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Extraction of Metronidazole and Furazolidone from industrial effluents by double salting out assisted liquid liquid extraction technique and their analyses by HPLC-UV method

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Abstract: We have developed a new analytical method for simultaneous determination of Metronidazole and Furazolidone by High Performance Liquid Chromatography (HPLC). The method is coupled to double salting-out assisted liquid-liquid extraction (SALLE) which facilitates the detection and quantification of Metronidazole and Furazolidone in different dosage forms by subsequent HPLC analysis. Extraction parameters such as solvent, pH and salt concentration are optimized and liquid liquid extraction showed efficiency up to 102% and 100% for Metronidazole and Furazolidone respectively in chloroform, at pH 3 and low salt concentration (0.5g KCl and 0.5 g$NaNO_3$). The designed simultaneous analytical method is further validated and Metronidazole and Furazolidone showed linearity upto $R^2$0.994 and 0.9936 respectively. LOD was 2.1µg/ml, 1.5µg/ml and LOQ was 21.1µg/ml, 15.2µg/ml for metronidazole benzoate and furazolidone. Precision of the method is 1.84% (RSD) and 1.72% (RSD) for Metronidazole and Furazolidine respectively. Moreover, accuracy is measured in terms of percent recovery which is more than 100% for both analytes under optimized conditions. Finally, robustness is evaluated by changing the flow rate and detection wavelength which is also obtained within permissible limit. This indicates that the proposed method is simple, efficient and shows excellent recoveries for simultaneous determination of Metronidazole and Furazolidone in different dosage forms and Industrial effluents.

Keywords: Metronidazole, Furazolidone, liquid liquid extraction, HPLC, robustness.

INTRODUCTION

Drugs have been an essential part of our life which are being used for the treatment of several diseases. Metronidazole benzoate (MNZ) "1-(2-benzoyloxyethyl)-5-nitro-2-methyl-imidazole" is still successfully used against wide varieties of anaerobic protozoal parasites (Jarrad *et al.,* 2016) and bacterial infections from 45 years in Human and animal orally and injectable form (Zamani *et al.,* 2010). It is also widely used in combination with other antibiotics and acid suppressing agents which process synergistic effect in eradication treatment of gastric *Helicobacter pylori* infections (Luther *et al.,* 2010). Its action and use is imidazole antibacterial (Nista *et al.,* 2006). Metronidazole Benzoate is the one of the top most persistent Drug and 10th most use drug in pregnancy (Sheehy *et al.,* 2015). Furazolidone, 3-(5nitrofurfury lideneamino)-2-oxo-oxazolidine, is a 3-nitiofuran used as an antibacterial agent against livestock diseases. Most nitrofurans are known to be mutagenic and carcinogenic (Cheng *et al.,* 2009), such as Metronodazole Benzoate. In some countries farmer mixed this in Animal feed (Kagambega *et al.,* 2018). Structures of both drugs are given in fig. 1.

The variety of methods and techniques are established for the estimation of Metronidazole benzoate such as HPLC-UV, LC-/MS-MS, UV-Visible Spectrophotometer, NMR, HPLC-Electrochemical Detector (Mishra *et al.,* 2014; Ghante *et al.,* 2012; Santos *et al.,* 2009). All these methods have certain advantages as well as disadvantages. Turbid metric in different type of samples like Pharmaceutical formulation, Animal tissues, Biological fluids which makes the identification of these drugs often difficult (Chen *et al.,* 2016). HPLC-UV is the most common, simple, easy, less expensive and accurate technique. This method is equally suitable for the simultaneously determination of Metronidazole benzoate and Furazolidone in oral liquid, Injection and industrial effluent using isocratic elution using HPLC-UV with column packing material C18 (Elkhoudary *et al.,* 2016).

Extraction plays crucial role before HPLC analysis because it minimizes the interferences and reduces the complexity, thus enabling better analysis of such compounds (Mohyuddin *et al.* 2019). Biological samples and compound separation has been using salting out assisted liquid-liquid extraction (SOLLE) with acetonitrile (Zhang *et al.,* 2019). The SOLLE has been used with organic and inorganic salts for extraction of drugs in biological samples and proven it is best to LLE, PPT, and SPE method (Alshishani *et al.,* 2018). Salt solubility is more in water and reducing the solubility of nonelectrolyte in water miscible phase thus separation it. The method has been adopted for organic compounds from water, food samples, biological samples, sea water, etc. Metal chelates salting out also investigated by the

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separation of acetone from water. More work reported on pesticide in fruits and drugs from blood and urine (Hyde *et al.,* 2017).

Few techniques are found in the literature for the simultaneous determination of metronidazole and furazolidone in bulk and pharmaceutical dosage forms. Elena and Milea in 2010 have developed HPLC method for determination of metronidazole and furazolidone. The chromatographic separation was also done by using C18 column (250 mm × 4.6 mm; 5µm particle size) with mobile phase consisted of methanol and 0.1% phosphoric acid (20:80 v/v), run at flow rate of 1mL/ min, at 317 nm. A stability-indicating HPLC method for the analysis of metronidazole, furazolidone and its degradation products was developed (Kumar *et al.* 2015). All these methods have certain advantages as well as disadvantages. Turbid metric in different types of samples like Pharmaceutical formulation, animal tissues, biological fluids makes the identification of these drugs often difficult (Jarrad *et al*. 2016).

