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Simultaneous quantification of amoxycillin and metronidazole in plasma using high-performance liquid chromatography with photodiode array detection ☆

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Abstract

A simple, robust HPLC method was developed to measure simultaneously the plasma concentrations of amoxycillin and metronidazole in order to assess their disposition in the eradication of *Helicobacter pylori*. Plasma samples were protein precipitated, pH adjusted and the supernatant injected onto the HPLC system which used a C_{18} column, paired-ion aqueous mobile phase and photodiode array detection of amoxycillin at 230 nm and metronidazole at 313 nm. Intra- and inter-day precision and inaccuracy were less than 10% for concentrations between 5 and 20 mg/l. The limit of quantification was 1 mg/l. Samples were stable on the HPLC injector for 48 h at room temperature and multiple freeze–thaw cycles led to no decomposition.

Keywords: Amoxycillin; Metronidazole; Antibiotics

1. Introduction

Amoxycillin, a broad spectrum amino-substituted β-lactam penicillin, and metronidazole, a synthetic 5-nitroimidazole with activity against protozoa and anaerobic bacteria, have widespread clinical use both individually and together. In particular, this combination is used commonly with acid suppressive therapy or bismuth salts, for the eradication of *Helicobacter pylori* infection. To examine the clinical pharmacology of these antibiotics, it is desirable to simultaneously quantify their plasma concentrations from different dosing regimens. Bioassay methods are time-consuming and non-specific, and when two or more antibiotics are present, one antibiotic needs to be inactivated or removed to allow for the specific measurement of the other. Several HPLC methods have been reported for the individual assay of these antibiotics [1,2]. A method for the measurement of seven antibiotics, but not including amoxycillin, in a single plasma sample has been reported [3]; however, it required a different UV wavelength and HPLC mobile phase for each analyte.

Whilst the HPLC assay of metronidazole is relatively straightforward, that for amoxycillin presents particular difficulties. Charles and Chaluvatnatol [4]

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have ascribed these problems with amoxycillin as being due to its amphoteric nature, thus causing it to elute among other endogenous, polar substances. In addition, its high polarity precludes the use of standard liquid extraction steps and it is unstable in media of high or low pH. These have been addressed by such techniques as deproteination with strong acid with immediate injection onto the HPLC, ultrafiltration and solid-phase or ion-pair column extraction, postcolumn derivatisation with fluorescence detection and column switching. Most of these HPLC methods are laborious, use complex instrumentation and are not suitable for batch processing.

We describe an HPLC method to assay amoxycillin and metronidazole simultaneously, which takes advantage of a photodiode array detection system to assay both drugs at different detector wavelengths with a single injection. Amoxycillin is stabilised by pH adjustment of samples prior to injection. The assay method is simple, specific, precise and requires minimal sample preparation.

2. Experimental

2.1. Chemicals

All reagents were of analytical grade and included hydrochloric acid (Ajax Chemicals, Auburn Australia), orthophosphoric acid, sodium dihydrogen orthophosphate monohydrate (BDH, Kilsyth, Australia) and sodium dodecylsulphate (SDS), (Sigma, St. Louis, MO, USA). Methanol and acetonitrile (BDH, Poole, UK) were of HPLC grade. Amoxycillin and metronidazole powder (Sigma) were of the highest grade available and were made up as 1 g/l stock solutions in 0.1 *M* sodium dihydrogen orthophosphate buffer, pH 4.9.

2.2. Instrumentation

The HPLC system consisted of a solvent delivery module (LC10AT), autoinjector (SIL10A), photodiode array (PDA) detector (SPD-M10A) and were all from Shimadzu (Kyoto, Japan). A pellicular ODS precolumn (1 cm) was positioned ahead of a 15 cm×4.6 mm I.D. stainless steel column packed with Spherisorb 5 μm ODS-2 packing material (Phase Separations, Queensferry, UK). The composition of the mobile phase was 24% acetonitrile and SDS (0.8 m*M* final concentration) in sodium dihydrogen orthophosphate buffer (20 m*M* final concentration) with pH adjusted to 2.1 with orthophosphoric acid. The flow-rate was 1.5 ml/min for the first 16 min, then linearly increased to 1.8 ml/min by 18 min and maintained at this rate for 17 min, then decreased to 1.5 ml/min for the next injection. The PDA detector wavelength was set at 230 and 313 nm for the determination of amoxycillin and metronidazole, respectively. Integration of the chromatography response was carried out using Shimadzu CLASS-LC10 software.

