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Simultaneous determination of metronidazole and spiramycin I in human plasma, saliva and gingival crevicular fluid by LC–MS/MS

Cyriaque Sagan[a,*], Arnaud Salvador[a,**], Didier Dubreuil[a], Pierre. P. Poulet[b], D. Duffaut[b], Ivan Brumpt[c]

[a] *CEPHAC Europe, 90 Avenue des Hauts de la Chaume, B.P. 28, 86281 Saint Benoît, France*
[b] *Faculté de Chirurgie Dentaire,3, chemin des Maraîchers, 31062 Toulouse, France*
[c] *Laboratoire AVENTIS, 46 quai de la Rapée, 75601 Paris Cedex 12, France*

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Abstract

An analytical validation of a new liquid chromatographic–mass spectrometric (LC–MS/MS) method for simultaneous determination of metronidazole and spiramycin I concentrations in human plasma, saliva and gingival crevicular fluid (GCF) is presented. Ornidazole was used as an internal standard, and sample pre-treatment consisted of a liquid–liquid extraction. Chromatographic separation was achieved on a 5 μm Kromasil C_{18} column (150 mm × 4.6 mm i.d., particle size 5 μm), with a gradient using acetonitrile, water and formic acid at a flow rate of 0.9 ml/min. The methods were validated in terms of intra- and inter-batch precision (<7.1% in plasma, 12% in saliva and 9.2% in GCF, respectively) and accuracy (within ±8.7% in plasma, within ±8.7%, except LDQ level within ±15.4% in saliva and within ±10.7% in GCF), linearity, specificity, recovery (extraction efficiency), matrix effect, dilution process, stability in human plasma and saliva after three freeze–thaw cycles, stability in human plasma and saliva at ambient temperature and stability of the extracts in the automatic injector of the HPLC system. The methods are applicable for accurate and simultaneous monitoring of the plasma, saliva and gingival crevicular fluid levels of metronidazole and spiramycin I from pharmacokinetic studies.

Keywords: Spiramycin; Metronidazole; Plasma; Saliva; Gingival crevicular fluid; LC–MS

1. Introduction

Spiramycin is a macrolide antibiotic isolated from *Streptomyces ambofaciens* and is a natural mixture of three components: spiramycin I, II, and III (Fig. 1) with a minimum of 85% of spiramycin I, and a maximum of 5% for II and 10% for III, respectively. The metabolism of spiramycin is not known; however, in vitro (in human plasma) spiramycin is rapidly transformed into a cysteinate derivative; this transformation is inhibited by the adjunction of formaldehyde. In the pig liver, neo-spiramycin derivatives by loss of one hexose residue can be found [1]. High concentrations of spiramycin, exceeding those measured in serum, have been measured in tissues, such as the lung, tonsils, infected sinus mucosa, the gingival mucosa and the mandibular bone [2,3].

Metronidazole is the reference agent of the nitroimidazole antibiotic family. It is well absorbed and serum concentrations reach high levels, with tissue concentrations being generally similar to or slightly lower than those measured in the serum [4]. Metronidazole is strongly metabolized to several derivatives. Its most important metabolites include an alcohol and an acid derivative [5], which, however, account for little of the antibacterial activity in vivo.

* Corresponding author. Tel.: +33 5 49 57 04 04; fax: +33 5 49 57 22 39.
** Co-corresponding author. Present address: Universite de Lyon 1, UFR Chimie-Laboratoire des sciences analytique, 43, Boulevard du 11 novembre 1918, 69622 Villeurbanne, France. Tel.: +33 4 72 43 11 52; fax: +33 4 72 44 62 02.
E-mail addresses: csagan@aster-cephac.com (C. Sagan), arnaud.salvador@univ-lyon1.fr (A. Salvador).

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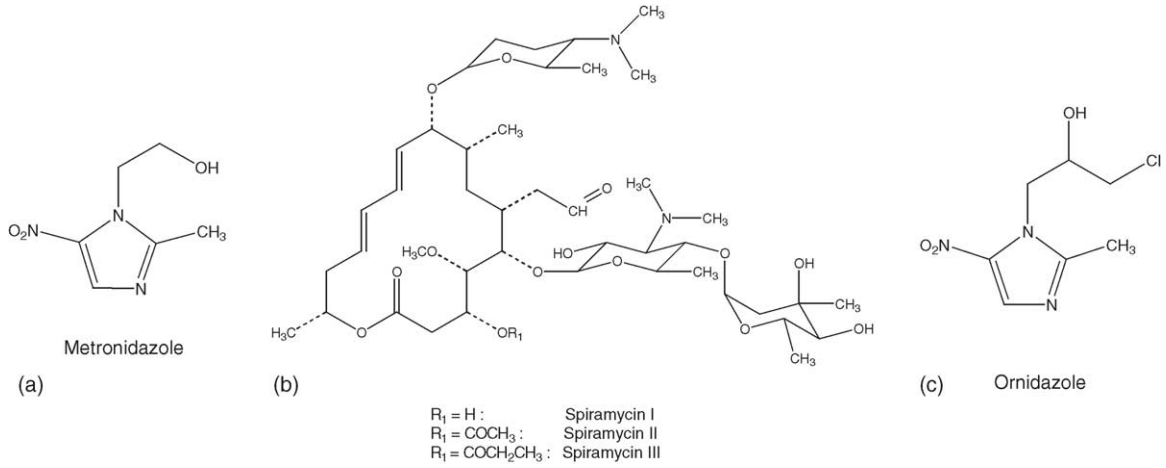