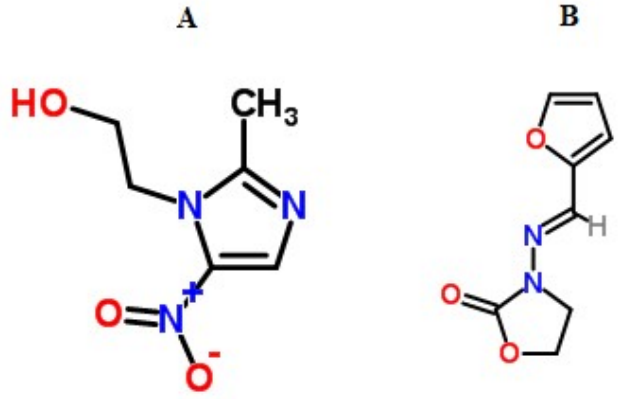

Fig. 1: Structures of Metronidazole and Furazolidone

Extraction plays crucial role before HPLC analysis because it minimizes the interferences and reduces the complexity, thus enabling better analysis of such compounds (Hussain *et al*. 2019). The SOLLE has been used with organic and inorganic salts for extraction of drugs in biological samples and proven it is best to LLE, PPT, and SPE method. Salt solubility is more in water and reducing the solubility of nonelectrolyte in water miscible phase thus separation it. The method has been adopted for organic compounds from water, food samples, biological samples, sea water (Goggin *et al.* 2019). In industrial effluents, large numbers of impurities are expected that is why in 20 minutes run time most of which get eluted without interfering the desired components. This method is more accurate, small LOD, LOQ, large linear range and applicable to real samples (industrial effluents), dosage form.

In this study an attempt has been made to develop a double salting out assisted liquid liquid extraction method for simultaneous determination of metronidazole and furazolidine from pharmaceutical dosage forms and industrial effluents.

MATERIALS AND METHODS

Chemicals

The highest purity analytical grade chemicals were used through out analysis. Metronidazole Benzoate and furazolidine of highest purity were purchased from local market. Methanol, Acetonitrile and Potassium dihydrogen phosphate (KH_2PO_4) was purchased from Merck (Germany). Dimethylformamide EP grade was purchased from Daejung (Korea). Metronidazole Benzoate and Furazolidone 99% purity were purchased from Sigma Aldrich (USA). Dependal-M suspension/ tablet product of GSK, Furalif (Life Pharmaceutical Company) was used as product for analysis. Chloroform, Hexane, Chloromethane, Diethyl ether, Sodium Nitrate, Potassium chloride, Sodium chloride, sodium sulphate, ammonium acetate were Analytical grade (Merk Germany).

Double salting out assisted liquid-liquid extraction

Industrial waste 2mL was spiked with 0.5mL containing metronidazole benzoate 15mg/mL and Furazolidone 5 mg/mL in stopered glass tube, add salts (Sodium Nitrate, potassium chloride, Sodium chloride, sodium sulphate, ammonium acetate) and shaken for 5 minutes with 5mL organic solvents (Chloroform, Hexane, Chloromethane, Diethyl ether). Then vortexed for 2 minutes and centrifuged at 5000 rpm for 20 minutes. Separation of the organic layer was done, solvent was evaporated to dryness, 5 mL N,N-dimethyl formamide was added, and the sample was analyzed by HPLC. The whole process was repeated three times and calculated the percentage recovery of each test accordingly.

Sample preparation

3.4g Potassium dihydrogen phosphate was dissolved in 500mL water. Adjusted the pH of this solution with dilute phosphoric acid (10% v/v) at 2.5±0.1. Then added 350mL of methanol and 100mL acetonitrile with continuous shaking. After making up to the volume upto 1000mL with water, sample was filtered through 0.45µm membrane filter.

Sample and standard solutions of Furazolidone and Metronidazole were prepared in area protected from light to avoid degradation of samples. 5mL of suspension (equivalent to 25mg of Furazolidone & 75mg of Metronidazole) after shaking well was taken in 50mL volumetric flask. 30mL of dimethyl formamide was added and stirred vigorously and added hot water at 3 different intervals (5mL each time), with gentle shaking and made the volume upto the mark with water to 50mL. Diluted 5mL of above solution to 25mL with mobile phase, stirred vigorously and filtered. Final concentrations of both samples was Furazolidone=100µg/mL and Metronidazole=300µg/mL.