2.3. Sample preparation

Plasma samples (100 μl) were aliquoted into microcentrifuge tubes, 150 μl methanol was added and samples were vortexed briefly to precipitate plasma proteins. A sodium dihydrogen orthophosphate pH adjustment buffer (50 μl, 0.1 *M*, pH 2.0) was added to the samples, the tubes were recapped, vortexed for 5 s and then allowed to stand for ≈20 min to ensure complete protein precipitation. Samples were then centrifuged (Eppendorf 5415C, Eppendorf-Netheler-Hinz, Hamburg, Germany) for 5 min at 14 000 rpm. The supernatant (140 μl) was then aliquoted into injection vials and 25 μl was injected onto the HPLC column.

2.4. Calibration, precision, accuracy and recovery

The stock solutions of amoxycillin and metronidazole were diluted in 0.1 *M* sodium dihydrogen orthophosphate buffer, pH 4.9, to prepare standards comprising six concentrations ranging from 5 to 250 mg/l. These were diluted in drug-free plasma on the day of assay to achieve concentrations ranging from 1 to 50 mg/l. Calibration curves for both compounds were constructed as the peak area versus amoxycillin or metronidazole concentration and linear unweighted least squares regression was performed to determine the slope, intercept, and coefficient of determination.

Validation of the method was performed by analysis of ten drug-free, spiked plasma samples of low (5 mg/l, $n=5$) and high (20 mg/l, $n=5$) concentrations

on a single assay day to determine intra-day precision and accuracy. Analysis of four quality control samples of low (5 mg/l, $n=2$) and high (15 mg/l, $n=2$) concentrations on each of 13 assay days was used to determine inter-day precision and inaccuracy. Recovery was determined at concentrations of 1, 10 and 50 mg/l for both analytes by comparing the peak areas of the analytes from deproteinated plasma samples to the peak area of the analytes from standard solutions in 0.1 *M*, pH 4.9 buffer of the same concentration. The limit of quantification for each analyte was determined as the lowest concentration at which the relative standard deviation (RSD) was $<20\%$ for precision and inaccuracy was within $\pm 20\%$ when four replicate standards were assayed in a single run. The stability of amoxycillin and metronidazole was determined in two ways. Firstly, the stability of both analytes on the injector was assessed by injecting replicate standards at concentrations of 5 and 20 mg/l, in spiked drug-free plasma, at evenly spaced intervals during a single HPLC assay run of 48 h. Secondly, the freeze–thaw stability for both analytes was assessed at concentrations of 5 and 20 mg/l in spiked drug-free plasma. The spiked drug-free plasma samples were assayed in duplicate and the remainder placed into a -80°C freezer until frozen. After approximately 15 min, samples were thawed and a further aliquot was assayed in duplicate. This was repeated until four freeze–thaw cycles were completed.

2.5. Assay application

Amoxycillin and metronidazole were administered simultaneously to a healthy, male subject by the intravenous route using loading doses of 130 and 480 mg, respectively, followed by continuous infusions at 80 and 50 mg/h, respectively for 5 h to maintain a plasma concentration of approximately 10 mg/l for each antibiotic. Venous blood samples were collected every 30 min for 5 h, centrifuged and the plasma stored at -20°C until assay.

