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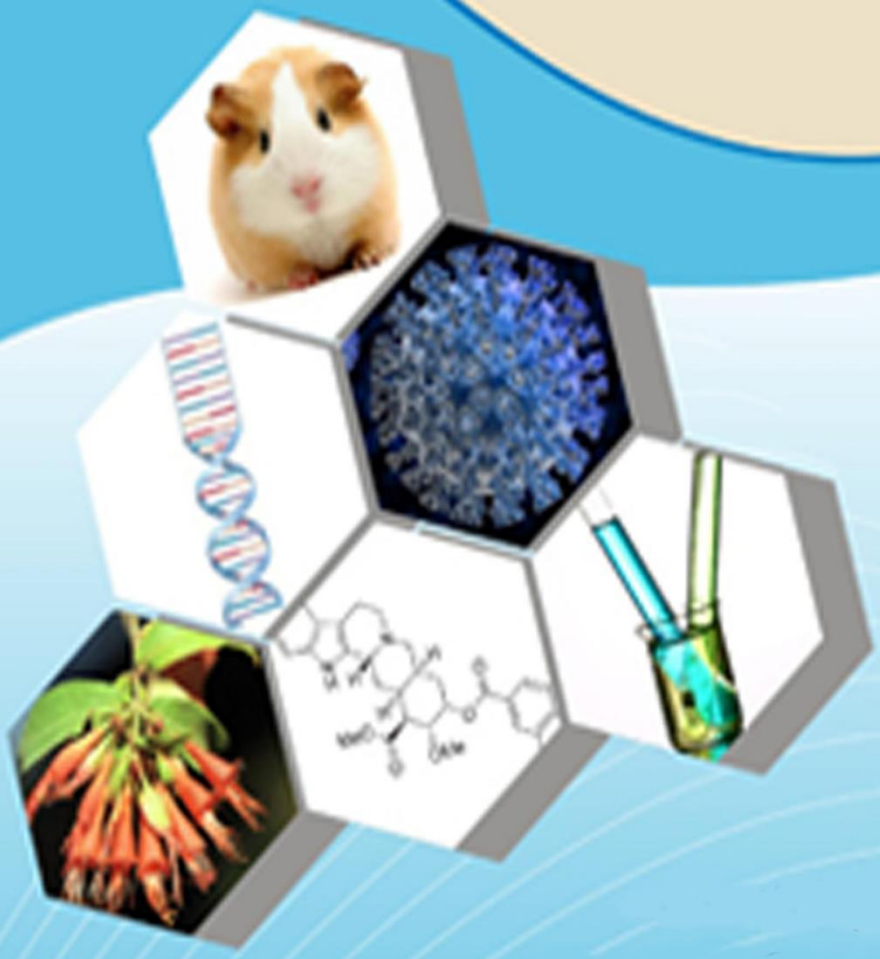

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Creating and testing an RP-HPLC technique to measure curcumin and metronidazole in a mixed dose form at the same time

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ABSTRACT

Purpose and Background: This work set out to create and test a straightforward RP-HPLC technique for the simultaneous measurement of curcumin and metronidazole in bulk, as well as their combination dose form. The compounds in question are natural.

Methods: A model combination system was created using an in situ gel formulation that included metronidazole and curcumin. Using UV-detection at 254 nm, the chromatographic separation was achieved isocratically on an Eclipse XDB-C18 column with dimensions 150 mm x 4.6 mm and a particle size of 5 µm. The mobile phase that was fine-tuned included a 50:50 (v/v) combination of Phosphate Buffer pH4.5 and Acetonitrile. The flow rate was adjusted to 1.0 mL/min, and the injection volume was 10 µL. The approach was utilized for quality control tests of their combination medication product and was verified in conformity with International Council for Harmonization (ICH) standards.

The findings showed that metronidazole had a retention duration of 1.40 minutes and curcumin of 8.60 minutes. The linear responses were seen for curcumin and metronidazole, respectively, throughout the concentration ranges of 3.0-80 and 4.8-128 µg/mL, with limits of detection (LOD) values of 0.62 and 1.03 µg/mL and limits of quantification (LOQ) values of 1.88 and 3.13 εg/mL. No formulation components interfered with the identification of the two active compounds, and the precision findings were within acceptable limits (RSD<2%).