Table 1: Optimization of pH, solvent and salt concentration

S. NO.	Solvent	pH	Salt 1 KCl	Salt 2 $NaNO_3$	% Recovery Metronidazole benzoate	% Recovery Furazolidone
1	Chloroform	3	0.5g	0.5 g	96.71-98.81%	97.66-99.05%
2	Chloroform	3	1.0 g	1.0 g	97.12-99.23%	97.82-99.41%
3	Chloroform	3	1.5 g	1.5 g	97.10-98.98%	97.11-99.26%
4	Chloroform	5	0.5g	0.5 g	84.00-87.19%	79.47-82.07%
5	Chloroform	5	1.0 g	1.0 g	83.94-87.89%	78.75-81.77%
6	Chloroform	5	1.5 g	1.5 g	85.50-88.49%	78.22-80.93%
7	Chloroform	7	0.5g	0.5 g	83.66-87.35%	71.52-73.33%
8	Chloroform	7	1.0 g	1.0 g	86.76-86.89%	71.74-73.07%
9	Chloroform	7	1.5 g	1.5 g	83.33-84.54%	70.51-73.14%
10	Chloroform	9	0.5g	0.5 g	74.00-75.23%	65.00-66.37%
11	Chloroform	9	1.0 g	1.0 g	72.12-74.67%	61.15-64.62%
12	Chloroform	9	1.5 g	1.5 g	73.55-74.48%	61.80-65.55%
13	Hexane	3	0.5g	0.5 g	70.77-71.76%	59.98-61.17%
14	Hexane	3	1.0 g	1.0 g	69.51-72.00%	62.45-65.03%
15	Hexane	3	1.5 g	1.5 g	71.00-73.25%	62.88-66.62%
16	Hexane	5	0.5g	0.5 g	63.78-65.32%	51.36-52.74%
17	Hexane	5	1.0 g	1.0 g	61.25-63.19%	51.54-52.88
18	Hexane	5	1.5 g	1.5 g	62.27-64.39%	51.11-52.39%
19	Hexane	7	0.5g	0.5 g	56.94-58.38%	40.25-41.62%
20	Hexane	7	1.0 g	1.0 g	56.22-57.15%	40.72-42.31%
21	Hexane	7	1.5 g	1.5 g	56.27-57.61%	41.16-43.34%
22	Hexane	9	0.5g	0.5 g	54.77-57.37%	39.93-41.48%
23	Hexane	9	1.0 g	1.0 g	53.68-55.30%	41.00-42.09%
24	Hexane	9	1.5 g	1.5 g	53.11-55.21%	39.64-41.17%
25	Chloromethane	3	0.5g	0.5 g	88.83-91.11%	80.37-81.15%
26	Chloromethane	3	1.0 g	1.0 g	87.39-91.16%	80.44-82.29%
27	Chloromethane	3	1.5 g	1.5 g	87.08-90.78%	81.02-82.06%
28	Chloromethane	5	0.5g	0.5 g	72.41-76.35%	69.00-71.66%
29	Chloromethane	5	1.0 g	1.0 g	72.79-76.31%	68.57-71.76%
30	Chloromethane	5	1.5 g	1.5 g	72.19-77.50%	61.11-62.55%
31	Chloromethane	7	0.5g	0.5 g	61.62-64.26%	49.40-50.32%
32	Chloromethane	7	1.0 g	1.0 g	62.60-65.28%	50.06-51.70%
33	Chloromethane	7	1.5 g	1.5 g	62.72-65.58%	50.71-52.44%
34	Chloromethane	9	0.5g	0.5 g	51.36-52.22%	36.50-36.88%
35	Chloromethane	9	1.0 g	1.0 g	50.21-53.70%	35.89-36.75%
36	Chloromethane	9	1.5 g	1.5 g	51.03-53.76%	35.04-36.41%
37	Diethylether	3	0.5g	0.5 g	93.48-95.04%	94.21-94.67%
38	Diethylether	3	1.0 g	1.0 g	94.07-95.88%	94.44-95.16%
39	Diethylether	3	1.5 g	1.5 g	94.46-96.01%	94.81-95.52%

Table 2: Precision of method developed for Metronidazole and Furazolidone analysis by HPLC

Sample #	Peak Area (Metronidazole)	Peak Area (Furazolidone)
1	4515768	4342590
2	4765869	4574603
3	4708414	4501518
4	4708948	4513596
5	4707087	4509152
6	4704228	4491967
Avg. Peak Area	4685052.33	4488904.33
Std Dev.	86207.33	77382.52
% RSD	1.84%	1.72%

Table 3: Concentration and Peak Area of standard solutions for the accuracy of method for Metronidazole

Standard ID	Concentrations	Peak Area
A	240µg/ml	3738423.33
B	270µg/ml	4121144.33
C	300µg/ml	4712832.33
D	330µg/ml	5220036.33
E	360µg/ml	5690954.00
F	390µg/ml	6017173.33

Table 4: Peak Area of sample solutions for the accuracy of method for Metronidazole

Concentration of Sample (µg)	Sample ID		Peak area	Peak Area (Mean)
240	A	Sample A1	3773283	3813568.66
		Sample A2	3823265	
		Sample A3	3844158	
300	B	Sample B1	4713357	4718127.00
		Sample B2	4717673	
		Sample B3	4723351	
360	C	Sample C1	5663807	5669024.66
		Sample C2	5669553	
		Sample C3	5673714	

Table 5: Concentration and peak area of standard solutions for the accuracy of method for Furazolidon

Standard ID	Concentrations	Peak Area (Mean)
A	80µg/ml	3464324.33
B	90µg/ml	3816907.00
C	100µg/ml	4368554.66
D	110µg/ml	4846708.33
E	120µg/ml	5272789.66
F	130µg/ml	5577640.66

Table 6: Peak Area of sample solutions for the accuracy of method for Furazolidone

Concentration of Sample (µg)	Sample ID		Peak Area	Peak Area (Mean)
80	A	Sample A1	3420558	3479886.33
		Sample A2	3506536	
		Sample A3	3512565	
100	B	Sample B1	4257083	4257578.66
		Sample B2	4253647	
		Sample B3	4262006	
120	C	Sample C1	5084312	5056931.66
		Sample C2	5049846	
		Sample C3	5036637	

Table 7: Accuracy of developed method for Metronidazole and Furazolidone

Conc. of Sample (µg)	Peak Area of Sample (Mean)	Peak Area of Standard (Mean)	Observed Amount (µm)	Theoretical Amount (µm)	%Recovery
% Recovery for Metronidazole					
240	3813568.66	3738423.33	244.82	240	102.01%
300	4718127.00	4712832.33	300.34	300	100.11%
360	5669024.66	5690954.00	358.61	360	99.61%
% Recovery for Furazolidone					
80	3479886.33	3464324.33	80.36	80	100.45%
100	4257578.66	4368554.66	97.46	100	97.46%
120	5056931.66	5272789.66	115.09	120	95.91%