Fig. 1. Chemical structures of metronidazole (a), spiramycin I (b) and ornidazole (c).

The combination of metronidazole and spiramycin has been developed on the basis of the complementarity of the antibacterial activity of both compounds in vitro on the range of bacteriae involved in dental infections, including facultative and strict anaerobes, along with the possibility of a synergical activity on some anaerobic strains [3,6–8]. The clinical activity of this combination in advanced periodontal diseases, a recognized indication of antibiotic therapy, has been published, although the data reported are limited [9]. Several studies have aimed at analyzing the in vitro MICs (minimal inhibition concentrations) of antibiotics directed against the bacteria involved in periodontitis. However, the results obtained are not consistently in agreement with those obtained from clinical studies. In order to better understand the relationship between the in vitro and the in vivo activity of the antibiotics, it is important to measure the in vivo concentrations of the antibiotics. The consideration of these concentrations together with the corresponding MICs of the pathogens should contribute to a more rational use of the antibiotics in periodontitis.

The determination of metronidazole concentrations in GCF, saliva and serum after oral administration has been performed previous studies [10,11]. Metronidazole and spiramycin concentrations were measured in one study after oral administration of the spiramycin–metronidazole combination [12]. In these studies, the gingival crevice fluid sample was collected either by aspiration through a capillary micropipette [10], or by adsorption on a paper point placed in the sub-gingival sulcus [11,12].

In the study by Rotzetter [12], the measurement of the concentrations of metronidazole and spiramycin in blood, saliva and crevicular fluid was done using an HPLC method (with UV detection) developed at Rhône-Poulenc-Rorer (Aventis) in Paris, France, after a simultaneous gradient of elution and gradient of absorption wavelength. The analytical method developed by Rhône-Poulenc-Rorer presents a lack of specificity (especially in plasma) and requires an analysis time exceeding 20 min. Moreover, in order to achieve a 50 ng/ml quantification limit for metronidazole and spiramycin in plasma, a 2 ml plasma volume and a concentration step with solid phase extraction were necessary. LC–MS/MS methods have general advantages over HPLC methods with UV detection: the need for a lower volume of clinical sample, a better specificity and a greater speed of analysis. Several methods have been published describing the individual determination of nitro-imidazole derivatives [13,14] and macrolide antibiotics [1,15,16]. As no method by LC–MS/MS was available for the simultaneous determination of metronidazole and spiramycin I, we developed and validated a method for the simultaneous determination of metronidazole and spiramycin I in human plasma, saliva and gingival crevicular fluid (GCF).

2. Experimental

2.1. Chemicals

Metronidazole and the internal standard (ornidazole) were provided by Sigma (St Louis, MO, USA) (purity 100 and 99%, respectively); spiramycin I was provided by Aventis Pharma (Vitry, France) (purity 88.6%). All reagents were of analytical grade. Acetonitrile and formaldehyde solution (37%) were obtained from J.T. Baker Co. (Phillipsburg, NJ, USA). Formic acid and sodium hydroxide 1N were obtained from Merck (Darmstat, Germany). Buffer solution pH 9 and ethyl acetate were purchased from SDS (Valdonne, France). Distilled water was purified in a Millipore system Milli Q (Molsheim, France).

2.2. Matrix

Human plasma, with heparin as anticoagulant, and saliva were obtained from CEPHAC volunteers. Human plasma and saliva samples were stored at −25 °C. Human GCF was obtained from volunteers by a dental practitioner (Pierre P. Poulet) according to the Cimasoni technique [12]. Briefly,

human GCF fluid was adsorbed on Whatman 1 strips of filter paper strips (length 6.5 mm, width 2 mm), contained in a closed plastic microtube). After gentle insertion in the subgingival crevice and a thorough local preparation in order to avoid any contamination by saliva, the strips were left in place for 3 min. Four impregnated strips were obtained. They were weighed before and after application on a microscale (Mettler H2OT), then re-introduced in the tube and stored at −70 °C.

