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Short communication

Application of nuclear magnetic resonance spectroscopy for quantitative analysis of miconazole, metronidazole and sulfamethoxazole in pharmaceutical and urine samples

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Abstract

Specific, accurate and precise NMR methods were developed for determining miconazole, metronidazole and sulfamethoxazole antibiotic drugs in authentic, pharmaceutical and urine samples. Proton nuclear magnetic resonance spectroscopy (^{1}H NMR) with maleic acid as an internal standard and DMSO-d_6 as NMR solvent were used. ^{1}H NMR signals at 9.0, 8.06, 7.50 and 6.26 ppm corresponding to miconazole, metronidazole, sulfamethoxazole and maleic acid were respectively used for calculating the concentrations of drugs per unit dose. Average percent recoveries of (97.54–101.10), (98.06–100.46) and (97.83–102.83) with average uncertainties of 1.02, 0.45 and 0.86 were respectively obtained for determining authentic samples of miconazole, metronidazole and sulfamethoxazole in the concentration range of 0.92–170 mg/0.6 ml DMSO-d_6. In pharmaceutical formulations and urine samples, average percent recoveries in the ranges of 97.50–101.33 and 94.46–100.86 were respectively obtained. Relative standard deviations (R.S.D.) $\leq$2.68 were obtained for analyzing the three drugs in authentic, pharmaceutical and urine samples.

Admixtures of the three drugs in authentic, pharmaceutical and urine samples were analyzed. Good precisions (0.79–2.99%) and recoveries (93.40–104.97%) were obtained indicating the high selectivity and resolving power of the developed NMR methods and no needs for separation steps.

Applying statistical Student *t*-test revealed insignificant difference between the real and measured contents at the 95% confidence level. *F*-test revealed insignificant difference in precisions between the developed NMR methods and HPLC methods reported for analyzing miconazole, metronidazole and sulfamethoxazole.

Keywords: Miconazole; Metronidazole; Sulfamethoxazole; NMR spectroscopy; Miconaz; Flagyl; Septazole; Urine; Quantitative analysis

1. Introduction

Because of its high selectivity under appropriate acquisition conditions and no needs for analytes standards, NMR spectroscopy can be particularly useful in quantitative analysis of antibiotics such as miconazole, metronidazole and sulfamethoxazole drugs in different matrices. These drugs are in use in treating severe bacterial, fungal or viral infections. Quantitative NMR determination is usually based on the integration ratio between a specific NMR signal of the analyte and a selected signal in NMR patterns of the internal standard. The possibility of overcoming sample pre-processing gives this method some advantages for in vivo and in vitro medical assays relative to other instrumental methods [1–7]. Compounds in seized drugs and mechanisms of postmortem decomposition have been identified using NMR [8,9].

The lack of absorbing chromophores for UV–vis detection and the needs for special chromatographic detectors as well as the difficulties in establishing highly efficient solid or liquid phase extraction procedures have promoted NMR spectroscopy as the suitable technique for urine analysis of many drugs. Tobramycin, hydrocortisone, adenine, butoxycaine, aesculin and some others have been identified by ^{1}H and ^{13}C NMR in national and international pharmacopoeias [10]. A wide range

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of Xenobiotics such as therapeutic agents, pesticides, solvents and alcohols have been also determined using ^{1}H NMR [11].

Miconazole nitrate (MIZ) "1-[2,4-dichloro-(b-(2,4-dichlorobenzyloxy) phenethyl] imidazole" pocesses a wide antifungal spectrum. It is administered by the troche dosage form or by the intravenous infusion in the treatment of severe systemic fungal infections. It is also applied as a 2.0% cream or powder in infections of nails and skin [12,13].

Metronidazole benzoate (MNZ) "1-(2-benzoyloxyethyl)-5-nitro-2-methyl-imidazole" is active against wide varieties of anaerobic protozoal parasites and bacterial infections. It is also widely used in combination with other antibiotics and acid-suppressing agents in eradication treatment of gastric *Helicobacter pylori* infections [12,14,15].

Sulfamethoxazole (SMZ) "*N*-(5-methyl-3-isoxazoly) sulphanilamide" belongs to the sulfonamide group of chemotherapeutics. Sulfonamide is still an important drug in treatment of acute urinary tract infections. The use of sulfonamides has greatly increased by the introduction of trimethoprim sulfamethoxazole mixtures, which possess synergistic antibacterial effects [16–18].