Curcumin and metronidazole in situ gel formulation total contents were determined using the suggested verified RP-HPLC technique. Routine quality control for their combined pharmaceutical use was shown to be possible by the validation findings, which also demonstrated that the suggested technique was simple, specific, and exact.

Developing an HPLC approach for the simultaneous measurement of curcumin and metronidazole

INTRODUCTION

Curcumin is an acronym for "curcuminoids," which are a class of compounds include 1,7-bis(4-hydroxy-3-METhoxy-phenyl)-1,6-heptadiene-3,5-ione. The primary component of Curcuma longa, often known as turmeric rhizome, is shown in Figure 1 (A). There is evidence of this herbal medication's therapeutic usage in Asian traditional medicine dating back more than a thousand years. No toxicities or bad side effects have been linked to CUR, even at extremely high dosages, and this has been confirmed in population-level studies. (Wächter et al., 2014; Berginc, Škalko-Basnet, Basnet, & Kristl, 2012; Basnet & Skalko-Basnet, 2011). Its photosensitivity, quick hydrolysis at alkaline pH, and fast systemic clearance, together with its poor water solubility, have restricted its therapeutic application. It is usual practice to increase CUR's effectiveness by using innovative drug delivery systems or by combining other medications (Yuan et al. 2012).

According to Chanda & Rakholiya (2011), Ejim et al. (2011), Lakshmi et al. (2016), and Sasidharan et al. (2014), CUR has the ability to improve the clinical results of several antibiotics and is therefore an attractive natural component in combination treatment. To minimize the risk of dosage-related side effects and defensive mechanisms,

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combination therapy is often the best option. According to Jain et al. (2016), a multi-target treatment approach may be made more effective by combining two or more therapeutically relevant drugs that work via different routes. Recent extensive study on CUR's anti-parasitic effects has shown that it might be an effective medicine against various parasites, either on its own or in conjunction with other drugs (Cheraghipour et al. 2018; Rangel-Castañeda et al. 2018).

Highly efficient in treating gastrointestinal infections and sexually transmitted diseases (STDs) including trichomoniasis, giardiasis, parasitic infections, and bacterial vaginosis, metronidazole (MET; Figure 1-B) is a broad-spectrum antibiotic.

In order to avoid the many side effects that may occur from systemic medication administration, topical distribution of MET has gained appeal (Held, 1987; Topal et al., 2015). Consequently, it is crucial to discover new therapies that are both more effective and less harmful. Combination therapy, which makes use of inhibitors of antibiotic resistance, is one example of such novel treatments. Recent research has shown that some naturally occurring and dietary substances may enhance

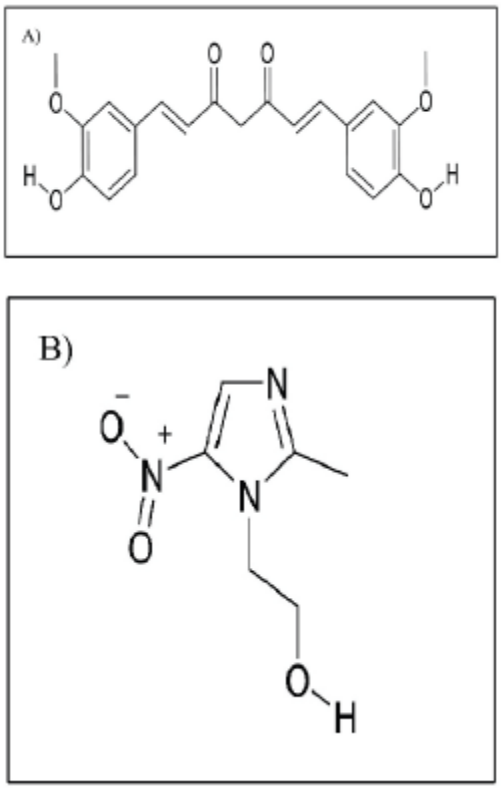

Figure 1. Chemical structures of CUR (A) and MET (B).

isacchi et al. (2012) and rangel-Castañeda et al. (2018) found that some medications, such metronidazole and artemisinin, have an antiparasitic effect. Furthermore, CUR may shield DNA from oxidative stress and damage caused by MET and other environmental and pharmaceutical pollutants (Singh & Giri 2013). While there have been research on CUR's biological activity, no one has looked at how this natural chemical interacts with different antibiotics (Teow & Ali 2015; Mun et al. 2013; Sasidharan et al. 2014).

