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ORIGINAL RESEARCH PAPER

HPLC-fluorescence detection for assay of tramadol binary mixtures with ibuprofen or chlorzoxazone in tablets and plasma: Analytical Eco-Scale and GAPI tools for green assessment

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ABSTRACT

Tramadol, a strong pain killer known for its addictive problems is either co-administrated or co-formulated with other analgesics or muscle relaxants. The power of fluorescence detection in HPLC is tested to resolve such mixtures in plasma matrix to reach the required sensitivity with simple sample treatment using just protein precipitation. The aim of this work was to develop an eco-friendly and sensitive HPLC method with fluorimetric detection for analysis of Tramadol in its two binary mixtures with Ibuprofen (mixture 1) and Chlorzoxazone (mixture 2) in two combined dosage forms and spiked plasma. Separation was done using a C_{18} column with mobile phase of acetonitrile and water (pH 3.5) in gradient elution and 1 mL/min flow rate. Detection was carried out with λ excitation/ λ emission of 220 and 307 nm, respectively. The method was applied to detect the two binary mixtures in real plasma samples after *in vivo* application to rats, to assure that the drugs' metabolites do not affect the sensitivity or selectivity of the assay. Evaluation of greenness of the proposed method was done using semi-quantitative Eco-Scale and new Green Analytical Procedure Index which showed that this method can be a greener alternative with higher sensitivity for analysis of both mixtures. The method (15 min-assay) was linear over concentrations of 0.1–10 $\mu\text{g/mL}$ and 0.1–33 $\mu\text{g/mL}$ in plasma. In addition, the proposed method was validated per ICH as well as FDA bioanalytical methods' validation guidelines.

KEYWORDS

tramadol, binary mixtures, HPLC-fluorescence detection, dosage form, green, plasma

1. INTRODUCTION

Different pain killers together with muscle relaxants are commonly used either over the counter or prescribed by physicians to relief moderate to severe pain especially associated with different musculoskeletal conditions. Thus, several combinations of analgesics (with or without muscle relaxants) are manufactured by different companies and launched in several markets. However, a lot of these combinations are not FDA approved nor tested for their pharmacological interactions. Therefore, it is necessary to find suitable analytical tools for simultaneous determination of these co-administered drugs in the same dosage form and especially in plasma to be available in hands for testing their pharmacokinetic interactions whenever needed.

Among these combinations found in the Indian market are two combinations of tramadol hydrochloride (TM). The first combination is TM with chlorzoxazone (CL) a muscle relaxant. The second combination is with ibuprofen (IB), especially designed for osteoarthritis patients, where TM would be an auxiliary to IB non-steroidal anti-inflammatory action. These two mixtures of TM have been co-formulated in tablets dosage forms to be used generally as a pain reliever, in cases of moderate and severe pain associated with musculo-

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Fig. 1. Chemical structures of (a) Tramadol (TM); (b) Ibuprofen (IB); and (c) Chlorzoxazone (CL)

skeletal spasms or cancer diseases. Since, there are not enough studies for the pharmacological interaction between TM and IB or CL when combined in the same dosage forms. It was important to develop a pharmacokinetic tool to analyze the three drugs in plasma for further pharmacological testing of the combined drugs when required in the future.

TM is an opioid analgesic drug centrally acting that has been used since 1977 for the relief of strong physical pain.[1] It is official in Indian [2], British [3] and United States [4] Pharmacopoeias. Chemically it is (1*RS*,2*RS*)-2-[(dimethylamino)methyl]-1-(3-methoxyphenyl)cyclohexanol hydrochloride (Fig. 1a). IB, chemically is (2*RS*)-2-[4-(2-methylpropyl)phenyl]propanoic acid (Fig. 1b), belongs to the NSAIDs (non-steroidal anti-inflammatory drugs) and is also official in the three pharmacopoeias.[2–4] CL is chemically, 5-Chloro-2-benzoxazolinone (Fig. 1c). It is a centrally acting muscle relaxant that is official in United States Pharmacopoeia [4].

Binary combinations of TM with IB or CL in their dosage forms challenging ratios (1:8, TM: IB and 1:5, TM: CL) were estimated in the literature by very few reported analytical methods including spectrophotometry [5, 6], HPLC [1, 6, 7], HPTLC [7, 8] and spectrofluorimetry [9]. However, by searching the literature, there were not any methods reported for the determination of the two binary mixtures in biological samples. In a resource limited settings, highly sensitive sophisticated instruments as mass spectroscopy would be not available and costly. An HPLC with fluorescence detector is a good choice to provide high sensitivity, compared to commonly used UV detection, and low quantitation limit (QL) which are essential for plasma analysis.

Therefore, the aim of this work was to establish a fast and simple HPLC method with fluorescence detection, due to its high sensitivity, for the simultaneous determination of TM, IB and CL in bulk form, dosage forms and plasma. Validation was fulfilled in accordance with the ICH “International Conference on Harmonization” [10] and the “FDA” Food and Drug Administration [11].

