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Development of a LC-MS method for simultaneous determination of amoxicillin and metronidazole in human serum using hydrophilic interaction chromatography (HILIC)

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ABSTRACT

A method was developed for the determination of amoxicillin and metronidazole in human serum. The procedure used was hydrophilic interaction chromatography (HILIC) followed by mass spectrometric (MS) detection. Chromatographic separation was achieved on a ZIC-HILIC column and the mobile phase consisted of a mixture of 0.1% (v/v) formic acid in water and 0.1% (v/v) formic acid in acetonitrile. The method was validated with regard to selectivity, accuracy, precision, calibration, lower limit of quantification (LOQ), extraction recovery and matrix effect. The LOQs were 0.0138 and 0.008 μg/ml for amoxicillin and metronidazole respectively, while for quantification purposes linearity was achieved in the range of 0.1 μg/ml to 6.4 μg/ml for both drugs with correlation coefficients > 0.9990. The intraday precision (expressed as %RSD) and the accuracy (expressed as the % deviation from the nominal value) was < 15% for both antibiotics at all QC levels. Extraction recoveries for both drugs and internal standards were > 80%, while a considerable matrix effect (< 60%) was observed for amoxicillin. Finally, the method was applied to the determination of amoxicillin and metronidazole concentrations in serum for 20 patients.

1. Introduction

Amoxicillin, a semi synthetic analogue of ampicillin, is a widely used, broad spectrum antibiotic and categorized in the aminopenicillin group. It acts mainly against Gram positive bacteria but is also active against some Gram negative bacteria [1]. Metronidazole (2-(2-methyl-nitro-1H-imidazole-1-yl) ethanol) is a synthetic 5-nitroimidazole; its spectrum of activity includes anaerobic Gram negative and Gram positive bacteria [2–4].

The rationale behind combining different classes of antibiotics as prophylaxis against surgical site infections is to cover a broad spectrum of potentially infecting organisms. The combination of amoxicillin, metronidazole and gentamicin is currently recommended by NHS Greater Glasgow and Clyde for adult patients undergoing colorectal surgery [5] but the dosage regimens used are largely empirical. Determination of the concentration of antibiotics during surgery would provide useful information on the optimal treatment schedules.

Numerous High Performance Liquid Chromatography (HPLC) methods have been published for the individual analysis of amoxicillin and metronidazole in biological samples [6–10]. Simultaneous determination of amoxicillin and metronidazole in a tablet formulation was reported by Tavakoli et al. using reversed phase HPLC with UV detection [7]. Amoxicillin is not strongly retained by reversed phase chromatography and it tends to elute with the polar endogenous compounds in plasma [8]. Several LC-MS methods have been developed for analysis of amoxicillin in plasma and the majority reversed phase chromatography [11–15] and many are run at low pH where amoxicillin has very little partitioning into a lipophilic stationary phase. Even if retention is achieved care has to be taken that high amounts of organic solvent used in does not cause the analyte to elute at the void volume [11]. Metronidazole has more lipophilicity and an LC-MS method for its determination by using reversed phase chromatography has been reported [16].

Hydrophilic interaction chromatography is more suitable than reversed phase chromatography for separating small polar ionisable molecules, providing reasonable retention without using derivatisation

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methods or ion pair reagents. A high organic aqueous mix mobile phase is highly compatible with an ESI (electrospray ionisation) MS detection system. A mobile phase containing some water, as used in HILIC, facilitates the solubility of polar compounds unlike the non-polar solvents used in normal phase chromatography. MS detection is more favoured with a high organic phase content in the mobile phase, which enables ready desolvation and droplet formation at the ESI interface and results in low back pressures in liquid chromatography. Hence, the ESI mode is very compatible with HILIC [17,18]. The high percentage of organic solvent used in HILIC increases the sensitivity of ESI-MS detection by producing sharp peaks that increase the signal to noise ratio [19]. In addition, there is no problem in focusing analyte peaks when the supernatants obtained from protein precipitation using a high level of organic solvent are injected since in HILIC mode organic solvents have weak eluting power. This makes reproducibility of injections in terms of sample focusing very robust.

The scope of the current study was to develop an analytical method to quantify metronidazole and amoxicillin in human serum using HILIC in combination with high resolution mass spectrometry. It was not possible to include gentamicin within the HILIC method because it was too polar to elute from the column thus for the purposes of the clinical study gentamicin was determined by homogeneous particle-enhanced turbidimetric inhibition immunoassay.

2. Experimental

2.1. Chemicals and reagents

Amoxicillin trihydrate, ampicillin anhydrous, metronidazole and metronidazole D4.HCl (metD4) were obtained from Sigma-Aldrich, Dorset UK. HPLC grade methanol, HPLC grade water, acetonitrile and formic acid were purchased from Fisher Chemicals, Leicestershire, UK.

