ELSEVIER

Journal of Chromatography B, 677 (1996) 374–379

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short communication

Rapid and selective high-performance liquid chromatographic method for the determination of metronidazole and its active metabolite in human plasma, saliva and gastric juice

M.J. Jessa[a], D.A. Barrett[a], P.N. Shaw[a,*], R.C. Spiller[b]

[a]*Department of Pharmaceutical Sciences, University of Nottingham, University Park,, Nottingham NG7 2RD, UK*
[b]*Division of Gastroenterology, University Hospital, Queen's Medical Centre,, Nottingham, NG7 2UH, UK*

Received 3 August 1995; revised 20 September 1995; accepted 28 September 1995

Abstract

A rapid and selective HPLC method has been developed for the separation and quantitation of metronidazole and its hydroxylated metabolite in human plasma, saliva and gastric juice. The assay requires a simple protein precipitation step prior to analysis and is selective, sensitive and reproducible. The limits of quantitation (0.5-ml sample) were at least 0.25 μg/ml for metronidazole and 0.20 μg/ml for its hydroxy metabolite. A Hypersil ODS 5 μm (150×4.6 mm I.D.) column was used with a mobile phase of acetonitrile–aqueous 0.05 *M* potassium phosphate buffer (pH 7) containing 0.1% triethylamine (10:90) delivered at a flow-rate of 1.0 ml/min.

Keywords: Metronidazole; Hydroxymetronidazole

1. Introduction

Metronidazole [1-(2 hydroxyethyl)-2-methyl-5-nitroimidazole, Fig. 1] is widely used for the treatment of protozoal and anaerobic bacterial infections [1], [2]. It is also used in combination therapy with other antibiotics and/or acid-suppressing agents for the treatment of gastric *Helicobacter pylori* infections. Metronidazole is metabolised primarily in the liver by oxidative processes, the two major metabolites being 1-(2-hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole (hydroxymetronidazole, Fig. 1) and 2-methyl-5-nitroimidazole-1-acetic acid [3]. The acetic acid metabolite is only found in urine and does not posses any pharmacological activity. However, hydroxymetronidazole has, against certain strains of bacteria, an antimicrobial potency approximately 30% that of metronidazole [4] and can be detected readily in the systemic circulation.

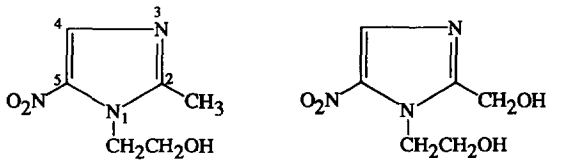

Fig. 1. Chemical structures of metronidazole and hydroxymetronidazole.

Methods reported previously have used perchloric acid [5,6] or trichloroacetic acid [7] as protein

*Corresponding author.

0378-4347/96/$15.00
SSDI 0378-4347(95)00421-1

precipitants prior to HPLC analysis. However, such a procedure may cause degradation of bonded silica HPLC packing materials due to the acidity (pH<1.5) of the injected sample. Other sample preparation methods have used liquid–liquid extraction [8–10] or dilution of samples with buffered aqueous mobile phase [11–13]. These methods are either complex or result in poor selectivity in the extraction process [14]. We describe herein an HPLC assay which permits the rapid and specific detection of metronidazole and hydroxymetronidazole in human plasma, saliva and gastric juice. This method is highly selective and results in an extract which has a neutral pH and therefore will prolong the working life of the HPLC column.

2. Experimental

2.1. Chemicals

Metronidazole and tinidazole were purchased from Sigma (Poole, UK) and hydroxymetronidazole was a gift from Dr. S Loft, University of Copenhagen. Potassium dihydrogen phosphate, orthophosphoric acid (85%, v/v) and triethylamine were all purchased from Fluka (Poole, UK). Di-sodium hydrogen phosphate, anhydrous potassium carbonate and HPLC grade acetonitrile were all purchased from Fisons (Loughborough, UK). Perchloric acid (70%, w/v) was obtained from BDH (Poole, UK). All chemicals were of analytical grade or better.

