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Simultaneous quantification of metronidazole, tinidazole, ornidazole and morinidazole in human saliva

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ABSTRACT

The aim of this study was to develop a rapid and sensitive method for the simultaneous quantification of metronidazole (MEZ), tinidazole (TNZ), ornidazole (ONZ) and morinidazole (MNZ) in human saliva. A reversed-phase high-performance liquid chromatography (HPLC) method with ultraviolet (UV) detection at 318 nm was carried out on a C18 column, using a mixture of potassium dihydrogen phosphate buffer, acetonitrile, and methanol (55:15:30, v/v/v) as a mobile phase with a flow rate of 1.0 ml/min. The saliva samples (100 μl) were firstly deproteinized by precipitation with methanol (400 μl), after which they were centrifuged and the supernatants were directly injected into the HPLC system. This method produced linear responses in the concentration ranges of 25.2–5040.0, 23.9–4790.0, 25.4–5080.0, 25.0–5000.0 ng/ml with detection limits of 6.0, 17.6, 10.0 and 11.3 ng/ml for MEZ, TNZ, ONZ and MNZ (S/N = 3), respectively. The methods were validated in terms of intra- and inter-batch precision (within 7.3% and 9.1%, respectively), accuracy, linearity, recovery and stability. The study proved that HPLC is both sensitive and selective for the simultaneous quantification of MEZ, TNZ, ONZ and MNZ in human saliva using a single mobile phase.

1. Introduction

Periodontal diseases are groups of infections and inflammatory conditions, including gingivitis and periodontitis that affect teeth-supporting structures. These diseases occur when bacteria from dental plaque invade surrounding tissues and from the accumulation of plaque at the gingival margin, which, in turn, induces an inflammatory response. The therapeutic goal is the removal of the bacteria responsible for the infection by way of mechanical cleaning and the use of topical application of antimicrobial agents, such as clindamycin, 5-nitroimidazole derivatives and ofloxacin [1].

Metronidazole (MEZ) is a first-generation member of 5-nitroimidazole derivatives with bactericidal activity against most anaerobic bacteria and protozoa. It is now used as the principal treatment for *Helicobacter pylori* infections, amebiasis, giardiasis, trichomoniasis, bacterial vaginosis, Crohn's disease and as a prophylactic antibiotic in surgical interventions. It is also widely used as an antibacterial compound in the treatment of some types of periodontal disease [2]. MEZ is one of the most globally used drugs and has been in use for over 43 years. Furthermore, it is among the top 100 most frequently prescribed drugs in the United States and among the top 10 prescribed during pregnancy [3].

Tinidazole (TNZ) and ornidazole (ONZ) are both second- and third-generation members of the 5-nitroimidazole group with selective activity against anaerobic bacteria and protozoa, such as *Trichomonas vaginalis*, *Entamoeba histolytica* and *Giardia lamblia*. They can also be used effectively against MEZ-resistant strains of *T. vaginalis* and recurrent periodontitis [4,5]. TNZ has been widely used in Europe as well as developing countries for over two decades with an established efficacy and an acceptable tolerability [6]. TNZ has also proven to be effective in the treatment of respiratory tract infections, intra-abdominal sepsis, obstetrical and gynecological infections, colonic and abdominal surgery, emergency appendectomy and amebic liver abscess [7–9].

With the development of bacterial resistance, there is an increasing need for the development of new 5-nitroimidazole derivatives as very selective agents against anaerobic bacteria. Morinidazole (MNZ, Fig. 1) is a new synthetic 5-nitroimidazole derivative that was developed specifically for the treatment of anaerobic bacterial infections, such as periodontal diseases and gynecological diseases, and is currently in Phase II clinical trials [10].

