extraction buffer and 200 μl 10% MeOH (*aq*) was added to two 0.5 g samples of faeces containing no metronidazole and extracted according to the sample preparation procedure described above. After reconstitution in 2 ml 10% MeOH (*aq*) the

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