2.2. Chromatographic system

The HPLC system consisted of an automatic sample injector 231, diluter 401, 303 solvent pump, pressure measurement unit 802C and computer software 715 (Gilson Medical Electronics, Villiers le Bel, France), an Applied Biosystems (Foster City, CA, USA) 759A UV absorbance detector set at a wavelength of 317 nm. The analytical column used was a Hypersil ODS 5-μm (Shandon HPLC, Runcorn, UK) (150×4.6 mm I.D.). A guard column (20×2 mm I.D.) packed with Hypersil ODS 5-μm material was placed between the injector and the analytical column. 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 was delivered at 1.0 ml/ min and the sample injection volume was 100 μl.

2.3. Calibration standards

Calibration samples (0.5 ml) of metronidazole and hydroxymetronidazole were prepared in blank plasma, saliva and gastric juice and were stored at −20°C. The plasma calibration was performed over concentration ranges of 0.05 to 12.5 μg/ml for metronidazole and 0.05 to 2 μg/ml for hydroxymetronidazole; saliva calibrations were from 0.05 to 12.5 μg/ml for metronidazole and from 0.10 to 2 μg/ml for hydroxymetronidazole; gastric juice calibrations were from 0.05 to 50 μg/ml for metronidazole and from 0.05 to 5 μg/ml for hydroxymetronidazole.

2.4. Extraction procedure

To a gastric juice sample (0.5 ml) in an Eppendorf tube (1.5-ml capacity) was added 20 μl of the aqueous tinidazole internal standard solution (10 μg/ml) and 50 μl of 50% w/v perchloric acid. The solution was vortex-mixed briefly and 1.5 g of solid anhydrous potassium carbonate was added, producing a mixture of neutral pH. Acetonitrile (300 μl) was added and the sample centrifuged at 11 600 *g* for 6 min. An aliquot of the supernatant (180 μl) was then transferred to a 2-ml glass HPLC vial and the solvent was removed by evaporation (50°C) under a flow of nitrogen gas. The sample was reconstituted in 0.5 ml of mobile phase before injection onto the HPLC system. Plasma and saliva samples were treated in an identical manner to gastric juice samples with the exception that the internal standard solution containing tinidazole was not added.

2.5. Validation

The peak-area ratios of metronidazole and hydroxymetronidazole to the internal standard were calculated and used to construct calibration lines of peak-area ratio against drug concentration in gastric

juice. Where no internal standard was used (plasma and saliva samples) the metronidazole or hydroxymetronidazole peak area was plotted against the respective sample concentration. Linear regression analysis was used to calculate the slope, intercept and the correlation coefficient of the calibration lines. The analyte recovery was calculated by comparing the peak area of the extracted samples to the peak area from unextracted standard solutions of equivalent concentration prepared in mobile phase.

2.6. Biological application

The assay procedure described above was used to determine the concentration–time profile of metronidazole and hydroxymetronidazole in eight healthy male volunteers who had received metronidazole 400 mg orally or intravenously. In all experiments plasma was collected at timed intervals over 28 h and saliva samples over 4 h. Gastric juice samples were collected at timed intervals by aspiration during the intravenous experiments.

3. Results and discussion

Representative chromatograms of blank gastric juice, plasma and saliva and for samples containing metronidazole and hydroxymetronidazole are shown in Fig. 2, Fig. 3 and Fig. 4. The retention times for metronidazole and hydroxymetronidazole were 6.7 and 5.1 min respectively, and that of the internal standard tinidazole (when used for the gastric juice samples) was 13.9 min. No interfering peaks were noticeable in the chromatograms of blank gastric juice, plasma or saliva or from those of samples obtained from the volunteers who were co-administered omeprazole in addition to metronidazole.

The assay was validated using eight point calibration lines in appropriate biological fluids and covering ranges outlined above. These calibration lines were found to be linear for both metronidazole (plasma: r^2= 0.991±0.012; gastric juice: r^2= 0.997±0.003; saliva: r^2=0.997±0.002) and hydroxymetronidazole (plasma: r^2 0.993±0.006; gastric juice: r^2=0.986±0.018; saliva: r^2= 0.996±0.003). The lower limits of quantitation for metronidazole (signal-to-noise ratio of 3:1) were