In the literature, many distinct HPLC detection techniques for CUR and MET have been suggested (Chaudhary et al. 2012; Ji et al. 2009; USP 2015; Venkateshwaran and Stewart 1995). There is currently no commercially available product that combines these medications, hence there is also no validated analytical technique for evaluating them all at once. The present investigation set out to create and verify an easy-to-use RP-HPLC technique for the determination of active compounds in both bulk and dose form that is sensitive, accurate, and repeatable. The current investigation made use of a single in situ gel system that was reflective of the aforementioned integrated model system by including CUR and MET.

MATERIAL AND METHODS

The company Merck in Darmstadt, Germany, was bought CUR. Ibrahim Etem Ulagay Menarini of Istanbul, Turkey, provided the MET. The following materials were supplied by Sigma-Aldrich: potassium dihydrogen phosphate, poloxamer 407 (PLX407), and poloxamer 188 (PLX 188). Ashland, located in Oregon, USA, supplied the Pharmasolve® (PHR). We bought HPLC grade phosphate buffer (PBS pH 4.5) and acetonitrile (ACN) from Merck in Darmstadt, Germany. We got our cellulose sterile acetate syringe filters from ISOLAB in Eshau, Germany. Their pores were 0.45 µm. Every experiment made use of double-distilled water.

Chromatographic setup and instrumentation

A diode array detector, auto sampler, solvent degasser, quaternary pump, and Agilent 1260 Infinity HPLC system (Wilmington, DE, USA) were all used to carry out the investigation. The instrument operations were managed and data processed using the Agilent ChemStation software. For the purpose of separation, the Eclipse XDB-C18 column (5 µm, 150 mm x 4.6 mm) was engaged. A 50:50 v/v combination of PBS pH 4.5 and ACN made up the optimum mobile phase.

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The injection volume was 10 µL, and the flow rate was set at 1.0 mL/min.

The analysis ran for 15 minutes in a column oven preheated to 37 °C. By using a DAD detector between 200 and 400 nm, the maximum wavelength (λ max) of 254 nm was found using conventional scanning for CUR and MET. With the mobile phase flowing through the system, the UV signal and back pressure were stabilized before each injection by equilibrating the column.

The samples were prepared and standard solutions were used.

For the preparation of the standard stock solution, 0.2 mg.mL-1 CUR and 0.32 mg.mL-1 MET were dissolved in ACN. An ultrasonic bath (Selecta Ultrason HD, Spain) was used to put the solution for 30 minutes min, aiming complete dissolution of the combination.

In situ gel preparation and analysis using CUR and MET

The in situ gel system was created using a cold method methodology, as described in previous studies (Baloglu et al., 2011b; Garala et al., 2013). The following ingredients were added to cooled and distilled water: PLX 407 (20%), PLX 188 (5%), PHR (15%), and a combination of 0.7% CUR and 0.7% MET (w/w). The mixture was stirred constantly while put in an ice bath (4 °C). To make sure the finished gel system (CUR-MET-Gel) was completely moist and free of air bubbles, it was kept at 4 °C for 24 hours. A drug-free in-situ gel sample was also prepared using the same procedure.

After the CURMET-Gel was physically and organoleptically examined, the HR-1 Discovery Hybrid Rheometer (TA Instruments, England) was used to investigate crucial parameters such as the gelation time, viscosity, and sol-gel transition temperature that had been measured using in situ gel systems. A steel probe measuring 40 mm in diameter, with a predetermined spacing of 500 µm and a constant frequency of 0.01 Hz, was used to carry out this examination. The samples were heated at a rate of 2 °C/minute between 15 and 50 °C in order to track the change in viscosity (Pa.s). According to Baloglu, Karavana, Senyigit, & Guneri (2011a) and Edsman, Carlfors, & Petersson (1998), the sol-gel transition temperature and gelation time were determined using the area where viscosity changes considerably. A pH-meter (Ohaus Starter 3100, USA) was used to measure the CUR-MET-Gel's pH.

