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Quantification of metronidazole in small-volume biological samples using narrow-bore high-performance liquid chromatography

J.I.D. Wibawa, P.N. Shaw, D.A. Barrett*

School of Pharmaceutical Sciences, University of Nottingham, University Park, Nottingham NG7 2RD, UK

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Abstract

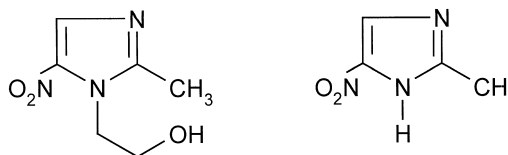
A rapid, selective and sensitive HPLC assay has been developed for the routine analysis of metronidazole in small volumes of rat plasma, gastric aspirate and gastric tissue. The extraction procedure involves liquid–liquid extraction and a protein precipitation step. A microbore Hypersil ODS 3 μm (150 \times 2.1 mm I.D.) column was used with a mobile phase consisting of acetonitrile–aqueous 0.05 M potassium phosphate buffer (pH 7) containing 0.1% triethylamine (10:90). The column temperature was at 25°C and the detection was by UV absorbance at 317 nm. The limit of detection was 0.015 $\mu\text{g ml}^{-1}$ for gastric juice aspirate and plasma and 0.010 $\mu\text{g g}^{-1}$ for gastric tissue (equivalent to 0.75 ng on-column). The method was linear up to a concentration of 200 $\mu\text{g ml}^{-1}$ for plasma and gastric juice aspirate and up to 40 $\mu\text{g g}^{-1}$ for tissue, with inter- and intra-day relative standard deviations less than 14%. The measured recovery was at least 78% in all sample matrices. The method proved robust and reliable when applied to the measurement of metronidazole in rat plasma, gastric juice aspirate and gastric tissue for pharmacokinetic studies in individual rats. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Metronidazole

1. Introduction

Metronidazole [1-(2 hydroxyethyl)-2-methyl-5-nitroimidazole, Fig. 1] is an antimicrobial drug that is used to treat protozoal and anaerobic bacterial infections [1,2]. It is now also widely used in a standard eradication treatment of gastric *Helicobacter pylori* infections, where it is combined with another antibiotic and an acid-suppressing agent [3].

In order to eradicate *H. pylori* effectively, metronidazole has to reach the minimum inhibitory concentration (MIC) of 2.0 $\mu\text{g ml}^{-1}$ in gastric juice



Metronidazole

2-Methyl-5-nitroimidazole

Fig. 1. Chemical structures of metronidazole and the internal standard, 2-methyl-5-nitroimidazole.

*Corresponding author. Tel.: +44-115-9515-062; fax: +44-115-9515-102.

E-mail address: david.barrett@nottingham.ac.uk (D.A. Barrett).

[4]. *H. pylori* resides in the gastric mucosa and hence the determination of metronidazole levels in gastric tissue and gastric aspirates is therefore crucial to ensure an effective pharmaco-therapeutic regimen. We have developed a rat model to study the exact transfer mechanisms of metronidazole from the systemic circulation to gastric juice. An analytical method was required to permit the collection of full pharmacokinetic data from single animal experiments where only small volumes of the biological matrix were available as timed samples.

No suitable high-performance liquid chromatography (HPLC) methods have been published that allow the rapid and accurate measurement of the metronidazole concentration in the small-volume samples (often less than 50 μ l) generated with these rat experiments. Methods reported previously describe a range of sample preparation procedures involving protein precipitation using perchloric acid [5] or trichloroacetic acid [6], liquid–liquid extraction [7,8], solid-phase extraction [9,10] and dilution with mobile phase prior to HPLC analysis [11]. The majority of the reports for the measurement of metronidazole in plasma or serum are designed for volumes of 0.5 ml or more, because of their use in human pharmacokinetic studies for which sample volumes of 1 ml or greater are readily obtainable [7,8,10,11,13]. There has been little need to develop a sensitive methods since metronidazole is present in relatively high concentrations in plasma. Both Pollak [12] and Jessa et al. [13] have reported rapid and specific HPLC assay methods for the detection of metronidazole in human plasma, saliva and gastric juice, neither of these methods was designed for gastric tissue samples.

