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Extractive spectrophotometric determination of terbinafine hydrochloride in pharmaceuticals and in human urine

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ABSTRACT

Two new simple, rapid and sensitive spectrophotometric methods have been developed for the assay of terbinafine hydrochloride (TFH) in bulk drug and its tablets. Method A is based on the formation of a orange red colored ion-pair complex (1:1 drug/dye) of TFH with calmagite (CGT) at pH 2.5 and extraction of the complex into dichloromethane followed by the measurement of the ion-pair complex at 500 nm. In method B, the drug-dye ion-pair is dissolved in ethanolic sulphuric acid and the resulting acid form of the dye is measured at 490 nm. Under the optimized conditions, Beer's law is obeyed over 1.5-30 and 1.0-20 µg mL^{-1} range for method A and method B, and the corresponding molar absorptivity values are 8.60 x 10^3 and 1.64 x 10^4 L mol^{-1} cm^{-1} respectively. The Sandell sensitivity, limits of detection and quantification values are also reported. The molar ratio of the formed ion-pair complex was found to be 1:1 as deduced by Job's method and the calculated stability constant is also reported. Over the linear ranges applicable, the accuracy and precision of the method were evaluated on intra-day and inter-day basis. Application of the proposed method to bulk drug, commercial pharmaceutical tablets, and spiked human urine is presented.

Keywords: Terbinafine hydrochloride, Calmagite, pharmaceuticals, spectrophotometry, assay.

1. INTRODUCTION

Terbinafine hydrochloride (TFH), chemically known as (E)-*N*-(6,6-dimethyl- 2 - hepten - 4 - ynyl) -*N*-methyl - 1 – naphthalene methanamine hydrochloride [1], is a potent antifungal agent of the allylamine class with broad spectrum activity against yeasts, dimorphic fungi, molds, and dermatophytes [2-5].

Various techniques have been used for the determination of TFH in body fluids and pharmaceuticals. The drug is official in European Pharmacopoeia [6], British Pharmacopoeia [7] and the United States Pharmacopoeia [8]. European Pharmacopoeia and British Pharmacopoeia describe acid-base titration in hydro-alcoholic medium, the end point being located potentiometrically [6,7]. United States Pharmacopoeia describes high performance liquid chromatographic method for the estimation of TFH [8]. High performance liquid chromatography (HPLC) has been applied for the determination of TFH and metabolites in human plasma [9,10], TFH and metabolites in human plasma, milk and urine [11], and the drug in tissues [12]. An improved high throughput liquid chromatographic/tandem mass spectrophotometric method for TFH in plasma [13] and ultra performance liquid chromatographic method for the drug and its metabolites in human plasma and urine [14] are the other chromatographic methods reported for body fluids. In addition, microbiological assays of the drug are also found in the literature [15, 16].

Several HPLC procedures [17-23] employing different columns and mobile phases have been reported for its assay in dosage forms when present alone [17-22] or in combination with bezafibrate [23]. High performance thin layer chromatography (HPTLC) has recently been applied for the assay of drug in tablets [24] and for the simultaneous determination of TFH and triamcinolone acetamide in compound tablets [25]. Non-aqueous titrimetry [26] and voltammetry [27] are the other analytical techniques available for the assay of TFH in its dosage forms.

Spectrophotometry is one of the simplest techniques routinely used in pharmaceutical quality control laboratories because of its sensitivity, speed, fair selectivity, low cost and ease of performance. However, the literature on TFH is poor with regard to spectrophotometric methods. In a method reported by Elazazy et.al [28] molybdenum (V) thiocyanate, orange G and

alizarin red as ion pairing complex agents for estimation of TFH in bulk drug and formulations. Ion pair complexes formed were extracted with organic solvents and measured at 469-471 nm, 498-500 nm and 425-426 nm with linear range of 5-75 µg mL^{-1}, 10-80 µg mL^{-1} and 5-55 µg mL^{-1} respectively. Florea, and Monciu [29] used methyl orange for ion pair formation with terbinafine at pH 2.6. The resulting yellow ion pair complex was extracted into chloroform and absorbance measured at 422 nm. This method shows linearity over the range 6-17 µg mL^{-1}. Chloroform extractable ion pair complexes of the drug with bromothymol blue, bromophenol blue and bromocresol green in acidic medium were used by Chennaiah et.al [30] for the assay of 2.0-25 µg mL^{-1} TFH with all the three dyes.