A number of methods have been reported for the determination of MIZ, MNZ and SMZ. Miconazole in creams was determined using second derivative spectrophotometry with methylene blue as an internal standard with precision $\leq$1.5 [13]. Metronidazole in parenteral and intravenous admixture with ciprofloxacin was analyzed by first derivative spectrophotometry in the range of 2.5–10 μg/ml [12]. First derivative spectrophotometry and reversed-phase HPLC were used for determining metronidazole and miconazole nitrate in ovules. Detection limits of 0.9 and 0.3 μg/ml were reported using derivative spectrophotometry whereas 4.0 μg/ml and 0.5 μg/l were reported using HPLC for both drugs, respectively [19].

Metronidazole (MNZ) and tinidazole (TNZ) in pure drug and pharmaceutical formulations were spectrophotometrically determined by interactions with 3-methylbenzothiazolin-2-one hydrazone (MBTH) and *N*-(1-naphthyl)ethylenediamine dihydrochloride (NEDA). Common excipients used as additives in pharmaceutical preparations did not interfere and highly reproducible results were obtained [18].

The bivariate calibration algorithm was applied to the spectrophotometric simultaneous determination of trimethoprim (TMP), sulfamethoxazole (SMZ) and sulphamethoxypyridazine (SMP) binary mixtures in pharmaceutical and veterinary products [16]. A reported selective spectrophotometric method based on forming a violet coloured azo product by the diazotization and coupling with iminodibenzyl was used for determining a number of sulfa-drugs include sulfamethoxazole (SMZ) in pharmaceutical preparations. Beer's law was obeyed in the concentration range of 0.05–6.0 μg/ml [20].

The aim of this work is to provide simple, selective and rapid NMR methods for the determination of MIZ, MNZ and SMZ antibiotics in authentic, pharmaceutical and human urine samples. The developed methods will be applied to analyze admixtures of the three investigated drugs in authentic, pharmaceutical and urine samples. The paper is an attempt to satisfy the growing demand for the need of highly specific determination methods for these drugs in different matrices and met with the very wide spread daily use of these drugs. This work is a continuation of our research work on NMR spectroscopic determination of organic compounds of pharmaceutical importance [21].

2. Experimental

2.1. Materials and reagents

The highest purity analytical grade substances were used throughout. Miconazole, metronidazole and sulfamethoxazole authentic samples were purchased from Sigma–Aldrich. Maleic acid (99%), dimethyl sulfoxide (DMSO-d_6) (99.99%) and spectroscopic grade methanol were purchased from Merck.

The following drugs were purchased from the local market:

Flagyl tablets contain 250 mg metronidazole manufactured by Alexandria Company for pharmaceuticals, Alexandria, Egypt.

Septazole tablets contain 400 mg sulfamethoxazole and 80 mg trimethoprim manufactured by Alexandria Company for pharmaceuticals, Alexandria, Egypt.

Miconaz powder contains 2.0% miconazole nitrate manufactured by MUP Company for Pharmaceuticals, Abou Sultan, Egypt.

2.2. NMR measurements

Proton NMR spectra of authentic drugs, tablets, and urine's extracts were measured using a 300 MHz, JEOL-NM-LA300 FT-NMR spectrometer operating at field strength of 7.1 T. Typically, 500 free induction decays (FIDs) were collected for each sample into 32,768 data points using a spectral width of 6009.6 Hz; digital resolution of 32768/6009.6 = 5.45 points/Hz and acquisition time of 5.453 s. A relaxation delay of 2.55 s enough to ensure T_1-relaxation between successive scans was applied. An exponential line-broadening factor of 0.18 Hz was applied prior to Fourier transformation. Chemical shifts were referenced internally to tetramethyl silane (TMS, $\delta = 0.0$). The NMR probe was maintained at a temperature of 25 °C throughout the whole measurements.

Symmetrical, well separated signals were automatically integrated using the belt-in integration protocol supported with the software package used for running the NMR spectrometer and quantifying the data. Integral regions of closely spaced signals were defined manually and integration was done by summing up the points within the integration range.

2.3. Procedures

2.3.1. General procedure (calibration graphs)

Successive amounts of accurately weighed pure miconazole (0.60–172.38 mg), metronidazole (0.50–231.40 mg) or sulfamethoxazole (0.50–155.38 mg) were thoroughly mixed with suitable amounts of maleic acid (1.90–7.80 mg) as internal standard. The mixtures were dissolved into 0.6 ml portions of DMSO-d_6. Each solution was thoroughly mixed using a vor-

tex mixer till complete dissolution and its ^{1}H NMR pattern was recorded under the experimental conditions given above. Signals at chemical shifts of 9.0 ppm for miconazole, 8.06 ppm for metronidazole, 7.5 ppm for sulfamethoxazole and 6.26 for maleic acid were integrated and used in quantifying the amounts of drugs.