2. EXPERIMENTAL

2.1. Chemicals and reagents

TM (99.0% purity), IB (99.9% purity) and CL (99.9% purity) were kindly provided by Fluka BioChemika (Switzerland), EIPICO Pharmaceutical Industries (Egypt) and

GlaxoSmithKline Co. (Egypt), respectively. Ibudol[®] tablets containing 400 mg IB and 50 mg TM and MUZO[®] tablets containing 250 mg CL and 50 mg TM (Stedman Pharmaceuticals Pvt Ltd., India) were purchased from the local Indian market. Acetonitrile HPLC-grade (Sigma-Aldrich Chemie GmbH, Switzerland), ortho-phosphoric acid (BDH Laboratory Suppliers, England) and double distilled water have been used.

2.2. Instrumentation

Chromatographic analysis was performed using Agilent 1260 device (USA) equipped with auto-injector, quaternary pump and fluorescence detector (G1321C). Agilent ChemStation Software also has been used for data analysis. A reversed phase Agilent Zorbax SB-C18 (150 × 4.6 mm) column thermostated at ambient temperature (25 °C) has been used for the chromatographic separation with mobile phase flow rate of 1 mL/min. The mobile phase used consisted of acetonitrile and water acidified with orthophosphoric acid to pH 3.5. Step wise gradient elution system has been used starting with 17 and 83% (v/v) acetonitrile and acidified water, respectively, from 0 to 2 min then switched to 70 and 30% (v/v) acetonitrile and acidified water, respectively, and this ratio was kept till the end (14 min). The fluorescence detector was set to 220/307 nm (λ excitation/ λ emission) for determination of the three studied analytes (TM, IB and CL).

2.3. Methods

2.3.1. Standard stock solutions. Standard stock solutions of TM, IB and CL (1000 µg/mL) were prepared by dissolving 100 mg, accurately weighed, of the authentic material of each drug in 100 mL methanol. Stability of the three prepared stock solutions was 14 days when stored at 4 °C protected from light.

2.3.2. Calibration graphs for TM and IB/CL analysis in dosage forms. TM, IB and CL standards (1000 µg/mL) were diluted by methanol to working solutions of 200 µg/mL each. The calibration standards were prepared by transferring accurate micro-volumes (5–500 µL) of each of TM, IB and CL working solutions into three separate sets of 10 mL volumetric flasks and diluted to volume with methanol to obtain a final calibration range of 0.1–10 µg/mL for all drugs. Triplicates HPLC injections (20 µL) of each drug calibration solution were injected using the chromatographic

conditions previously mentioned (Section 2.2). The obtained peak area means were plotted against their corresponding concentrations so the calibration curves and regression equations would be obtained.

2.3.3. Calibration graphs for TM and IB/CL analysis in plasma. A series of working standards for plasma calibration curve and quality control samples "QCs" were prepared. Five different volumes of each of TM, IB and CL standard stock solutions (1000 µg/mL) were transferred into three series of five 10 mL volumetric flasks separately and completed to mark with methanol to achieve a final working concentration range of (1–330 µg/mL). Aliquots of 250 µL plasma were spiked separately with 25 µL of each of the TM and IB or TM and CL working solutions (1–330 µg/mL) and 25 µL of IS solution, from working solution of 100 µg/mL, (CL as IS for TM/IB mixture and IB as IS for TM/CL mixture) followed by 500 µL of acetonitrile to achieve concentrations in the range of 0.1–33 µg/mL plasma for the three concerned drugs with fixed IS concentration of 10 µg/mL plasma in all samples. After vortex mixing for 2 min, centrifugation was done for 15 min in a cooling centrifuge (–4 °C) at 15,000 rpm. The supernatant was then filtered by a 0.45 µm syringe adapter. A 20 µL volume was then injected in triplicate and chromatographed. In addition, QCs were prepared in the same way, 0.1, 0.3, 10 and 30 µg/mL plasma, by spiking 25 µL of their respective working standards in blank plasma to be LLOQ "lower limit of quantification", LQC "low QC", MQC "mid QC" and HQC "high QC", respectively. The ratios of peak areas of analyte/IS against corresponding drug concentrations were plotted for calibration graphs construction.

2.3.4. Analysis of synthetic mixtures. Accurate volumes of standard solutions of TM, IB or CL were transferred into a set of five 10 mL volumetric flasks then dissolved to volume with methanol. Portions of 20 µL of each mixture in triplicates were chromatographed and the % recoveries were calculated.

