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Short communication

Simple and rapid liquid chromatography method for determination of moxifloxacin in saliva

A. K. Hemanth Kumar, V. Sudha, R. Srinivasan, Geetha Ramachandran*

Tuberculosis Research Centre (Indian Council of Medical Research), Chetput, Chennai, India

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ABSTRACT

A high performance liquid chromatographic method for determination of moxifloxacin in human saliva was developed. The method involved deproteinisation of the sample with perchloric acid and analysis of the supernatant using a reversed-phase C18 column (150 mm) and fluorescence detection at an excitation wavelength of 290 nm and an emission wavelength of 460 nm. The assay was specific for moxifloxacin and linear from 0.25 to 10.0 μg/ml. The relative standard deviation of intra- and inter-day assays was lower than 10%. The average recovery of moxifloxacin from saliva was 101%. Due to its simplicity, the assay can be used for pharmacokinetic studies of moxifloxacin.

1. Introduction

Moxifloxacin is a new 8-methoxyquinolone which has potent activity against an extensive spectrum of bacteria. It has been shown to have promising antimycobacterial activity, and has the potential to shorten the duration of treatment of tuberculosis [1]. Early bactericidal activity studies using moxifloxacin have demonstrated the ability of this drug to kill slowly replicating persistent bacilli in the tissues, and this is regarded as an important characteristic to shorten tuberculosis treatment [2,3]. Controlled clinical trials using moxifloxacin along with first-line anti-tuberculosis drugs in pulmonary tuberculosis patients are being carried out in an attempt to shorten the duration of treatment for tuberculosis [4]. Hence monitoring of moxifloxacin concentrations may be useful to study its pharmacokinetics and understand drug–drug interactions when coadministered with other anti-tuberculosis drugs. The determination of drug concentrations in saliva has gained widespread acceptance in a variety of settings [5]. Salivary concentrations of drugs have been employed for therapeutic drug monitoring and for calculation of pharmacokinetic variables [6]. Collection of saliva is non-invasive, involves minimal discomfort and can be collected at multiple time points. It is particularly suitable in geriatric and pediatric populations. Information on the diffusion of moxifloxacin into saliva is scarce; investigations were therefore undertaken to determine the concentration of moxifloxacin in time matched plasma and saliva collected from healthy subjects. We developed and validated a simple and rapid assay procedure for estimation of moxifloxacin in saliva using the method that we had earlier developed for plasma estimation [7], and applied this method to determine its correlation with plasma concentrations.

2. Experimental

2.1. Chemicals

Pure moxifloxacin powder (purity ~99%) was a kind gift from Bayer, India. Ofloxacin from Sigma Chemical Company, MO, USA, acetonitrile (HPLC grade) from Merck (India), potassium dihydrogen orthophosphate and perchloric acid from Qualigens (India) were used. Deionized water was processed through a Milli-Q water purification system (Millipore, USA). Pooled human saliva was obtained from healthy volunteers, Chennai, India.

2.2. Chromatographic system

The HPLC system (Shimadzu Corporation, Kyoto, Japan) consisted of two pumps (LC-10ATvp), fluorescence detector (RF-10AXL) and auto sampler (SIL-HTA) with built in system controller. Class VP-LC workstation was used for data collection and acquisition. The analytical column was a C18, 150 mm × 4.6 mm ID, 5 μm particle size (Lichrospher 100 RP-18e, Merck, Germany) protected by a compatible guard column. The mobile phase consisted of 50 mM phosphate buffer, pH 2.6 (adjusted with 1 N HCl) and acetonitrile (80:20, v/v). Prior to preparation of the mobile phase, the phosphate buffer and acetonitrile were degassed separately using a Millipore vacuum pump. The fluorescence detector was set at

* Corresponding author at: Department of Clinical Research, Tuberculosis Research Centre, Mayor V.R. Ramanathan Road, Chetput, Chennai 600 031, India. Tel.: +91 44 28369648; fax: +91 44 28362528.
E-mail address: geetha202@rediffmail.com (G. Ramachandran).

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an excitation wavelength of 290 nm and an emission wavelength of 460 nm. The chromatogram was run for 8 min at a flow rate of 1.5 ml/min at ambient temperature. Unknown concentrations were derived from linear regression analysis of the peak height ratios (analyte/internal standard) vs. concentration curve. The linearity was verified using estimates of correlation coefficient (r).

2.3. Preparation of standard solution

A stock standard (1 mg/ml) was prepared by dissolving moxifloxacin in 0.1 N HCl. The working standards of moxifloxacin in concentrations ranging from 0.25 to 10.0 μg/ml were prepared in pooled saliva.