Calibration graphs were constructed by plotting normalized areas of drugs' selected signals with respect to maleic acid's internal signal versus the milligram amounts of drugs. Linear graphs were obtained and used for calculating drugs in pharmaceutical and urine samples. Detection limits were calculated as the drug amounts at which the signal over noise ratio for the selected quantifiable signals is equals to or greater than 5 ($S/N \geq 5$). At least three replicate samples were analyzed to check the repeatability of our results.

2.3.2. Analysis of pharmaceutical preparations

Into a series of stoppered tubes, accurately weighed portions of finely ground Miconaz powder equivalents to 18.00, 31.30 and 60.20 mg miconazole nitrate were transferred.

Ten tablets of Flagyl or Septazole were thoroughly ground into finely divided powders. Portions equivalents to 10.26, 23.50 and 39.50 mg of metronidazole or 16.92, 18.90 and 39.93 mg of sulfamethaxzaole were accurately weighed and transferred into another series of stopperd tubes.

Appropriate amount of accurately weighed maleic acid (7.00–19.00 mg) and 0.6 ml portion of DMSO-d_6 were added to each tube. Solutions were thoroughly mixed using a vortex mixer. The NMR patterns were then recorded for each mixture under the experimental conditions given in Section 2.2.

Again, signals at 9.0, 8.06, 7.5 and 6.2 ppm corresponding to miconazole, metronidazole, sulfamethoxazole and maleic acid were integrated and used for the drugs quantifications. The precision and recovery were tested by at least three determinations of each sample.

2.3.3. Analysis of urine samples

Into six freeze dryer vessels containing 10 ml human urine portions each, 8.30, 29.90 or 54.70 mg amount of pure metronidazole and 6.70, 55.80 or 104.70 mg amount of pure sulfamethoxazole were dissolved. Each solution was thoroughly mixed using vortex mixer till complete dissolution and then freeze dried. Resulting residues were dissolved into 0.6 ml DMSO-d_6 portions. Accurate weight (6.00–7.00 mg) of maleic acid internal standard was added to each vessel and thoroughly mixed. Resulting solutions were well mixed for complete dissolutions and their NMR patterns were recorded using the experimental conditions mentioned in Section 2.2. Signals at 8.06, 7.5 and 6.2 ppm corresponding to metronidazole benzoate, sulfamethoxazole and maleic acid were integrated and quantified. At least three replicate samples were analyzed to validate results obtained.

2.3.4. Analysis of admixtures in authentic, pharmaceutical and urine samples

Mixtures of authentic miconazole nitrate (3.30–16.65 mg), metronidazole benzoate (2.40–12.80 mg) and sulfamethoxazole (2.45–13.12 mg) were thoroughly mixed with 3.58 mg portions of maleic acid. The mixtures were dissolved into 0.6 ml portions of DMSO-d_6. Resulting solutions were well mixed for complete dissolutions and their NMR patterns were recorded using the experimental conditions mentioned in Section 2.2.

Finely ground portions of miconaz, flagyl and septazole powders equivalents to 6.85, 6.84 and 3.32 mg of miconazole nitrate, metronidazole benzoate and sulfamethoxzaole, respectively, were accurately mixed with 6.50 mg of maleic acid and dissolved into 0.6 ml portions of DMSO-d_6. Solutions were thoroughly mixed using a vortex mixer. The NMR patterns were then recorded for each mixture under the experimental conditions given in Section 2.2.

A mixture of 17.30, 12.70 and 12.95 mg of authentic miconazole, metronidazole and sulfamethoxazole respectively were dissolved into 10 ml urine. The solution was thoroughly mixed using vortex mixer till complete dissolution and then, freeze dried. Resulting residue was dissolved into 0.6 ml DMSO-d_6. A 3.627 mg of maleic acid was added and thoroughly mixed. Resulting solution was well shaken for complete dissolution and its NMR pattern was recorded using the experimental conditions mentioned in Section 2.2.

The precision and recovery were tested by at least three determinations for each mixture.

2.4. Calculations

Maleic acid's signal at 6.26 ppm was used as a reference signal. This signal is not interfering with any of the three signals selected for quantifying the three drugs and represents the foundation for the high selectivity of our NMR quantitations.