Table 8: Summary of validation parameters for Metronidazole

Validation parameters	Result (Metronidazole)		Acceptance criteria
Linearity	Correlation coefficient = 0.994		Correlation coefficient Not less than 0.97
Precision	1.84 % RSD		% RSD Not more than 2.0
Accuracy	Concentration	% Recovered	% Recovery within 90% --- 110%
	240 µg/ml	102.01%	
	300 µg/ml	100.11%	
	360 µg/ml	99.61%	
Robustness	Change in Flow rate	% RSD	% RSD Not more than 1.5
	1.3ml/min	0.75%	
	1.7ml/min	1.41%	
	Change in Wavelength	% RSD	
	250nm	1.50%	
	270nm	1.19%	

Table 9: Summary of validation parameters for Furazolidone

Validation parameters	Result (Furazolidone)		Acceptance criteria
Linearity	Correlation coefficient = 0.9936		Correlation coefficient Not less than 0.97
Precision	1.72% RSD		% RSD Not more than 2.0
Accuracy	Concentration	% Recovered	% Recovery within 90% --- 110%
	80 µg/ml	100.45%	
	100 µg/ml	97.46%	
	120 µg/ml	95.91%	
Robustness	Change in Flow rate	% RSD	% RSD Not more than 1.5
	1.3ml/min	1.47%	
	1.7ml/min	0.97%	
	Change in Wavelength	% RSD	
	250nm	1.35%	
	270nm	1.37%	

Table 10: Robustness of developed method for the detection of Metronidazole and Fuazolidone

Sample #	Flow Rate		Wavelength	
	Peak Area at 1.3 mL/min	Peak Area at 1.7 mL/min	250 nm	290 nm
Robustness of Metronidazole				
1	5596811	3948845	8186967	9074172
2	5513904	4046399	8314092	8904360
3	5549401	4047891	8435980	9103464
Mean	5553372.0	4014378.33	8312346.33	9027332.0
Std. deviation	41595.90	56758.43	124515.68	107499.26
% RSD	0.75%	1.41%	1.50%	1.19%
Robustness of Furazolidone				
1	4849463	3271642	4095187	2091244
2	4854612	3298097	4150333	2088581
3	4976735	3335433	4207090	2139952
Mean	4893603.33	3301724.0	4150870.0	2106592.0
Std. deviation	72040.15	32049.79	55953.43	28920.98
% RSD	1.47%	0.97%	1.35%	1.37%

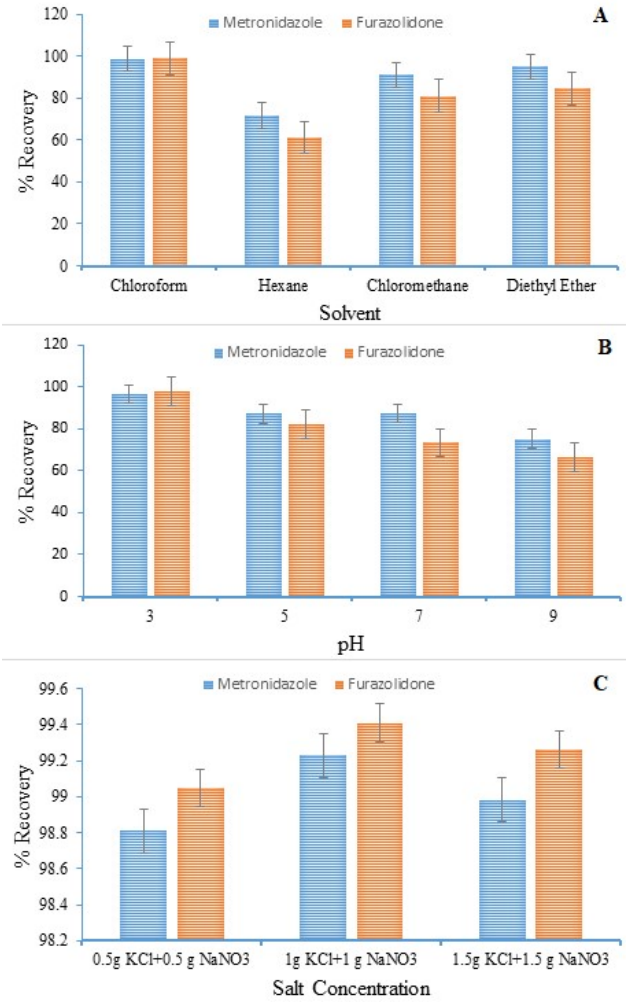

Fig. 2: Optimization of extraction parameters (A) Solvent, (B) pH and (C) Salt concentration for KCl and $NaNO_3$

Standard solution was also prepared using similar procedure. Briefly, 25mg accurately weighed quantity of Furazolidone working standard and 121mg of Metronidazole benzoate (equivalent to 75mg of Metronidazole) was taken in 50ml volumetric flask. To dissolve the stanards, 30mL of dimethyl formamide was added while stirring for three minutes and added hot water in 3 steps 5mL each, with shaking every time. Dilute 5mL of above solution to 25mL with Mobile phase, mixed well and filtered. Following the final concentrations of Furazolidone was 100µg/mL and Metronidazole was 300µg/mL.

HPLC Analysis

Shimadzu (Japan) HPLC having LC-20AT pump and Detector SPD-20A and HPLC Column (4.6mm x 250mm, 5µm) C18 (Promosil) was used for analysis after calibration. Detector Wavelength was used 270nm. Flow rate was kept at 1.5ml per minute and approximate run time was 25 minutes.