The reported methods [28-30] suffer from the twin disadvantages of poor sensitivity and narrow linear dynamic range. In continuation to our work on the use of ion pair reactions for the sensitive and selective determination of several pharmaceuticals [31-38], an attempt was made to apply this reaction for the assay of the TFH. In this present work the ion pair formed between TFH and CGT in acidic buffer medium was extracted into dichloromethane and measured the absorbance at 500 nm in method-A. Additionally, in the method-B drug-dye ion pair complex was broken in the ethanolic H_2SO_4 and the acid form of the dye was measured at 490 nm. Both the methods were applied to bulk drug, tablets and spiked human urine and excellent recovery with good precision was obtained.

2. Materials and methods

2.1. Apparatus

A Systronics model 166 digital spectrophotometer (Systronics, Ahmedabad, Gujarat, India) with matched 1-cm quartz cells was used for absorbance measurements. A digital pH meter Model Elico L1 120 was used for pH measurements.

2.2. Materials

Pharmaceutical grade terbinafine hydrochloride (TFH) was received from Dr. Reddy's laboratories limited, Hyderabad, India, as gift sample and used as received. Zimig-250 (Glaxo Smith Kline Pharmaceuticals Limited, India), Terbiforce-250 (Lifestar Pharma Pvt. Ltd.) and Sebifin (Ranbaxy Laboratories Ltd, India) tablets were purchased from local commercial sources. All reagents and chemicals used were of analytical reagent grade, spectroscopic grade organic solvents and distilled water was used throughout the study.

2.3. Calmagite, CGT (0.025 %)

Prepared by shaking 25 mg of CGT dye (Qualigens fine chemicals, Mumbai, India) with water in a 100 mL calibrated flask.

2.4. Sodium acetate-hydrochloric acid buffer (pH 2.5)

Prepared by mixing 1 M solutions of sodium acetate and hydrochloric acid (Merck Pvt. Ltd., Mumbai, India, sp. gr. 1.18) and the pH was adjusted to 2.5 by drop wise addition of sodium acetate/HCl solution.

2.5. Ethanolic sulphuric acid, H_2SO_4 (1 %)

One mL of conc. H_2SO_4 (Merck Pvt. Ltd., Mumbai, India, sp. gr. 1.84) was dissolved in 100 mL of ethanol.

2.6. Standard TFH solution

TFH standard solution (300 µg mL^{-1}) was prepared by dissolving 15 mg pure TFH in a 50 mL standard flask with water. From this, 30 µg mL^{-1} solution was prepared by dilution with water.

2.7. General procedures

2.7.1. Preparation of calibration graph

2.7.1.1. Method A

Aliquots of 30 µg mL^{-1} TFH standard solution 0.0, 0.5, 3.0, 5.0, 7.0, and 10.0 mL were measured accurately and transferred into a series of 125 mL separating funnels and the total volume was brought to 10 mL by adding water. To each funnel were added 6 mL NaOAc-HCl buffer of pH 2.5 and 4 mL of 0.025 % CGT dye solution, mixed well and kept aside for 1 min. The drug-dye ion-pair was then extracted with 10 mL of dichloromethane by shaking for 1 min and the layers were allowed to separate for 2 min. The organic layer was then passed over anhydrous sodium sulphate and absorbance measured at 500 nm against the reagent blank.

2.7.1.2. Method B

Into a series of 10 mL volumetric flasks, 0.0, 0.25, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 mL of TFH-CGT complex (40 µg mL^{-1} in TFH prepared as in method A) and equivalent to 1-20 µg mL^{-1} TFH were transferred. The total volume in each flask was brought to 5 mL with dichloromethane. After the addition of 1 mL 1 % ethanolic H_2SO_4, then diluted upto the mark with ethanol and absorbance measured at 490 nm against the reagent blank.

In both methods, standard graph was prepared by plotting the absorbance *vs* TFH concentration, and the concentration of the unknown was read from the calibration graph or computed from the respective regression equation derived using the absorbance-concentration data.

2.7.2. Procedure for tablets

Twenty tablets each containing 250 mg of TFH were weighed ground into a fine powder and mixed well. An accurately weighed quantity of finely ground tablet powder equivalent to 15 mg of TFH was accurately weighed into a 50 mL calibrated flask, 30 mL of water added, and the flask was shaken for 20 min; and finally made upto the mark with water. The flask was kept aside for 5 min, and filtered using Whatman No 42 filter paper. First 10 mL portion of the filtrate was discarded and a suitable aliquot of the filtrate (containing 300 µg mL^{-1} TFH) was diluted with water to get a working concentration of 30 µg mL^{-1} TFH and used for the assay by method A. The ion-pair complex TFH-CGT (40 µg mL^{-1} TFH) from tablet powder was used for the assay by applying the procedure described under method B. The results of assay are presented in table 3.