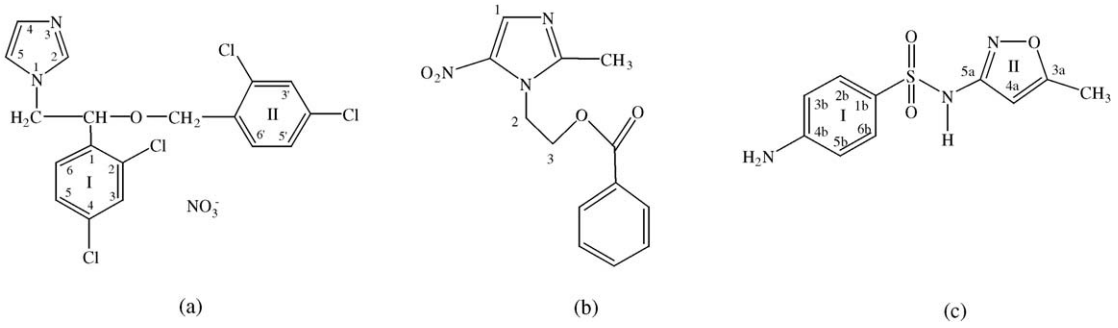

Fig. 1. Chemical structures of miconazole nitrate (a), metronidazole benzoate (b) and sulfamethoxazole (c).

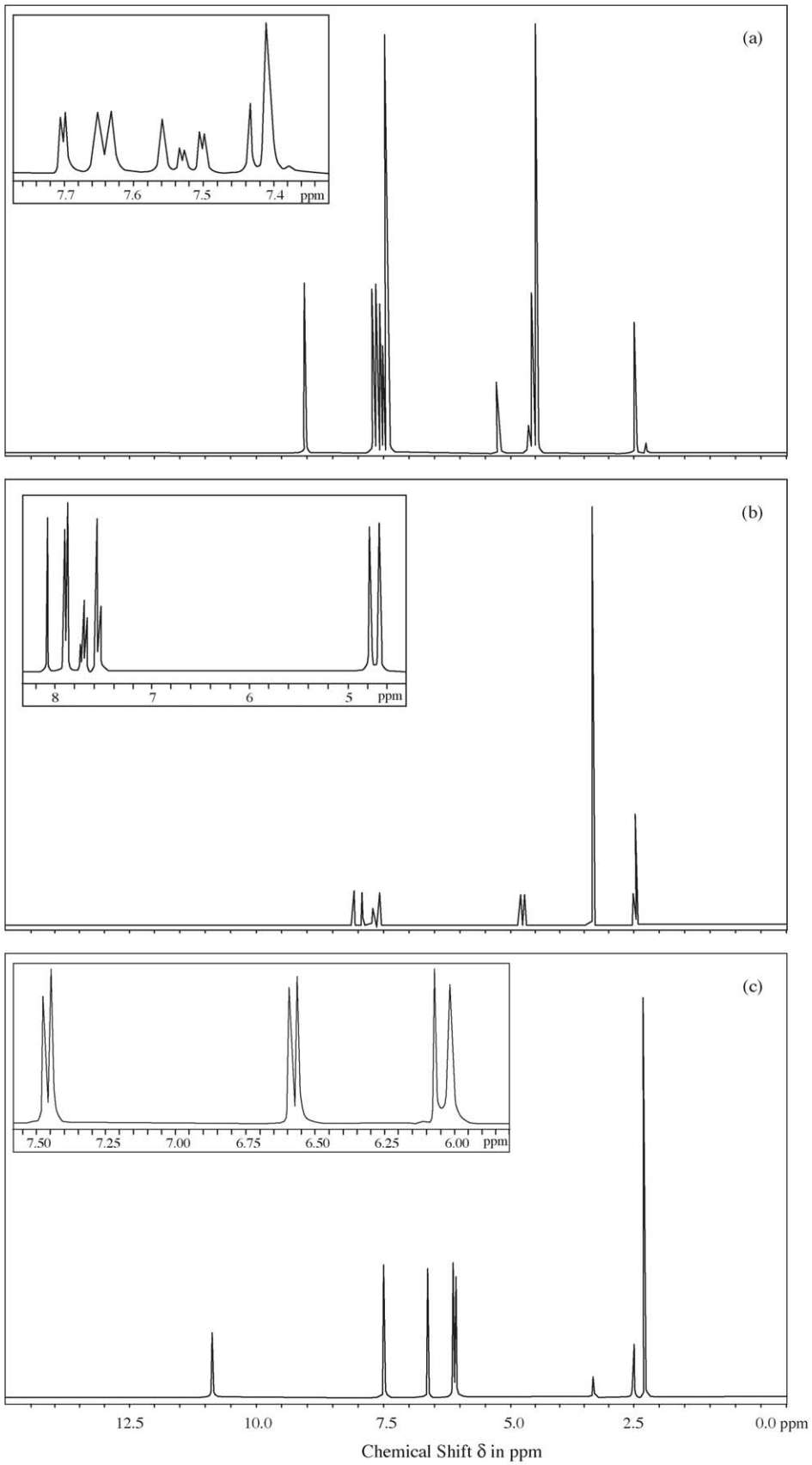

Fig. 2. Three hundred mega hertz proton NMR spectra of miconazole nitrate (a), metronidazole benzoate (b) and sulfamethoxazole (c) in DMSO-d_6.