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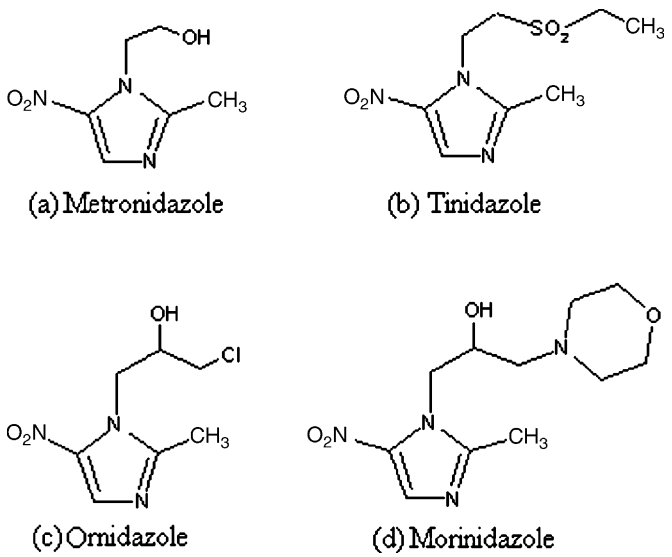

Fig. 1. Chemical structure of metronidazole (MEZ, a), tinidazole (TNZ, b), ornidazole (ONZ, c) and morinidazole (MNZ, d).

To date, only a few articles have reported quantification methods for MEZ, TNZ and ONZ. These methods include electrochemical methods, high-performance thin-layer chromatography, high-performance liquid chromatography (HPLC), ultraviolet (UV) spectrophotometry as well as liquid chromatography with tandem mass spectrometry (LC–MS/MS) [11–15]. The extraction methods used include ethyl acetate extraction [12], methanol precipitation [13,16] and perchloric acid [17]. To the best of our knowledge, there have been no recently published studies regarding the simultaneous quantification of all four of these imidazole compounds. The aim of this study was to develop and validate a method for the simultaneous quantification of MEZ, TNZ, ONZ and MNZ in human saliva.

2. Materials and methods

2.1. Materials

MEZ, TNZ and ONZ were supplied by the National Institute for the Control of Pharmaceutical and Biological Products, China. MNZ (99.6% purity, Lot 20050805) was supplied by Hansoh Pharmaceutical Group (China). Acetonitrile and methanol (Merck, Germany) were HPLC grade. Capillary micropipettes were supplied by Zhejiang Sorfa Medical Plastic Co. Ltd., China.

2.2. Equipment and chromatographic conditions

An LC-2010-CHT series HPLC instrument (Shimadzu Corp., Japan) consisting of a UV detector at 318 nm and an automatic injector with 50-μl loop were used. Chromatography was performed on a Kromasil C18 reversed phase column (250 mm × 4.6 mm, 5 μm, EKA Chemicals Inc., Sweden). The mobile phase consisted of potassium dihydrogen phosphate (0.02 mol/l), acetonitrile and methanol (55:15:30, v/v/v, pH 4.51). The flow-rate was set at 1.0 ml/min and the column was kept at a temperature of 40 °C.

2.3. Sample preparation

A 100-μl aliquot of human saliva sample was firstly transferred to a 1.5-ml test tube which was then spiked with 400 μl methanol. After vortexing for 2 min and centrifugation at 14,500 × *g* (rotor capacity 0.5/2 ml × 12, ShangHai Anting Scientific Instrument Factory) for 10 min, the supernatant was next transferred to an autosampler vial and 20 μl was injected into the HPLC system.