2.7.3. Procedure for spiked human urine

Ten mL of 100 µg mL^{-1} TFH solution was taken in a 50 mL calibrated flask using a micro burette, the flask was made up to the mark with human urine. The content was mixed well for two minutes and suitable aliquots of this spiked urine sample was analyzed by using the procedure described earlier in method A or method B.

3. Results and discussion

3.1. Spectral characteristics

The absorption spectrum of the orange red colored TFH-CGT ion-pair complex shown in fig.1 has a maximum absorbance (λ_{max}) at 500 nm. This drug-dye ion-pair complex was broken in ethanolic acid to yield orange colored acid form of dye with λ_{max} at 490 nm, respectively (fig.1). In both cases, the blanks had negligible absorbance.

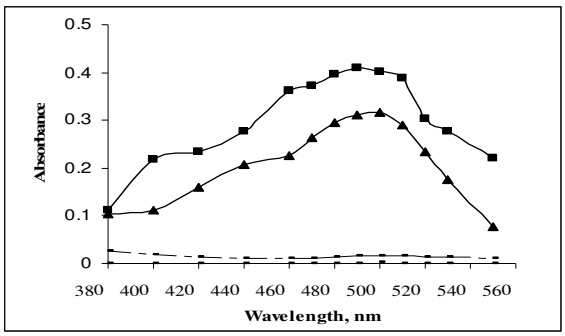
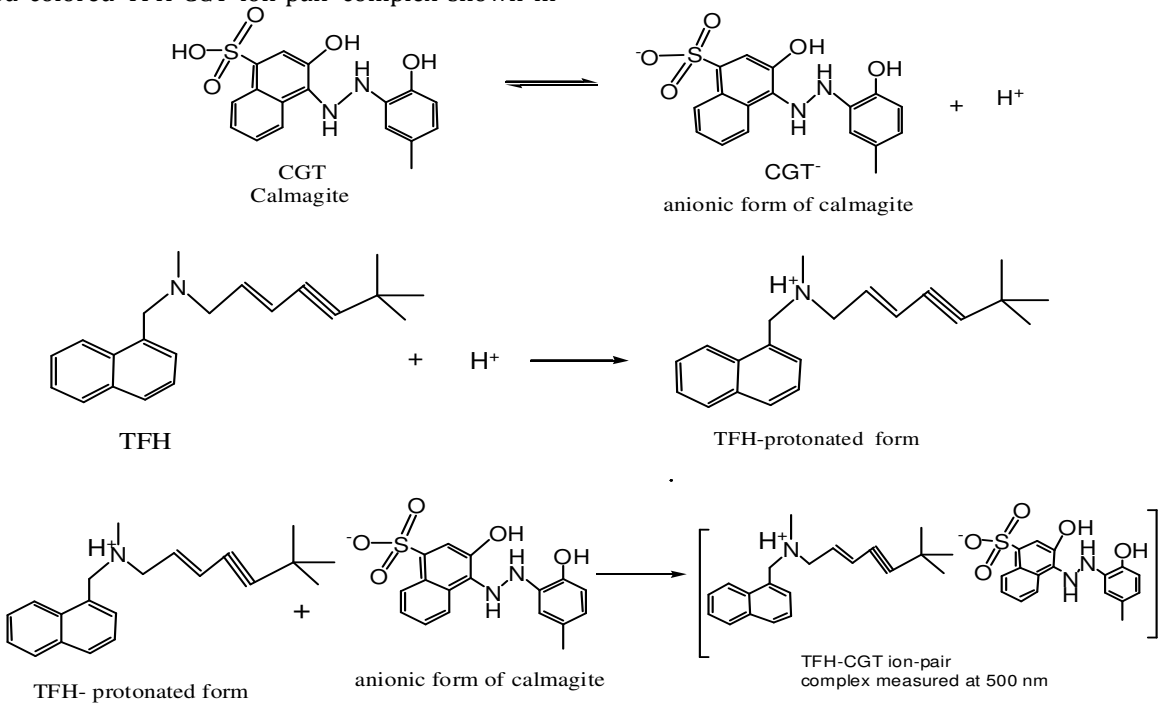


