THE SPECTROFLUORIMETRY METHOD FOR DETERMINATION OF NALIDIXIC ACID IN HUMAN PLASMA

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Abstract

This paper describes a precise and sensitive method for analysis of Nalidixic Acid (NA) in plasma following the oral administration of a therapeutic dose in humans. Most procedures used for quantitation of NA are based on its fluorescence. The spectrofluorimetry is the best choice for a fluorescent drug, regarding its specificity and sensitivity of the method. Also, it is recommended for the fluorescent drug assay in pharmaceutical companies as a cheaper, more routin and time saving right way in comparison with chromatographic methods. For the practical determination, a new formulation of nalidixic acid tablet was used as a modle and compared with a work standard by invitro and invivo evalutions as a crossover study in 12 normal volunteers. The friability, weight variation, assay and dissolution test for both products were almost the same and approved by USP requirements.The plasma samples and pharmacokinetic parameters were determined. Results of the tests and their statistical analysis indicated no significant differences ($P<0.05$) between the products. Since the extent and rate of absorption of the new and work standard tablets are comparable and the plasma 90% confidence intervals for Cmax and Tmax ratios lie within 80-125% of their respective mean values, therefore the spectrofluorimetry is approved to be the best selection for a fluorescent drug , chemically and pharmaceutically.

Keywords:

Spectrofluorimetry, Fluorescent Drug, Nalidixic Acid, Formulation, Bioavailability.

Introduction

Nalidixic acid [1-Ethyl-1,4-dihydro-7-methyl-4-oxo-1,1,8-naphthyridine-3-carboxylic acid] is a 4-Quinolone antibacterial agent, active against eterobacteriaceae and given by mouth mainly to treat urinary-tract infections. It is rapidly and almost completely absorbed from the gastro-intestinal tract and about 93% of Nalidixic acid is bound to plasma protein (1-4). Most procedure used for quantitation of Nalidixic acid (NA) in biological materials involved either acid and base titration (5), microbiological techniques (6), polarography (7), ultraviolet spectrophotometry (8-9), gas (10-12), tin layer (13-14) and high-performance liquid chromatography (15-17) or Fluorimetry (4,18,19). A few of these methods has been applied only to quantitation of Nalidixic acid base on its fluorescence (4,19,20).

These are specific for the initial (essential) drug (NA), but not toward the metabolites. In the present work, we need just to compare initial drug and therefore spectrofluorimetric method is a specific and a selective way with less cost for this evaluation.

Apparatus

A Shimadzu spectrofluorimeter (Japan), with the detection limit of 10ng/ml, operating at exitation wavelength of 355.2 nm and emission wavelength of 472 nm. A Shimadzu spectrophotometer UV 160 (Japan), at 257 nm, and Pharma test dissolution model D2 611 (West Germany) were used.

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Materials

The commercial Nalidixic acid work standard tablets (Negram®) were supplied by the Stering Winthrop Research Institute (Rensselaer , N.Y., U.S.A) and the Generic Nalidixic acid, was a new formulation which had been developed for the Iranian Rouzdarou Laboratories in Tehran. Dimethyl formamide, lithium methoxide, ethanol, chloroform, (distilled before use) sodium hydroxide mono basic potassium phosphate, citric acid, hydrochloric acid, potassium chloride and concentrated phosphoric acid, were of analytical grade (Merck).

Methods

***A.In vitro*:** To prepare dissolution standard curve, 64 mg of Nalidixic acid powder was dissolved in 0.01N sodium hydroxide to make 200ml of 320µg/ml solution, and from this solution , a stock solution with 160 µg/ml was prepared and applide to make other concentrations . A quantity of 100ml from the stock solution was diluted to 200 ml with 0.01 N sodium hydroxide , to prepare a solution of 80µg/ml drug . In order to make solutions with 10, 20, 40 µg/ml for the standard curve, 12.5, 25 and 50ml of the last stock solution were mixed with 0.01N sodium hydroxide solution to make 200ml, respectively (3,20).