2.2. Drug-free serum and clinical samples

Drug-free human serum samples were provided by the Scottish National Blood Transfusion Service (SNBTS). Serum samples were collected from 20 patients for a pilot study involving the quantification of antibiotic concentrations in patients undergoing bowel resection surgery at Glasgow Royal Infirmary. The study was approved by the East Midlands NHS Research Ethics Committee (ref 16/EM/0209) and the Greater Glasgow and Clyde Health Board (ref GN16OR139). Each patient was given an intravenous dose of 1 g amoxicillin and 500 mg metronidazole before surgery. Amoxicillin was re-dosed if the operation lasted > 4 h, and both amoxicillin and metronidazole were re-dosed if the intra-operative blood loss was > 1.5 l. Serum samples were collected pre-dose, 1 and 2 h after administration and at skin closure. In patients who were re-dosed, additional samples were withdrawn before and 1 h after re-dosing. Samples were stored under −80 °C until the start of analysis and under −20 °C during the analysis.

2.3. Instrumentation and chromatographic conditions

The method was developed and validated using an UltiMate 3000 HPLC system coupled with an Exactive benchtop Orbitrap mass spectroscopy system (Thermo Scientific, USA). Samples were introduced to the column using a DIONEX UltiMate 3000 autosampler. A ZIC-HILIC column (150 × 4.60 mm, 3.5 µm, HiChrom, Reading UK) fitted with the metal free guard column ZIC-HILIC (20 × 2.1 mm) was used. The aqueous phase (A) consisted of 0.1% v/v formic acid in water and the organic phase (B) consisted of 0.1% v/v formic acid in acetonitrile. Gradient elution was performed as detailed in Table 1. The sample injection volume was 10 µl and the sample tray was operated at 4 °C.

The method utilised ampicillin and metronidazole D4 as internal standards. The analytes were quantified by using extracted ion chromatograms with 0.02 amu mass windows as follows: metronidazole *m/z* 172.06–172.08, metronidazole D4 *m/z* 176.08–176.1, ampicillin *m/z* 350.11–350.13 and amoxicillin *m/z* 366.1–366.12. The ESI interface was operated in a positive ion mode with a spray voltage of 3.8 kV. The capillary temperature was 250 °C and the flow rates of the sheath and auxiliary gases were 50 and 17 arbitrary units respectively. The full scan range was *m/z* 75 to 1200. The data was recorded using Xcalibur 2.2 SP 1.48 software (Thermo Fisher Scientific). Signals at *m/z* 83.06037, 129.10224, 167.03388, 195.08765 and 391.28428 were selected as lock masses for positive ion mode during each analytical run.

Table 1
Gradient programme of the HPLC method.

Time (minutes)	% mobile phase A	% mobile phase B	Flow rate (ml/min)
0	20	80	0.300
12	80	20	0.300
14	80	20	0.300
15	20	80	0.600
20	20	80	0.600

2.4. Preparation of standard stock solutions and spiked samples

2.4.1. Preparation of standard stock solutions

Standard stock solutions (500 µg/ml) of amoxicillin, ampicillin, metronidazole and metD4 were prepared in 0.1% v/v formic acid in a methanol:water (1:1) mixture and stored at 4 °C during the period of study (five weeks).

2.4.2. Preparation of calibration and quality control standards

Calibration samples and quality control (QC) samples were prepared by spiking varying amounts of the analytes and fixed amounts of the internal standards into drug-free human serum. Calibration samples were prepared over the concentration range of 0.1–6.4 µg/ml and concentrations of the internal standards were kept constant at 1 µg/ml in each calibration sample. Three QC levels, 0.1, 0.8 and 6.4 µg/ml were selected covering the low, middle and upper levels of the calibration range for both drugs which was selected to ensure that both early and late post-dose sampling points remained within the calibration range. Five replicates of the three different concentrations of amoxicillin and metronidazole were prepared in the same way as the calibration samples using 100 µl of serum. Calibration samples were analysed in duplicate before and after the test sample run.

2.4.3. Preparation of quality control samples and clinical samples

Serum samples from patients were thawed well and vortexed for 30 s before preparation. Clinical and quality control samples were prepared by combining 100 µl of serum and 20 µl of each internal standard (50 µg/ml). Acetonitrile 860 µl was then added, samples were vortexed for 1 min, centrifuged at 5000 rpm for 10 min and then injected. Concentrations were determined according to the calibration curves constructed over the range 0.1–6.4 µg/ml.

2.5. Method validation

Method validation was carried out according to the FDA guidelines: (www.fda.gov/downloads/Drugs/Guidance/ucm070107.pdf).

2.5.1. Selectivity

Selectivity was determined by analysing drug-free human serum. A volume of 100 µl of drug-free serum was mixed with 900 µl of acetonitrile for protein precipitation (PPT). This was vortexed for 1 min and centrifuged at 7000*g* for 10 min. The clear supernatant was then injected into the LC-MS system. Chromatograms of drug-free serum samples were compared with spiked samples at the lower limit of quantification (LOQ) of each antibiotic and internal standard to identify

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