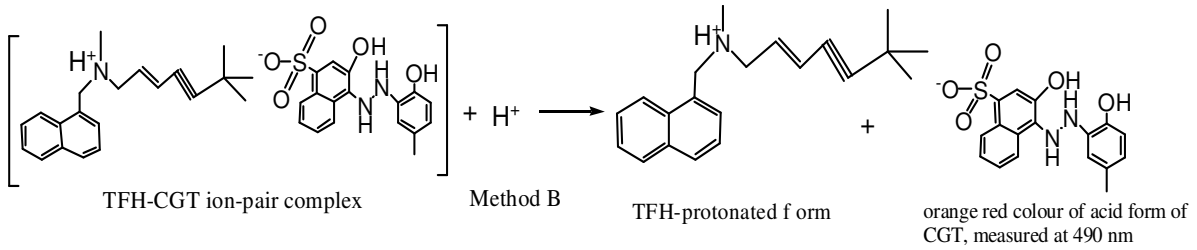
Figure - 1: Absorption spectra of: TFH-CGT ion-pair complex (-▲-), acid form of dye (-■-), and blanks (-(-□-)-).

3.2. Reaction pathway

TFH forms ion-pair complex with CGT since the drug contains a tertiary amino group which is protonated in acid medium. Each drug-dye complex molecule, with two oppositely charged ions, behaves as a single unit held together by an electrostatic force of attraction (scheme 1). In alcoholic acid medium, this ion-pair complex gets disturbed and it breaks to form orange red colored acid form of the dye and the drug. The mechanism of this breaking is shown in scheme 2.



Scheme -1



Scheme - 2

3.3. Optimization of reaction conditions

The optimization of the methods was carefully performed to achieve complete ion-pair complex formation, quantitative extraction of the ion-pair complex and maximum sensitivity. For the ion-pair complex formation and its extraction conditions such as pH, type of buffer and organic solvent, volume of the dye, and shaking and equilibration time for the extraction of ion-pair complex were optimized. In method B, ethanolic H_2SO_4 required for complete breaking of the complex was optimized.

3.4. Selection of the extracting solvent

A number of organic solvents such as chloroform, dichloromethane, 1, 2-dichloroethane, carbon tetrachloride, hexane, toluene and benzene was examined for extraction of the ion-pair complex in order to provide an applicable extraction procedure. Dichloromethane was preferred for its efficient and quantitative extraction of ion-pair complex and the stability of the extracted ion-pair, its high sensitivity, and very low absorbance of the reagent blank and shortest time to reach the equilibrium between both phases.

3.5. Effect of pH on the ion-pair formation

The effect of pH of the aqueous phase was studied by extracting the colored complex at pH 0.5-5.0. It was noticed that the maximum absorbance of complex and minimum absorbance of the reagent blank were observed at pH 2.5. The results are shown in Fig.2. At pH values greater than 2.5, a decrease in absorbance of the ion-pair complex was observed and at pH values below than 2.5 also decrease in absorbance of the sample and reagent blank was observed. Hence pH 2.5 was fixed in all subsequent work. Effect of volume of buffer also studied and it was found that 6.0 mL buffer of pH 2.5 was optimum Fig.3.

Different buffers systems of pH 2.5 such as Walpole (1M sodium acetate-1 M hydrochloric acid), Clark and $Lubs_1$ (0.2 M potassium chloride-0.1 M hydrochloric acid), Sorenson (0.1 M sodium chloride-0.1 M hydrochloric acid), Clark and $Lubs_2$ (0.1 M potassium hydrogen phthalate-0.1 M hydrochloric acid), and Mc Ilvaine (0.2 M disodium hydrogenphosphate-0.1 M citric acid) were tried, it was found that Walpole buffer of pH 2.5 was the best for complex formation as well as extraction.

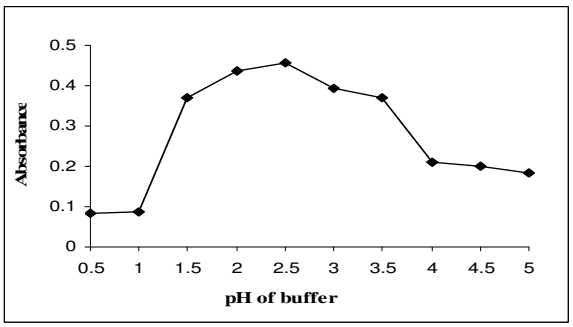


Figure - 2: Effect of buffer pH (method A-Drug conc. 10 µg mL^{-1}).