2.3. Chromatography

The HPLC system consisted of a Perkin-Elmer (Norwalk, CT, USA) 200 series pump and a Perkin-Elmer 200 series auto-sampler with a Peltier tray cooler set at 8 °C. Chromatographic separations were performed on a 5 μm Kromasil C_{18} column, 150 mm × 4.6 mm i.d. (Interchim, Montluçon, France), operated at ambient temperature with a flow rate of 0.9 ml/min. A gradient elution method was used with two solvents: solvent A was a mixture of acetonitrile/water/formic acid (15:85:0.1, v/v/v) and solvent B was a mixture of acetonitrile/water/formic acid (50:50:0.1, v/v/v). The initial composition of the mobile phase was 100% solvent A. An isocratic gradient was used for 2 min, followed by a linear gradient over 2 min to 100% solvent B. The gradient was returned to the initial conditions over 2 min and held in those conditions for 6 min prior to the next injection, to equilibrate the analytical column. The total run time for a LC–MS/MS analysis was 10 min per sample.

2.4. Mass spectrometry

A Sciex API 3000 triple quadrupole mass spectrometer (MDS Sciex, Toronto, Canada) was coupled to the HPLC system through a Sciex TurboIonSpray source (TIS) operated in positive mode. Instrument control, data acquisition and processing were performed using Analyst 1.1 sofware. The mass spectrometer was initially calibrated using polypropylene glycol as standard (Applied Biosystems, Foster City, CA, USA), setting the resolution, as peak width at half height, in the range 0.7 ± 0.1 u.m.a.

The nebulizer and the curtain gas flows (nitrogen) were set at 14 and 8 arbitrary units (AU), respectively. The TurboIonSpray source was operated at 500 °C with the auxiliary gas flow (nitrogen) set at 8 l/min. The TurboIonSpray voltage was set at 5000 V and the orifice voltage and ring voltages at 41 and 200 V for metronidazole, 46 and 210 V for spiramycin I and 41 and 220 V for ornidazole (IS), respectively. Multiple reaction monitoring (MRM) experiments in the positive ionization mode were performed to detect ion transitions at *m/z* 422.6/174.2 $[M + 2H]^{2+}$ (spiramycin I), 172/127.9 $[M + H]^{+}$ (metronidazole), and 219.9/127.9 $[M + H]^{+}$ (ornidazole) with a dwell time of 250 ms per transition. Product ions used for monitoring were selected based on their significance in the MS/MS spectra. For spiramycin I, the doubly protonated parent ion was used. The collision energy was optimized for each analyte, using the autotune feature of the software (31 V for spiramycin, 21 V for metronidazole, and 23 V for ornidazole).

2.5. Extraction

For plasma, the samples were thawed at room temperature and vortexed, then centrifuged for 5 min at 3500 rpm at approximately +4 °C. To 500 μl of plasma, 50 μl of 0.1N sodium hydroxide solution, and 0.1N 1 ml of pH 9 buffer solution were added, followed by 6 ml of ethyl acetate after vortexing for a few seconds. The tubes were capped and shaken (5 min at 4 cpm on a reciprocal shaker, then centrifuged for 5 min at 3500 rpm at approximately +4 °C. The aqueous phase was frozen on a bed of cardice. The upper organic phase was transferred to a 10 ml polypropylene tube, and then evaporated to dryness under a gentle stream of nitrogen at approximately 40 °C. The dried extract was then reconstituted with 600 μl acetonitrile, vortex mixed and diluted with 3.4 ml of water containing 0.1% of formic acid, and a 40 μl aliquot was injected into chromatographic system.

For saliva, the extraction procedure was essentially the same, except that formaldehyde was not added to the matrix: volumes of 400 μl of water, 50 μl of sodium hydroxide solution 0.1N and 1 ml of buffer solution pH 9 were added to all samples, then 6 ml of ethyl acetate after vortexing; after evaporation, the residues were reconstituted with 150 μl of acetonitrile, 850 μl water containing 0.1% of formic acid and vortexing. A 40 μl aliquot was injected into chromatographic system.

For the human GCF, the samples were thawed at room temperature. Twenty microlitres internal standard at 0.4 ng/μl was added, followed by 50 μl of acetonitrile. The tubes were capped and centrifuged for 1 min at 3500 rpm at approximately +4 °C, put into an ultrasonic bath for 5 min, then centrifuged again. The liquid phase was transferred to 1.5 ml click-cap polypropylene microtubes. Five hundred and ten microlitres of water containing 0.1% of formic acid was added. The tubes were then vortexed for a few seconds and the contents were transferred to glass autosampler vials. A 40 μl aliquot was injected into chromatographic system.