2.4. Sample preparation

To 100 μl each of calibration standards and test samples, 10 μl of ofloxacin (internal standard) was added at a concentration of 2 μg/ml. This was mixed with 50 μl of 7% perchloric acid, the contents were vortexed vigorously, centrifuged at 10,000 rpm for 10 min. Seventy-five microlitres of the clear supernatant was directly injected to the HPLC column.

2.5. Accuracy and linearity

The accuracy and linearity of moxifloxacin standards were evaluated by analysing a set of standards ranging from 0.25 to 10.0 μg/ml. The within day and between day variations were determined by processing each standard concentration in duplicate for six consecutive days.

2.6. Precision

In order to evaluate the precision of the method, saliva samples containing three concentrations of moxifloxacin at different levels

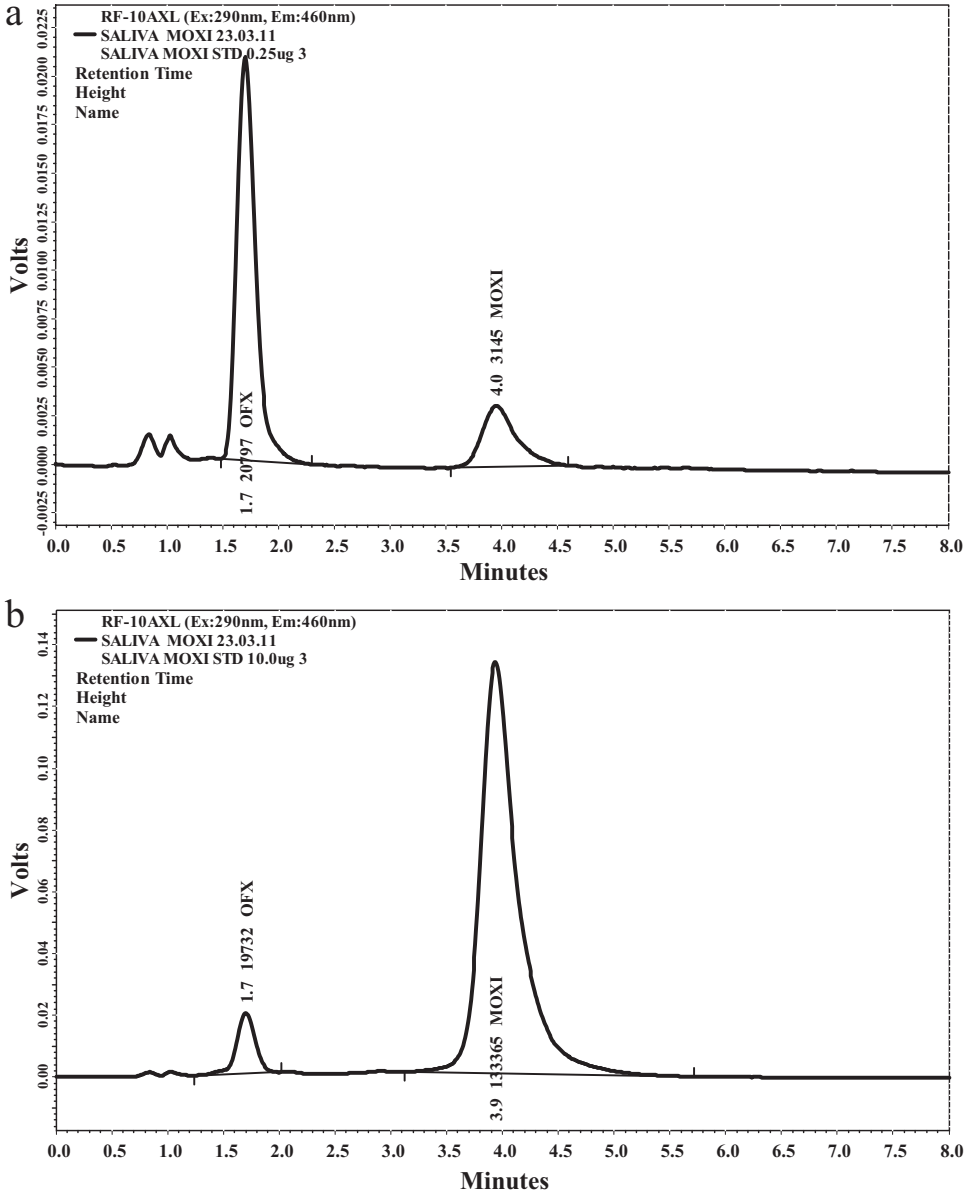

Fig. 1. (a) Chromatogram of extracted moxifloxacin saliva standard 0.25 μg/ml (contains internal standard—10 μl of ofloxacin added at a concentration of 2.0 μg/ml). (b) Chromatogram of extracted moxifloxacin saliva standard 10.0 μg/ml (contains internal standard—10 μl of ofloxacin added at a concentration of 2.0 μg/ml). (c) Chromatogram of extracted blank saliva (contains internal standard—10 μl of ofloxacin added at a concentration of 2.0 μg/ml). (d) Chromatogram of extracted blank saliva.

