طريقة جديدة باستخدام كروماتوغرافيا الطبقات الرقيقة TLC مع مقياس الكثافة الضوئية للتحديد الكمي المتزامن للمركبات المضادة للميكروبات المُصاغة حديثًا المكونة من مينوسيكلين وسيبروفلوكساسين وميترونيدازول

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□ ملخّص □

تم استخدام مزيج من المضادات الحيوية المعتمدة حديثًا (مشاركة دوائية) من مينوسكلين (MIN) وسيبروفلوكساسين هيدروكلورايد (CIP) وميترونيدازول (MET)، المستخدمة في علاج التهاب الأسنان العميق وتجديد القناة اللبية في جذر الأسنان. فمن الضروري تطوير طرائق تحليلية لتركيبة الأدوية الجديدة. في العمل الحالي؛ تم تطوير طريقة كروماتوغرافيا الطبقات الرقيقة باستخدام مقياس الكثافة الضوئية من أجل التحديد المتزامن لـ MIN وCIP وMET للمزائج الدوائية المحضرة في المختبر. تعتمد الطريقة المقترحة على تحديد النطاقات المرئية للأشعة فوق البنفسجية بعد إجراء فصل لـ MIN وCIP وMET على طبقات TLC. تم فصل الأدوية المدروسة كمياً على طبقات TLC الجاهزة من نوع Merck مغطاة بطبقة من السيليكاجل 60 F_{254} قياس (20 × 20) سم كطور ساكن، وباستخدام طور متحرك مكون من مزيج (أسيتونيتريل: ميثانول: ماء: أمونيا: ثنائي كلورو ميثان) بنسب (17:12:4:42:25) حجم / حجم. تم إجراء مسح ضوئي للبقع المفصولة باستخدام جهاز ماسح الكثافة الضوئية في مجال الأشعة فوق البنفسجية عند طول موجة 220 نانومتر. حيث نتج عن هذا النظام الكروماتوغرافي قمم متناظرة ومضغوطة عند قيم R_f تبلغ 0.208 ± 0.003 و0.454 ± 0.004 و0.841 ± 0.004 لمينوسيكلين وسيبروفلوكساسين وميترونيدازول، على التوالي. تم التحقق من صحة الطريقة المقترحة وفقًا لإرشادات مؤتمر التنسيق الدولي لمواءمة المتطلبات الفنية الخاصة بالمستحضرات الصيدلانية المعدة للاستخدام البشري (ICH). يمكن للطريقة المقترحة أن تحلل كميًا MIN وCIP وMET في المزائج المحضرة في المختبر دون تدخل من السواغات. الطريقة المقترحة جديدة وسريعة ودقيقة وقابلة للتكرار ومنخفضة التكلفة، وبالتالي، يمكن استخدامها في الاختبارات والتحاليل الروتينية لمراقبة الجودة لهذه التركيبات الصيدلانية.

الكلمات المفتاحية: مينوسكلين هيدروكلوريد، سيبروفلوكساسين هيدروكلوريد، ميترونيدازول، كروماتوغرافيا الطبقة الرقيقة TLC، جهاز ماسح الكثافة الضوئية، علاج التهاب الأسنان العميق.

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A Novel TLC Densitometry Method for Simultaneous Quantification of the Newly Co-formulated Antimicrobial Agents Minocycline, Ciprofloxacin and Metronidazol

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□ ABSTRACT □

A newly approved Antibiotics mixture (combination) of minocycline (MIN), ciprofloxacin HCl (CIP) and metronidazole (MET), has been used for dental root canal medicaments in pulp regeneration therapy. It is necessary to developing analytical method for the new drug combination.

In the present work; an accurate, precise and simple thin-layer chromatography densitometry method (TLC – densitometry) has been developed for simultaneous determination of MET, CIP and MIN in their laboratory made dosage form. The proposed method based on determination of the UV-visualized bands after TLC separation of MET, CIP and MIN. The studied drugs were quantitatively separated on Merck aluminum TLC plates covered with silica gel 60 F254 plates (20 × 20 cm) as stationary Phase, using mobile phase consists of (acetonitrile:methanol:H_2O:ammonia:dichloromethane) (25:42:4:12:17)v/v. Densitometric scanning of the separated spots was done at 220 nm. with UV detection. This chromatographic system results in symmetric, compact peaks at R_f values of 0.208 ±0.003, 0.454 ±0.004 and 0.841 ±0.004 for MIN, CIP and MET, respectively. The proposed method has been validated according to the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines.

The proposed method can quantitatively analyze MIN, CIP and MET in their laboratory prepared combinations without interference from excipients.