Validation of analytical methods

Precision, accuracy, specificity, selectivity, linearity, limits of detection (LOD) and quantitation (LOQ), system appropriateness, and stability were all tested to ensure the technique fulfilled ICH standards (ICH 2005).

A linear model

For both medicines, linear calibration curves were created by diluting stock solutions using a mobile phase mixture of PBS pH 4.5 and ACN (50:50) (v/v). The concentrations for CUR were 3, 5, 10, 20, 40, 60, and 80 µg/mL, while for MET, the concentrations were 4.8, 8, 16, 32, 64, 96, and 128 µg/mL. The least-squares regression analysis was used to test for linearity.

Specificity

By analyzing chromatograms of the excipient(s) interference with CUR and MET determination, the specificity was established. Chromatograms of drug-free in-situ gel solution, bulk solution containing 10 µg/mL of CUR and 20 µg/mL of MET, as well as mobile phase, were introduced into the chromatographic apparatus in order to achieve this.

Accuracy

The comparison between the actual and theoretical results validated the analytical method's correctness. We introduced three sets of CUR and MET doses of 4 µg/mL, 12 µg/mL, and 30 µg/mL, 19.2 µg/mL, and 48 µg/mL, respectively, to the matrix samples of pH 4.5 phosphate buffer and acetonitrile (50:50) medium in order to achieve this purpose. Percent recovery was used to report the findings.

Precision

System accuracy was confirmed by measuring its repeatability, intermediate accuracy, and reproducibility. In order to ensure consistency, six separate samples were produced and introduced into the HPLC system at a concentration of 30 µg/mL for CUR and 48 µg/mL for MET.

In addition, two separate analyzers checked six solutions with the same concentration (CUR: 30 µg/mL and MET: 48 µg/mL) on two separate days to validate the intermediate precision. We used SD and RSD, or relative standard deviation, to assess all of the outcomes.

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The LOD and the LOQ are the limits of detection and quantification, respectively.

The quantification value and limits of detection were established using the slope (S) and standard deviation (SD) of the responses. Data for LOD and LOQ were computed using the following equations:

$$LOD = 3.3\ SD/S \quad (Eq.1)$$

$$LOQ = 10\ SD/S \quad (Eq.2)$$

How Curcumin and Metronidazole Solvent Holds Up in the Near Future

We developed and evaluated a solution for short-term stability that included 70 µg/mL of CUR and MET. We kept the prepared solution at 37 °C for 48 hours. We took three samples at0,24, and 48 hours and studied them.

In situ gel formulation assay protocol

Dissolving a precisely measured amount of gel (about 500 mg) in ACN allowed us to ascertain the drug contents of the CUR and MET in gel formulations. The solution was then moved to a 50 mL volumetric flask and diluted using the mobile phase, which consisted of PBS pH 4.5 and ACN in a 50:50 v/v ratio. To get the active pharmaceutical ingredients (API) completely dissolved, the solution was subjected to ultrasound for 20 minutes. The yield concentrations for CUR and MET were 70 µg/mL. Before going on to HPLC analysis, the solution that was produced was filtered using 0.45 µm syringe membrane filters from ISOLAB in Eshau, Germany.

RESULTS AND DISCUSSION

Investigation of in situ gels using CUR and MET: preparation and analysis

The cold approach proved effective in preparing the in situ gel system. The resulting gel was exquisite in appearance, had a homogenous texture, and without any roughness. Its hue was light orange. Figure 2 shows the results of the sol-gel transition, gelation time, viscosity, and pH. When applied a stimulus, the rheological characteristics of a stimulus-sensitive gel change from Newtonian/elasto-viscous to viscoelastic behavior, a phenomenon known as the sol-gel transition temperature.