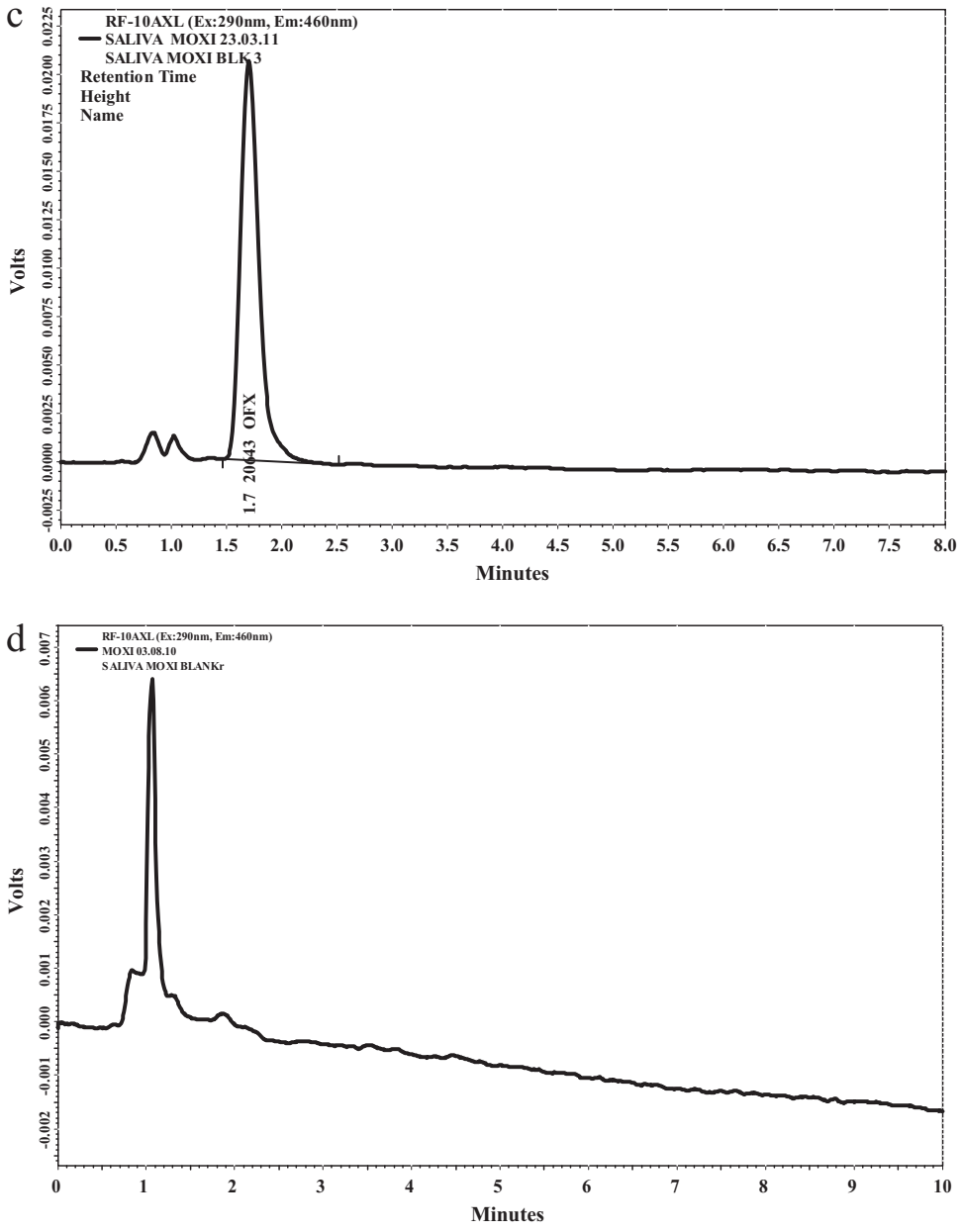

Fig. 1. *(Continued.)*

(0.1, 4.5 and 9.0 μg/ml) were prepared and analysed in duplicate on three consecutive days.