The determination of metronidazole in tissue samples has been reported by Venkateshwaran and Stewart, requiring long HPLC run times [9], and by Chacko et al. [14]. However, neither of these methods were either practical or sensitive enough to be applied to the smaller samples required for single animal pharmacokinetic studies.

In this paper, we describe a rapid, sensitive and selective narrow-bore (2.1 mm I.D.) HPLC assay for the determination of metronidazole in small volumes of rat plasma, gastric juice aspirate and gastric tissue samples.

2. Experimental

2.1. Chemicals

Metronidazole and 2-methyl-5-nitroimidazole were purchased from Sigma (Poole, UK). Omeprazole sodium was kindly donated (Astra Hässle, Mölndal, Sweden). Potassium dihydrogenphosphate, sodium chloride and anhydrous potassium carbonate were purchased from Fluka (Poole, UK). Disodium hydrogenphosphate, orthophosphoric acid (85%, v/v), triethylamine and perchloric acid (60%, v/v) were all obtained from Fisons (Loughborough, UK). HPLC-grade acetonitrile was purchased from Fischer Scientific (Loughborough, UK) and pentagastrin was bought from Cambridge Labs. (Newcastle upon Tyne, UK). All chemicals were of analytical grade or better. Water obtained from an Elga Maxima water purification system (Elga, High Wycombe, UK) was used. Blank rat plasma and blank rat stomach tissue were obtained from untreated rats.

2.2. Instrumentation

The HPLC system consisted of a Gilson 231XL automatic sample injector, a Gilson 401 dilutor, a Gilson 305 solvent pump, a computer running 715 software (Gilson Medical Electronics, Villiers le Bel, France), and an Applied Biosystems 759A UV absorbance detector set at a wavelength of 317 nm (Applied Biosystems, Foster City, CA, USA) fitted with a 2.4 μ l, 6 mm optical pathlength flowcell from Perkin-Elmer (Warrington, UK). A guard column (20 \times 1.0 mm I.D.) packed with 5 μ m pellicular ODS material from Whatman (Maidstone, UK) and a Hypersil ODS 3 μ m (150 \times 2.1 mm I.D.) (HiChrom, Reading, UK) analytical column were used. The column temperature was maintained at 25°C using a Model 7990 column oven from Jones Chromatography (Hengoed, UK). The Jouan microcentrifuge sample concentrator/evaporator system consisted of the RC 10.22 concentrator coupled to the RCT 120 cold trap and an Edwards 5 vacuum pump. The mobile phase consisted of acetonitrile–aqueous 0.05 *M* phosphate buffer (10:90, v/v). The phosphate buffer contained 0.1% (v/v) triethylamine and was adjusted to pH 7.0 using orthophosphoric acid. The

mobile phase solvents were filtered through a 0.45- μm nylon or nitro-cellulose membrane filter (Whatman), mixed and degassed using helium. The flow-rate was 0.2 ml min^{-1} and the sample injection volume was $20 \mu\text{l}$.

2.3. Calibration standards

Calibration samples of metronidazole were prepared in blank plasma ($50 \mu\text{l}$), saline ($300 \mu\text{l}$) and in mobile phase (for determination of % recovery of metronidazole). Saline was used to sample the stomach contents (see Section 2.6) and was therefore also used as a blank for gastric juice aspirate. Stomach tissue calibration samples were prepared by adding blank stomach tissue obtained from rats (0.25 g), to stock solutions of metronidazole in saline. The plasma, gastric aspirate and stomach tissue calibrations samples were prepared by adding known concentrations of metronidazole dissolved in water to give a metronidazole concentration range of 5.0 to $200 \mu\text{g ml}^{-1}$ for plasma and gastric juice aspirate and 1.0 to $40 \mu\text{g g}^{-1}$ for tissue.