2.3.5. Analysis of TM and IB/CL dosage forms. Five Ibuprofen® tablets were weighed and powdered. Into a 25 mL volumetric flask, a powder weight equivalent to 25 mg IB and 3.125 mg TM was transferred using 15 mL methanol. Five MUZOX® tablets were also weighed and powdered. Into another 25 mL volumetric flask, accurate powder weight equivalent to 25 mg CL and 5 mg TM was transferred. After 15 min sonication, the flasks were completed to volume with methanol and filtered using filter paper (Whatman). Aliquots of 80 and 50 µL from each of the Ibuprofen® tablet sample solution (mixture 1) and the MUZOX® tablet sample solution (mixture 2), respectively, were transferred separately into two 10 mL volumetric flasks and diluted to mark with methanol. The concentrations achieved after dilution were 8 µg/mL of IB and 1 µg/mL of TM (mixture 1) and 5 µg/mL CL and 1 µg/mL TM (mixture 2). Aliquots of 20 µL were injected and chromatographed as previously. The percentage recoveries were calculated using the previously computed regression equation (section 2.3.2).

2.3.6. In vivo application. Two groups each of six male Wistar rats weighing 200–250 gm were used for the in vivo study. Animals were housed under controlled conditions (25 ± 10 °C, RH 55 ± 10%). They were allowed to adapt to the housing environment for at least 1 week before the study. Diet was prohibited for 12 h before the experiment but water was freely available. Group (1) was treated with 20 mg/kg TM [12] and 17.8 mg/Kg IB [13] and group (2) was treated with 20 mg/kg TM [12] and 15 mg/Kg CL [14]. All drugs were orally administered once, and then six blood samples were collected from each group by retro-orbital bleeding at 3 specific time intervals for each drug in its mixture, which are $t_{\max}$, t_{average} (average time interval between $t_{\max}$ and $t_{1/2}$) and $t_{1/2}$ of each drug. The blood samples were immediately collected to K₂-EDTA tubes and centrifuged at 4000 rpm (10 min) to obtain plasma. The plasma samples were then stored at –80 °C until analysis time.

3. RESULTS AND DISCUSSION

3.1. Chromatographic method development and optimization

Several factors affected the separation and resolution of TM in its binary mixtures with IB and CL. All these factors were optimized independently to achieve the best chromatographic system suitability parameters. For selection of the stationary phase, some columns have been tried such as C8 (250 × 4.6 mm), C18 (150 × 4.6 mm) and C18 (250 × 4.6 mm). However, the best separation in terms of sensitivity, resolution and baseline noise was achieved using the Zorbax SB-C18 (150 × 4.6 mm) column. Different mobile phases were tried in order to achieve the best separation between TM and IB and/or CL in a reasonable analysis time. Mobile phases of different organic modifiers in different ratios (methanol, ethanol or acetonitrile) were tried. Acetonitrile gave the best results in terms of separation and peak symmetry. A high acetonitrile ratio was essential to elute IB but that caused TM to co-elute with the solvent front. Several isocratic trials were attempted to try to reach a mobile phase ratio that separated both TM and IB within reasonable retention times and also away from early retention times where the plasma co-eluting peaks may interfere. However, none of these trials succeeded due to the huge difference in retention times of TM and IB, so the only solution was to go for gradient programming. After numerous trials to reach the optimum gradient system to achieve the required separation between the drugs of interest, the system of choice was to start with 17 and 83% (v/v) acetonitrile and acidified water, respectively, till 2 min then switched to 70 and 30% (v/v) acetonitrile and acidified water, respectively. Different buffers such as phosphate, acetate and formate buffers at different pH values (pH 3.0–7.0) were tried in the beginning of the study. Using buffers resulted in peaks tailing and distortion of the baseline. Also, pure water caused broadening of the peaks due to its neutral pH value so water with the addition of phosphoric acid to maintain the pH acidic (3.5) was used.

Table 1. System suitability parameters of the HPLC system used

Mixture	Analyte	Retention time (R _t), min	Capacity factor (k')	Selectivity (α)	Resolution (R _s)	Asymmetry (A _f)	Efficiency (plates/m)
Mixture 1	TM	4.92	2.04	2.96	49.01	0.92	25,978.79
	IB	11.38	6.03	-----	-----	1.11	103,488.39
Mixture 2	TM	4.92	2.04	2.04	27.6	0.91	25,809.41
	CL	8.37	4.17	-----	-----	1.10	68,952.58

k' (2–10), $\alpha > 1$, R_s > 2, A_f (0.8–1.2) and plates.m⁻¹ (> 2000)

It was important to optimize the pH of acidified water so pH 3 to 9 was tried. The optimum pH was 3.5 as it gave the best separation and least tailing for TM and CL and also after optimizing the gradient system and flow rate, this pH was also optimum for IB separation and peak symmetry.

Flow rate was also optimized to enhance the resolution of peaks. The flow rate has been adjusted over the range of 0.5–2 mL/min. The optimum chosen flow rate was 1 mL/min, which gave the best separation in reasonable retention time. Flow rates below 1 mL/min caused prolonged retention time. Increasing the flow rates caused resolution and retention time to decrease. After optimization of chromatographic parameters, all system suitability parameters have been measured and compared to reference values as they are essential for validation of the analytical method (Table 1).