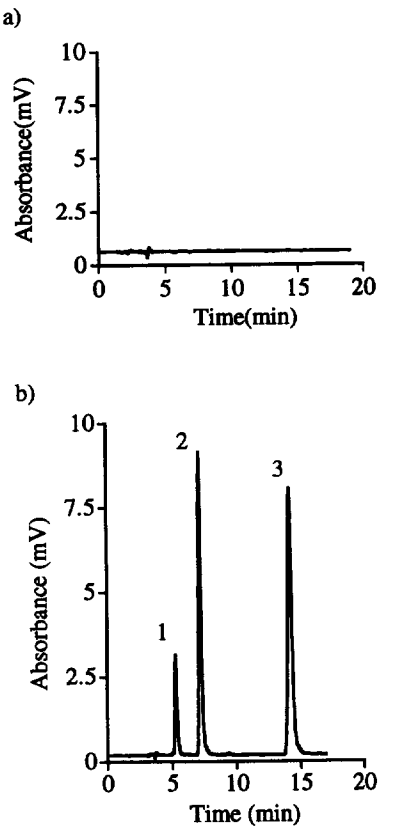

Fig. 2. (a) Chromatogram of an extracted blank human gastric juice sample. (b) Chromatogram of an extract of human gastric juice, containing: 1, hydroxymetronidazole (1.4 μg/ml); 2, metronidazole (4.8 μg/ml); 3, tinidazole (internal standard). The sample was taken 195 min after the start of the intravenous administration of metronidazole (400 mg).

found to be 0.25 μg/ml (plasma and gastric juice) and 0.1 μg/ml (saliva) and for hydroxymetronidazole was 0.20 μg/ml (plasma, gastric juice and saliva) and these were suitable for the determination of the two analytes in the pharmacokinetic study outlined above.

The recoveries of metronidazole and hydroxymetronidazole in each of the biological fluids were reproducible with the absolute values being consistently higher for the metabolite than for the parent drug (Table 1). The inter-day accuracy and precision results (expressed as the coefficient of variation of at least six replicate concentrations) for metronidazole (Table 2) and hydroxymetronidazole (Table 3) at a

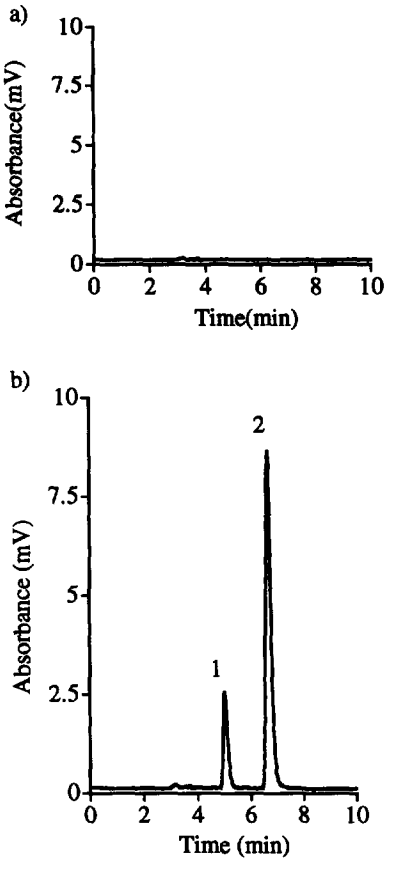

Fig. 3. (a) Chromatogram of an extracted blank human plasma sample. (b) Chromatogram of an extract of human plasma, containing: 1, hydroxymetronidazole (0.9 μg/ml); 2, metronidazole (4.3 μg/ml). The sample was taken 6 h after the start of the intravenous administration of metronidazole (400 mg).

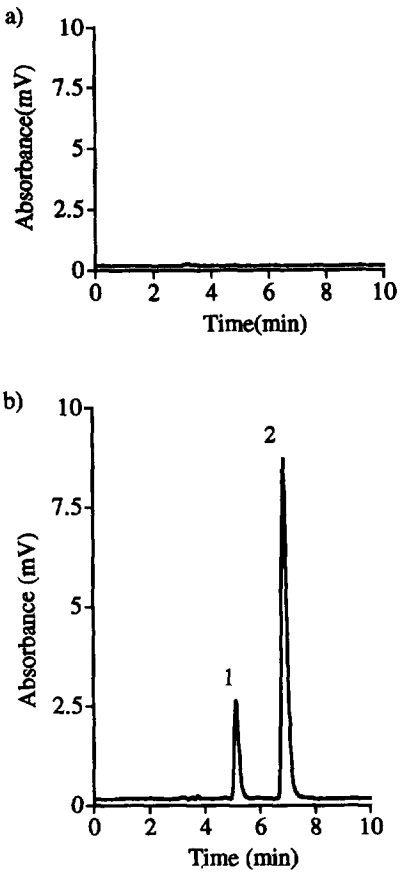

Fig. 4. (a) Chromatogram of an extracted blank human saliva sample. (b) Chromatogram of an extract of human saliva, containing: 1, hydroxymetronidazole (0.5 μg/ml); 2, metronidazole (7.7 μg/ml). The sample was taken 2 h after the start of the intravenous administration of metronidazole (400 mg).

range of concentrations were all within acceptable limits.