B.In vivo: The in vivo standard curve of Nalidixic acid contained two separate parts:

1. Standard curve of drug in the solvent: To 10mg of NA powder in a 100ml volumetric flusk was added 100ml buffer (0.2M boric acid, 0.2M potassium chloride, 0.176M sodium hydroxide), pH 9, to make a solution with 100µg/ml. Then, 50ml of the above solution was added to 50ml of the borate buffer, to make the stock solution. Serially, 1, 2, 4, 8 and 10ml of the stock solution were diluted with the buffer up to 20ml, to make the proper solutions for the standard curve(4).

2. Standard curve of drug in the plasma: 25mg of NA powder was dissolved in 0.01N sodium hydroxide to make 100ml of 0.25µg/µl solution. A volume of 50 microliters of above solution was added to 0.5ml of plasma to have a solution of 25µg/ml. To make solutions with 2.5, 5, 10 and 20µg/ml drug, respectively 5,10,20 and 40 ml of the last solution were added to 0.5ml of plasma and mixed thoroughly(19).

Procedures

A.In vitro

1.Friability and weight variation: 20 tablets of the new formulation and the commercial were weight, separately and both tests were down as required (3,20). The average, standard divation and coefficient variation percent (CV%) were calculated (21).

2.Assay of Nalidixic acid tablets: 20 tablets were milled and some powder containing 150mg NA was mixed with 100ml chloroform for 5 min and extracted with 20ml of 1 N NaOH. Extractive was diluted to 200ml with 1N NaOH.and 10ml of this solution was added to sufficient distilled water to make 100ml. Using a Shimadzu spectrophotometer, absorbance of the solution was recorded against of the standard at 258nm. The amount of NA was calculated using the flowing equation (3):

$$C_u = 20C_s\ (A_u/A_s) \qquad (1)$$

3.Dissolution test: A buffer solution (pH 5.6) containing 2.5 volumes of 0.2 molar NaOH., 2.5 volume of 0.2 molar monobasic potassium phosphate, 2 volumes of methanol and QS, to 10 volumes of water, was needed for this procedure. Dissolution test was carried out at 5, 10, 20, 30, 40, 50 and 60-minute intervals, at 60 rpm. Standard solution of NA (USP) in 0.01N NaOH. was prepared using definite concentration of NA and the blank solution, was a suitable composition of the buffer and 0.01N NaOH.. In this test a common tolerance in the USP/NF is not less than 80% dissolved in 30 min (3).

B. In vivo

1.Subject: 12 normal healthy male volunteers 21 to 27 year old weighing 51 to 74Kg were employed after passing all clinical pathologic tests screening for the liver, kidney and hematology function: BUN ,Creatinine , SGOT and SGPT.

2.Experimental Design: This study was designed as a crossover investigation. All subjects were fasted overnight for 6 hours and 5ml blood of each volunteer was taken before receiving any drug as the control. Each subject received 500mg of Nalidixic acid with a full glass of water.

3.Blood sampling: Blood samples were collected into heparinized glass tubes just prior and at 0.5, 1, 2, 3, 5, 5.5, 7.5 and 9.5 hours after administration of the drug. Plasma was separated immediately after collection from heparinized blood and kept frozen at –20C until analysis (4).

4.Sample preparation: To 0.5ml of human plasma in a 15ml glass centrifuge tube, were added 1.5ml of a 0.1 N hydrochloric acid and 4 ml of chloroform. The tube was stoppered and placed on a rotating shaker (60 rpm) for 10 min. After centrifugation (10 min at 1200g), the chloroform layer was transferred to another tube and extracted with 3ml borate buffer mentioned above, for 2 min. After recentrifugation (10 min), the aqueous phase was separated and to 2ml of each sample one drop of concentrated sulfuric acid was added to make fluorescence properties for Nalidixic acid (4,19).