2.4. Extraction procedure

To $50 \mu\text{l}$ of a plasma sample in an Eppendorf tube (1.5 ml capacity), $10 \mu\text{l}$ of the 2-methyl-5-nitroimidazole internal standard solution (0.25 mg ml^{-1}) and $500 \mu\text{l}$ of an ice cold acetonitrile–perchloric acid (5% , w/v in water) ($50:50$) mixture were added. The solution was vortex-mixed briefly and 0.5 g of solid anhydrous potassium carbonate was added to produce a mixture of neutral pH. The suspension was then vortex-mixed for 5 min and centrifuged for 10 min at $11\,600 \text{ g}$. The organic (top) layer was transferred to a clean Eppendorf tube and evaporated in vacuo for 40 min at 50°C using the Jouan microcentrifuge evaporator. The residue was reconstituted in $200 \mu\text{l}$ mobile phase, transferred onto an Eppendorf filter device (Vectaspin micro, $0.45 \mu\text{m}$ pore size polypropylene filter inserts) (Whatman) and centrifuged at $11\,600 \text{ g}$ for 10 min to remove undissolved particulate matter. The filtrate was analysed for metronidazole content by HPLC.

The rat stomach was snap-frozen in liquid nitrogen and transferred into a polyethylene sample bag. The

frozen stomach was then crushed using a table-top vice to a homogenous mass. This was then re-frozen in the bag in liquid nitrogen, transferred into a pre-weighed Eppendorf tube (1.5 ml capacity) and weighed. Phosphate-buffered saline ($200 \mu\text{l}$) was added to obtain a homogenised stomach tissue sample for each stomach analysed. Gastric aspirate was treated in an identical manner to plasma. The extraction of homogenised stomach tissue was performed twice by admixture and removal of an extra aliquot of acetonitrile ($200 \mu\text{l}$) to the tissue homogenate after the initial removal of the organic layer. The two organic layers were subsequently pooled and treated identically to the extracts of the plasma and gastric aspirate samples.

2.5. Validation

The peak-area ratio of metronidazole to the internal standard was calculated and used to construct calibration lines of peak-area ratio against drug concentration in plasma, gastric aspirate and stomach tissue by linear regression analysis. Slope, intercept and correlation coefficient of the calibration lines were determined. The analyte recovery was calculated by comparing the peak area of the extracted samples to the peak area from the unextracted standard solutions of equivalent concentration prepared in mobile phase. Quality control samples of fixed concentration prepared from the appropriate blank matrix (see Table 1) were prepared to determine the intra- and inter-day variability. Intra-day assay variations were determined by analyzing replicate samples ($n=6$ at each concentration) on a single day. Inter-day assay variations were determined by analyzing samples ($n=6$ at each concentration) on 6 separate days.

2.6. Metronidazole pharmacokinetic study in rats

Male Wistar rats, weighing $250\text{--}370 \text{ g}$ were fasted 24 h prior to experimentation. Following anaesthesia, the stomach was cannulated to sample the gastric juice produced by securing a cannula into the antral part of the stomach via the duodenum and pylorus. Gastric acid secretion was either suppressed, by administering a single intravenous (i.v.) bolus of

Table 1
Validation of the determination of metronidazole in rat plasma, gastric aspirate and gastric tissue: recovery, intra- and inter-day precision and accuracy for the assay