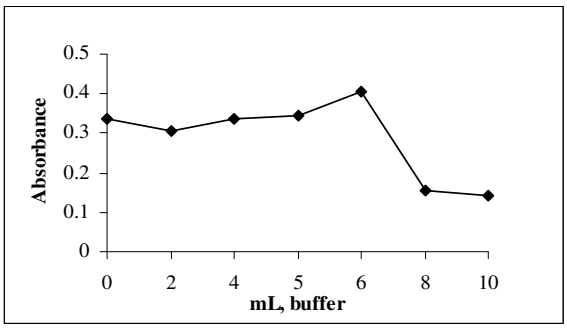


Figure - 3: Effect of volume of buffer (method A Drug conc. 10 µg mL^{-1}).

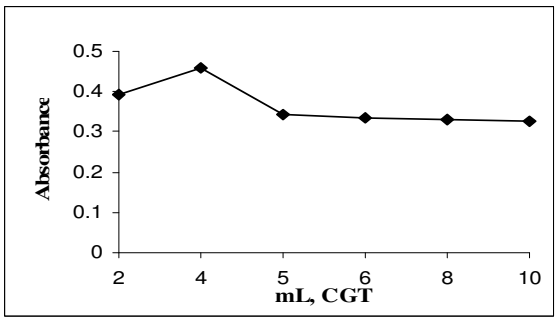


Figure - 4: Effect of volume of CGT dye (method A Drug conc. 10 µg mL^{-1}).

3.6. Effect of dye concentration

The effect of the dye concentration was studied in method A by measuring the absorbance of solutions containing a fixed concentration of TFH (10.0 µg mL^{-1}) and varied amounts of BCG. It

is clear from Fig.4 that the maximum absorbance was found with 4.0 mL of 0.025 % BCG. Thus, 4.0 mL of 0.025 % BCG in a total volume of 20 mL aqueous phase was used for ion-pair formation throughout the investigation (Fig.4).

3.7. Effect of the volume of aqueous phase

The effect of volume of aqueous phase was studied by using different volumes of aqueous phase (including drug, BCG and buffer) such as 15, 20, 25, 30 35 and 40 mL and extracting with 10 mL of dichloromethane (Fig.5). The use of 20 mL of aqueous phase was found to be sufficient to achieve maximum absorbance of measured species and minimum absorbance of reagent blank and hence an aqueous phase of 20 mL was fixed throughout.

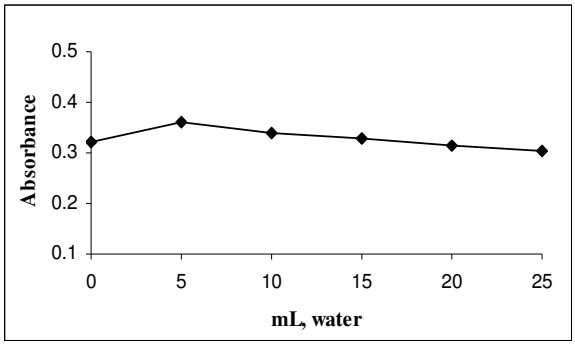


Figure - 5: Effect of volume of water (aqueous phase) (method A Drug conc. 10 µg mL^{-1}).

3.8. Effects of contact and shaking time and sequence of addition

The effect of contact time between TFH and CGT in the presence of buffer was studied in the time range 0-30 min before extraction and it was found that 5 min was sufficient to achieve maximum absorbance at 500 nm. Shaking times of 0.5–3 min produced a constant absorbance in method A, and hence a shaking time of 1 min was used throughout the investigation. In method B, the effect of the time required to break the complex was studied after the addition of ethanolic H_2SO_4 to the complex and breaking was found to occur instantly. There was no appreciable change in the absorbance or color of the measured species if the order of addition of the reactants was varied.

3.9. Effect of study of equilibration time and number of extractions

The time required for the two layers to separate was studied by keeping all other parameters constant and two minutes was adequate for the complete separation of aqueous and organic phases.

The number of extractions required for complete removal of the complex from the aqueous phase was examined. One extraction with 10 mL of $CHCl_3$ was sufficient and second extraction gave the absorbance same as the blank absorbance.

3.10. Effect of volume of ethanolic H_2SO_4

The volume of alcoholic H_2SO_4 required to break the complex was studied in method B by taking a fixed concentration of TFH-CGT complex and it was found that 1.0 mL of acid in method B gave maximum absorbance. (Fig.6)

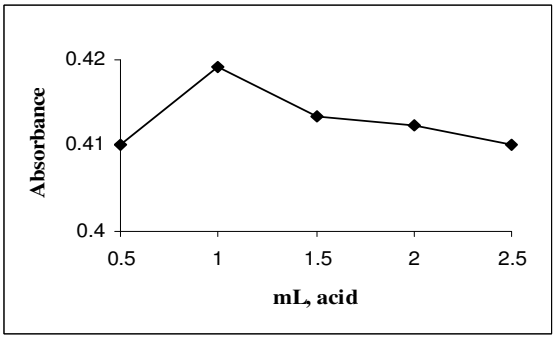


Figure - 6: Effect of volume of acid (method B Drug conc. 8 µg mL^{-1}).