Amounts of miconazole, metronidazole or sulfamethoxazole per unit dose were calculated using the following equation:

$$\text{weight of drug } (W_{\text{drug}}) = W_{\text{int. stand}} \times (E_{\text{drug}}/E_{\text{int. stand}}) \times (A_{\text{drug}}/A_{\text{int. stand}})$$

A_{drug} is the integral value for the drugs selected ^{1}H NMR signals at 9.0, 8.06 or 7.5 pmm. $A_{\text{int. stand}}$ is the integral value of the maleic acid absorbing ^{1}H NMR signals at 6.2 ppm. E_{drug} is the formula weight of each drug divided by one absorbing protons in case of miconazole and metronidazole and divided by two in case of sulfamethoxazole. $E_{\text{int. stand}}$ is the formula weight of maleic acid divided by the two absorbing protons (116.07/2 = 58.04). $W_{\text{int. stand}}$ is the milligram weight of maleic acid used in the assay [21].

3. Results and discussion

3.1. Assignment of ^{1}H NMR signals of the drugs

Structural formula of miconazole nitrate, metronidazole benzoate and sulfamethoxazole are shown in Fig. 1. Proton NMR spectra were measured with 500 scans collected for each sample into 32,768 data points using a spectral width of 6009.60 Hz; digital resolution of 32768/6009.6 (5.45 points/Hz) and acquisition time of 5.45 s. Relaxation times of 1.15, 2.09 and 0.62 s were determined for miconazole nitrate, metronidazole benzoate and sulfamethoxazole, respectively. Therefore, a relaxation time of 2.55 s was enough to ensure T_1-relaxation between successive scans for the three investigated compounds.

Fig. 2a shows the ^{1}H NMR spectra for miconazole. The doublets appeared at 7.61 and 7.62 ppm can be attributed to the splitting of H_4 and H_5 protons of the immidazole ring due to their coupling with each other. The isolated singlet sharp signal at 9.0 ppm is attributed to the H_2 proton on the immidazole ring. This signal was selected for quantifying miconazole in different matrices. The signals appeared at 7.7 and 7.55 ppm can be attributed to the H'_3 and H_3 protons. The signals appeared at 7.52 and 7.49 ppm are attributed to the H'_5 and H'_6 proton, while that appeared at 7.40 ppm represent H_5 and H_6. The signal appeared at 7.43 ppm can be attributed to the aliphatic CH proton, while that appeared between 4.44 and 4.54 ppm may be attributed to the two aliphatic CH_2 protons. The singlet at 2.49 ppm is due to the DMSO-d_6. Thus, the isolated, sharp singlet signal appeared at 9.0 ppm was selected for the NMR quantitative determination of miconazole (Table 1) [22].

The ^{1}H NMR spectrum of metronidazole is shown in Fig. 2b. The singlet appeared at 8.06 ppm is attributed to the hydrogen on carbon number 1 (H1), which was selected for quantifying metronidazole in different matrices. The doublets at ~4.68 and 4.77 ppm are attributed to protons at carbons' 2 and 3, respectively. The singlet at 2.52 ppm is due to the hydrogens of the methyl group. The signal at 2.49 ppm is attributed to DMSO-d_6. Signals appeared between 7.52 and 7.89 ppm can be attributed to the hydrogen on the phenyl ring of benzoic acid (Table 1).

In Fig. 2c, ^{1}H NMR spectrum of sulfamethoxazole is shown. The N–H proton appeared as a broad signal at 10.86 ppm. The signals appeared at 6.08 and 6.00 ppm could be attributed to the protons attached to the carbon C(4a) and NH_2, respectively. The signal at 6.6 ppm is due to the protons of carbon 3b and 5b. Doublet signal at 7.5 ppm can be attributed to protons on carbons 2b, 6b and was selected for the NMR quantitative determination of sulfamethoxazole. The sharp singlet at 2.3 ppm is due to the protons of methyl group on ring II (Table 1) [23].

In the NMR spectrum of maleic acid, an isolated sharp singlet signal appeared at 6.26 ppm was attributed to protons on methylene groups and was used as the standard signal for the NMR quantitative determination of miconazole, metronidazole and sulfamethoxazole. Signals appeared at 2.06 and 2.49 ppm were attributed to the OH and DMSO residual protons, respectively. (Table 1) [21].