	Spiked metronidazole ($\mu\text{g ml}^{-1}$) or ($\mu\text{g g}^{-1}$)	Recovery ($n=3$) (%)	Intra-assay ($n=6$)			Inter-assay ($n=6$)		
			Measured (mean \pm SD) ($\mu\text{g ml}^{-1}$) or ($\mu\text{g g}^{-1}$)	RSD (%)	Accuracy (%)	Measured (mean \pm SD) ($\mu\text{g ml}^{-1}$) or ($\mu\text{g g}^{-1}$)	RSD (%)	Accuracy (%)
Gastric aspirate	5.1	81.9 \pm 3.3	4.9 \pm 0.25	5.1	96.3	5.06 \pm 0.39	7.7	99.3
	20.4	81.2 \pm 3.5	19.2 \pm 1.1	5.6	94.3	20.2 \pm 0.37	1.8	99.0
	203.7	88.8 \pm 3.0	199.7 \pm 2.7	1.4	98.6	202.6 \pm 4.6	2.2	99.5
Plasma	5.1	82.2 \pm 3.5	5.0 \pm 0.15	2.9	98.8	5.0 \pm 0.24	4.7	98.5
	10.2	89.3 \pm 7.5	10.0 \pm 0.16	1.6	98.4	10.1 \pm 0.12	1.1	99.1
	20.4	81.3 \pm 2.4	20.9 \pm 0.38	1.8	102.4	20.6 \pm 0.54	2.6	98.8
Tissue	8.2	77.6 \pm 5.8	7.56 \pm 0.76	10.1	92.8	8.3 \pm 0.56	6.7	102.0
	16.3	86.5 \pm 8.8	N/A	N/A	N/A	16.6 \pm 0.43	2.6	102.0
	20.4	N/A	20.9 \pm 1.3	6.4	102.9	N/A	N/A	N/A
	40.7	86.5 \pm 2.0	38.6 \pm 5.2	13.4	94.7	40.8 \pm 0.98	2.4	100.1

Metronidazole concentrations in gastric juice aspirate and plasma in $\mu\text{g ml}^{-1}$ and gastric tissue concentrations in $\mu\text{g g}^{-1}$.

10 $\mu\text{mol kg}^{-1}$ omeprazole sodium dissolved in 0.9% saline via the tail vein, or stimulated, by administering i.v. boluses of 25 $\mu\text{g kg}^{-1}$ pentagastrin every 15 min. Either drug was started immediately after placement of the duodenal cannula.

The experiment was started by administering a 9 mg kg^{-1} i.v. bolus and starting an i.v. infusion (3.6 $\text{mg kg}^{-1} \text{h}^{-1}$) of metronidazole. Blood plasma samples were taken at 15 min intervals for 2 h. The complete gastric contents was aspirated at the same time points. Gastric tissue samples were removed at the end of the experiment. Plasma, gastric juice aspirate and tissue samples were immediately snap-frozen in liquid nitrogen and stored at -80°C pending analysis.

3. Results and discussion

3.1. Chromatography

Representative chromatograms of blank plasma, gastric juice aspirate and stomach tissue and for samples containing metronidazole are shown in Fig. 2. The retention times for metronidazole and that of internal standard 2-methyl-5-nitroimidazole were 3.7 and 3.0 min, respectively. No interfering peaks were noticeable in the chromatograms of blank plasma, gastric juice aspirate and gastric tissue and there was no interference from other drugs (omeprazole and pentagastrin) used in the *in vitro* experiments. Table 1 summarises the recovery after extraction, the accuracy and the reproducibility of the analysis. The pH of the mobile phase (pH 7.0) was chosen so that metronidazole, a weak base with $\text{p}K_a=2.52$, was not ionised under the analytical conditions used. The analysis of metronidazole in its unionised form and the addition of triethylamine to the mobile phase ensured stable chromatography conditions and an absence of peak tailing.

Eight-point calibration lines were used to validate the assay in the aforementioned biological matrices over the concentration ranges outlined above. Corresponding correlation coefficients (r^2) were 0.9997 ± 0.0002 for gastric juice aspirate, 0.9987 ± 0.001 for plasma and 0.9986 ± 0.0011 for tissue ($n=6$), confirming the linearity of the assay. The lower limits of detection (signal-to-noise ratio of

3:1) were 0.015 $\mu\text{g ml}^{-1}$ for gastric juice aspirate and plasma, and 0.010 $\mu\text{g g}^{-1}$ for gastric tissue. Inter- and intra-day accuracy and precision at a range of concentrations were all within acceptable limits (Table 1).