3.11. Composition of the ion-pair complex

The composition of the ion-pair complex formed between TFH and CGT in method A was established by applying Job's method of continuous variations [39]. In this method, 1.22 x 10^{-4} M solutions of TFH and CGT were used and mixed in varying volume ratios in such a way that the total volume of each mixture was the same. The absorbance of each solution was measured and plotted against the mole fraction of the drug (Fig.7). The plot reached a maximum value at a mole fraction of 0.5 indicating that a 1:1 (TFH:CGT) ion-pair complex is formed through the electrostatic attraction between protonated TFH and CGT anion. The conditional stability constant (K_f) of the ion-pair complex was calculated [40] from the data of continuous variations method and the log K_f was found to be 6.92.

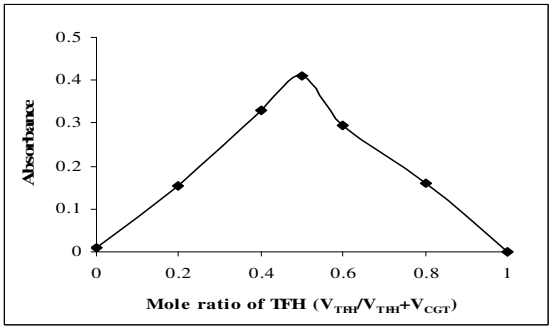


Figure - 7: Jobs plot for TFH-CGT ion-pair complex (1.22 x 10^{-4} M solutions of TFH and CGT).

3.12. Stability of the measured species

The formation of the ion-pair was rapid and the orange red color extract was stable for at least 45 min without any change in color intensity at room temperature. Also, the absorbance of the orange red color of acid form of the dye in method B was stable for more than 30 min.

3.13. Method validation

3.13.1 Linearity and sensitivity

At described experimental conditions for TFH determination, the absorbance – concentration plots were found to be linear over the concentration ranges stated in Table 1. The regression parameters given in the regression equation calculated from the calibration graphs along with the standard deviations of the slope (S_b) and the intercept (S_a) are also given in Table 1. The linearity of calibration graphs was proved by the high values of the correlation coefficient(r) and the small values of the y-intercepts of the regression equations. The apparent molar absorptivity, Sandell sensitivity, limits of detection and quantification of all the methods were also calculated and recorded.

3.13.2. Precision and accuracy

In order to evaluate the precision of the proposed methods, solutions containing three different concentrations of the TFH were prepared and analyzed in seven replicates. The results obtained from this investigation are summarized in Table 2. The low values of the relative standard deviation (% RSD) and percentage relative error (% RE) also indicate the high precision and the good accuracy of the proposed methods. The assay procedure was repeated seven times, and percentage relative standard deviation (% RSD) values were obtained within the same day to evaluate repeatability (intra-day precision) and over five different days to evaluate intermediate precision (inter-day precision).

3.13.3. Selectivity study

Selectivity was evaluated by placebo blank and synthetic mixture analyses. A placebo blank consisting of starch (20 mg), acacia (25 mg), hydroxyl cellulose (20 mg), sodium citrate (30mg) talc (20 mg), magnesium stearate (25 mg) and sodium alginate (20 mg) was prepared by thorough mixing and its solution was prepared as described under "procedure for tablets", by taking about 20 mg, and then subjected to analysis.

A synthetic mixture was prepared by adding 10 mg of pure TFH to 10 mg of the above mentioned placebo blank, and the mixture was homogenized. Following the procedure employed for tablets, the synthetic mixture solution was prepared, and a suitable aliquot was subjected to analysis by the methods, after appropriate dilution and no significant interference was observed from these excipients.