3.2. Analysis of authentic miconazole, metronidazole and sulfamethoxazole

Symmetrical, well separated signals were automatically integrated. Integral regions of closely spaced signals were defined

Table 1
Proton chemical shifts in ppm for miconazole, metronidazole and sulfamethoxazole at room temperature in DMSO-d_6

Miconazole		Metronidazole		Sulfamethoxazole		Maleic acid	
Carbon atom #	Chemical shift (ppm)	Carbon atom #	Chemical shift (ppm)	Carbon atom #	Chemical shift (ppm)	Carbon atom #	Chemical shift (ppm)
(Imidazole) H2[a]	9.00[a]	H1[a]	8.06[a]	N–H	10.86	O–H	2.06
(Imidazole) H4	7.61	H2	4.68	C(4a)–H	6.08	DMSO	2.49
(Imidazole) H5	7.62	H3, CH_3	4.77, 2.52	C(2b)–H[a], C(3b)–H	7.50[a], 6.60	C–H[a]	6.26[a]
(2,4-Dichlorophenyl)		Ar-CH	7.52–7.89	NH_2	6.00		
(Ring I) H3	7.55			CH_3	2.30		
(Ring I) H5	7.40						
(Ring I) H6	7.41						
(Ring II) H3′	7.70						
(Ring II) H5′	7.52						
(Ring II) H6′	7.49						
Aliphatic-CH	7.43						
2-Aliphatic-CH_2	4.44–4.54						

[a] Signals selected for integration and quantification.

Table 2
Analysis of miconazole, metronidazole and sulfamethoxazole in authentic, pharmaceutical formulations and human urine samples using developed ^{1}H NMR methods

Miconazole						Metronidazole						Sulfamethoxazole					
Taken (mg)	Found (mg)	Recovery (%)	S.D. (R.S.D.)	*t*-value	*F*-value	Taken (mg)	Found (mg)	Recovery (%)	S.D. (R.S.D.)	*t*-value	*F*-value	Taken (mg)	Found (mg)	Recovery (%)	S.D. (R.S.D.)	*t*-value	*F*-value
A. Miconazole, metronidazole and sulfamethoxazole in authentic samples																	
0.99	0.98	99.00	0.01 (1.02)	1.73	1.21, [19]	0.93	0.92	98.92	0.01 (1.09)	1.73	1.00, [19]	0.92	0.90	97.83	0.02 (2.22)	1.73	2.04, [24]
10.35	10.25	99.03	0.33 (3.22)	0.53	2.72, [19]	7.90	7.87	99.62	0.20 (2.54)	0.26	4.00, [19]	9.48	9.55	100.74	0.06 (0.63)	2.02	5.44, [24]
37.20	37.61	101.10	0.46 (1.22)	1.54	1.14, [19]	34.00	33.50	98.53	0.58 (1.73)	1.49	3.09, [19]	36.48	36.66	100.49	0.49 (1.34)	0.64	1.26, [24]
60.20	60.06	99.77	0.55 (0.92)	0.44	1.53, [19]	60.30	59.13	98.06	0.71 (1.20)	2.85	1.45, [19]	62.70	63.10	100.64	0.42 (0.67)	1.65	5.12, [24]
120.90	120.33	99.53	1.50 (2.25)	0.66	1.20, [19]	131.20	131.80	100.46	0.40 (0.30)	2.60	5.50, [19]	103.70	105.20	101.45	2.39 (2.27)	1.09	2.29, [24]
166.80	162.70	97.54	2.60 (1.60)	2.73	1.98, [19]	170.10	169.60	99.71	1.15 (0.68)	0.75	2.13, [19]	155.40	159.80	102.83	2.68 (1.68)	2.84	1.25, [24]
B. Miconazole in miconaz powder, metronidazole in flagyl tablets and sulfamethoxazole in septazole tablets.																	
18.00	17.55	97.50	0.31 (1.77)	2.51	1.75, [19]	10.26	10.07	98.15	0.25 (2.48)	1.32	5.17, [19]	16.92	16.90	99.88	0.06 (0.36)	0.58	4.00, [24]
31.30	30.65	97.92	0.32 (1.04)	3.52	4.92, [19]	23.50	22.64	96.34	0.50 (2.98)	1.66	4.00, [19]	18.90	18.60	98.41	0.20 (1.08)	2.60	2.37, [24]
60.20	59.20	98.34	0.52 (0.88)	3.33	5.33, [19]	39.50	38.51	97.50	0.50 (1.30)	3.43	1.50, [19]	39.93	40.46	101.33	0.62 (1.53)	1.48	4.80, [24]
C. Metronidazole and sulfamethoxazole in human urine samples																	
–	–	–	–	–	–	8.30	7.84	94.46	0.14 (1.79)	5.69	1.36, [25]	6.70	6.64	99.10	0.05 (0.75)	5.20	4.00, [25]
–	–	–	–	–	–	29.90	28.40	94.98	0.45 (1.58)	5.77	1.10, [25]	55.80	57.90	103.76	1.14 (1.97)	3.19	1.72, [25]
–	–	–	–	–	–	54.70	53.50	97.81	0.47 (0.88)	4.42	2.90, [25]	104.70	105.60	100.86	1.38 (1.31)	1.13	1.31, [25]

Average of at least three determinations: $t = (\bar{X} - Xt)\sqrt{N}/\text{S.D.}$, $F = \text{S.D.}_1^2/\text{S.D.}_2^2$. *F*-values: based on S.D. values of our NMR method and HPLC method [19,24,25].