5.Analysis: Dissolution curve and release of drug versus time were drived. D30%, D60%, t15% and t50% were calculated. The area under the plasma concentration-time curve (AUC) was estimate using the trapezoidal method and extrapolate to infinity. The eliminate half-life values were determined by the method of last squares. The maximum plasma concentration (C_{max}), the time of C_{max} (T_{max}), Ka, Kel and Ft% were also calculated (21).

Results

A. In vitro:

1. Friability and weight variation: result of the variations for the new generic and commercial work standard formulations are summarized in table 1.

2. Assay: the result of assay for the new generic formulation and work standard tablets is illustrated in table2.

Table 1: Friability and weight variation results

Specifications	Generic New Formulation	Commercial Standard
X	0.625(g)	0.677(g)
SD	0.007	0.008
CV%	1.1	1.18
FT%	0.16	0.15
WV/USP	Approved	Approved

X:Mean SD:Standard deviation CV: Coefficient of voriation

Table 2: Assay of tablets

Product	Batch No.	NA% (mean ± SD)
New Formulation	NL- 1-77-3	95.27±1.25
Work Standard	IPN 273	95.28±1.35

3. Dissolution of tablets: The results summarized in table 3 show that more than 80% of labeled drug is dissolved within 30min, therefore both tablets are approved by USP requirements.

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Table 3: Dissolution test results for the new generic (G) and work standard (S) tablets

Time (Minute)	Test No.1 (%)		Test No.2 (%)		Test No.3 (%)		Mean±SD (%)	
	G	S	G	S	G	S	G	S
5	42.3	41.3	42.8	40	42.5	41.8	42±0.17	41±0.93
10	65	66	65.5	66	65	70	65.2±0.29	67.3±2.3
20	72	73	74.00	76.3	75	75	73.7±1.53	75.4±0.75
30	85.5	80.81	87	80.71	88	80.91	86.8±1	80.8±0.1
40	87.5	85.75	89.5	85.80	88.85	85.85	88.4±0.85	85.8±0.05
50	90	89.5	90	89.9	92	89.5	90.6±1.15	89.6±0.23
60	95	96.5	94	95.7	97	95.7	95.5±1.5	96±0.46

B. In vivo

1. Recovery and Specificity: The data in table 4 show that NA is clearly extracted (separated) from plasma by this method.

Table 4: Specificity and recovery for extraction method of NA from plasma

Concentration (µg/ml)	Drug fluorescent in solvent	Drug Fluorescent in plasma	Extractable (%)
2.5	5.50	5.21	94.8
5	10.45	9.96	95.3
10	17.78	16.75	94.2
20	35.80	34.37	96
25	44.95	72.70	95

2. Reproducibility: In order to check the reproducibility of the extraction procedures used various spiked plasma samples were repeatedly analyzed. Table 5 shows the results obtained for several concentration of NA. The overall mean coefficients of variation for the between - day and within-day were 2% and 1.7%, respectively.

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Table 5: Reproducibility of the assay of NA on Plasma

Concentration (µg/ml)	Coefficient of variation whitin-day(%)	Coefficient of variation between- day(%)
2.5	1.06	1.24
5	2.02	2.03
10	1.50	1.70
20	2.30	3.10

3. Plasma Pharmacokinetic analysis: the mean Plasma levels of NA in 12 healthy volunteers following oral administration of 500mg Nalidixic acid of the new Generic and work standard formulations are platted in figure 1.