Table - 1: Sensitivity and regression parameters

Parameter	Method A	Method B
λmax, nm	500	490
Colour stability, min	upto 45	30
Linear range, μg mL^{-1}	1.5–30	1.0-20
Molar absorptivity (ε), L mol^{-1} cm^{-1}	8.60×10^3	1.64×10^4
Sandell sensitivity*, μg cm^{-2}	0.038	0.02
Limit of detection (LOD), μg mL^{-1}	0.12	0.03
Limit of quantification (LOQ), μg mL^{-1}	0.38	0.09
Regression equation, Y**		
Intercept (a)	-0.0154	0.0109
Slope (b)	0.0279	0.0470
Standard deviation of a (S_a)	9.98×10^{-2}	9.98×10^{-2}
Standard deviation of b (S_b)	4.06×10^{-3}	5.60×10^{-2}
Regression coefficient (r)	0.9986	0.9994

*Limit of determination as the weight in μg mL^{-1} of solution, which corresponds to an absorbance of A = 0.001 measured in a cuvette of cross-sectional area 1 cm^2 and l = 1 cm. **Y=a+bX, Where Y is the absorbance, X is concentration in μg mL^{-1}, a is intercept and b is slope.

Table - 2: Evaluation of Intra-day and inter-day accuracy and precision

Method	TFH taken (µg mL^{-1})	Intra-day accuracy and precision (n=7)			Inter-day accuracy and precision (n=7)		
		TFH Found[a] (µg mL^{-1})	RSD[b] %	RE[c] %	TFH found (µg mL^{-1})	RSD[b] %	RE[c] %
A	9.0	9.10	0.92	1.11	9.12	0.84	1.34
	15.0	14.82	1.63	1.20	14.74	1.32	1.73
	21.0	20.72	1.54	1.34	21.49	1.36	2.34
B	8.0	8.07	0.79	0.88	8.10	0.83	1.25
	12.0	11.89	1.26	0.92	12.12	1.37	1.00
	16.0	15.74	1.47	1.62	16.23	1.14	1.44

[a] Mean value of seven determinations; [b] Relative standard deviation (%); [c] Relative error (%)

Table - 3: Method robustness and ruggedness expressed as intermediate precision (% RSD)

Method	TFH Taken (µg mL^{-1})	Robustness		Ruggedness	
		Parameters altered		Inter-analysts (%RSD), (n=4)	Inter-instruments (%RSD), (n=4)
		Volume of buffer / ethanolic H_2SO_4[**]	Reaction / breaking time[Ψ]		
A	9	1.20	0.92	0.97	2.36
	15	0.72	1.36	1.54	1.66
	21	1.27	0.85	0.79	2.65
B	8	0.98	2.10	1.36	1.97
	12	1.63	0.96	2.10	1.54
	16	0.81	1.06	0.63	2.22

[**]In method A, the volume of buffer was 5, 6 and 7 mL, in method B the volumes of ethanolic H_2SO_4 added were 0.8, 1.0 and 1.2 mL, [Ψ]In methods A, and B, the reaction times / breaking times were 0, 1 and 2 min.

Table - 4: Results of analysis of tablets by the proposed methods and statistical comparison of the results with the official method

Tablets brand name[Ψ]	Nominal amount (mg/tablet)	Found[*] (Percent of label claim ± SD)		
		Official method	Method A	Method B
[a]Zimig	250	100.9±1.20	101.5±1.32 *t*= 0.75 *F*= 1.21	100.3±0.93 *t*= 0.89 *F*= 1.66
[b] Terbiforce	250	98.7 ±1.07	99.5±0.92 *t*= 1.26 *F*= 1.35	98.1±1.27 *t*= 0.81 *F*= 1.41
[c] Sebifin	250	101.5±1.25	102.3±1.72 *t*= 0.84 *F*= 1.89	100.6±1.35 *t*= 1.09 *F*= 1.17

[*]Mean value of five determinations, (Tabulated t-value at the 95 % confidence level and for four degrees of freedom is 2.77). (Tabulated F-value at the 95 % confidence level and for four degrees of freedom is 6.39). [Ψ]Marketed by : [a] Glaxo Smith Kline Pharma Ltd., India, [b] Lifestar Pharma Pvt. Ltd., India, [c]Ranbaxy Laboratories Ltd, India.

3.13.4. Robustness and ruggedness

The robustness of the methods was evaluated by making small incremental changes in two selected variables (volumes of buffer and reaction time in method A ; volumes of ethanolic H_2SO_4 and the breaking times in method B) and the effect of changes was studied on the basis of absorbance of colored systems. The changes had negligible influence on the results as revealed by small RSD % as intermediate precision. The results are tabulated in table 3.

Method ruggedness was studied by having the analysis done by four different analysts, and also by a single analyst performing analysis on four different instruments in the same laboratory. Intermediate precision values in all the methods were in the range 0.63-2.65 indicating acceptable ruggedness. These results are given in table 3.

