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SIMPLE SPECTROFLUORIMETRIC AND MICROBIOLOGICAL ASSAY METHODS FOR THE ESTIMATION OF OFLOXACIN IN BIOLOGICAL FLUIDS

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SUMMARY

Objective: To evolve simple methods for the assay of ofloxacin in biological fluids.

Methods: Simple methods for the estimation of ofloxacin in plasma, saliva and urine employing microbiological assay using plate diffusion technique and by fluorimetric method based on the measurement of native fluorescence emitted by ofloxacin, have been described.

Results: The recovery of ofloxacin from all the three biologial fluids was 93-98% and the sensitivity was 0.5 µg/ml on all 5 different occasions by both the methods. Anti-TB drugs viz., rifampicin, ethambutol, isoniazid and pyrazinamide and also anti-leprosy drugs *viz.,* dapsone and clofazimine at concentrations of 10 and 20 µg/ml did not interfere with the estimation of ofloxacin by either method. Ofloxacin is stable in biological fluids for a period of at least 8 days at -20°C.

Conclusion: Both the methods described are simple, involve very few steps and do not need either costly chemicals or sophisticated equipments.

KEY WORDS Ofloxacin E-coli fluorescence anti-TB drugs anti-leprosy drugs

INTRODUCTION

Ofloxacin is a synthetic fluorinated carboxy quinolone which is reported to have a broad antimicrobial spectrum[1-4]. Pharmacological studies of ofloxacin require sensitive and specific methods for the estimation of the drug in biological fluids such as blood, saliva and urine. HPLC methods using fluorescence detector[5,6] are available but they involve a series of steps for preparation of the sample before analysis. More over, all laboratories cannot afford to have such a sophisticated equipment. Since *E. coli* is sensitive to ofloxacin, we used it as test organism for the microbiological assay. The zone of inhibition exhibited by ofloxacin against *E.coli* is proportional to the concentration of the drug present. Ofloxacin, by nature is fluorescent and that the intensity of its native fluorescence depends on the amount of the drug.

Making use of the above two properties of ofloxacin, we have standardised the estimation of the drug in plasma, saliva and urine by microbiological and fluorimetric methods. The degree of interference, if any, of certain anti-TB and anti-leprosy drugs in these methods and the stability of ofloxacin in blood, saliva and urine upon storage over a period of time were also studied.

MATERIALS AND METHODS

Pure rifampicin, pyrazinamide, ethambutol and ofloxacin from Sigma Chemical Company, pure isoniazid from May & Baker Ltd., nutrient agar (ingredients per litre are beef extract 3g, peptone 5g, agar 15g) and nutrient broth (ingredients per litre are beef extract 3g, peptone 5g) from DIFCO, were used. All other chemicals used were of analytical grade. The strain of *E.coli* used was a clinical isolate.

Estimation of ofloxacin in plasma, saliva and urine

Microbiological assay: *E.coli* cultures were maintained in nutrient broth. Stock cultures of *E.Coli* from the clinical isolates were maintained on nutrient agar plates and stored at 4°C. Before each assay run, the organism was sub-cultured onto nutrient broth. At least two transfers in nutrient broth were made before the test assay. The nutrient broth cultures were kept at 37°C for 24 h. On the day of the assay, turbid-

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Table 1. Serial ofloxacin concentrations by microbiological method

Concentration of ofloxacin (µg/ml)	zone of inhibition* of *E.coli* against ofloxacin in		
	Plasma (mm)	Saliva (mm)	Urine (mm)
0.5	5.82 ± 0.58	5.40 ± 0.61	4.89 ± 0.65
1.0	7.44 ± 1.03	7.28 ± 0.98	6.69 ± 0.74
2.0	9.17 ± 1.04	9.15 ± 1.09	8.92 ± 0.95
4.0	10.98 ± 0.97	10.78 ± 1.08	10.73 ± 0.80
8.0	12.33 ± 0.92	12.42 ± 0.83	12.20 ± 0.87
18.0	14.04 ± 0.69	14.05 ± 0.80	13.94 ± 0.59

* Values are the results of 5 different occasions (mean ± SD) and the samples on each occasion in duplicate (n=10)