2.6. Preparation of standards and quality control samples

Two separate weighings of each compound (metronidazole and spiramycin I) were made to prepare stock solutions at 500 μg/ml dissolved in acetonitrile. One stock solution of each compound was used to prepare working solutions for the calibration curve standards (STD) and was called the STD stock solution. The other stock solution of each compound was used to prepare working solutions for quality control (QC) samples and was called QC stock solution. Standard spiking solutions of metronidazole were prepared from serial dilutions of the STD stock solution in acetonitrile. Standard spiking solutions of spiramycin I was prepared from serial

dilutions of the STD stock solution in acetonitrile containing 0.25% formaldehyde solution.

For plasma, the preparation of calibration standards, as well as for the preparation of the quality control samples, validation and tests samples were performed using formolated human plasma to insure stability of spiramycin I. A 20 μl volume of metronidazole spiking solution was first added, evaporated to dryness under a gentle stream of nitrogen at approximately 40 °C, then the residue was reconstituted with a 20 μl volume of spiramycin I spiking solution and a 20 μl volume of internal standard at 10 ng/ml. After vortexing for a few seconds, a 500 μl volume of matrix was added.

In plasma, the standard concentrations of metronidazole and spiramycin I ranged from 50 to 5000 ng/ml and 15 to 2000 ng/ml, respectively. Quality control (QC) samples in plasma were prepared at the concentrations (metronidazole/spiramycin I) of 50/15, 150/45, 2000/800 and 4000/1600 ng/ml.

For saliva, the calibration standards preparation was essentially the same, but the volume of the matrix was 100 μl and the concentration of internal standard was 400 ng/ml. In saliva, the standard concentrations of metronidazole and spiramycin I ranged from 50 to 5000 ng/ml and 15 to 5000 ng/ml, respectively. Quality control (QC) samples in saliva were prepared at the concentrations (metronidazole/spiramycin I) of 50/15, 150/45, 2000/2000 and 4000/4000 ng/ml.

For human gingival fluid, the calculations of the volumes were performed assuming a theoretical volume of 2 μl of matrix (based on the volume collected during clinical study). A 20 μl volume of metronidazole spiking standard solution was first added, then 20 μl volume of spiramycin I spiking solution, and then a 20 μl volume of internal standard at 400 ng/ml plus a 30 μl volume of diluent (acetonitrile) was added. Two microlitres volume of quality control stock solution was deposited on standardized filter papers impregnated with human gingival fluid and evaporated to dryness under a low gentle stream of nitrogen. For the preparation of test samples, the same volumes of diluent and internal standard were added.

In human gingival fluid, the standard concentrations of metronidazole and spiramycin I ranged from 15 to 50,000 ng/ml, respectively. Quality control (QC) samples in gingival fluid were prepared at the concentrations of metronidazole and spiramycin 0.15, 0.5, 20 and 40 μg/ml.

3. Results and discussion

3.1. Method development

It had been shown previously [1] that spiramycin is unstable in biological matrix due to a reaction with cysteine present in the plasma and transformation into a cysteinate derivative after reaction of the aldehyde group (Fig. 1). To prevent this reaction and restore stability in the biological matrix, we added formaldehyde to all solutions containing spiramycin and to blank matrix. Under these circumstances, formaldehyde is preferentially transformed with L-cysteine. Prior to validation, we ensured that formaldehyde did not compromise the determination of metronidazole in human plasma.

As the structures of metronidazole and spiramycin I (Fig. 1) are different, it was not possible to resolve all the compounds in the same run under isocratic conditions. Consequently, an elution gradient was used. Under these conditions, all compounds were eluted in less than 8 min and the reproducibility of the chromatography is acceptable (Fig. 2). The retention times of metronidazole and spiramycin I in human were approximately 3.4 and 7.3 min, respectively, and