3. Results and discussion

Metronidazole and amoxycillin had retention times of $\approx$7.3 and 12.6 min, respectively, using the chromatographic conditions described above. The total chromatography run time was prolonged to 35 min to allow for a late eluting peak. Calibration curves were linear over the 1 to 50 mg/l concentration range with mean r^2 values greater than 0.99 for both analytes and the RSD of the slopes was $<5\%$ for both analytes ($n=10$). Inaccuracy and precision data for five replicates at 5 and 20 mg/l were less than 5% and for inter-day of thirteen analytical runs were $<10\%$ at 5 and 15 mg/l (Table 1). The limit of quantification was 1 mg/l for both analytes. At this concentration, intra-day precision and mean inaccuracy for amoxycillin were 5.6% and

Table 1
Intra- and inter-day (13 consecutive assays) accuracy and precision data expressed as mean calculated concentration and relative standard deviation (RSD) of spiked drug-free plasma and quality control samples

Analyte	Nominal conc. (mg/l)	Calculated conc. (mg/l)	RSD[a] (%)	Nominal conc. (mg/l)	Calculated conc. (mg/l)	RSD[a] (%)
Intra-day ($n=5$)						
Amoxycillin	5[b]	5.11	3.0	20[b]	20.35	1.0
Metronidazole	5[b]	5.00	3.1	20[b]	20.19	3.1
Inter-day ($n=26$)						
Amoxycillin	5[c]	4.94	6.3	15[d]	16.10	6.8
Metronidazole	5[c]	4.91	7.6	15[d]	15.37	6.3

[a] RSD=(SD/mean calculated concentration)×100.
[b] Spiked drug-free plasma sample.
[c] Low quality control.
[d] High quality control.

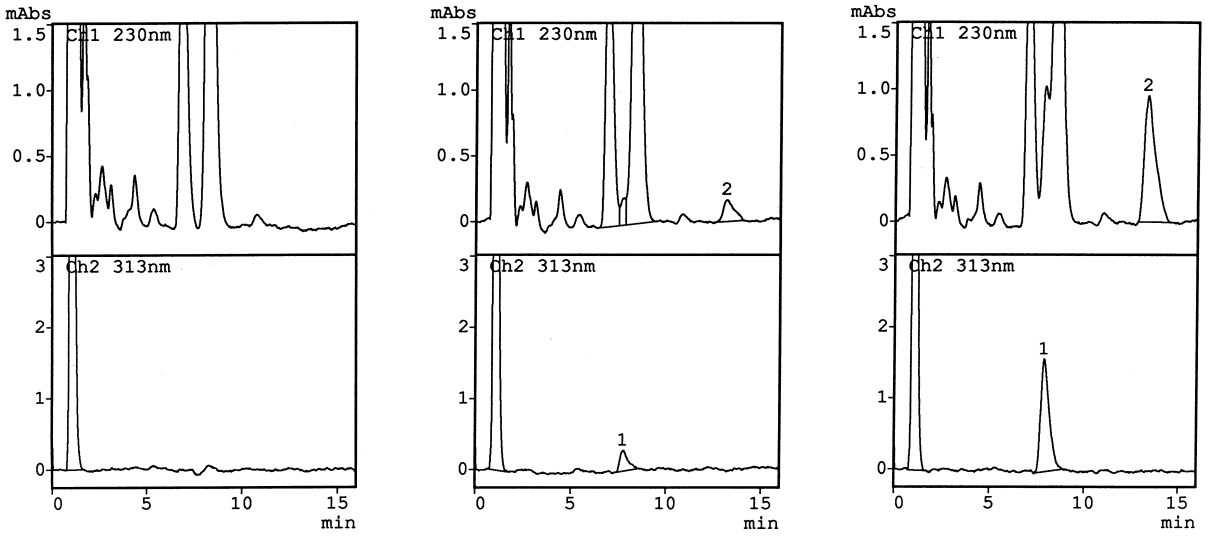

Fig. 1. Chromatograms of a blank plasma sample (left hand panel), the lowest plasma calibration standard (1 mg/l) (middle panel) and a plasma sample containing 5 mg/l of metronidazole (1) at 313 nm (lower panels) and amoxycillin (2) at 230 nm (upper panels).