Separately injected equal volumes (about 20 µL) of the standards and sample solutions into the HPLC injection system, recorded the chromatograms, and measured the peak areas for the major peaks. And then, % age contents of Furazolidone and Metronidazole were calculated by following formulas:

$(FC_S / FC_U) \times (FA_U / FA_S) \times 100$ = Percentage of Furazolidone (1)

$(MC_S / MC_U) \times (MA_U / MA_S) \times 100$ = Percentage of Metronidazole (2)

Where FA_S is Peak Area of Furazolidone in Standard solution, FA_U is Peak Area of Furazolidone in sample solution, FC_S=100µg/mL is concentration of Furazolidone in standard solution, FC_U=100µg/mL concentration of Furazolidone in sample solution, $MA_S$is the Peak Area of Metronidazole in standard solution, MA_U is the Peak Area of Metronidazole in sample solution, MC_S=300µg/mL is the concentration of Metronidazole in standard solution and MC_U=300µg/mL is the concentration of Metronidazole in sample solution.

STATISTICAL ANALYSIS

Validated Microsoft Office Excel 2016 was used for the calculation of mean, SD, RSD and graphs for this research work

RESULTS

Optimization of extraction parameters

Initially extraction parameters such as extraction solvent, *pH* and concentration of salts were optimized and % recovery of each drug was calculated by HPLC. Four different extraction solvents were used for the extraction purpose and their extraction efficiency was evaluated. These solvents were tested at different pH (3, 5, 7 and 9) and at different salt concentration 0.5g, 1g and 1.5g of KCl and $NaNO_3$. During the optimization of certain parameter, all other parameters are varied one by one and percentage recovery was calculated. Detailed information about the parameters and their percentage recovery is given in Table 1. During solvent optimization maximum recovery for both Metronidazole and Furazolidone was observed in case of chloroform (fig. 2A). Maximum calculated recovery of Metronidazole is upto 98.81% and Furazolidone upto 99.05% using chloroform as extraction solvent. In case of diethyl ether as extraction solvent, obtained recovery is 95.04% and 87.64% for Metronidazole and Furazolidone respectively. Lowest recovery was observed in case of hexane as extraction solvent and 71.76% and 61.17% for Metronidazole and Furazolidone, respectively.

Similarly, pH of all four extraction solvents also optimized, in order to achieve best possible extraction

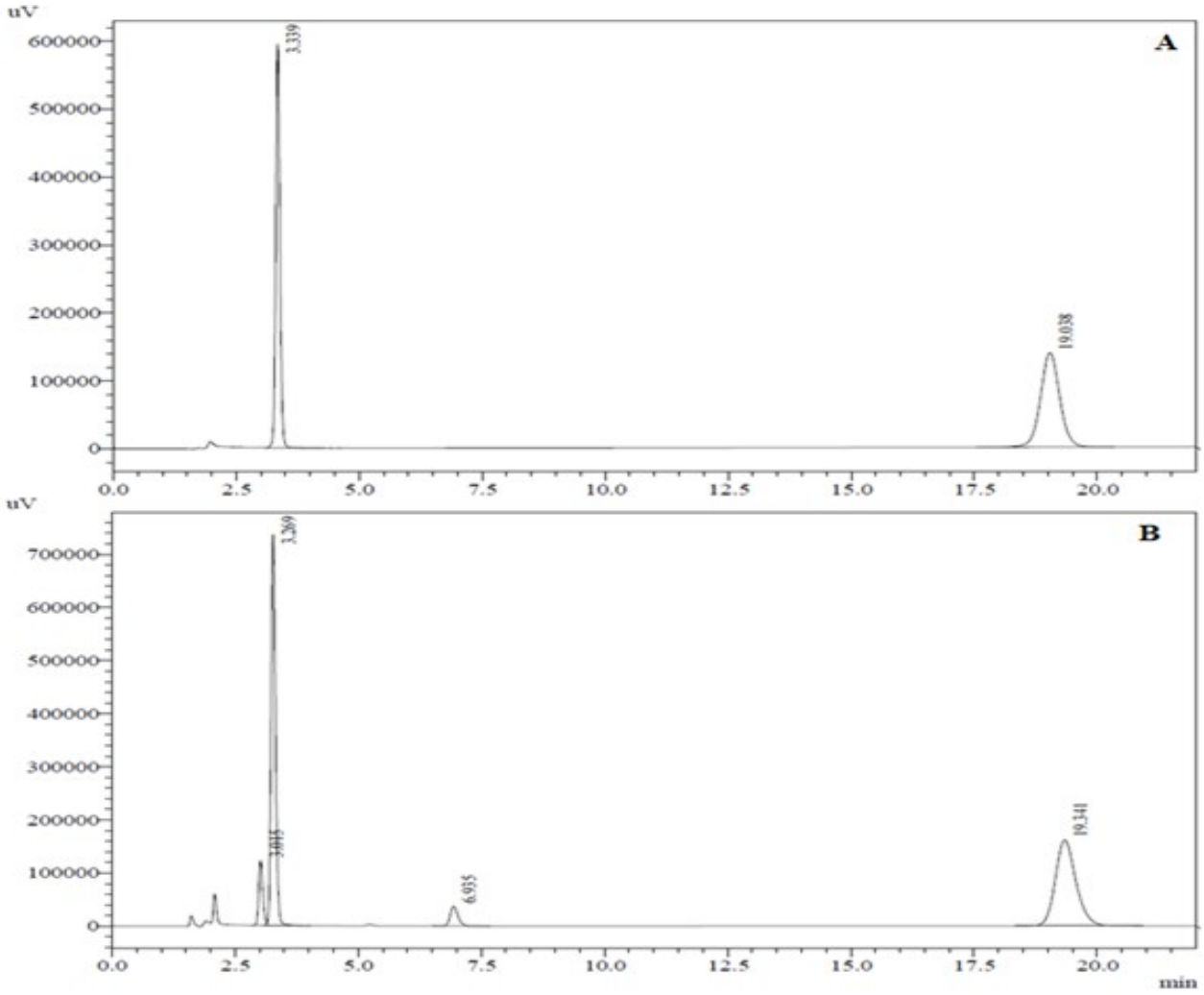

Fig. 3: HPLC Chromatograms of Metronidazole and Furazolidone in (A) Standard solution and (B) Sample solution