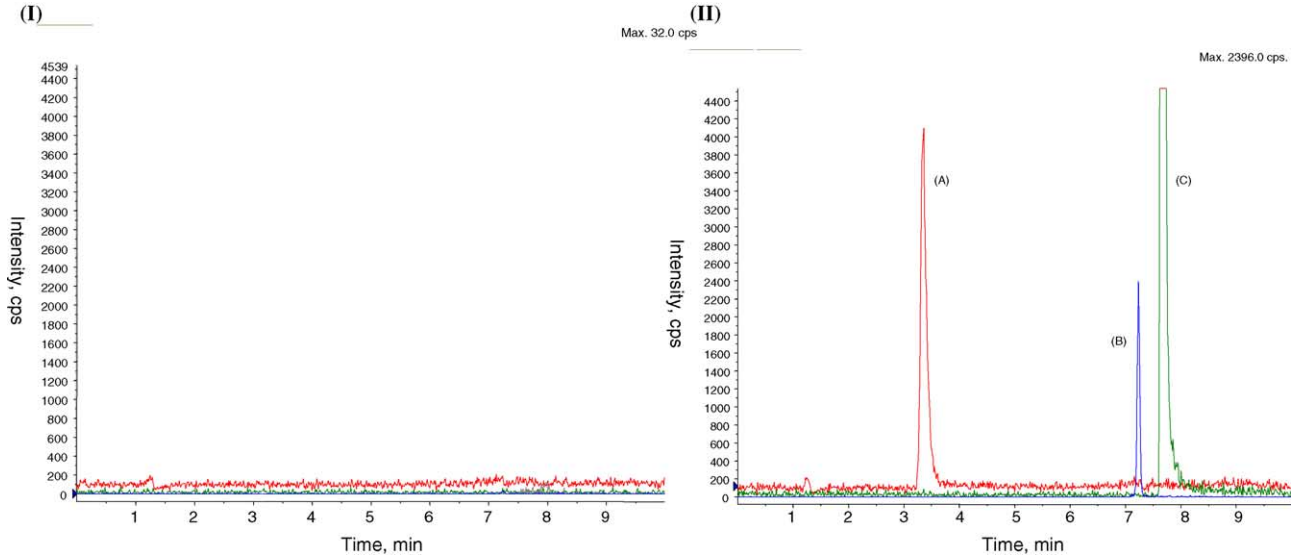

Fig. 2. Representative chromatogram of (I) blank human plasma, (II) human plasma spiked at the LLOQ with metronidazole (A), spiramycin I (B) and ornidazole (C).

Table 1
Intra- and inter-day precision and accuracy for metronidazole and spiramycin I in plasma

Nominal concentration (ng/ml)	Intra-day variation ($n = 6$)			Inter-day variation ($n = 6$)			Recovery ($n = 6$) (%)[c]
	Determined concentration (mean ± S.D.)	Precision (%)[a]	Accuracy (%)[b]	Determined concentration (mean ± S.D.)	Precision (%)[a]	Accuracy (%)[b]	
Metronidazole							
50	49.6 ± 1.88	3.79	−0.80	46.7 ± 1.93	4.13	−6.60	–
150	144 ± 3.41	2.37	−4.00	139 ± 7.87	5.66	−7.33	77.29
2000	1940 ± 47.2	2.43	−3.00	1940 ± 41.7	2.15	−3.00	–
4000	3860 ± 35.0	0.91	−3.50	3940 ± 121	3.07	−1.50	78.79
Spiramycin I							
15	16.2 ± 0.801	4.94	8.00	16.3 ± 0.746	4.58	8.67	–
45	45.7 ± 2.13	4.66	1.56	45.0 ± 2.68	5.96	0.00	79.61
800	864 ± 35.7	4.13	8.00	829 ± 43.3	5.22	3.63	–
1600	1700 ± 44.5	2.62	6.25	1730 ± 122	7.05	8.13	76.31
Internal standard[d]							92.54

[a] Expressed as R.S.D.: (S.D./mean) × 100.
[b] Expressed as % difference: [(concentration found − concentration added)/concentration added] × 100.
[c] Expressed as mean peak area ratio of extracted samples/mean peak area ratio of the unextracted samples.
[d] At a plasma concentration of 400 ng/ml.

7.8 min for the internal standard ornidazole. Representative chromatograms in plasma are presented in Fig. 2. The developed method is shorter than the LC-UV method developed by Rhone-Poulenc-Rorer (10 min instead of 20 min).

Positive electrospray ionization mode was selected, since it produced a stronger signal than positive APCI ionization for spiramycin. The mass spectra of metronidazole showed a major ion, corresponding to the protonated molecule $[M + H]^+$ ($m/z = 172$), whereas spiramycin I showed a double protonated molecule $[M + 2H]^{2+}$ ($m/z = 422.6$). Multiple-reaction monitoring was based on the fragmentation of the two types of protonated molecules.