3.2. Sample extraction optimization

Protein precipitation is the method of choice for extraction in several reports [15, 16] due to its simplicity and greenness. Protein precipitation does not involve extraction with amounts of organic solvents nor huge number of steps like liquid-liquid or solid-liquid extractions. Both methanol and acetonitrile were tried; but acetonitrile was a stronger protein precipitating agent. There was no need to add strong acids nor pH adjustment nor evaporating the solvent to dryness. The 2:1 acetonitrile/plasma ratio was sufficient to precipitate the proteins and obtain good recovery for all drugs with the required sensitivity without breaching the rules of green analysis.

3.3. Fluorescence sensitivity and internal standard selection

Fluorescence detection offers higher selectivity and sensitivity compared to UV. So this advantage was used in this study, as the three drugs have native fluorescent nature. Several excitation and emission wavelengths were tried. The best λ excitation was found to be 220 nm that gave highest fluorescence signals for the three drugs especially the minor component (TM) and the three drugs had acceptable corresponding emission at 307 nm. Regarding internal standard selection, it was difficult to find a fluorescent sensitive compound at an appropriate retention time, with minimum interference of the plasma matrix and high resolution under the proposed chromatographic conditions. Thus, CL was chosen as IS for TM/IB mixture and IB was chosen as an IS for TM/CL mixture.

3.4. Method Validation

The ICH [10] and the FDA [11] for bioanalytical method validation guidelines have been followed for validation.

3.4.1. Linearity and range validation. Linearity has been studied to determine the range in which the analytes' responses are linear with their concentrations ($n = 5$). By plotting peak areas (for dosage form analysis) or peak areas/IS peak area (for plasma analysis) against drugs' concentrations (Table 2), the graphs obtained showed linear relationships. The slopes, intercepts, and correlation coefficients obtained by the linear least squares regression treatment of the results together with the standard deviations of intercept (S_a), slope (S_b) and residuals ($S_{y/x}$), indicating the closeness of the points to the line, are presented for each compound in Table 2. All obtained results were within the acceptable limits indicating the validity of the method. The correlation coefficients ($r \geq 0.9990$) together with the low F-values indicate the good linearity of the calibration graphs.

Generally, the proposed method offered a LLOQ 0.1 $\mu\text{g/mL}$ in plasma for the three drugs. The reported C_{max} of TM, CL and IB after oral administration in plasma is within the linearity range of the three drugs [17–19] and higher than the LLOQ of this assay. Thus, this method is enough sensitive for the simultaneous quantitation of TM and IB or TM and CL in routine pharmacokinetic studies. Representative chromatograms of plasma spiked with LLOQ levels are shown in Fig. 2.

3.4.2. Detection Limit (DL) and Quantitation Limit (QL) validation. Signal to noise ratio "S/N" method was used to calculate DL and QL where the S/N ratio is 3/1 and 10/1 for DL and QL, respectively. The DL/QL values of the proposed HPLC method are proposed in Table 2.

3.4.3. Accuracy and precision validation. In order to assess the accuracy and precision of the proposed method in separating and resolving the two studied binary mixtures in different ratios. Five different synthetic mixtures were chosen and prepared as mentioned above in section 2.3.4. including the dosage form ratio of the two mixtures and analysed. The mean percentage recoveries, the relative error values and relative standard deviation values indicate excellent accuracy and precision (Table 3) and proved no matter the concentration of each drug relative to the other, the method separate the drugs accurately and precisely.

Table 2. Parameters of regression for TM/IB and TM/CL determination by the proposed method

Parameter	For dosage form analysis			For plasma analysis			
	TM	IB	CL	TM	IB	TM	CL
Linearity range, ($\mu\text{g/mL}$)	0.1–10	0.1–10	0.1–10	0.1–33*	0.1–33*	0.1–33*	0.1–33*
QL ($\mu\text{g/mL}$)	0.10	0.10	0.10	0.10*	0.10*	0.10*	0.10*
DL ($\mu\text{g/mL}$)	0.03	0.03	0.03	0.03*	0.03*	0.03*	0.03*
Intercept	35.76	21.29	8.03	0.11	0.06	0.24	0.03
Slope	122.98	97.98	49.28	0.07	0.15	0.15	0.03
Correlation Coefficient, r	0.9995	0.9991	0.9996	0.9990	0.9993	0.9991	0.9995
S_a	12.14	12.65	3.92	0.03	0.05	0.06	9.21×10^{-3}
S_b	2.34	2.43	0.75	1.89×10^{-3}	3.24×10^{-3}	3.75×10^{-3}	5.50×10^{-4}
$S_{y/x}$	18.40	19.17	5.94	0.05	0.08	0.10	0.01
F	2770.69	1620.59	4270.17	1561.72	2093.67	1669.57	2871.82
Significance F	1.51×10^{-5}	3.37×10^{-5}	7.90×10^{-6}	3.57×10^{-5}	2.30×10^{-5}	3.23×10^{-5}	1.43×10^{-5}

* S_a standard deviation of intercept, S_b standard deviation of slope and $S_{y/x}$ standard deviation of residuals (* $\mu\text{g/mL}$ plasma)

Fig. 2. HPLC with fluorescence detection chromatograms of TM and IB (mixture 1) and CL (IS) (a) and TM and CL (mixture 2) and IB (IS) (b) in extracted plasma spiked at the LLOQ levels

In plasma, validation of accuracy and precision was done at the four QC levels. Replicates ($n=6$) of each concentration were analyzed on same day or on several days for intra- and inter-day evaluation of precision, respectively (Table 4).