conditions. Results showed that with increasing pH from 3 to 9, extraction efficiency decreased for all solvents (Table S1). Maximum extraction efficiency was observed in acidic pH in chloroform for Metronidazole (96.71%) and Furazolidone (97.11%). With increasing pH from to 3 to 5, extraction efficiency decreased significantly. Minimum recovery 75.23% and 66.37% for both Metronidazole and Furazolidone was observed at basic pH (fig. 2B).

Furthermore, effect of amount of salts (KCl and $NaNO_3$) was also evaluated on the extraction efficiency of Metronidazole and Furazolidone. Different amounts of salts were tested during the extraction experiments and it resulted in different recovery of Metronidazole and Furazolidone. 0.5g KCl and 0.5 g $NaNO_3$showed best recovery for both analytes. With increasing the salt amount from 0.5g to 1 gram each, efficiency and recovery decreased (fig. 2C). These results indicated that higher salt concentration was not feasible for extraction.

Method validation

HPLC chromatograms of both Metronidazole and Furazolidone in standard as well as in sample are shown in fig. 3A and 3B. Figures show that retention time of both analytes Furazolidone (3.339minutes) and Metronidazole (19.03) minutes.

System suitability criteria was RSD (within ± 2%) which was obtained by six replicates 1.84 % and 1.74 % for metronidazole benzoate and furazolidone respectively. Specificity of method was monitored by using blank, placebo, analytes standard solution separately; no peak was detected near to desired analytes, which proved the high specificity of method.

Linearity of the method for Metronidazole has been evaluated on different concentrations (240µg/ml, 270µg/ml, 300µg/ml, 330µg/ml, 360µg/ml and 390µg/ml). Fig. 4A shows the linearity of Metronidazole and straight line with correlation coefficient (R^2) of 0.994.

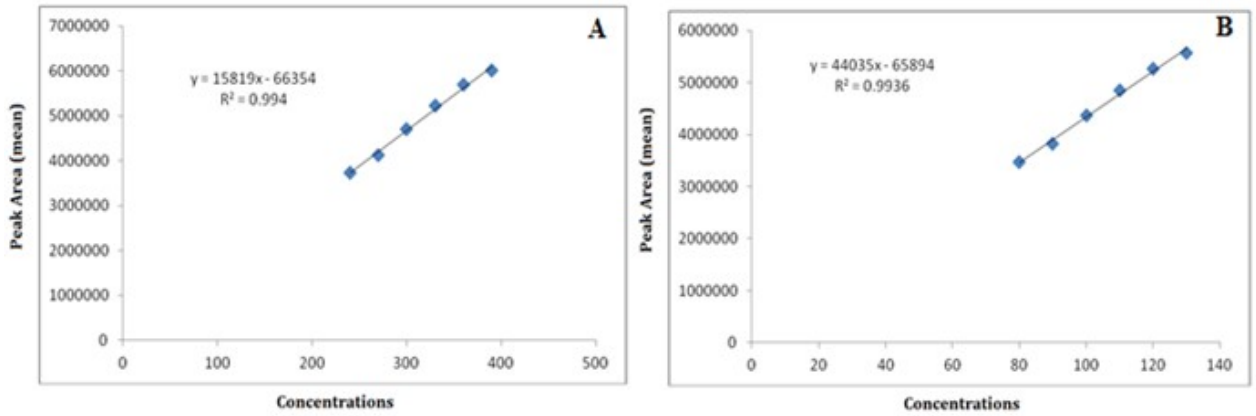

Fig. 4: (A) Linearity Curve for Metronidazole, (B) Linearity Curve for Furazolidone

This indicates that extraction, followed by HPLC method for Metronidazole. Similarly, linearity of furazolidone is also evaluated at different concentrations (80µg/ml, 90µg/ml, 100µg/ml, 110µg/ml, 120µg/ml and 130µg/ml) and graph is plotted against their peak areas. The results show that linearity of method for Furazolidone is also good with R^2 value of 0.9936 (fig. 4B). Signal -to- noise ratio3:1 for LOD were found to be 2.1µg/ml, 1.5µg/ml for metronidazole benzoate and furazolidone while 10:1 ratio for LOQ were found to be 21.1µg/ml, 15.2µg/ml for metronidazole benzoate and furazolidone.

For precision, six samples of Metronidazole and Furazolidone of same concentration 300µg/mL and 100µg/mL respectively, were prepared for assessment of precision. Standard deviation and relative standard deviation were calculated from the peak area of each analyte at each HPLC run (Table 2). The results indicate that relative standard deviation (RSD) for Metronidazole and Furazolidone is 1.84% and 1.72% respectively.

Then accuracy of method for Metronidazole was assessed by analyzing the standard solutions at different concentrations (240µg/ml, 270µg/ml, 300µg/ml, 330µg/ml, 360µg/ml and 390µg/ml) and peak area is calculated, same as in case of linearity experiment (Table 3). Slope and intercepts for Metronidazole (Y= 15819x - 66354) and R^2=0.994 are calculated. Similarly, 3 samples A, B and C were also analyzed in comparison to standard solution and peak area was calculated. All the reading were taken in triplicate measurements and results are presented in Table 4. For Furazolidone slope and intercept values are Y=44035x -65894 and correlation coefficient (R^2) is 0.9936. The detail of peak areas of standard solution and sample solutions is given in Table 5 and 6. % recovery of Furazolidone at 80µg concentration is 100.45%, while 100µg and 120µg 97.46 % and 95.91 % recovery is obtained (Table 7).