Table 3
Analysis of miconazole, metronidazole and sulfamethoxazole in admixtures using developed ^{1}H NMR methods

Miconazole						Metronidazole						Sulfamethoxazole					
Taken (mg)	Found (mg)	Recovery (%)	S.D. (R.S.D.)	*t*-value	*F*-value	Taken (mg)	Found (mg)	Recovery (%)	S.D. (R.S.D.)	*t*-value	*F*-value	Taken (mg)	Found (mg)	Recovery (%)	S.D. (R.S.D.)	*t*-value	*F*-value
A. Admixtures of miconazole, metronidazole and sulfamethoxazole authentic samples																	
3.30	3.35	101.50	0.10 (2.99)	0.87	6.25, [19]	2.40	2.37	98.70	0.07 (2.95)	0.74	5.44, [19]	2.45	2.38	97.50	0.033 (1.26)	3.67	1.19, [24]
11.60	11.80	101.70	0.16 (1.36)	2.17	1.40, [19]	8.45	8.27	97.90	0.09 (1.09)	3.46	1.27, [19]	8.72	8.23	94.40	0.24 (2.92)	3.53	3.81, [24]
16.65	16.75	100.60	0.25 (1.49)	0.69	1.73, [19]	12.80	12.26	95.80	0.16 (1.31)	5.84	1.78, [19]	13.12	12.68	96.60	0.10 (0.79)	7.62	3.61, [24]
B. Admixtures of miconaz, flagyl and septazole pharmaceutical formulations																	
6.85	7.05	102.91	0.18 (2.55)	1.92	1.22, [19]	6.90	7.18	104.97	0.10 (1.39)	4.85	1.73, [19]	3.31	3.13	94.56	0.05 (1.60)	6.23	5.16, [19]
C. Admixtures of miconazole, metronidazole and sulfamethoxazole in human urine samples																	
17.30	17.86	103.20	0.28 (1.57)	3.46	1.08, [25]	12.70	11.90	93.40	0.20, 1.68	6.92	1.24, [25]	12.95	13.08	100.90	0.11, 0.84	2.05	3.31, [25]

Average of at least three determinations: $t = (\bar{X} - Xt)\sqrt{N}/\text{S.D.}$, $F = \text{S.D.}_1^2/\text{S.D.}_2^2$. *F*-values: based on S.D. values of our NMR method and HPLC method [19,24,25].

manually and integration was done by summing up the points within the integration range. Quantification limits (QL) were imposed by the sensitivity of the NMR spectrometer detection for miconazole, metronidazole and sulfamethoxazole. Upper quantification limits of 172.38, 231.4 and 155.38 mg, respectively were imposed by the saturation limits of the three drugs into the 0.6 ml of DMSO-d_6.

A straight line calibration graphs were obtained by plotting the normalized signal areas ($A_{drug}/A_{int.\ stand}$) versus the milligram drug amounts using the 9.0, 8.06 and 7.5 ppm signals for miconazole, metronidazole and sulfamethoxazole, respectively. Regression equations of $Y = 0.119X + 0.049$, $Y = 0.209X + 0.031$ and $Y = 0.234X - 0.124$ with correlation coefficients of 1.00 were respectively, obtained for the three drugs. Average recoveries of 97.54–101.10, 98.06–100.46 and 97.83–102.83 with average uncertainties of 1.02, 0.45 and 0.86 were respectively obtained for authentic miconazle, metronidazole and sulfamethoxazole (Table 2).

Similar to chromatography, detection limits were defined by the drug's concentration at which the signal over noise ratio for the selected quantified signal is greater than or equals to 5 ($S/N \geq 5$). Consequently, detection limits of 0.6, 0.5 and 0.5 mg for miconazole nitrate, metronidazole benzoate and sulfamethoxazole per 0.6 ml of DMSO-d_6 were obtained.

Applying Student *t*-test on analytical results obtained for the investigated compounds gave *t*-values $\leq$2.85 indicating insignificant differences between measured and real contents at 95% confidence level (Table 2). Applying the statistical *F*-test revealed insignificant difference in precisions between the developed NMR methods and HPLC methods reported for analyzing miconazole, metronidazole and sulfamethoxazole [19,24] (Table 2).