Figure 2 (A) shows that at 37 °C, the formulation's viscosity profile dropped as the sliding speed went up. Mucosal compositions are best gelled at temperatures between 30 and 36 degrees Celsius. Gelation temperatures above 37 °C cause gel to remain in a liquid state, leading to rapid elimination after administration (Giuliano et al., 2018), while temperatures below 30 °C facilitate gel formation at room temperature, which creates difficulties in manufacturing, handling, and administration. Achieving optimal clinical effectiveness is influenced by the rheological performance of topical gel compositions, which affects both the ease of administration and re-

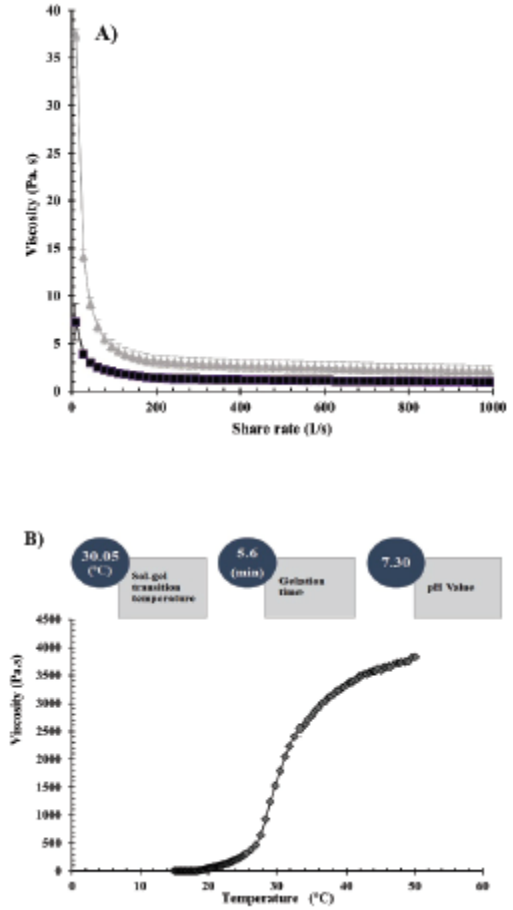

Figure 2. Measurements of flow rheograms at 37 °C (-▲-) and 25 °C (-■-) (A); a graph showing the relationship between viscosity and temperature (B), the sol-gel transition temperature (C), the time it takes for the gel to set, and the pH value.

stress on the surface of the vagina. Within the bounds of physiological limits, the prepared gel sample exhibited appropriate characteristics for mucosal delivery, including optimal sol-gel transition temperature and gelation duration (30.05 °C; 5.6 min)

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and pH (7.30 ± 0.08) (Baloglu, et al., 2011b; Yu et al., 2011).

Developing and optimizing HPLC methods

Several high-performance liquid chromatography (HPLC) techniques have been developed for the individual active components; a great deal of trial and error has gone into developing the best chromatographic approach for the simultaneous measurement of CUR and MET. In order to achieve this goal, the separation conditions were optimized by experimenting with numerous combinations of solvents used as a mobile phase. These included tetrahydrofuran: water, 0.1% ortho phosphoric acid: ACN, and 0.01 M monobasic potassium phosphate buffer pH 4.5: methanol. Isocratic elution is a common method for drug combinations, and one common way is to use a mixture of buffered solution and organic solvents that are miscible with water.

The organic phase ACN was selected over tetrahydrofuran or methanol due to its low viscosity, high eluting power, and extreme selectivity for the curcuminoids (Chaudhary et al., 2012; Galmier et al., 1998; Jangle and Thorat, 2013; Jayaprakasha et al., 2002; Ji et al., 2009; Venkateshwaran & Stewart, 1995). The best separation was seen from all of the tested combinations of PBS pH 4.5 and ACN (90:10, 80:20, 70:30, 60:40, 50:50, 40:60, and 30:70 v/v).

The peaks' shape and symmetry dictated that a 50:50 mixture of PBS pH 4.5 and ACN yielded the optimum results.

In this setup, the peaks for the MET and CUR APIs were precisely characterized, and the elution times were 1.40 and 8.60 minutes, respectively. According to Chaudhary et al. (2012) and Jayaprakasha et al. (2002), the present method offers the benefit of a quick run time, which enables greater output. According to Jangle and Thorat (2013), the column and system were designed to have a longer lifespan by reducing the acid concentration in the mobile phase, using just two solvents, and maintaining a lower flow rate of 1.0 mL/min. Additionally, this study's use of isocratic elution instead of gradient elution was cost-effective, easy to implement, and maintained consistency throughout the testing period. Table 1 displays the HPLC settings, retention time, and symmetry factor summary.