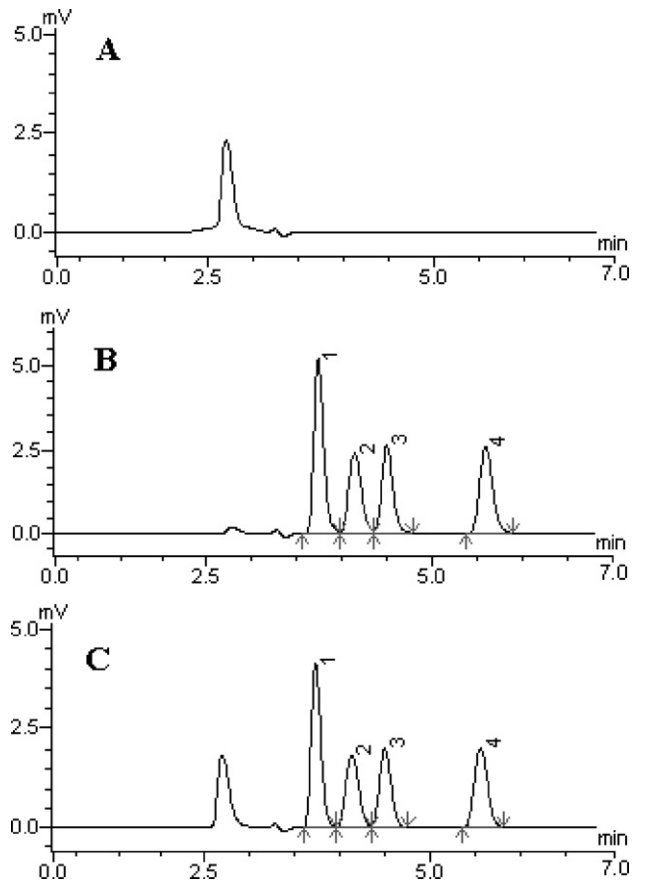

Fig. 2. Typical chromatograms of blank saliva (a), aqueous solution with MEZ, TNZ, MNZ and ONZ (b), and blank saliva spiked with analyte controls (c). 1: MEZ, 2: TNZ, 3: MNZ, 4: ONZ.

2.4. Collection of saliva

The study population consisted of 142 outpatient subjects (80 women and 62 men) all of whom suffered from periodontal diseases. Written consent was obtained from those who agreed to participate voluntarily and ethical clearance was obtained from the hospital's ethics committee. One milliliter of saliva and gingival crevicular fluid (GCF) was collected from the gingival crevice fluid samples from patients with periodontal diseases by way of aspiration through a capillary micropipette into 1.5-ml tubes prior to and again after i.v. infusion of ONZ (500 mg) or MNZ (500 mg) at intervals of 15 min. After collection, the saliva samples were immediately stored at −70 °C until analyzed.

3. Results

3.1. Specificity (selectivity)

Representative reversed-phase HPLC chromatograms of saliva samples are shown in Fig. 2. Retention times were approximately 3.7, 4.1, 4.5 and 5.6 min for MEZ, TNZ, MNZ and ONZ, respectively. The peaks of interest were well separated and free from interference of any endogenous substances.

Table 1
Results from regression analysis of the calibration curves, LOQ and LOD.

Parameters	Linear regression equations	Correlation coefficient, r	Linearity (ng/ml)	LOQ (ng/ml)	LOD (ng/ml)
MEZ	$C = 0.0836A - 13.308$	0.9998	25.2–5040.0	25.2	6.0
TNZ	$C = 0.1409A + 5.7038$	0.9998	23.9–4790.0	23.9	17.6
MNZ	$C = 0.141A - 12.082$	0.9997	25.4–5080.0	25.4	10.0
ONZ	$C = 0.1197A - 8.0669$	0.9997	25.0–5000.0	25.0	11.3

LOD, limit of detection; LOQ, limit of quantification; R.S.D., relative standard deviation.

Table 2
The extraction recoveries of MEZ, TNZ, MNZ and ONZ ($n = 3$).

Extraction recovery	MEZ (%)	TNZ (%)	MNZ (%)	ONZ (%)
50 (ng/ml)	95.3 ± 1.1	96.3 ± 12.8	89.7 ± 4.3	80.7 ± 1.5
500 (ng/ml)	89.5 ± 1.1	87.9 ± 1.1	90.7 ± 0.5	96.7 ± 1.3
4750 (ng/ml)	88.0 ± 0.3	86.9 ± 1.3	87.2 ± 3.2	88.0 ± 0.3

3.2. Linearity and calibration curves

Calibration curves were constructed by plotting peak areas versus concentrations of MEZ, TNZ, MNZ and ONZ, and the regression equations were calculated. Calibration curves were plotted over the concentration range of MEZ (25.2, 50.4, 100.8, 252.0, 504.0, 1000.8, 2520.0, 5040.0 ng/ml), TNZ (23.9, 47.9, 95.8, 239.5, 479.0, 958.0, 2395.0, 4790.0 ng/ml), MNZ (25.4, 50.8, 101.6, 254.0, 508.0, 1016.0, 2540.0, 5080.0 ng/ml) and ONZ (25.0, 50.0, 100.0, 250.0, 500.0, 1000.0, 2500.0, 5000.0 ng/ml). Each solution was analyzed five times. The least-squares method was used for the calculation of the slope, intercept and correlation coefficient (r). The results are listed in Table 1.