It has been observed that with increasing mobile phase upto 1.7mL/min, peak area decreased slightly as compared to 1.3mL/min but there was not much difference. RSD values were very low (0.75%) as compared to 1.41% at 1.7mL/min flow rate (Table 10).

Similarly, robustness of method was also evaluated for Furazolidone also at different flow rates and different wavelengths. Results are shown in Table 10. RSD values at 1.3mL/min, 1.7mL/min, 230 nm and 270 nm are 1.47%, 0.97%, 1.35% and 1.37%, respectively. These results were also indicated the robustness of our proposed method. The overall summary of performance and validation of the method for Metronidazole and Furazolidone is given Table 8 and Table 9 respectively.

DISCUSSION

Method development is an important area of analytical research. Easy, low cost and reproducible methods for the analysis of drugs pesticides and drugs by HPLC are always demanded in pharmaceutical and industrial research (Ashok *et al.,* 2016). In this study, we propose a simple and efficient method for simultaneous detection and quantification of Metronidazole and Furazolidone by facile liquid liquid extraction approach followed by HPLC analysis for industrial effluents and pharmaceutical dosage forms. Standard solutions of both drugs were also analyzed. Optimization of extraction parameters was carried out in the study and the results show that, chloroform was the best extraction solvent for these two analytes which provide excellent extraction medium, so recovery of both analytes was upto 99%. So, chloroform was selected for the extraction process for further experiments. The study showed that acidic medium is best suited for the extraction of Metronidazole and furazolidone (Elkhoudary *et al.,* 2016).

The retention time for both sample and standard was same but peak area of analytes was increased in sample as compared to standard which indicated that liquid liquid extraction has reduced the complexity of sample, thus, facilitated the detection of Metronidazole and

furazolidone from different dosage forms of pharmaceutical products and industrial effluents. After initial optimization of parameters, method is validated in terms of system suitability, specificity, linearity, limit of quantification, limit of detection, precision, accuracy and robustness. Linearity of the method is considered as the first step in validation of an analytical method (Mishra *et al.,* 2014). The precision was in acceptable range and much better than previously reported methods for these analytes. This improved precision of the analysis may be attributed to the liquid liquid extraction step, prior to HPLC analysis.

Accuracy of method validation was also carried out. By the comparison of peak area of standard solution and sample solution, percentage recovery was calculated for each concentration of Metronidazole. Results indicated that maximum recovery was obtained at 240µg concentration (102.01%). At 300 µg and 360 µg, obtained recoveries were 100.11% and 99.61% respectively. These results showed that our proposed method was accurate at different concentrations with excellent recovery of Metronidazole. Similar procedure was adopted for the accuracy of method for furazolidone using standard solution concentrations (80µg/ml, 90µg/ml, 100µg/ml, 110µg/ml, 120µg/ml and 130µg/ml). It indicated that simultaneous extraction and analysis of both Metronidazole and Furazolidone by this developed method was accurate, reproducible with high recovery from different type of samples under optimized conditions.

Finally robustness was evaluated by changing the detection wavelength as well as changing the flow rate of mobile phase. For metronidazole, initially the flow rate changed from 1.5mL/min to 1.3mL/min and then from 1.5mL/min to 1.7mL/min. This can be attributed that at higher flow rate, sample passed through the system very quickly, without much retention, which resulted in smaller peak area (Ibrahim *et al.,* 2018). But RSD values at this high flow rate were still in acceptable range. Moreover, detection wavelength varied from 250nm to 270nm and from 270nm to 290nm to access the robustness of metronidazole. With changing the detection wavelength not much difference is observed in relative standard deviation of Metronidazole i.e. 250 nm (1.50%) and 290 nm (1.19%). This shows that the developed method showed robust performance at different wavelengths.

CONCLUSION

A newly developed HPLC-UV method is proposed for determination of Metronidazole and Furazolidone from different pharmaceutical sample and industrial effluents. This method is based on pretreatment of both of these analytes by liquid-liquid extraction, followed by HPLC analysis. By the addition of liquid liquid extraction step, overall detection performance for Metronidazole and Furazolidone improved in pharmaceutical dosage form using a single mobile phase. This method is simple and more efficient in terms of recovery of analytes, as compared to previously reported methods. Extraction parameters are optimized and method shows maximum efficiency in chloroform at pH 3 in lower salt concentration. Method is validated by checking the system suitability, specificity, linearity, limit of quantification, limit of detection, precision, accuracy and robustness analysis under different experimental conditions. Metronidazole and Furazolidone both are detected and quantified with high recovery and low standard deviation with excellent linearity. The developed method has proved specific, precise and accurate for assaying two drugs either individually or in combined form. So, this method can be used at commercial scale in pharmaceutical industry for the detection and quantification of Metronidazole and Furazolidone from different dosage forms of pharmaceutical products. The proposed (SALLE) method has shown excellent results for simultaneous determination of metronidazole and furazolidine in industrial effluents as well.

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REFERENCES

Alshishani A, Salhimi SM and Saad B (2018). Salting-out assisted liquid-liquid extraction coupled with hydrophilic interaction chromatography for the determination of biguanides in biological and environmental samples. *J. Chromatogr. B.*, **1073**(1): 51-59.

Ashok K, Kishore L, Kaur N and Nair AP (2012). Method development and validation: Skills and tricks. *Chronicles. Young Scientists*, **3**(1): 3-11.