3.4.4. Recovery validation. Recoveries of TM, IB and CL spiked to plasma, at LQC and HQC levels, were calculated by comparing the results of the spiked samples to the standards' drugs solutions. All extraction recoveries were between 96 and 99% and the mean recovery of IS of mixture 1 was 98% and of mixture 2 was 96.5%. These results indicate successful complete extraction just after simple protein precipitation pretreatment step.

3.4.5. Robustness validation. Robustness of the proposed method was assessed through analysis of synthetic mixtures with deliberate variation of selected chromatographic parameters including pH of the acidified water and its percentage, flow rate and temperature. All deliberate variations showed no influence on resolution or recoveries of the studied drugs (Table 5).

3.4.6. Selectivity validation. Selectivity of the proposed HPLC method for TM and IB/CL combined tablets analysis, was previously assessed in the results where the percentage relative errors $< 2\%$ proves the selectivity well. The absence of interference from the tablets' excipients, also demonstrates the selectivity of the method (Table 6).

The HPLC peak purity of the TM, IB and CL was also checked. The purity angle was within the purity threshold limit for all drugs. In addition, the emission spectra of TM, IB and CL were recorded at different time intervals across their peaks indicates the purity of the peaks in their dosage forms (Fig. 3) and also in plasma samples (Fig. 4).

In plasma samples, no evidence of interfering peaks was noticed throughout the analysis. Chromatograms showing blank plasma and plasma spiked only with the ISs of the two mixtures are shown in Fig. 5a and b, respectively where no interference is noticed.

3.4.7. Stability. The stability results of TM, IB and CL were obtained at LQC and HQC ($n=6$) under freeze-thaw, postpreparative, short-term and long-term stability conditions in Table 7. The stock solutions and working standard

Table 3. Precision and accuracy assessment of the proposed method for determination of TM/IB and TM/CL in synthetic mixtures

Mixture 1						Mixture 2					
TM	IB	TM		IB		TM	CL	TM		CL	
Concentration (µg/mL)		Mean % Recovery ± %RSD	%E _r	Mean % Recovery ± %RSD	%E _r	Concentration (µg/mL)		Mean % Recovery ± %RSD	%E _r	Mean % Recovery ± %RSD ^a	%E _r
<i>Results of accuracy and Intra-day precision, (n = 5)</i>											
1.00	8.00	99.20 ± 1.30	−0.80	100.97 ± 0.99	0.97	1.00	5.00	101.07 ± 1.50	1.07	98.10 ± 1.20	−1.90
0.10	2.00	100.30 ± 1.20	0.30	100.50 ± 1.79	0.50	0.10	2.00	100.77 ± 1.00	0.77	100.04 ± 1.99	0.04
3.50	10.00	100.05 ± 1.94	0.05	99.99 ± 1.02	−0.01	3.50	10.00	101.09 ± 0.89	1.09	100.75 ± 1.80	0.75
2.00	0.10	98.20 ± 1.00	−1.80	100.19 ± 0.90	0.19	2.00	0.10	99.87 ± 0.99	−0.13	99.05 ± 1.09	−0.95
8.00	0.50	100.55 ± 1.25	0.55	100.50 ± 1.99	0.50	5.00	0.50	99.90 ± 0.87	−0.10	100.65 ± 0.60	0.65
Grand mean		99.66 ± 1.34	−0.34	100.43 ± 1.34	0.43	Grand mean		100.54 ± 1.05	0.54	99.72 ± 1.34	−0.28
<i>Results of accuracy and Inter-day precision, (n = 5)</i>											
1.00	8.00	98.22 ± 1.80	−1.78	99.59 ± 0.90	−0.41	1.00	5.00	100.99 ± 1.00	0.99	100.40 ± 1.74	0.40
0.10	2.00	99.10 ± 0.89	−0.90	99.67 ± 1.87	−0.33	0.10	2.00	99.91 ± 1.50	−0.09	99.55 ± 1.99	−0.45
3.50	10.00	101.30 ± 0.99	1.30	98.30 ± 1.20	−1.70	3.50	10.00	102.00 ± 1.99	2.00	99.90 ± 1.23	−0.10
2.00	0.10	100.44 ± 0.88	0.44	100.09 ± 0.85	0.09	2.00	00.10	100.50 ± 1.98	0.50	100.55 ± 1.83	0.55
8.00	0.50	99.99 ± 1.78	−0.01	99.50 ± 1.11	−0.50	5.00	00.50	101.35 ± 1.01	1.35	101.59 ± 0.99	1.59
Grand mean		99.81 ± 1.27	−0.19	99.43 ± 1.19	−0.57	Grand mean		100.95 ± 1.50	0.95	100.40 ± 1.56	0.40