The suggested method is novel, rapid, accurate, reproducible and of low cost, so; thus, it can be used for quality control analysis of these formulations.

Keywords: Minocycline HCl, Ciprofloxacin HCl, Metronidazole, Thin-layer chromatography–densitometry, deep tooth inflammation Treatment.

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INTRODUCTION:

Ciprofloxacin Hydrochloride (CIP), is 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid]; Fig(1,a) [1,2]. Ciprofloxacin hydrochloride exerts its bactericidal effect by interfering with the bacterial DNA gyrase, thereby inhibiting the DNA synthesis and preventing bacterial cell growth [3,4]. Ciprofloxacin has been linked to rare but convincing instances of liver injury that can be severe and even fatal.

Metronidazole (MET), is 2-(2-methyl-5-nitro-1H-imidazol-1-yl) ethanol Fig (1,b). It is used as antibacterial and antiamaebiasis [1,2]. It is a commonly used antibiotic, belonging to the nitroimidazole class of antibiotics. It is frequently used to treat gastrointestinal infections as well as trichomoniasis and giardiasis, and amebiasis which are parasitic infections [5]. Metronidazole has been used as an antibiotic for several decades, with added antiparasitic properties that set it apart from many other antibacterial drugs, allowing it to treat a wide variety of infections. It is available in capsule form, tablet form, and topical form, and suppository preparations for the treatment of various infections [6].

Minocycline Hydrochloride (MIN) is the (4*S*,4*aS*,5*aR*,12*aR*)-4,7-bis(dimethylamino)-1,10,11,12*a*-tetrahydroxy-3,12-dioxo-4*a*,5,5*a*,6-tetrahydro-4*H*-tetracene-2carboxamide; hydrochloride Fig (1,c) [1,2]. It is a minocycline hydrochloride (Min) is recommended to treat various bacterial infections, such as urinary, respiratory, and skin infections. It may also be used with other medications to treat severe acne.

This medication belongs to a class of drugs known as tetracycline antibiotics. It works by stopping the growth of bacteria [7].

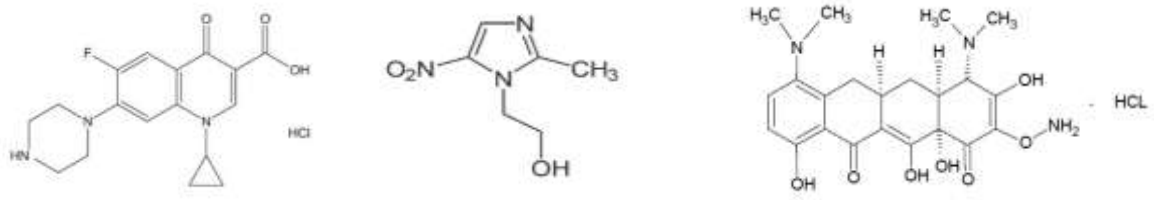
The novelty of this article, is a new TLC – densitometry method has been developed for simultaneous quantitation of a triple antibiotic mixture of minocycline. HCl, ciprofloxacin. HCl, and metronidazole. which was used as an intracranial (intracanal) medicament in an attempt to disinfect the root canal system for revascularization of a tooth with a necrotic pulp. However, discoloration developed after applying the triple antibiotic mixture [8-12].

The literature survey reveals that many methods concerning the determination of separate formulations containing either MIN, CIP and MET. Various analytical methods have been reported for the estimation of MIN, CIP and MET as alone as well as in combination with other drugs. The three drugs were determined separately using various techniques and in combination with other drugs., no method has been reported to date for the simultaneous estimation of MIN, CIP and MET in pharmaceutical mixture.

Several methods were reported for the determination of each component of this formulation. for CIP there are spectrophotometry [13–21], TLC [22-24], HPTLC [25,26], HPLC [27,28], CE [29], electrochemistry [30-32]. the methods for MET determination include ion selective electrodes [33,34], HPLC [35-38], HPTLC [39,40], spectrophotometric methods [41-43], fluorescence [44], photo-fenton oxidation technology [45], glassy carbon electrode modified with gold-copper nanoparticles as novel electrochemical sensor for determination of metronidazole [46], and cerium doped magnetite nanoparticles for highly sensitive detection of metronidazole via chemiluminescence [47]. various methods for MIN determination have been reported in the literature, such as several analytical techniques were previously reported for the determination of MIN including spectroscopic methods [48], and chromatographic separation [49–54]. Additionally, a few electrochemical methods were addressed for the determination of Min [55,56], CE [57,58].