3.3. Limit of detection

The limit of detection (LOD) was separately determined at a signal to noise ratio (S/N) of 3, with a relative standard deviation value of less than 10%, LODs for MEZ, TNZ, ONZ and MNZ were found to be 6.0, 17.6, 11.3 and 10.0 ng/ml, respectively (Table 1).

3.4. Extraction recovery

The extraction recovery (R) of mixtures at three concentration levels (50, 500, 4750 ng/ml) was determined by comparing two different sets of samples. In set 1, blank saliva was spiked with the analytes and prepared as described in Section 2.3, and the obtained peak areas of the analytes were defined as A. In set 2, the analytes were resolved in extracting solution, which was obtained from blank saliva, and the obtained peak areas of the analytes were defined as B. The extraction recovery rate was calculated using the formula: $R\ (\%) = A/B \times 100$. The extraction recoveries of MEZ, TNZ, MNZ and ONZ are shown in Table 2.

3.5. Precision and accuracy

The intra-day and inter-day precisions of the proposed methods were determined by estimating the corresponding responses for three different concentrations (50, 500, 4750 ng/ml) of MEZ, TNZ, MNZ and ONZ five times during the same day and over three consecutive days, respectively. The intra-day precision was ≤7.3%, and inter-day precision was ≤9.1% for each level of MEZ, TNZ, MNZ and ONZ. The results demonstrate that the values were within the acceptable range and that the method is both accurate and precise (Table 3).

3.6. Stability

The stability data obtained from three different concentration levels of 50, 500, 4750 ng/ml demonstrates the suitability of the

Table 3
Results of accuracy and precision.

Parameters	MEZ (ng/ml)	TNZ (ng/ml)	MNZ (ng/ml)	ONZ (ng/ml)
Accuracy (%)	84.8–101.3	89.2–101.1	81.6–103.2	99.5–104.1
Precision (R.S.D., %)				
Intra-day ($n = 5$)	2.8	7.3	3.1	2.9
Inter-day ($n = 3$ runs, 5 replicates per run)	9.1	7.5	5.7	5.0

R.S.D., relative standard deviation.

Table 4
Stability data of MEZ, TNZ, MNZ and ONZ in human saliva under various storage conditions ($n = 3$).

Storage conditions		Short-term stability (8 h at 25 °C)			Long-term stability (6 weeks at −70 °C)		
MNZ (ng/ml)	Added	50.9	508.5	4830.8	50.9	508.5	4830.8
	Found	52.6 ± 1.4	494.8 ± 12.5	4856.5 ± 50.5	54.5 + 2.2	448.6 + 14.4	4685.2 + 121.8
	R.S.D. (%)	2.7	2.5	1.0	2.2	3.2	2.6
ONZ (ng/ml)	Added	50.0	500.0	4750.0	50.0	500.0	4750.0
	Found	50.0 ± 0.9	490.4 ± 6.9	4772.4 ± 46.9	55.3 + 2.9	446.8 + 17.4	4688.3 + 147.3
	R.S.D. (%)	1.8	1.4	1.0	5.3	3.9	3.1
MEZ (ng/ml)	Added	50.4	504.0	4788.0	50.4	504.0	4788.0
	Found	52.2 ± 1.4	495.7 ± 8.4	4811.7 ± 47.4	47.7 + 2.9	494.4 + 23.2	4668.3 + 102.7
	R.S.D. (%)	2.7	1.7	1.0	6.1	4.7	2.2
TNZ (ng/ml)	Added	47.9	479.0	4550.5	47.9	479.0	4550.5
	Found	50.5 ± 2.9	469.0 ± 7.5	4530.8 ± 89.2	49.4 + 4.7	453.1 + 26.3	4432.2 + 172.9
	R.S.D. (%)	5.7	1.6	2.0	9.5	5.8	3.9

Table 5
The concentration of MNZ and ONZ in saliva after finishing a 50-min period i.v. infusion at the interval of 15 min.