•“%E_r” percentage of relative error

•“%RSD” percentage of relative standard deviation

Table 4. Precision and accuracy assessment of the proposed method for determination of TM/IB and TM/CL in plasma

Concentration	Theoretical concentration (µg/mL)	Intra-day				Inter-day			
		Calculated concentration (µg/mL)	Mean % Recovery ± SD*	% RSD	%E _r	Calculated concentration (µg/mL)	Mean % Recovery ± SD*	% RSD	%E _r
<i>TM</i>									
LLOQ	0.1	0.301	100.33 ± 1.00	1.00	0.33	0.305	101.67 ± 1.68	1.65	1.67
LQC	0.3	0.605	101.83 ± 1.35	1.33	1.83	0.609	101.50 ± 1.22	1.20	1.50
MQC	10.0	10.110	101.10 ± 1.25	1.24	1.10	10.255	102.55 ± 0.88	0.86	2.55
HQC	30.0	30.051	100.17 ± 1.29	1.29	0.17	30.005	100.02 ± 1.77	1.77	0.02
<i>IB</i>									
LLOQ	0.1	0.318	102.25 ± 1.55	1.52	2.25	0.350	105.00 ± 1.89	1.80	5.00
LQC	0.3	0.656	102.55 ± 1.99	1.94	2.55	0.625	102.00 ± 1.25	1.26	2.00
MQC	10.0	5.355	103.04 ± 0.85	0.82	3.04	10.077	101.55 ± 0.97	0.96	1.55
HQC	30.0	30.089	100.07 ± 1.00	1.00	0.07	30.187	104.10 ± 1.34	1.29	4.10
<i>CL</i>									
LLOQ	0.1	0.321	103.88 ± 1.90	1.83	3.88	0.334	100.50 ± 1.00	1.00	0.50
LQC	0.3	0.678	101.66 ± 0.99	0.97	1.66	0.622	102.60 ± 1.69	1.65	2.60
MQC	10.0	10.550	104.07 ± 0.23	0.22	4.07	10.902	101.99 ± 1.25	1.23	1.99
HQC	30.0	30.099	100.67 ± 1.50	1.49	0.67	30.561	100.44 ± 1.00	1.00	0.44

*standard deviation of six determinations

Table 5. Robustness assessment for determination of TM/IB and TM/CL

Parameters tested	HPLC–FD method*					
	TM		IB		CL	
	RSD % of peak areas	R _t ± SD	RSD % of peak areas	R _t ± SD	RSD % of peak areas	R _t ± SD
1) Mobile phase ratio [±1 % organic phase]	0.50	4.92 ± 2.0 × 10 ⁻²	1.50	11.38 ± 1.5 × 10 ⁻²	1.80	8.36 ± 2.2 × 10 ⁻²
2) Flow rate [1 ± 0.1 mL/min]	0.41	4.91 ± 2.5 × 10 ⁻²	0.80	11.38 ± 3.9 × 10 ⁻²	1.00	8.37 ± 4.5 × 10 ⁻²
3) Column temperature [25 °C ± 2 °C]	0.12	4.92 ± 3.4 × 10 ⁻²	1.50	11.36 ± 5.5 × 10 ⁻²	1.97	8.36 ± 5.5 × 10 ⁻²
4) pH of the aqueous phase [3.5 ± 0.5]	0.90	4.93 ± 3.6 × 10 ⁻²	0.65	11.39 ± 3.1 × 10 ⁻²	1.55	8.37 ± 1.54 × 10 ⁻²

* Mean of 3 concentrations levels 0.5, 5 and 10 µg/mL of TM, IB and CL

of TM, IB and CL were stable for 14 days at refrigerator temperature and for at least 6 h at ambient temperature. The RSD% not exceeding 4% and Er% not exceeding 10% indicate good stability of the drugs under all the studied conditions. Further forced degradation studies will be conducted by the authors in a future study to investigate the stability of the combinations under different conditions as done in their previous assays [20].

3.5. Application for determination of TM and IB/CL in their combined tablets dosage form

The proposed method was implemented for the analysis of TM in its co-formulated tablets with either IB or CL. Figure 6 shows the HPLC chromatograms, of the assayed pharmaceutical preparations with no interferences. The obtained results were not only in agreement with the labeled claims but also compared with those of the comparison methods [1, 6] (Table 6).

3.6. Application for determination of TM and IB/CL in plasma samples

The developed method was applied successfully for determination of TM and IB/CL in plasma with only simple protein precipitation step with excellent correlation coefficients for the calibration curves (0.1–33 µg/mL plasma) and very low LLOD below the reported C_{max} of the three studied drugs.