2.7. Recovery

For the recovery experiment, known concentrations of moxifloxacin (0.3, 3.0 and 6.0 μg/ml) were prepared in pooled human saliva samples and were spiked with 1.0 and 3.0 μg/ml moxifloxacin and assayed after addition of the internal standard. The percentage of recovery was calculated by dividing sample differences with the added concentrations. Recovery experiments were carried out on three different occasions.

2.8. Specificity

Interference from endogenous compounds was investigated by analysing blank saliva samples obtained from six each of male and female subjects. Interference from certain anti-tuberculosis drugs such as rifampicin, isoniazid, pyrazinamide, ethambutol and streptomycin at a concentration of 10 μg/ml was also evaluated.

2.9. Limits of quantitation (LOQ) and detection (LOD)

These values were estimated mathematically from the standard curve equations. The LOQ was obtained by multiplying the standard deviation (SD) of the *Y*-axis intercepts by 10. The LOD was equal to 3.3 times the SD of the *Y*-axis intercepts [11].

2.10. Samples

Forty-eight paired saliva and plasma samples were collected from 24 healthy volunteers at different time points after they were administered a single oral dose of 400 mg moxifloxacin. Two milliliters of blood was collected in heparinised vacutainer tubes, followed by collection of saliva. To facilitate salivary secretion, the individuals were asked to chew a piece of unsweetened, unflavored

Table 1
Linearity and reproducibility of saliva moxifloxacin standards.

Standard concentration (μg/ml)	Within day ($n = 6$)		Between day ($n = 6$)	
	Mean peek height ratio ± SD (RSD %)	Measured concentration (μg/ml)	Mean peek height ratio ± SD (RSD %)	Measured concentration (μg/ml)
0.25	0.14 ± 0.01 (3.6)	0.26 ± 0.01	0.14 ± 0.01 (7.8)	0.26 ± 0.01
0.5	0.30 ± 0.02 (5.6)	0.52 ± 0.03	0.29 ± 0.02 (7.3)	0.53 ± 0.03
1.0	0.58 ± 0.02 (4.3)	1.03 ± 0.04	0.56 ± 0.02 (2.7)	1.03 ± 0.06
2.5	1.41 ± 0.11 (7.8)	2.68 ± 0.09	1.52 ± 0.07 (4.7)	2.40 ± 0.11
5.0	2.80 ± 0.17 (6.1)	4.97 ± 0.12	2.58 ± 0.14 (5.3)	4.90 ± 0.14
10.0	5.62 ± 0.14 (2.5)	9.79 ± 0.15	5.43 ± 0.37 (6.8)	9.97 ± 0.17

chewing gum and spit out the initial salivary secretion. About 2 ml of saliva was collected over 5–10 min in a universal container. The blood samples were centrifuged immediately and plasma was separated and stored at −20 °C. The saliva samples were frozen at −20 °C over night. The following day, samples were thawed and centrifuged. The residue containing mucoproteins were discarded and the clear supernatants were stored at −20 °C. Estimation of plasma moxifloxacin was undertaken according to a previously validated method [6]. The study was approved by the Institutional Ethics Committee, and all the healthy subjects gave informed, written consent before sample collections.

3. Results and discussion

Use of saliva instead of blood for pharmacokinetic investigations has obvious practical advantages, particularly in children. It is a non-invasive procedure which avoids venipuncture and is amenable for collection of multiple specimens. It has been suggested that saliva can serve as an alternative body fluid for pharmacokinetic studies of certain drugs [8,9]. A few studies have undertaken estimation of moxifloxacin in saliva by high performance liquid chromatography methods [10,11]. These studies have employed the method described by Stass et al. [12], which has used gradient elution and on-column focusing.

In this study, sample preparation required a simple one-step deproteinisation method and analysis using a C18 column and an isocratic mobile phase. The present method has the advantages of being rapid (run time is only 8 min) and using a small sample volume (100 μl), without any loss of analyte. The use of ofloxacin as internal standard helped in monitoring the recovery of moxifloxacin from plasma. Under the chromatographic conditions described above, moxifloxacin was well separated as seen in the representative chromatograms (Fig. 1a and b). The retention times of the internal standard and moxifloxacin were 1.7 and 3.9 min, respectively. Blank saliva samples did not give any peak at the retention times of moxifloxacin (Fig. 1c) and ofloxacin (Fig. 1d). The lowest concentration of moxifloxacin gave a discrete peak at 4.0 min (Fig. 1a). Specificity experiments showed that the method was highly specific for moxifloxacin, and that no endogenous substances or first-line anti-tuberculosis drugs such as rifampicin, isoniazid, pyrazinamide, ethambutol and streptomycin interfered with the moxifloxacin chromatogram.