	Concentration (ng/ml)
MNZ (n = 34)	4267.5 ± 2307.2
ONZ (n = 29)	4108.1 ± 2388.1

The values are given as the mean ± standard deviation (S.D.).

method. One sample from each concentration level of standard solutions was kept at room temperature for 8 h and another sample from each level was stored at −70 °C for 6 weeks. These were then compared with that of a freshly prepared solution. The results are shown in Table 4.

3.7. Concentration of MNZ and ONZ in saliva

The concentrations of MNZ and ONZ in saliva from patients with periodontal diseases are shown in Table 5. After finishing a 50-min i.v. infusion of MNZ and ONZ, both concentrations reach almost the same saliva levels, at more than 4100 ng/ml.

4. Discussion and conclusions

The concentrations of MNZ and ONZ found in crevice fluid after a 50-min i.v. infusion reach a saliva level of more than 4100 ng/ml, which can inhibit or kill most of the *Bacteroides thetaiotaomicron*.

This is the first method which describes the simultaneous determination of MEZ, TNZ, ONZ and MNZ in human saliva via HPLC-UV detection, using one mobile phase. The presence of each of the four compounds did not interfere with the measurement of the other endogenous compounds in the saliva and the limit of quantity (LOQ) was 25.2, 23.9, 25.4 and 25.0 ng/ml for MEZ, TNZ, MNZ and ONZ, respectively, using 0.1 ml of human saliva. The proposed HPLC method was validated by the evaluation of the following parameters: LOD, LOQ values, relative standard deviation of slope and intercept, correlation coefficient, intra- and inter-day precision, and selectivity. The proposed method has acceptable precision, accuracy and adequate sensitivity.

The main advantage of the method described here is not only its simplicity but also its excellent and consistent recovery with the methanol precipitation. Moreover, the lack of any elaborate or time-consuming sample preparation procedures eliminated the need of an internal standard without compromising the accuracy or precision of the method. It was successfully applied to the analysis of clinical samples obtained from saliva and gingival crevicular fluid (GCF) in Phase II clinical trials of MNZ within a short analysis time (6 min).

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References

[1] L. Perioli, V. Ambrogi, D. Rubini, S. Giovagnoli, M. Ricci, P. Blasi, C. Rossi, J. Control. Release 95 (2004) 521.
[2] K. Patel, I.G. Hopkins, S. Lu, A.R. Tunkel, Int. J. Infect. Dis. 12 (2008) e111.
[3] C. Akay, S.A. Ozkan, Z. Senturk, S. Cevheroglu, II Farmaco 57 (2002) 953.
[4] L.Y. Zhang, Z.J. Zhang, K. Wu, J. Pharm. Biomed. Anal. 41 (2006) 1453.
[5] A. Mombelli, F.A. Gusberti, N.P. Lang, J. Clin. Periodontol. 16 (1989) 38.
[6] H.B. Fung, T.L. Doan, Clin. Ther. 27 (2005) 1859.
[7] M. Bakshi, S. Singh, J. Pharm. Biomed. Anal. 34 (2004) 11.
[8] M.N. Austina, L.A. Meyna, S.L. Hillier, Anaerobe 12 (2006) 227.
[9] C.E. Nord, L. Kager, Infection 11 (1983) 54.
[10] W.X. Lv, Talanta 80 (2010) 1406.
[11] T. Saffaj, M. Charrouf, A. Abourriche, Y. Abboud, A. Bennamara, M. Berrada, II Farmaco 59 (2004) 843.
[12] C. Sagan, A. Salvador, D. Dubreuil, P.P. Poulet, D. Duffaut, I. Brumpt, J. Pharm. Biomed. Anal. 38 (2005) 298.
[13] T.G.D. Nascimento, E.D.D.J. Oliveira, R.O. Macedo, J. Pharm. Biomed. Anal. 37 (2005) 777.
[14] E.R. Pähkla, T. Koppel, M. Saag, R. Pähkla, J. Clin. Periodontol. 32 (2005) 163.
[15] C. Ho, D.W.M. Sin, K.M. Wong, H.P.O. Tang, Anal. Chim. Acta 530 (2005) 23.
[16] J.I.D. Wibawa, P.N. Shaw, D.A. Barrett, J. Chromatogr. B 761 (2001) 213.
[17] T.G. Venkateshwaran, J.T. Stewart, J. Chromatogr. B 672 (1995) 300.