3.7. In vivo application

The applicability of the method to real clinical practice was assessed by therapeutic drug monitoring of the two binary mixtures in plasma samples from laboratory rats orally treated with the concerned drugs. The proposed method was applied for the determination of TM and IB or CL in plasma after a single oral dose of TM in combination with IB or CL. Real plasma samples were obtained at different intervals as

Table 6. Assay results for the determination of TM, IB and CL in their drug products using the proposed method

	% Found $\pm$ SD ^a , <i>n</i> = 5			
	TM		IB	
	Proposed HPLC method	Comparison HPLC-UV method [6]	Proposed HPLC method	Comparison HPLC-UV method [6]
Mixture 1 (TM/IB) Ibudol [®] tablets	99.71 $\pm$ 0.87	100.78 $\pm$ 0.54	100.39 $\pm$ 0.91	100.51 $\pm$ 0.49
Students' <i>t</i> -test (<i>t</i>) *	0.07		0.82	
Variance ratio <i>F</i> -test (<i>F</i>) *	2.62		3.47	
	% Found $\pm$ SD ^a , <i>n</i> = 5			
	TM		CL	
	Proposed HPLC method	Comparison HPLC-UV method [1]	Proposed HPLC method	Comparison HPLC-UV method [1]
Mixture 2 (TM/CL) MUZOX [®] tablets	101.13 $\pm$ 0.83	101.56 $\pm$ 0.96	100.72 $\pm$ 1.18	100.48 $\pm$ 1.06
Students' <i>t</i> -test (<i>t</i>) *	0.58		0.81	
Variance ratio <i>F</i> -test (<i>F</i>) *	0.20		0.13	

* Theoretical values, at 95% confidence limit, of *t* and *F* are 2.31 and 6.39, respectively

Fig. 3. Emission spectra illustrating peak purity of (a) TM and (b) IB (mixture 1) and (c) TM and (d) CL each is obtained from their tablets solutions for the HPLC method

Fig. 4. Emission spectra illustrating peak purity of (a) TM and (b) IB (mixture 1) and (c) TM and (d) CL in plasma samples for the HPLC method

Fig. 5. HPLC with fluorescence detection chromatograms for treated blank plasma (A) and treated blank with IS plasma (B)

Table 7. Stability tests of TM, IB and CL in plasma ($n = 6$)

Stability	QC samples	TM		IB		CL	
		Mean % Recovery $\pm$ %RSD	%E _r	Mean % Recovery $\pm$ %RSD	%E _r	Mean % Recovery $\pm$ %RSD	%E _r
Short-term room temperature (6 h)	LQC	103.02 $\pm$ 1.90	3.02	99.00 $\pm$ 1.67	−1.00	102.43 $\pm$ 0.70	2.43
	HQC	102.22 $\pm$ 0.90	2.22	103.89 $\pm$ 1.70	3.89	100.50 $\pm$ 1.20	0.50
Freeze-thaw (3 cycles, at −70 °C)	LQC	102.40 $\pm$ 1.77	2.40	100.34 $\pm$ 1.09	0.34	99.00 $\pm$ 0.71	−1.00
	HQC	101.09 $\pm$ 1.50	1.09	101.99 $\pm$ 1.20	1.99	101.76 $\pm$ 0.50	1.76
Post preparative 5 °C for 24 h	LQC	101.56 $\pm$ 1.45	1.56	98.50 $\pm$ 0.90	−1.50	100.40 $\pm$ 1.99	0.40
	HQC	100.21 $\pm$ 0.87	0.21	97.20 $\pm$ 0.99	−2.80	99.01 $\pm$ 1.98	−0.99
Long-term 45 days at −70 °C	LQC	103.44 $\pm$ 1.12	3.44	100.60 $\pm$ 0.92	0.60	97.54 $\pm$ 0.98	−2.46
	HQC	100.46 $\pm$ 1.00	0.46	101.16 $\pm$ 1.66	1.16	100.09 $\pm$ 0.43	0.09

collected in Table 8. At the day of analysis, calibration standards and QC samples were analyzed in parallel with the real plasma samples. Figure 7 shows representative chromatogram of real rat plasma samples with no interference from any metabolites that may have not appeared in spiked plasma samples. The peak shape and chromatographic resolution of the analytes were identical to those obtained from spiked plasma samples. The obtained results in Table 8 and Fig. 7 ensures the ability of the method to assay the drugs after their administration with no interference and with the required selectivity and accuracy. All determined concentrations were within the proposed calibration range. In addition the $C_{\max}$ of all drugs obtained either from group

one (treated with TM/IB) or group two (treated with TM/CL) were in compliance with the previously reported pharmacokinetic studies for each single drug. [12–14, 21, 22].