3.2. Linearity

Standard curves were prepared in duplicate by injecting the extracts from seven known concentrations of metronidazole and spiramycin I in matrix with internal standard (ornidazole). The calibration curves were obtained by $1/x^2$ least-square linear regression analysis of known drugs concentrations ratios versus peak area ratios. Sample concentrations were calculated by using the regression equation of the straight-line $y = ax + b$, where y is the peak area ratio, a the slope, b the intercept and x the ratio of unknown concentration to ornidazole internal standard concentration. The

Table 2
Intra- and inter-day precision and accuracy for metronidazole and spiramycin I in saliva

Nominal concentration (ng/ml)	Intra-day variation ($n = 6$)			Inter-day variation ($n = 6$)			Recovery ($n = 6$) (%)[c]
	Determined concentration (mean ± S.D.)	Precision (%)[a]	Accuracy (%)[b]	Determined concentration (mean ± S.D.)	Precision (%)[a]	Accuracy (%)[b]	
Metronidazole							
50	50.0 ± 0.997	1.99	0.00	53.9 ± 5.57	10.33	7.80	–
150	145 ± 2.64	1.82	−3.33	147 ± 6.90	4.69	−2.00	81.93
2000	2020 ± 56.8	2.81	1.00	2060 ± 117	5.68	3.00	–
4000	4320 ± 319	7.38	8.00	4440 ± 526	11.85	11.00	67.72
Spiramycin I							
15	12.7 ± 0.344	2.71	−15.33	13.8 ± 0.769	5.57	−8.00	–
45	44.0 ± 1.06	2.41	−2.22	45.1 ± 1.82	4.04	0.22	87.58
2000	2030 ± 29.9	1.47	1.50	2030 ± 103	5.07	1.50	–
4000	4260 ± 330	7.75	6.50	3920 ± 203	5.18	−2.00	82.88
Internal standard[d]							76.53

[a] Expressed as R.S.D.: (S.D./mean) × 100.
[b] Expressed as % difference: [(concentration found − concentration added)/concentration added] × 100.
[c] Expressed as mean peak area ratio of extracted samples/mean peak area ratio of the unextracted samples.
[d] At a plasma concentration of 400 ng/ml.

Table 3
Intra-day precision and accuracy for metronidazole and spiramycin I in gingival crevicular fluid

Nominal concentration (ng/ml)	Intra-day variation ($n = 6$)			Recovery ($n = 6$) (%)[c]
	Determined concentration (mean ± S.D.)	Precision (%)[a]	Accuracy (%)[b]	
Metronidazole				
150	134 ± 12.3	9.18	−10.67	–
500	498 ± 31.6	6.35	−0.40	103.34
20,000	21,400 ± 1670	7.80	7.00	–
40,000	42,700 ± 1720	4.03	6.75	102.74
Spiramycin I				
150	166 ± 7.96	4.80	10.67	–
500	530 ± 27.5	5.19	6.00	128.57
20,000	21,700 ± 1340	6.18	8.50	–
40,000	43,800 ± 1970	4.50	9.50	112.95
Internal standard[d]				–

[a] Expressed as R.S.D.: (S.D./mean) × 100.
[b] Expressed as % difference: [(concentration found − concentration added)/concentration added] × 100.
[c] Expressed as mean peak area ratio of extracted samples/mean peak area ratio of the unextracted samples.
[d] At a plasma concentration of 400 ng/ml.

plasma standard curves for metronidazole and spiramycin I were found to be linear in the range of 50–5000 ng/ml and 15–2000 ng/ml with slope of (reported as mean ± S.D., $n = 5$) 0.0024845 ± 0.00010271 and 0.0021975 ± 0.00015351, respectively. The volume of plasma required to achieve the measurement of 50 ng/ml metronidazole and 15 ng/ml spiramycin concentrations is lower than the previously developed Rhone-poulenc-Rorer method (0.5 ml of plasma

Table 4
Stability of metronidazole and spiramycin I in plasma

Nominal concentration (ng/ml)	Determined concentration (mean ± S.D.)	Precision (%)[a]	Accuracy (%)[b]
Freeze–thaw stability			
Metronidazole			
150	139 ± 3.97	2.86	−7.33
4000	3870 ± 215	5.56	−3.25
Spiramycin I			
45	46.3 ± 1.45	3.13	2.89
1600	1720 ± 62.3	3.62	7.50
Short-term stability (4 h at room temperature)			
Metronidazole			
150	140 ± 4.76	3.40	−6.67
4000	3920 ± 136	3.47	−2.00
Spiramycin I			
45	44.5 ± 1.52	3.42	−1.11
1600	1680 ± 130	7.74	5.00
Long-term stability (−75 °C for 8 months)			
Metronidazole			
150	157 ± 3.41	2.17	4.67
4000	4260 ± 63.1	1.48	6.50
Spiramycin I			
45	46.1 ± 1.03	2.23	2.44
16,000	1650 ± 18.6	1.13	3.13
Stability of extract (101 h)			
Metronidazole			
150	144 ± 4.97	3.45	−4.00
4000	3960 ± 50.9	1.29	−1.00
Spiramycin I			
45	44.6 ± 1.35	3.03	−0.89
1600	1670 ± 36.7	2.20	4.38

[a] Expressed as R.S.D.: (S.D./mean) × 100.
[b] Expressed as % difference:[(concentration found − concentration added)/concentration added] × 100.