Table 2. Serial ofloxacin concentrations by fluorimetric method

Concentration of ofloxacin (µg/ml)	Fluorescence units* of ofloxacin in		
	Plasma	Saliva	Urine
0.5	30.10 ± 2.70	32.10 ± 3.75	25.40 ± 1.91
1.0	64.20 ± 4.04	63.30 ± 2.79	55.90 ± 3.53
2.0	120.90 ± 3.94	127.10 ± 3.21	109.80 ± 4.86
4.0	252.00 ± 4.27	257.00 ± 5.54	220.80 ± 10.26
8.0	501.40 ± 7.75	507.00 ± 8.09	436.20 ± 20.60
16.0	974.90 ± 13.87	971.00 ± 6.97	849.70 ± 43.30

*Values are the results of 5 different occasions (mean ± S.D) and the samples on each occasion in duplicate (n=10)

ity measurement of the broth culture was done at 630nm in a spectrophotometer. Only those cultures which showed an optical density ranging from 0.275 to 0.325 were used.

Nutrient agar (11.5gm) was dissolved in 500ml of distilled water and pH adjusted to 7.0 using 0.4N sodium hydroxide solution. The nutrient agar solution was auto-claved for 20 min. at 15 lbs. pressure. The agar solution was then brought to 37 °C to 40 °C and the organism kept in nutrient broth was seeded (0.4ml culture/500ml solution). This was then poured into sterile petri plates. Wells of 2mm diameter were cut.

A 1 mg/ml stock solution of ofloxacin was prepared by dissolving it in a minimal amount of sterile 0.1N hydrochloric acid solution (0.2ml acid/10mg. ofloxacin). Ofloxacin in concen trations of 0, 0.5, 1.0, 2.0, 4.0, 8.0 and 16.0 µg/ml in pooled plasma, saliva and urine was set up in quadruplicate. Ten microlitres of the standard solutions were loaded onto the wells. The plates were incubated at 37°C for 24h. The zone of inhibition was measured and its diameter was found to be proportional to the amount of drug. The concentration of the drug in unknown samples can be calculated from the regression line of the log concentration of the standard and the diameter of the zone of inhibition.

Fluorimetric method: One millilitre of plasma, saliva or urine containing ofloxacin ranging from 0.5-16.0µg/ml was deproteinised with 1.O ml. of 0.7M perchloric acid. The contents were centrifuged at 2500 rpm for 30 minutes. The resulting protein free filtrate containing ofloxacin was read in a Perkin Elmer LS 2B filter fluorimeter with an excitation wavelength at 310 nm and an emission wavelength at 510 nm. The relationship between ofloxacin concentration and

the fluorescence units was linear. The concentration of ofloxacin in unknown samples can be calculated using a set of standard solutions containing known concentrations of the drug.

Aqueous solutions containing the same concentrations of ofloxacin were processed simultaneously to check for recovery of the drug from plasma, urine and saliva.

The assays were undertaken on five different occasions after coding the samples independently for both methods.

Interference of anti-TB and anti-leprosy drugs: On each of 3 occasions, the interference of anti-TB drugs like rifampicin, ethambutol, isoniazid and pyrazinamide and anti-leprosy drugs like dapsone and clofazimine in the assay of ofloxacin by both the methods was tested individually. These drugs in concentrations of 10 and 20 µg/ml were added to ofloxacin concentrations of 0, 0.5, 1.0, 2.0, 4.0, 8.0 and 16.0 µg/ml set up in quadruplicate and in duplicate for microbiological and fluorimetric methods respectively, in pooled plasma.

Stability of ofloxacin: Blood, saliva and urine were collected from 2 patients after administration of 300 mg of ofloxacin. Plasma was separated and aliquots of plasma, saliva and urine were stored at -20°C. Ofloxacin was estimated by microbiological method on days 0, 1, 3, 6 and 8.

RESULTS

Recovery of ofloxacin from plasma, saliva and urine: The mean zones of inhibition and the mean fluorescence units obtained with plasma, saliva and urine are presented in Tables 1 and 2 respectively.

Results showed that with the microbiological assay, the zones of inhibition increased with increasing concentrations of ofloxacin over the range tested. Comparison of plasma, saliva and urine standards with aqueous standards processed similarly showed that recoveries of ofloxacin were quantitative (93-98%). The co-efficients of variation for replicate estimations were 10.4, 11.2 and 13.3 % for a concentration of 0.5 µg/ml and 4.9, 5.7 and 4.3 % for a concentration of 16.0 µg/ml with plasma, saliva and urine respectively. Similar co-efficients of variation were obtained for all the three biological fluids when repeated on five different occasions.