The apparent recovery of metronidazole added to blank gastric tissue was in the range of 78–87%, but this approach could not confirm that metronidazole in a real tissue sample would be extracted from gastric cells. To verify that metronidazole is readily extracted from tissue from which it is incorporated (i.e., tissue derived from experiments) we undertook repetitive extraction of the tissue until no more metronidazole could be recovered. Exhaustive extraction of the tissue homogenates indicated that 95% of the total amount that could be recovered was extracted after the first repetition of the liquid–liquid extraction (data not shown)

Tinidazole, as used by Holt et al. [15] and Pollak [12], was initially evaluated as internal standard but showed a small but variable degradation during the extraction process as reported previously by Jessa et al. [13], and therefore proved unsuitable. It was found that the 2-methyl-5-nitroimidazole was more stable than tinidazole and gave less variable results and hence was more suitable for the purposes of this assay. Other reported methods for metronidazole have used sample preparation with perchloric acid [5,13], trichloroacetic acid [6] or solvent extraction [7,8]. We compared 10% (w/v) trichloroacetic acid, 5% (w/v) perchloric acid and 50% acetonitrile and found that perchloric acid was superior in terms of the % recovery of metronidazole, linearity of calibration and reduction of interference from endogenous compounds on the chromatogram. The optimised extraction procedure itself was easy, economical and could be entirely carried out in a 1.5 ml Eppendorf vial. Other methods using less than 200 μl plasma [6,16,17] were unsuitable due to either non-specific sample preparation procedures or insufficient sensitivity. The method described here has a detection limit of 0.75 ng on-column which is at least 10-fold more sensitive than other published methods [6,16,17].