Table 5
Stability of metronidazole and spiramycin I in saliva

Nominal concentration (ng/ml)	Determined concentration (mean ± S.D.)	Precision (%)[a]	Accuracy (%)[b]
Freeze–thaw stability			
Metronidazole			
150	149 ± 9.95	6.68	−0.67
4000	4400 ± 257	5.84	10.00
Spiramycin I			
45	46.3 ± 4.74	10.24	2.89
4000	3870 ± 151	3.90	−3.25
Short-term stability (4 h at room temperature)			
Metronidazole			
150	167 ± 9.18	5.50	11.33
4000	4500 ± 164	3.64	12.50
Spiramycin I			
45	44.1 ± 1.76	3.99	−2.00
4000	4170 ± 90	2.16	4.25
Long-term stability (−75 °C for 9 months)			
Metronidazole			
150	154 ± 3.06	1.99	2.67
4000	3940 ± 36.7	0.93	−1.50
Spiramycin I			
45	43.8 ± 2.26	5.16	−2.67
4000	3790 ± 152	4.01	−5.25
Stability of extract (152 h)			
Metronidazole			
150	155 ± 3.20	2.06	3.33
4000	4380 ± 117	2.67	9.50
Spiramycin I			
45	38.2 ± 1.24	3.25	−15.11
4000	3700 ± 243	6.57	−7.50

[a] Expressed as R.S.D.: (S.D./mean) × 100.
[b] Expressed as % difference: [(concentration found − concentration added)/concentration added] × 100.

instead of 2 ml). The good signal to noise obtained at these concentrations (Fig. 2II) allows to still decrease the volume of matrix used. The saliva standard curve for metronidazole and spiramycin I was found to be linear in the range of 50–5000 ng/ml and 15–5000 ng/ml with slope (reported as mean ± S.D., $n = 5$) of 0.0031691 ± 0.000761351 and 0.0041799 ± 0.0010876, respectively. The GCF standard curve for metronidazole and spiramycin I was found to be linear in the range of 150–50,000 ng/ml and 150–50,000 ng/ml with a slope (reported as mean) of 0.00026209 and 0.00027474, respectively.

3.3. Precision and accuracy

Six replicates of the QC samples at each concentration level were used to evaluate the intra-day precision and accuracy. Two replicates of the QC samples at each concentration level from three separate batches were used to evaluate the inter-day precision and accuracy. The intra- and inter-assay mean accuracy in plasma was between −7.33 and −0.80% for metronidazole and between 0.00 and 8.67% for spiramycin I (Table 1). The intra- and inter-assay precision in plasma was between 0.91 and 5.66% for metronidazole, and between 2.62 and 7.05% for spiramycin I (Table 1). The intra- and inter-assay mean accuracy in saliva was between −3.33 and 11.00% for metronidazole and between −15.33 and 6.50% for spiramycin I (Table 1). The intra- and inter-assay precision in saliva was between 1.82 and 11.85% for metronidazole and between 1.47 and 7.75% for spiramycin I (Table 2). Due to the difficulty in obtaining blank human GCF, only intra assay was evaluated. The intra mean accuracy in GCF was between −10.67 and 7.00% for metronidazole and between 6.00 and 10.67% for spiramycin I and the intra-assay precision was between 4.03 and 9.18% for metronidazole and between 4.50 and 6.18% for spiramycin I (Table 3).

3.4. Recovery

The extraction recovery of analytes from the three different matrices was determined at two QC levels by comparing the peak area ratios of analytes to internal standard in samples that had been spiked with analytes prior to extraction with samples to which the analytes have been added post-extraction. The internal standard was added to both sets of samples post-extraction. The extraction recoveries of metronidazole, spiramycin I, and internal standard from human plasma were greater than 77, 76 and 92%, respectively (Table 1), and the dependence on concentration was negligi-

ble. The extraction recoveries of metronidazole, spiramycin I, and internal standard from human saliva were higher than 67, 82 and 76%, respectively (Table 2).

The extraction recoveries of metronidazole, spiramycin I, and internal standard from human GCF were approximately 100%. In GCF, the values obtained higher than 100% are likely due to the preparation process (2 μl of matrix used).

3.5. Stability

The stability of metronidazole and spiramycin I in plasma and saliva were investigated. The stability experiments were aimed at testing all possible conditions that the samples might experience between collection and to analysis. Stability results are summarized in Tables 4 and 5. Three freeze–thaw cycles and 4 h room temperature storage for low- and high-quality controls samples indicated that metronidazole and spiramycin I were stable in human plasma and saliva under these conditions. QC samples were stable when stored frozen at −75 °C for at least 8 or 9 months, for plasma and saliva, respectively. Testing of autosampler stability of quality control extract samples indicated that metronidazole and spiramycin I were stable when kept in the autosampler for up 101 h and 152 h for plasma and saliva, respectively.