The fluorescent units were proportional to the concentration of ofloxacin employed and the recoveries were also quantitative (95-97%) for all the three biological fluids when compared with water standards. The co-efficients of variation for replicate estimations for a concentration of 0.5 µg/ml were 9.0, 11.7 and 7.5 % and 1.4, 0.7 and 4.3 % for a concentration of 16.0 µg/ml with plasma, saliva and urine respectively. The intraassay variations obtained for the three biological fluids by the fluorimetric method on five occasions tested were almost same.

These results indicate that with ofloxacin, Beer's law is followed over the range 0.5-16.0 µg/ml in all the three biological fluids by the fluorimetric method.

The fluorescent units obtained with plasma, saliva and urine blanks were 9, 13 and 116 respectively compared to a reading of 3 for water blank. This suggests that it is essential to employ pooled plasma, saliva and urine blanks for estimating ofloxacin concentrations in the corresponding samples.

Sensitivity: Sensitivity of the method has been regarded as the lowest concentration where the co-efficient of variation for replicate estimations is 10 to 15 percent. In the estimation of ofloxacin by the microbiological method, the lowest concentration with which the zone of inhibition could be read is 0.5 µg/ml. In the case of fluorimetric method, the co-efficients of variation obtained for plasma, saliva and urine at a concentration of 0.25 µg/ml were 15.5, 17.3 and 15.4 % respectively, at a concentration of 0.5 µg/ml, the co-eff icients of variation were 9.0, 11.7 and 7.5 % which is lower than those obtained with 0.25 µg/ml. The sensitivity of both the methods is, therefore, 0.5 µg/ml.

Interference of anti-TB and anti-leprosy drugs: The zones of inhibition for the microbiological method and the fluorescence units for the fluorimetric method were determined after randomisation of the specimens. Results indicated that none of the drugs interfered in the estimation of ofloxacin by both the methods.

Stability of ofloxacin: Results of the stability experiments have shown that samples containing ofloxacin can be stored upto 8 days at -20° C before the assay is undertaken. (Table 3)

Table 3. Stability of ofloxacin

Day	Ofloxacin (µg/ml)					
	Plasma		Saliva		Urine	
	1	2	1	2	1	2
0	7.44	0.91	3.98	0.89	195.41	108.40
1	8.14	1.48	4.64	0.86	219.79	147.46
3	6.28	0.91	5.74	0.83	251.11	133.90
6	8.44	0.84	5.41	0.74	201.47	120.92
8	8.70	0.97	4.57	0.78	192.95	103.02

Note: 1 & 2 indicate patients from whom samples were collected after drug administration.

DISCUSSION

The methods described here for the estimation of ofloxacin in plasma, saliva and urine are fairly simple and specific. Although the fluorimetric method is less time consuming (i.e., the results will be available within 2 hours), the volume of test sample required is 0.5 ml (500 µl). On the other hand, with the microbiological method, although the volume of the test sample required is only 10 - 50 µl, the results will be available only after 24 hours. Depending upon the volume of the sample available, any one of the two methods can be chosen. The sensitivity by both the methods is 0.5 µg/ml for the estimation of ofloxacin in either plasma, saliva or urine but this is not a serious limitation as this concentration is well below the minimal inhibitory concentration of 2.0 µg/ml in L.J. medium[7].

In the estimation of ofloxacin reported here, urine blanks gave higher fluorescence units than plasma or saliva blanks. However, this higher fluorescence in the urine blank, does not have any quenching effect when pooled urine containing varying concentrations of ofloxacin were estimated. There was linearity in the fluorescence units in relation to varying concentrations tested in urine.

Other anti-TB and anti-leprosy drugs do not interfere in the estimation of ofloxacin by both the methods. It is, therefore, not necessary to withhold any drugs when pharmacological investigations with ofloxacin are carried out. The two methods, can thus be used to estimate ofloxacin, even when the latter is given in combination with other drugs. Ofloxacin is quite stable in plasma, saliva and urine upto a period of 8 days when stored at -20 °C.

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