Table 1. Data for optimized HPLC method.

Parameters	
Mobile phase:	Isocratic mixture: Phosphate buffer pH 4.5: acetonitrile (50:50, v/v)
Flow rate:	1.0 mL/s
Injection volume:	10 µL
Wavelengths:	254 nm
Dilution solvent:	Mobile phase
Retention time for MET:	1.40 min
Retention time for CUR:	8.60 min
Symmetry factor for MET:	0.75
Symmetry factor CUR:	0.80

Validation of analytical methods

Following ICH standards (ICH 2005), the method was examined for linearity, system appropriateness, precision, accuracy, specificity, and selectivity, as well as limits of detection (LOD) and quantitation (LOQ). Additionally, short-term solution stability was taken into consideration.

Due to the lack of interference between the chromatograms of the active compounds inside the gel formulation excipients and mobile phase components, the specificity methodology was determined to be accurate (Figure 3).

A linear model

The linear regression equations y=21.946x+0.738 (R^2=0.999) and y=12.975x+9.434 (R^2=0.999) were used to draw the standard lines within the concentration ranges of 3-80 and 4.8-128 µg/mL, respectively.

CUR and MET stand for themselves. Equipment response is directly proportional to drug concentrations in the analysis, as shown by the low standard deviation and near values of the determined coefficients of determination for CUR and MET. As you can see in Figure 4, the calibration analyses are shown.

Precision and restoration

In Table 2, you can see the recovery % findings for CUR and MET. The method's accuracy and precision were shown by the 95% confidence interval and RSD values for both APIs.

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Precision

The fact that the CUR and MET peak regions did not fluctuate by more than 2% indicates that the procedure is quite reproducible.

Over the course of two days, six studies were conducted to assess the intermediate precision.

The precision criterion were met as all tests had RSD readings below 2% (Çelebier et al. 2010; Chaudhary et al. 2012). Key findings are summarized in Tables 3 and 4.

The LOD and the LOQ are the limits of detection and quantification, respectively.

The method's sensitivity was evaluated by determining the LOD and LOQ values using Eq. 1 and 2. Table 5 displays the results, which suggest that the approach is reasonable enough to assess CUR and MET together.

Curcumin and metronidazole solution stability in the short term

Neither the retention duration nor the peak features of the identified HPLC peaks were affected by the short-term stability tests conducted on the CUR and MET solutions. With an RSD of less than 2%, both medications were stable at 37 °C for 48 hours.

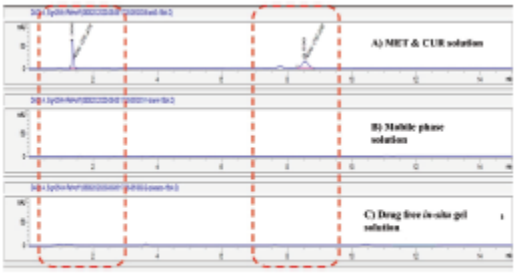

Figure 3. Chromatogram of (A) CUR and MET injection, (B) mobile phase solution-placebo and C) drug free *in situ* gel solution.

Implementation of the API Assay Procedure Intended for use in in-situ gel formulation

The total drug content of CUR and MET for the developed in situ gel formulation (CUR-MET-Gel) was effectively determined using the suggested validated technique. Table 6 shows that when the indicated quantities were same, the outcomes were also identical.

CONCLUSION

The importance of the validation procedure in developing the analytical technique is well acknowledged. The created