Tinidazole has previously been used as an internal standard [15] in the assay of metronidazole, and in our method of extraction it performs well when used to quantitate metronidazole and hydroxymetronidazole from gastric juice samples. However, when used with plasma or saliva samples, small but variable degradation of tinidazole was observed during the extraction process. These samples were therefore analysed using an external standardisation procedure. Other potential internal standards which were examined but were found to be unsuitable included ornidazole and 2-methyl-4-nitro-imidazole propionitrile; these were acid-labile and 2-methyl-5-nitroimidazole eluted with the same retention time as hydroxymetronidazole.

Metronidazole is completely absorbed after oral administration and is distributed in total body water, and it has been reported that the concentration of metronidazole in saliva is equivalent to that observed in plasma [16]. The metronidazole concentrations in saliva samples were compared with those obtained from plasma samples taken at the same time. Our data demonstrate a strong linear relationship between saliva and plasma concentration of both analytes [Fig. 5(a) and Fig. 5(b)] which compares well with that reported previously [16]. It is therefore possible

Table 1
Recovery of metronidazole and hydroxymetronidazole from biological fluids

Sample	Recovery (%)		
	Metronidazole	Hydroxymetronidazole	Tinidazole
Plasma (n=8)	66.22 ± 6.90	88.90 ± 2.62	N/A
Gastric juice (n=8)	74.31 ± 9.25	104.78 ± 12.78	83.74±11.96
Saliva (n=8)	73.60 ± 8.66	97.84 ± 7.34	N/A

Results are expressed as mean ± standard deviation. N/A = not applicable. Tinidazole was not added to plasma or saliva samples. See text for details.

Table 2
Inter-day precision and accuracy for the metronidazole assay

Sample	Nominal concentration (μg/ml)	Measured concentration (μg/ml)	n	Bias (%)	C.V. (%)
Plasma	10	9.67±0.86	8	−3.3	8.9
	5	4.98±0.48	8	−0.4	9.7
	0.5	0.54±0.05	7	+8.0	8.6
Gastric juice	50	50.05±0.49	6	+0.1	1.0
	5	5.02±0.23	6	+0.4	4.5
	0.5	0.55±0.09	6	+10.0	17.1
Saliva	10	9.91±0.39	8	−0.9	3.9
	5	5.23±0.35	8	+4.6	6.7
	0.5	0.45±0.07	8	−10.0	16.2

Results are expressed as mean ± standard deviation together with the coefficient of variation.

to state that the measured saliva concentration may be used as a non-invasive marker of the plasma concentration of both metronidazole and the active metabolite, hydroxymetronidazole.

Acknowledgments

We thank Dr. A. F. Goddard, Division of Gastroenterology University Hospital, Nottingham for

Table 3
Inter-day precision and accuracy for the hydroxymetronidazole assay

Sample	Nominal concentration (μg/ml)	Measured concentration (μg/ml)	n	Bias (%)	C.V. (%)
Plasma	2	2.03 ± 0.07	8	+1.5	3.5
	1	0.97 ± 0.05	8	−3.0	5.2
	0.5	0.48 ± 0.05	8	−4.0	10.8
Gastric juice	5	4.89 ± 0.19	6	−2.2	3.8
	1.5	1.57 ± 0.09	6	+4,7	5.6
	0.5	0.53 ± 0.07	6	+6.0	13.2
Saliva	2	2.00 ± 0.04	8	0	1.8
	1	1.02 ± 0.04	8	+2.0	3.5
	0.5	0.51 ± 0.02	8	+2.0	4.6

Results are expressed as mean ± standard deviation together with the coefficient of variation.