3.6. Specificity and matrix effect

Due to the nature of electrospray ionization, matrix components eluted from HPLC column into the mass spectrometer at the same time as the analytes and/or internal standard may affect the ionization of the compounds of interest. This effect may be seen as either a suppression or enhancement of the analyte and/or internal standard response, even if the matrix component is not present in the MRM channel monitored for the analytes or IS. The ion suppression or enhancement caused by plasma or saliva was evaluated. The matrix effect of the method was considerably reduced through elimination of a number of endogenous components from plasma and saliva extracts during sample preparation. No matrix effect or interferences from endogenous compounds were detected from six different sources of human plasma and saliva (Tables 1–3). With the previously described Rhone-Poulenc-Rorer HPLC-UV method, interferences chromatographic peaks from endogenous source were present in chromatograms.

3.7. Application

To investigate the potential of the validated method for clinical studies, it was applied to determine metronidazole and spiramycin I in GCF after an oral administration three times a day of 250 mg of metronidazole and 1.5 MUI of spiramycin I during 6 days, in patients with a chronic periodontitis or a rapid progressive periodontitis. GCF samples were collected before the first dose of treatment (day 0), then again at days 2 and 6 (3 h after the administration of the morning dose), and again at days 7 and 30. Fig. 3 shows the mean GCF concentration at days 2, 6 and 7.

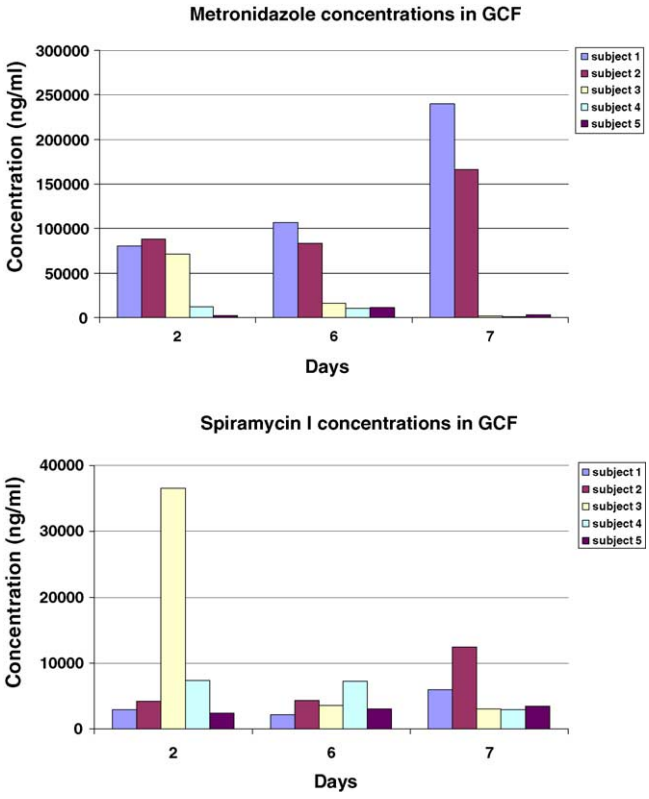

Fig. 3. Mean GCF concentration–day profile of metronidazole and spiramycin I after an oral administration three times a day of 250 mg metronidazole and 1.5 MUI of spiramycin I.

4. Conclusion

This is the first method describing the simultaneous extraction and quantitation of metronidazole and spiramycin I in plasma, saliva and GCF with LC–MS/MS detection. The method has shown acceptable precision, accuracy and adequate sensitivity. A Turbo IonSpray LC–MS/MS method was successfully developed and validated for the simultaneous determination of metronidazole and spiramycin I in human plasma, human saliva and human GCF. The method has an LLOQ of 50 ng/ml (metronidazole) and 15 ng/ml (spiramycin I) using 0.5 ml of human plasma and 0.1 ml of human saliva, and of 150 ng/ml (metronidazole and spiramycin I) using 2 μl of human GCF, which are lower than for the HPLC-UV method developed by Rhone-Poulenc-Rorer. This method enables the determination of two compounds with different polarities in a single, rapid, and reliable gradient elution analytical run. In opposition to the method developed by Rhone-Poulenc-Rorer, the same chromatographic conditions could be used whatever the matrix used due to specificity of mass spectrometry. The sample extraction is simple and can be transposed to several matrices (plasma, saliva) with satis-

factory specificity, recoveries, precision and accuracy. This method could be used for the analysis of clinical samples obtained from tissue diffusion studies after administration of the spiramycin–metronidazole combination.

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