3.8. Assessment of greenness of the method

Green chemistry gained huge concern recently as it can be perceived as a contribution of analysts to the sustainable development trend. The analytical methods' green evaluation can be challenging due to the many factors to be considered in method development as eliminating or reducing the use of reagents and decreasing energy

Fig. 6. HPLC with fluorescence detection chromatograms of (a) Ibudol[®] tablet sample solution containing 1 µg/mL of TM and 8 µg/mL of IB (mixture 1) and (b) MUZOX[®] tablet sample solution containing 1 µg/mL of TM and 5 µg/mL CL (mixture 2)

Table 8. Data regarding drug posology and the corresponding plasma concentration of TM, IB & CL collected from male Wistar rats after applying the developed method

Mixture	Monitored Drug	Dose (mg/Kg) (single oral dose)	Time sample collection (min)	Mean plasma concentration ± SD (µg/mL)
Mixture 1 (TM, IB) (n = 6)	TM	20	T _{max} (60)	1.15 ± 2.09
			T _{average} (180)	0.91 ± 1.98
			t _{1/2} (300)	0.50 ± 2.50
	IB	17.8	T _{max} (32)	35.0 ± 1.50
			T _{average} (70)	25.56 ± 0.99
			t _{1/2} (108)	11.67 ± 1.50
Mixture 2 (TM, CL) (n = 6)	TM	20	T _{max} (60)	1.18 ± 1.22
			T _{average} (180)	1.03 ± 2.00
			t _{1/2} (300)	0.55 ± 2.66
	CL	15	T _{max} (20)	1.60 ± 0.87
			T _{average} (60)	1.09 ± 0.99
			t _{1/2} (99)	0.77 ± 1.09

consumption and wastes. Some measures may be adopted to make greener approaches such as using safer and more degradable solvents and reducing hazardous wastes and number of steps in analytical procedures. However, in

some cases, it is very difficult to reach full green chemistry in certain aspects. For example, one of the main challenges is the multi-step nature of most analytical processes. Also, Tobiszewski et al. [23] pointed out that the

Fig. 7. HPLC with fluorescence detection chromatograms of the two binary mixtures in extracted real rat plasma samples at 60 min after oral administration of 20 mg/kg TM and 17.8 mg/Kg IB (a) and 20 mg/kg TM and 15 mg/Kg CL (b), each spiked with its respective IS

Table 9. Points of penalty according to the Eco Scale of the proposed HPLC method

Reagents	Penalty points
Acetonitrile	4
Phosphoric acid	2
Instrumentation	Penalty points
LC	1
Occupational hazard	0
Waste	6
Centrifuge	1
Sonicator	1
Sum of penalty points	15
Final score	85

Fig. 8. Green assessment profile for the proposed HPLC with fluorescence detection method using the GAPI tool

least green step in analytical process is sample preparation step which is sometimes would be difficult to simply or eliminate.

To assess greenness of analytical methods, in a qualitative approach, several Internet databases are useful such as the NEMI “National Environmental Methods Index”, or the Green Chemical Alternatives Wizard developed by Massachusetts Institute of Technology.

Van-Aken et al. [24] developed an interesting semi-quantitative approach to greenness evaluation of organic synthesis, that is called the Eco-Scale, this scales assumes that an “ideal” system, has a score of 100, which is 100% safe for

both the analyst and the environment. Penalty points are used for parameters deviating from the ideal value are which lower the overall score. The higher the score, the greener the procedure is. In 2012, an Eco-Scale for evaluation of green analysis was developed in the same approach.[25] The Eco-Scale result of the proposed HPLC method is 85 showing excellent greenness (Table 9). This high score is due to using only one organic modifier in a very low percentage which is acetonitrile, a low toxicity solvent, instead of the commonly used hazardous combination solvents. In addition, the sample pretreatment was done by simple protein precipitation using also acetonitrile only.

Another new green assessment tool is the Green Analytical Procedure Index “GAPI”. It uses pentagrams to evaluate the environmental impact of each step in the procedure using colors of three: red, yellow and green to represent high, medium and low impacts, respectively. This visual presentation allows researchers to make judgments about conflicting green criteria and compare between different methods to choose the greenest one for a particular purpose.[9] Figure 8 shows the pentagrams of the proposed method having low environmental impact based on the used reagents and instrumentation.

4. CONCLUSION

This work proposes a robust and valid HPLC method with fluorescence detection for simultaneous analysis of TM and IB (mixture 1) and TM and CL (mixture 2) either in their combined tablets or in] plasma samples. The HPLC method proposed showed many advantages, the main of which is the novelty of the method; as the literature lacks the presence of any method for analysis of these two mixtures in plasma matrix and/or using fluorimetric detector. It was proved that the suggested method can be applied for future pharmacokinetics of these drugs due to its low sensitivity which was indicated by lower DL and QL values and in vivo rat application. In addition the proposed method is eco-friendly and green. The greenness of the method has been assessed by two different scales which are the new tool GAPI and the analytical Eco-Scale. These advantages make this method superior for the routine analysis of such combination in commercially available tablets and also plasma samples.

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