3.2. Metronidazole pharmacokinetic study in rats

A steady-state concentration of metronidazole in

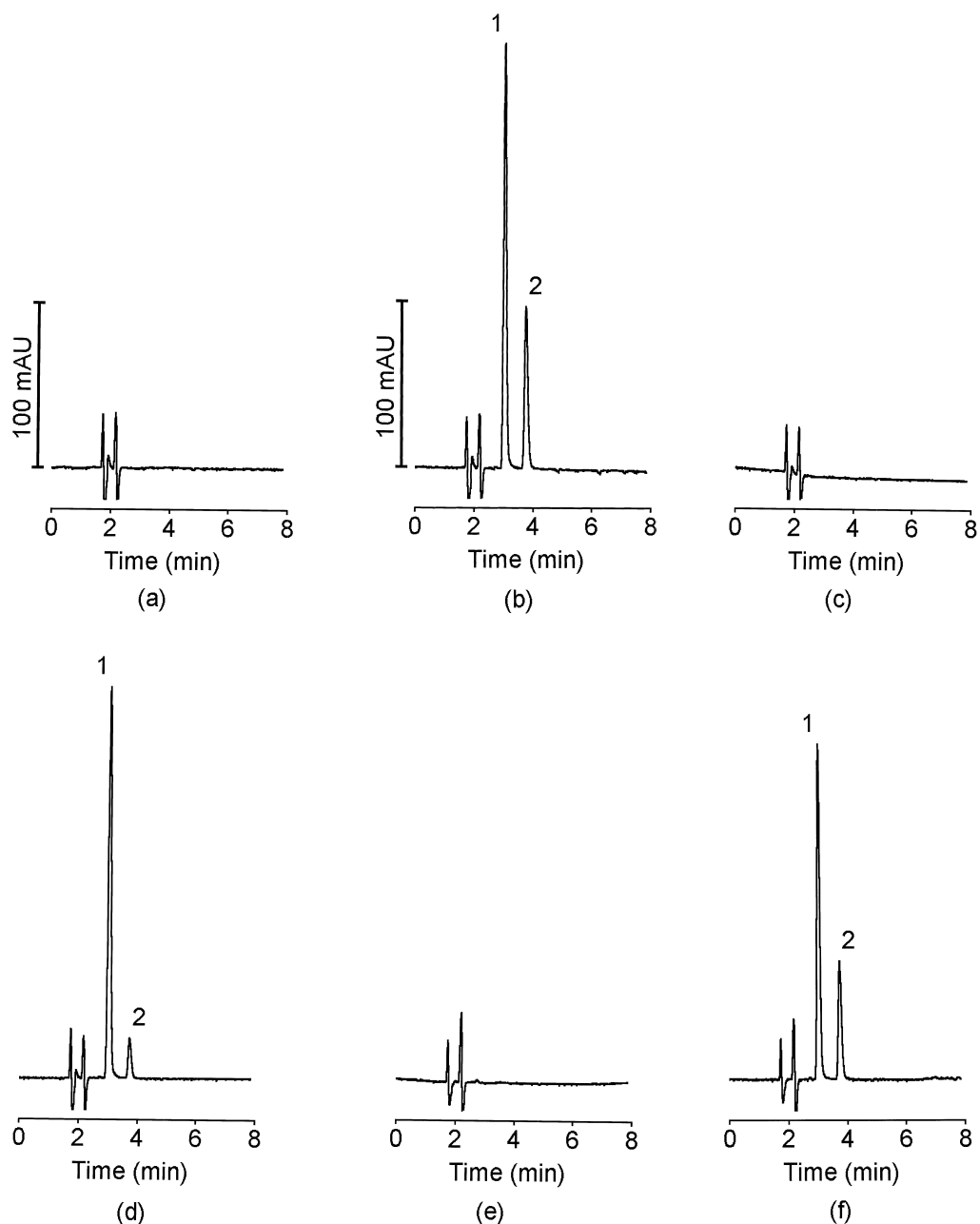


Fig. 2. Chromatograms of extracts of: blank gastric aspirate (a), gastric aspirate spiked with metronidazole ($20.37 \mu\text{g ml}^{-1}$) (b), blank plasma (c), plasma spiked with metronidazole ($5.09 \mu\text{g ml}^{-1}$) (d), blank gastric tissue (e) and gastric tissue spiked with metronidazole ($4.07 \mu\text{g g}^{-1}$) (f). Peaks: 1=2-methyl-5-nitroimidazole and 2=metronidazole.

plasma was reached within 15 min as depicted in Fig. 3 for a typical rat treated with pentagastrin. Fig. 3 also shows the metronidazole concentration time

profile in gastric aspirate, with the metronidazole concentration being higher in gastric aspirate after 30 min than in plasma. The tissue concentration of

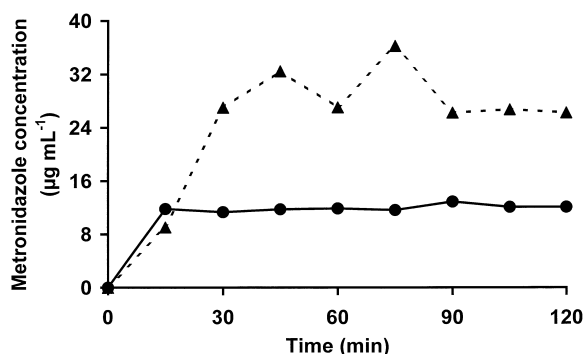


Fig. 3. Typical metronidazole concentration vs. time profiles following intravenous infusion of metronidazole for gastric aspirate (broken line) and plasma (solid line) from a rat treated with pentagastrin.

metronidazole at the end of the experiment was found to be $11.3 \mu\text{g g}^{-1}$, slightly less than the plasma steady-state concentration ($11.9 \mu\text{g ml}^{-1}$). The assay method has been used to determine metronidazole in more than 1000 samples over a period of 1 year and has proved to be robust and reliable in use. The results were subsequently used to calculate the amount of metronidazole transferred into the gastric juice (gastric transfer fraction) and other pharmacokinetic parameters [18].

4. Conclusion

We have developed a rapid, practical and sensitive HPLC assay for the determination of metronidazole in small volumes of rat plasma, gastric aspirate and gastric tissue. The short time of analysis, sensitivity and reproducibility of the method make it particularly useful for the pharmacokinetic studies in individual rats for which it was developed.

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