In the present method, moxifloxacin concentrations ranging from 0.25 to 10.0 μg/ml were checked for linearity. The calibration curve parameters of moxifloxacin from six individual experiments for standard concentrations ranging from 0.25 to 10.0 μg/ml showed a linear relationship between peak height ratio and concentrations. The mean (+SD) correlation coefficient, intercept and slope values were 0.9990 + 0.0007, 0.0260 + 0.0105 and 0.6890 + 0.2504, respectively. The linearity and reproducibility of the various standards used for constructing calibration graphs for saliva moxifloxacin are given in Table 1. The within-day and between-day relative standard deviation (RSD) for standards containing 0.25–10.0 μg/ml ranged from 2.5 to 7.8% and 2.7 to 7.8%, respectively. The reproducibility of the method was further evaluated by analysing three saliva samples containing different concentrations of moxifloxacin. The RSD for these samples ranged from 2.9 to 6.0% (Table 2). The % variations from the actual concentrations ranged from 97 to 105%. The LOD and LOQ estimated mathematically from the standard curve equation [13] were 0.03 μg/ml and 0.10 μg/ml, respectively. The method reliably eliminated interfering material from plasma, yielding a recovery for moxifloxacin that ranged from 96% to 106%.

Table 2
Precision of plasma moxifloxacin assay.

Actual conc. (μg/ml)	Found conc. (μg/ml) mean ± SD (% RSD)
9.0	9.13 ± 0.26 (2.9)
4.5	4.62 ± 0.14 (3.1)
0.1	0.11 ± 0.01 (4.6)

The method described was applied for the determination of moxifloxacin concentration in saliva, and to determine its correlation with time-matched plasma concentrations collected from 24 healthy subjects who received a single oral dose of 400 mg moxifloxacin. Moxifloxacin concentrations in the 48 paired plasma and saliva samples were highly correlated ($r^2 = 0.847$; $p < 0.001$) (Fig. 2). This finding suggests that pharmacokinetic variables of moxifloxacin can be determined using saliva concentrations instead of plasma. The mean saliva and plasma concentrations were 2.01 and 3.65 μg/ml, respectively, the saliva to plasma ratio being 0.54. This

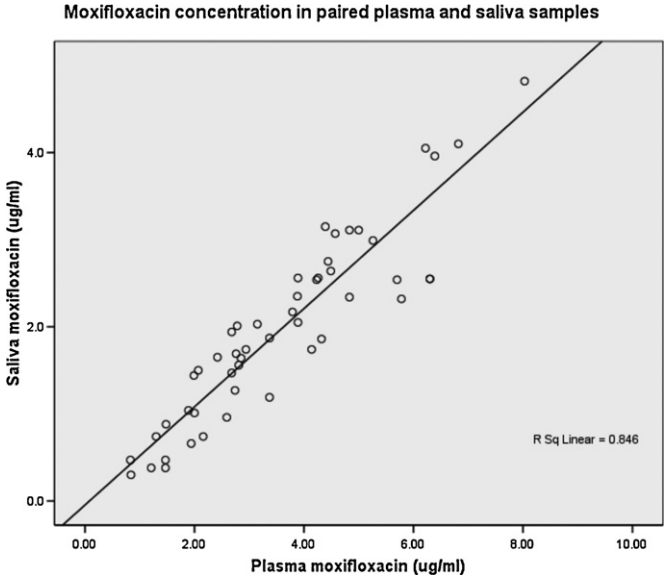

Fig. 2. Moxifloxacin concentration in paired plasma and saliva samples.

study data has shown that the plasma protein binding of moxifloxacin was about 46%, which is different from that reported by Ostergaard et al. (54%) [14] and Burkhardt et al. (20%) [12]. Contrary to all these reports, Muller et al. observed saliva and plasma moxifloxacin concentrations to be similar [10].

In conclusion, a sensitive, specific and validated method for quantitative determination of moxifloxacin in saliva is described. This rapid, accurate and reproducible method utilises a single step extraction. The chromatogram yields a well resolved peak for moxifloxacin with good intra- and inter-day precision. A good correlation between plasma and saliva concentrations of moxifloxacin, suggests that estimation of salivary levels could be used in therapeutic monitoring and in pharmacokinetic studies.

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