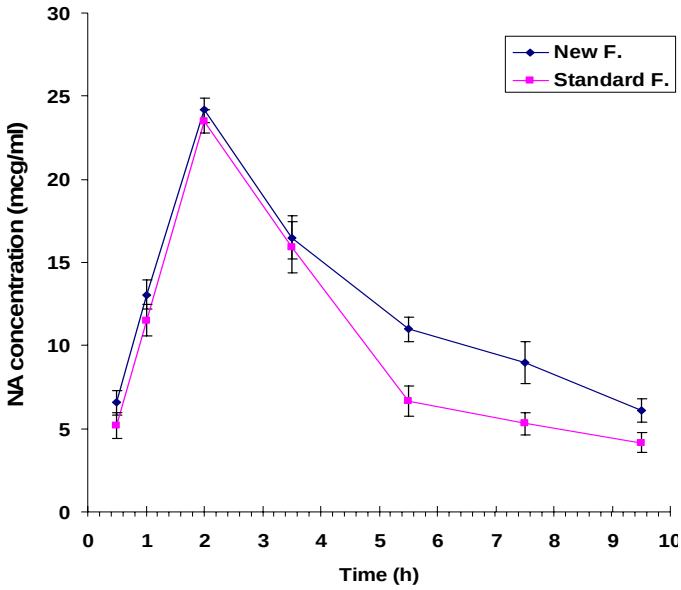

Fig. 1: Mean plasma nalidixic acid (NA) concentration in 12 helthy volunteers following oral administration of 500 mg of the new and standard formulation tablets.

Table 6: Average pharmacokinetic parameters (Mean±SD) following administration of 500mg Nalidixic acid tablets of formulations to 12 volunteers.

Formulation	Kel	Ka	T_{max}	C_{max}	$T_{1/2}$	AUC 0–9.5	AUC 0-∞
New Formulation	0.189 ± 0.02	1.27 ± 0.26	1.79 ± 0.16	25.3 ± 2	3.51 ± 0.7	108.96 ± 4.6	141.13
Work Standard	0.2 ± 0.04	1.17 ± 0.13	0.8 ± 0.18	23.9 ± 1.36	3.3 ± 0.44	101.5 ± 7.2	123.10

Discussion

Most procedures used for quantitation of NA are based on its fluorescence. The spectrofluorimetry is the best choice for a fluorescent drug , regarding its specificity and sensitivity of the method. Also , it is recommended for the fluorescent drug assay in pharmaceutical companies as a cheaper , more routin and time saving right way in comparison with chromatographic methods.(4,19). Statistical analysis of the data for assay, friability and weight variation indicated no significant differences between both formulations in terms of t-test student ($p<0.05$). The results summarized in table No 3 show that more than 80% of labeled drug is dissolved within 30min, therefore both tablets are approved by USP requirements. The data in table 4 show that NA is clearly extracted (separated) from plasma by this method.So, these results indicated a good recovery and specificity for the products. The overall mean coefficients of variation for the between-day and within-day were 2% and 1.7%, respectively.These results showed a good reproducibility of the assay of NA on

plasma. The results indicated that the plasma concentration versus time curve of various Nalidixic acid dosage forms are almost the same. Peak plasma concentration (Cmax) and the time necessary to reach peak plasma concentration (Tmax) are the two pharmacokinetic parameters which have been vitalized for the rate of drug absorption. Average peak plasma concentration (Cmax)and the average time of peak plasma level. (Tmax) for both formulations are very similar, and statistical analysis of the data indicated no significant differences between different formulations of NA ($p<0.05$). Area under the plasma concentration time curve (AUC) is used to evaluate the extent of absorption of the dosage forms. A comparison of the mean of area under the plasma concentration curve (AUC) indicated a 5% difference, higher for the new generic formulation, which is not scientifically important because, this difference is still less and within the WHO requirements (80-125%) of mean value acceptable interval.

Conclusion

Theoretically, Spectrofluorimetry method describes a simple, robust and easy way to automate analysis of the fluorescent drugs in laboratories. Practically, in pharmaceutical companies which have to assay and control many batches routinely, it is very important to use methods and procedures which could be run and done very fast and precise, specially for clinical pharmacokinetic stodies. Many methods have been applied to evaluate Nalidixic acid but, it seems that spectrofluorimetry is more economicall for a pharmaceutical company to determine fluorescent drugs in blood. Although the specificity, sensitivity and simplicity of the procedure are the most important factors which introduce it as a selective method.

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