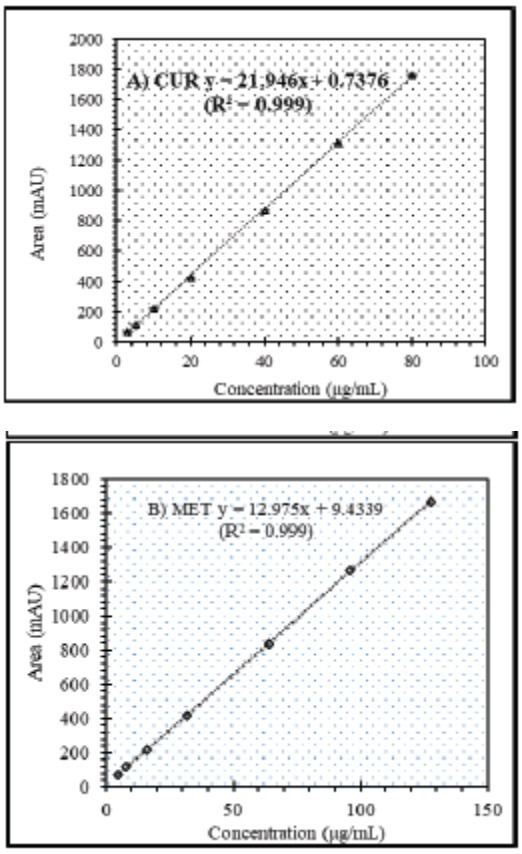

Figure 4. Calibration curves of CUR (A) and MET (B).

Table 2. Recovery results for CUR and MET.

API	n	Theoretical Concentration (µg/mL)	Practical Concentration (µg/mL)	Recovery (%)	SD	RSD (%)	CI (95%)
CUR	6	4.00	3.95	98.70	0.42	0.17	98.22-99.18
	6	12.00	12.09	100.78	2.58	1.05	98.37-104.29
	6	30.00	29.71	99.04	0.95	0.39	98.27-100.27
MET	6	6.40	6.81	106.42	1.10	0.45	105.15-107.68
	6	19.20	19.28	100.46	1.68	0.68	98.53-102.39
	6	48.00	49.21	102.52	1.07	0.44	101.29-103.75

SD: Standard Deviation; RSD: Relative standard deviation; CI: Confidence Interval (95%, Lower and Upper Limit)

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Table 3. Precision test results of CUR and MET in pH 4.5 phosphate buffer and acetonitrile (50:50) medium.

Sample number	AUC for CUR (30 µg/mL)	AUC for MET (48 µg/mL)
1	649.00	654.20
2	650.40	650.00
3	648.90	642.20
4	652.80	640.30
5	645.90	646.40
6	660.70	650.00
AVR	651.28	647.35
SD	4.68	5.05
RSD (%)	1.91	1.96
CI (95%)	645.89-656.67	641.54-653.15

SD: Standard Deviation; RSD: Relative standard deviation; CI: Confidence Interval (95%, Lower and Upper Limit)

Table 4. Intermediate precision checked by two analysts and on two different days.

API		1. Analyst	2. Analyst	1. Day	2. Day
CUR (%)	Concentration: 30 µg/mL (n=6)	99.31	98.90	99.86	100.01
	SD	0.62	0.77	0.59	0.45
	RSD (%)	0.25	0.31	0.24	0.18
	CI (95%)	98.65-99.96	98.09-99.70	99.24-100.48	99.55-100.48
MET (%)	Concentration: 48 µg/mL (n=6)	104.74	103.27	102.84	101.88
	SD	1.98	0.51	0.51	1.34
	RSD (%)	0.81	0.21	0.21	0.55
	CI (95%)	103.48-106.57	102.79-103.75	102.25-103.44	100.64-103.12

SD: Standard Deviation; RSD: Relative standard deviation; CI: Confidence Interval (95%, Lower and Upper Limit)

Table 5. Limits of detection (LOD) and quantitation (LOQ) for CUR and MET.

	CUR (µg/mL)	MET (µg/mL)
Limits of detection - LOD	0.62	1.03
Limits of quantitation - LOQ	1.88	3.13

Table 6. *CUR and MET* assay for prepared *in situ* gel dosage form.

Prepared *in-situ* gel formulation	n	Recovery for CUR (%) ± RSD (%)	Recovery for MET (%) ± RSD (%)
CUR-MET-GEL	6	94.00±0.16	98.00±1.25

RSD: Relative standard deviation

The approach was evaluated using the protocols established by the ICH.

The suggested approach was determined to be easy to use, specific, accurate, and exact based on the validation findings. It was also able to quantify CUR and MET in bulk solutions and in situ gel formulations. In addition, the approach works well for quantitative testing and regular examination of pharmacological dosage forms including mixtures of CUR and MET.

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