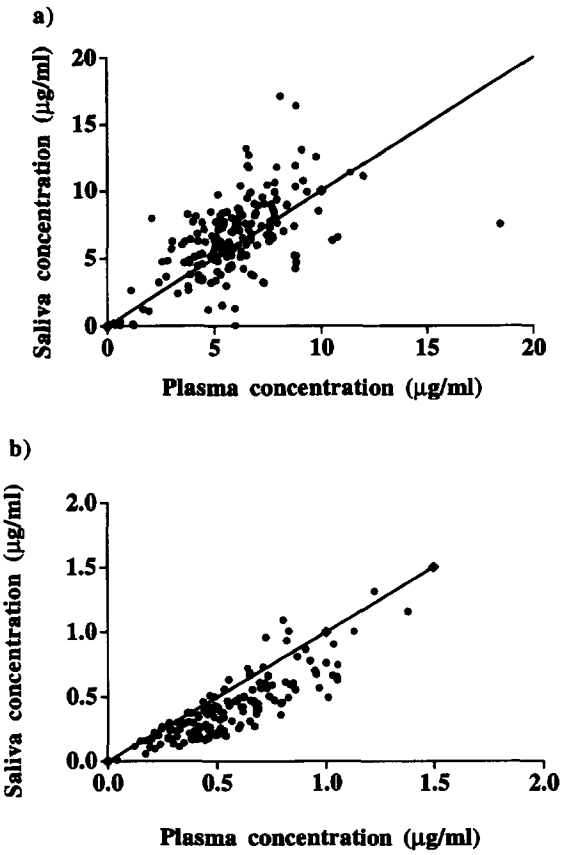

Fig. 5. (a) Relationship between saliva and plasma concentrations of metronidazole in human volunteers. Equation of line of best fit $y = 0.77x - 2.50$. Pearson correlation coefficient $= 0.600$ ($p < 0.0001$, $n = 179$). Solid line is the line of identity. (b) Relationship between saliva and plasma concentrations of hydroxymetronidazole in human volunteers. Equation of line of best fit $y = 0.82x + 0.60$. Pearson correlation coefficient $= 0.846$ ($p < 0.0001$, $n = 147$). Solid line is the line of identity.

his invaluable contribution to the running of the volunteer study and Dr. Stefen Loft, Department of Pharmacology, University of Copenhagen for kindly supplying the hydroxymetronidazole.

References

[1] R.N. Brogden, R.C. Heel, T.M. Speight and G.S. Avery, Drugs, 16 (1978) 387–417.
[2] E.D. Ralph, Clin. Pharmacokin., 8 (1983) 43–62.
[3] J.E. Stambaugh, L.G. Feo and R.W. Manthei, J. Pharmacol. Exp. Ther., 161 (1968) 373–381.
[4] E.D. Ralph and M.M. Kirby, J. Infect. Dis., 132 (1975) 587–591.
[5] I. Nilsson-Ehle, B. Ursing and P. Nilsson-Ehle, Antimicrob. Agents Chemother., 19 (1981) 754–760.
[6] B. Davis, D.D. Glover and B. Larsen, Am. J. Obstet. Gynecol., 149 (1984) 802–804.
[7] H.R. Rabin, R.C. Urtasun, J. Partington, D. Koziol, M. Sharon and K. Walker, Canc.Treat. Report., 64 (1980) 1087–1095.
[8] L.P. Hackett and L.J. Dusci, J. Chromatogr., 175 (1979) 347–349.
[9] R.A. Marques, B. Stafford, N. Flynn and W. Sadee, J. Chromatogr., 146 (1978) 163–166.
[10] W. Siegmund, M. Zschiesche, G. Franke and A. Wilke, Pharmazie, 47 (1992) 522–524.
[11] L.A. Wheeler, M. DeMeo, M. Halula, L. George and P. Heseltine, Antimicrob. Agents Chemother., 13 (1978) 205–209.
[12] J. Adamovics, J.Chromatogr., 309 (1984) 436–440.
[13] K. Róna and B. Gachályi, J.Chromatogr., 420 (1987) 228–230.
[14] G.A. Woollard, J.Chromatogr., 303 (1984) 222–224.
[15] D.E. Holt, J. DeLouvois, R. Hurley and D. Harvey, J. Antimicrob. Chemother., 26 (1990) 107–115.
[16] S. Loft, H.E. Poulsen, J. Sonne and M. Dossing, Clin. Pharmacol. Ther., 43 (1988) 420–428.