These values are fairly good figures of merit for the developed NMR methods and allow their use for determining miconazole, metronidazole and sulfamethoxazole in pharmaceutical and urine samples.

3.3. Analysis of pharmaceutical samples

An advantage of the developed NMR methods is their capabilities for simultaneous determination of the identity and quantity of investigated drugs in dosage forms. Miconazole, metronidazole and sulfamethoxazole in miconaz, flagyl and septazole dosage forms were determined using the developed NMR methods. Average recoveries of 97.50–98.34%, 96.34–98.15% and 98.41–101.33% were respectively, obtained (Table 2).

The results were statistically evaluated using Student *t*-test. *t*-Values $\leq$3.52, $\leq$1.10 and $\leq$2.60 for miconazole, metronidazole and sulfamethoxazole were respectively obtained indicating insignificant difference between the measured and real contents at 95% confidence level (Table 2). *F*-values $\leq$5.33 revealed insignificant differences in precisions between the developed NMR methods and HPLC methods reported for analyzing miconazole, metronidazole and sulfamethoxazole [19,24] (Table 2).

3.4. Analysis of urine samples

Urine samples containing different amounts of metronidazole and sulfamethoxazole were analyzed using the developed NMR methods described in Section 2.3.3. Average recoveries in the range 94.50–97.80% and 99.10–103.80% with standard deviations in the range of 0.14–0.47 and 0.05–1.38 were respectively obtained for metronidazole and sulfamethoxazole (Table 2).

Applying Student test for the analytical results of metronidazole and sulfamethaxzole in urine gave Student *t*-values in the ranges of 4.42–5.77 and 1.13–5.20 indicating insignificant differences between the measured and real contents at 95% confidence level. *F*-test again revealed insignificant difference in precisions between the developed NMR methods and HPLC methods reported for analyzing miconazole, metronidazole and sulfamethoxazole [25] (Table 2).

3.5. Analysis of admixtures in authentic, pharmaceutical and urine samples

Developed NMR methods were used for determining miconazole nitrate, metronidazole benzoate and sulfamethoxazole in admixtures of authentic, pharmaceutical and urine samples. Recoveries in the ranges of 100.60–101.70%, 95.80–98.70% and 94.40–97.50% were respectively, obtained (Table 3). Corresponding average recoveries of 102.91%, 104.97% and 94.56% were obtained for admixtures of miconaz, flagyl and septazole powders (Table 3). Admixtures of the three drugs in urine samples gave recoveries of 103.20%, 93.40% and 100.90%, respectively (Table 3).

These results were statistically evaluated using Student *t*-test. Average *t*-values of 1.24, 3.35 and 4.94 were respectively obtained for admixtures of authentic miconazole, metronidazole and sulfamethoxazole. Corresponding *t*-values of 1.92, 4.85, 6.23 were obtained for admixtures of miconaz, flagyl and septazole powders whereas values of 3.46, 6.92, 2.05 were obtained for admixtures of the three drugs in urine samples. Theses values indicate insignificant difference between the measured and real values at 95% confidence level (Table 3).

F-test at 95% confidence level, gave average *F*-values $\leq$5.16 for admixtures of the three drugs in authentic, pharmaceutical and urine samples. These results confirm the absence of significant differences in precisions between the developed NMR methods and HPLC reference method [19,24,25] (Table 3).

4. Conclusion

Newly developed NMR methods were developed for determining miconazole, metronidazole and sulfamethoxazole in authentic, pharmaceutical dosage and urine samples using maleic acid as an internal standard. The developed methods have proved specific, precise and accurate for assaying the three drugs either individually or in admixtures. The high specificity and resolving power of the developed NMR methods enabled us to analyze admixtures of the three investigated drugs without the need for pre-separation steps.

Detection limits of 0.6, 0.5 and 0.5 mg per 0.6 ml of DMSO-d_6 were respectively obtained for miconazole, metronidazole and sulfamethoxazole. These detection limits are less than reported pharmacopeial methods. Since about 20.0% of metronidazole and 30.0% of sulfamethoxazole are excreted in urine as unchanged, our detection limits are far less than the amounts of these drugs excreted in urine.

Statistical Student *t*-test applied for the three individual drugs as well as their admixtures in authentic, pharmaceutical and urine samples indicated insignificant differences between the real and measured values at the 95% confidence level. *F*-test revealed insignificant difference in precisions between the developed NMR methods and HPLC methods reported for analyzing miconazole, metronidazole and sulfamethoxazole.

Compared to HPLC analysis, the developed methods could assure accuracy, precision and high quality control standards. Consequently, they can serve for identification and stability indicating assays of miconazole, metronidazole and sulfamethoxazole. They can also be adopted as alternatives to existing pharmacopoeial methods.

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