3.13.5. Application

The proposed methods were applied for the determination of TFH in commercial tablets and spiked human urine. The results were compared with these obtained using a official method [6]. Statistical analysis of the results did not give any significant difference between the performance of the proposed methods and official method with respect to accuracy and precision as revealed by Student's *t*-value and variance ratio *F*-value. The results of assay are given in table 4. The proposed methods were also applied to the determination of TFH in spiked human urine sample and the results are presented in table 5.

Table - 5: Terbinafine hydrochloride determination in spiked urine sample, n = 5

Method	Spiked concentration (µg mL^{-1})	Concentration found* (µg mL^{-1})	% Recovery ±SD*
A	9	9.32	103.5±0.72
	15	15.34	102.3±0.91
	21	21.52	102.5±1.21
B	8	8.42	105.3±1.93
	12	12.43	103.6±1.27
	16	16.43	102.7±1.54

*Mean value of five determinations.

Table – 6: Results of recovery study *via* standard-addition method.

Method	Tablets studied	TFH in formulation, µg mL^{-1}	Pure TFH added, µg mL^{-1}	Total found, µg mL^{-1}	Pure TFH recovered (Percent±SD*)
A	Zimig-250	12.18	6.0	18.22	100.21±1.12
		12.18	12.0	23.89	98.80±0.55
		12.18	18.0	29.94	99.20±0.75
	Terbiforce-250	11.94	6.0	17.81	99.27±1.29
		11.94	12.0	24.14	100.83±0.93
		11.94	18.0	29.73	99.29±0.65
	Sebifin-250	12.28	6.0	18.18	99.43±1.29
		12.28	12.0	24.72	101.8±0.45
		12.28	18.0	29.73	98.20±0.95
B	Zimig-250	8.02	4.0	12.18	101.37±1.29
		8.02	8.0	16.47	102.8±0.45
		8.02	12.0	19.87	99.25±0.75
	Terbiforce-250	7.85	4.0	11.75	99.17±1.29
		7.85	8.0	16.15	101.9±0.85
		7.85	12.0	20.27	102.1±0.54
	Sebifin-250	8.05	4.0	11.82	98.07±1.63
		8.05	8.0	16.02	99.83±0.98
		8.05	12.0	20.29	101.21±1.23

*Mean value of three determinations.

Table – 7: Comparison of performance of the present methods with the existing methods.

Reagent/s used	Methodology	λ_{max} (nm)	Linear range (µg mL^{-1}) ε (L $mol^{-1}cm^{-1}$)	Remarks	Ref. No.
Orange G, alizarin Red, Mo(v)SCN	Absorbance of ion-pair complex in suitable organic solvents measured	470	5-75	Less sensitive, narrow ranges	28
		425	10-80		
		499	5-55		
Methyl orange	Ion-pair complex measured in acidic pH	422	NA	--	29
*BPB, BTB and BCG	Absorbance of ion-pair complex measured in chloroform	410	2-25	--	30
*CGT	a) Measurement of absorbance of extracted TFH-CGT ion pair in CH_2Cl_2	500	1.5-30 8.60×10^3	Sensitive and selective with wide linear dynamic ranges, Applicable to spiked urine	Present work
	b) Acid form of the BCG measured	490	1.0-20 1.64×10^4		

*BPB-bromophenol blue, BTB-bromothymol blue, BCG-bromocresol green and CGT-calmagite

3.13.6. Recovery study

To ascertain the accuracy of the proposed methods, recovery experiment was performed *via* standard addition technique. To a fixed and known amount of TFH in tablet powder (pre-analyzed), pure TFH was added at three levels 50, 100 and 150% of the level present in the tablets and the total was found by the proposed methods. Results of this study are presented in table 6. In all the cases, the percent found ranged from 98.07 to 102.8 with SD values in the range 0.45 to1.63 and indicate that the co formulated substances did not interfere in the assay.

4. Conclusions

A significant advantage of the extractive spectrophotometric methods is that it can be applied for the determination of individual compounds in a multi component mixture. The proposed methods make use of simple reagent which an ordinary analytical laboratory can afford, and the procedures do not involve any critical reaction conditions or tedious sample preparation. The methods are highly reliable owing to the stability of the ion-pair complex and acid form of the dye, which are ultimately measured. Moreover, the methods are accurate, reproducible, adequately sensitive and free from interference caused by the excipients expected to be present in tablets. The methods were successfully applied to the spiked human urine, and both the methods were demonstrated to be both robust and rugged. The methods offer several advantages over the existing methods in terms of sensitivity, selectivity, and linear dynamic range as indicated in table 7.

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