Chen D, Hu YN, Hussain D, Zhu GT, Huang YQ and Feng YQ (2016). Electrospun fibrous thin film micro extraction coupled with desorption corona beam ionization-mass spectrometry for rapid analysis of antidepressants in human plasma. *Talanta*, **152**(15): 188-195.

Cheng CC, Hsieh KH, Lei YC, Tai YT, Chang TH, Sheu SY, Li WR and Kuo TF (2009). Development and Residue Screening of the Furazolidone Metabolite, 3-

Amino-2-oxazolidinone (AOZ), in Cultured Fish by an Enzyme-Linked Immunosorbent Assay. *J. Agri. Food. Chem.*, **57**(27): 5687-5692.

Elena GO and Milea B (2014). Development and validation of a RP-HPLC method for the quantisation studies of metronidazole and furazolidone from product Enteroguard-M M*edicamentul Veterinar* **4**(2):1-4.

Elkhoudary MM, Abdel Salam RA and Hadad GM (2016). Development and optimization of HPLC analysis of metronidazole, diloxanide, spiramycin and cliquinol in pharmaceutical dosage forms using experimental design. *J. Chromatographic Sci.,* **54**(23): 1701-1712.

Ghante MR, Pannu HK, Loni A and Shivsharan T (2012). Development and validation of a RP-HPLC method for simultaneous estimation of metronidazole and norfloxacin in bulk and tablet dosage form. *Intl. J. Pharm. Pharm. Sci.,* **40**(2): 241-250.

Goggin MM, Janis GC (2019). Salt-Assisted Liquid-Liquid Extraction of Meconium for Analysis of Cocaine and Amphetamines by Liquid Chromatography-Tandem Mass Spectrometry. LC-MS Drug Analysis, pp.199-209.

Hussain D, Naqvi STR, Ashiq MN and Najam-ul-Haq M (2019). Analytical sample preparation by electrospun solid phase microextraction sorbents. *Talanta.,* p.120413.

Hyde AM, Zultanski SL, Waldman JH, Zhong L, Shevlin M and Peng F (2017). General principles and strategies for salting-out informed by the hofmeister series. *Org. Process. Res. Develop.,* **21**(03): 1355-1370.

Ibrahim F, Wahba ME and Magdy G (2018). Analytical method development and validation of spectro-fluorimetric and spectrophotometric determination of some antimicrobial drugs in their pharmaceuticals, Spectrochimica Acta Part A: *Mol. Biomol*., **188**(25): 525-536,

Jarrad AM, Debnat A, Miyamoto Y, Hansford KA, Pelingon R, Butler MS, Bains T, Karoli T, Blaskovich M A, Eckmann L and Cooper MA (2016). Nitroimidazolecarboxamides as antiparasitic agents targeting *Giardia lamblia, Entamoeba histolytica* and *Trichomonas vaginalis*. *Europe. J. Med. Chem*., **120**(9): 353-362

Kagambèga A, Lienemann T, Frye JG, Barro N and Haukka K (2018). Whole genome sequencing of multidrug-resistant *Salmonella entericaserovar* Typhimurium isolated from humans and poultry in Burkina Faso. *Trop. Med. Health.,* **46**(3): 46-52.

Kumar MS, Anjaneyulu A, Mangilal T (2015). Simultaneous Determination of Metronidazole and Furazolidone in Combined Tablet Dosage Form: Development and Validation of a Stability Indicating HPLC Method. *World J. Pharm. Pharma. Sci.,* **4**(5): 1944-46.

Luther J, Higgins PD, Schoenfeld PS, Moayyedi P, Vakil N and Chey WD (2010). Empiric quadruple vs. triple therapy for primary treatment of Helicobacter pylori infection: systematic review and meta-analysis of efficacy and tolerability. *American J. Gastroenterol.,* 105(11): 65-76.

Mishra AK, Kumar A, Mishra A and Mishra HV (2014). Development of ultraviolet spectroscopic method for the estimation of metronidazole benzoate from pharmaceutical formulation. *J. Nat. Sci. Biol. Med.,* **25**(09): 261-264.

Mohyuddin A, Hussain D, Fatima B, Athar M, Ashiq M N and Najam-ul-Haq M (2019). Gallic acid functionalized UiO-66 for the recovery of ribosylated metabolites from human urine samples. *Talanta,* **201**(1): 23-32.

Nista EC, Candelli M, Zocco MA, Cremonini F, Ojetti V, Finizio R, Spada C, Cammarota G, Gasbarrini G and Gasbarrini A (2006). Levofloxacin-based triple therapy in first-line treatment for *Helicobacter pylori* eradication. *Am. J. Gastroenterol.,* **101**(22): 1985-1997.

Sheehy O, Santos F, Ferreira E and Berard A (2015). The use of metronidazole during pregnancy: A Review of evidence. *Curr. Drug Saf.,* **10**(2): 170-179.

Santos AL, Takeuchi RM and Stradiotto NR (2009). Electrochemical, spectrophotometric and liquid-chromatographic approaches for analysis of tropical disease drugs. *Current. Pharm. Analysis,* **5**(20): 69-88.

Zamani M, Morshed M, Varshosaz J and JannesariM (2010). Controlled release of metronidazole benzoate from poly ε-caprolactoneelectrospun nanofibers for periodontal diseases. *Europe J. Pharm. Biopharm.,* **75**(3): 179-185.

Zhang J and Xiong X (2019). Salting out Assisted Liquid-Liquid Extraction (SALLE) in LC MS Bioanalysis. *Sample Preparation in LC MS Bioanalysis,* pp.68-70.