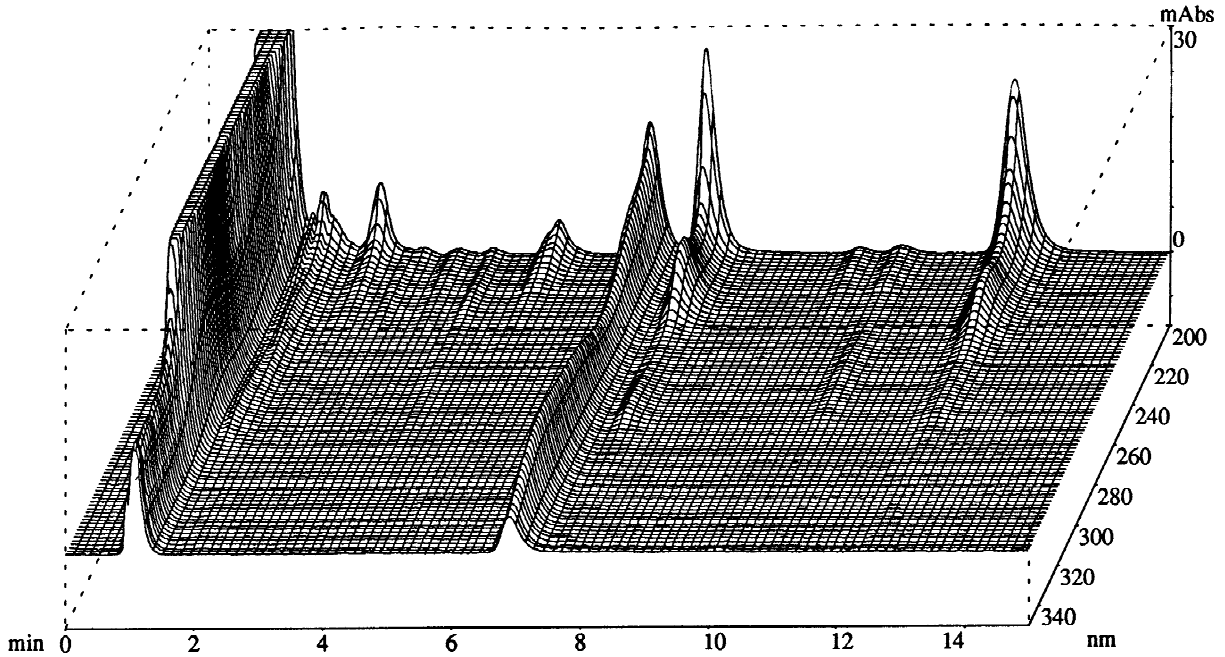

Fig. 2. Three-dimensional chromatogram from assaying a plasma sample containing 5 mg/l metronidazole (peak at 7 min) and amoxycillin (peak at 12 min). The figure illustrates the selectivity of metronidazole at 313 nm compared with 230 nm.

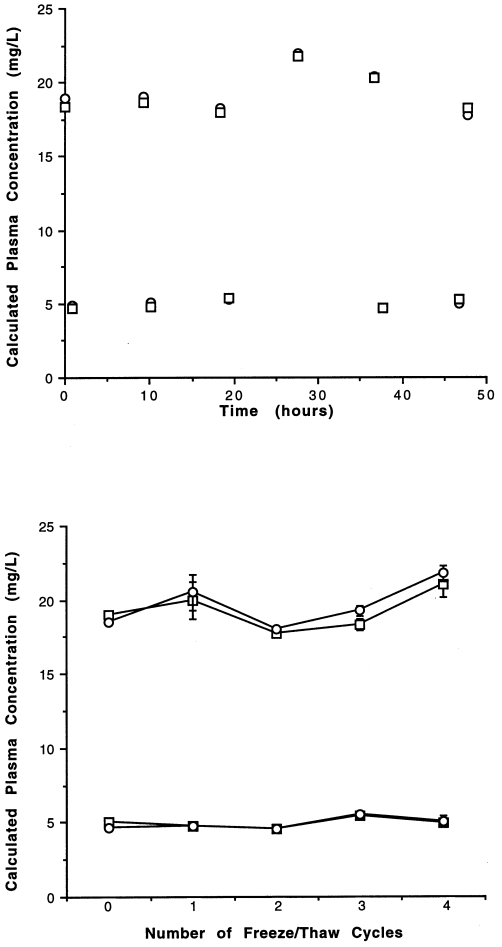

Fig. 3. Upper figure: stability of amoxycillin (5 and 20 mg/l □) and metronidazole (5 and 20 mg/l ○) on the HPLC injector over 48 h. Samples were injected at time 0, approximately 10, 18, 28, 38 and 48 h together with a calibration curve. Lower figure: effect of freeze–thaw cycles on the stability of plasma samples containing amoxycillin and metronidazole (mean±SD; $n=2$). Samples were frozen at −80°C, thawed then refrozen for a total of four cycles.

−3.2%, respectively, and for metronidazole 7.7% and +15.3%, respectively ($n=4$ for both analytes). Inter-day precision and mean inaccuracy for amoxycillin at 1 mg/l was 17.8% and +3.5%, respectively, and for metronidazole 12.6% and +4.6%, respectively, for 13 consecutive assays. This limit of quantification (1 mg/l) could possibly be improved by injecting more than the stated 25 μl onto the HPLC column.

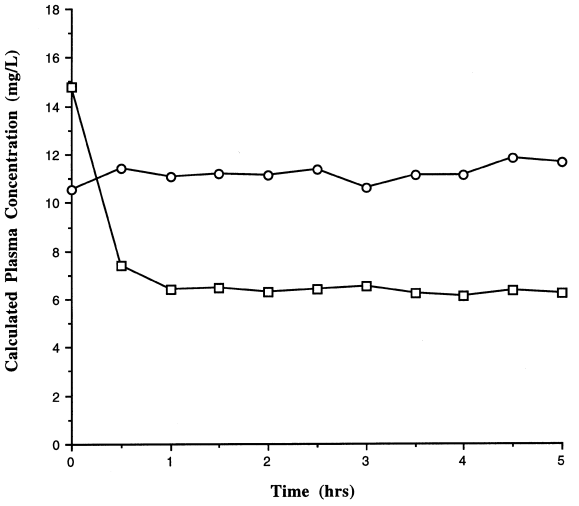

Fig. 4. Plasma concentration–time profile for amoxycillin (□) and metronidazole (○) in a healthy male volunteer receiving intravenous loading doses followed by continuous infusions of amoxycillin and metronidazole to achieve a target concentration of approximately 10 mg/l.

Fig. 1 shows chromatograms obtained for amoxycillin at 230 nm (upper panels) and metronidazole at 313 nm (lower panels) for a blank plasma sample (left hand panel), the lowest calibration standard of 1 mg/l (middle panel), and a sample containing 5 mg/l of both compounds (right hand panel). Fig. 2 shows a three-dimensional chromatogram for both drugs and illustrates the ability of the PDA detector to quantify metronidazole early in the chromatogram at a longer wavelength where co-eluting endogenous substances would normally have interfered at 230 nm, the wavelength for amoxycillin. The use of the ion-pairing agent SDS in a mobile phase of low pH also prevented early elution of the amphoteric amoxycillin among other polar, endogenous substances. The mobile phase proved to be very robust such that minor fluctuations in pH and organic modifier did not significantly alter retention times or lead to chromatographic interference by endogenous substances.

Mean recoveries from plasma samples at concentrations of 1, 10 and 50 mg/l were 97.7, 98.7 and 95.6%, respectively, for amoxycillin and 103.1, 102.7 and 100.1%, respectively, for metronidazole ($n=4$ for both compounds at each concentration).

The addition of pH adjustment buffer, which adjusted the pH of the supernatant to approximately 4.9, resulted in high recoveries for amoxycillin which degrades at extremes of pH [4]. Fig. 3A illustrates the stability of amoxycillin and metronidazole on the injector at room temperature for at least 48 h. The lack of instability allowed a large batch of samples to be processed in the one assay. Fig. 3B shows that neither drug was degraded after four freeze–thaw cycles.

This method was used to estimate the concentrations of amoxycillin and metronidazole in a subject following an intravenous bolus and infusion of both antibiotics. Fig. 4 shows the plasma concentration–time profile of each analyte in which the steady-state target concentration of 10 mg/l was achieved for metronidazole but for amoxycillin, the concentrations were lower; no interfering peaks were observed in the chromatography. The assay is precise and accurate, allows for the simultaneous quantification of both compounds and can be used for pharmacokinetic and bioequivalence studies.

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