Literature review indicated that numbers of analytical methods like UV spectroscopy, HPLC and derivative UV-spectroscopic method are available for estimation of

ciprofloxacin. HCl. minocycline and metronidazole in single dosage form and in combination with other drugs. there is no TLC method have been reported for this combination of drug so it was thought of interest to develop simple, sensitive, specific, accurate and precise TLC method for estimation of minocycline. HCl, ciprofloxacin. HCl and metronidazole in their combined dosage form. the major advantage of TLC is that several samples can be run in chorus using small amount of mobile phase and samples; in contrast to HPLC, thus time saving and cost effective. the aim of this study is to develop and validate of analytical methods as per the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines. [59], for simultaneous estimation of minocycline. HCl, ciprofloxacin. HCl and metronidazole in their combined dosage form.



(a) (b) (c)

Figure 1: The chemical structure of (a) ciprofloxacin hydrochloride, (b) metronidazole, and (c) minocycline hydrochloride

To date, there is no reported thin-layer chromatography TLC – densitometry method for the simultaneous determination mixture of CIP, MIN and MET. The TLC method has the advantages over the HPLC method of being less cost and time-saving and not requiring pH adjustment of the mobile phase or tedious cleanup procedures. Thus, the aim of our present work was to conduct TLC – densitometry for the simultaneous determination of the 3 co-formulated drugs in bulk powder and lab made pharmaceutical preparation.

RESEARCH MATERIAL AND METHODS:

1.Apparatus:

Shimadzu "dual wavelength flying spot scanning" densitometer CS-9301 PC (Tokyo, Japan, 2000) (program version 2.00) was used for TLC plates scanning. UV-254 nm chamber was used for UV experiments. pre-coated TLC plates, silica gel 60 GF254 (20 × 20 cm) (Merck, Germany). 10µL syringe hamilton (switzerland) was used to apply samples on TLC plates. glass TLC developing chamber (20 × 20 × 10 cm).

2. Materials and Reagents:

Pharmaceutical grade ciprofloxacin HCl, metronidazole and minocycline. HCl (99%) (supplied by Lyphar, Shaanxi, China). methanol, isocratic HPLC grade (scharlo S.L., spain). dichloromethane, acetonitrile, amonia (Merck, Germany). all the reagents used were of AR grade.

Procedure:

1. Preparation of Standard Stock Solutions

Stock solutions prepared by dissolving 250 mg of CIP, MET, and Min separately in 25mL volumetric flask, in least amount of methanol and complete to 25 mL, to obtain solutions contain (10 mg/mL) of MIN, CIP and MET.

2. Preparation of Standard Mixture Solutions:

Suitable amounts of the last mentioned standard stock solutions, were taken in series of 10 mL volumetric flasks, made up to the mark with methanol, to prepare a standard

mixture solutions of MIN, CIP and MET in the concentration range (1.5-8.0) mg/mL for each drug.

1. Method Development

1.1. Selection of Wavelength for Mixture

5 µl volume of each standard mixture solutions of MIN, CIP and MET, were spotted in form of spots of width 3 mm using a 10 µl syringe on precoated silica gel aluminum plate 60F254 (20×20 cm) then all plates are scanned in the reflectance-absorption mode using UV-detector in the range of 200 – 400 nm.

1.2. Selection of Mobile Phase

Initially prewashing of TLC plate was done using methanol and activated in hot air oven for 5 min at 60 ^{o}C. 5 µl amount of each standard stock solutions of MIN, CIP and MET, were applied separately on TLC plate by 10 µl syringe and allowed it to dry for about 5 min at room temperature**.** The mobile phase as displayed in Table 1, was taken in CAMAG glass chamber and allowed it to saturate for 20 min. After saturation period the spotted plate was allow to run and develop in saturated mobile phase. afterwards plate was carefully removed from glass chamber and let to dry. spots were observed in CAMAG TLC visualizer for band tailing, band shape, band separation …etc.

various trails were performed by changing the mobile phase composition and their ratio in order to obtain good resolution and good R_f value. The optimized mobile phase was number (7), selected after number of trials using different reagents as mentioned in Table (1).

Table (1): TLC Mobile Phase Optimization

No.	Mobile Phase Ratio (V/V) %
1	isopropanol: NaOH: ammonia: ethyle acetate (20:30:30:20) v/v
2	chloroform: methanol: ammonia (45:45:10) v/v
3	dichloromethane: methanol: H_2O (57:35:8) v/v
4	acetonitrile: methanol: H_2O: isopropanol (40:50:5:5) v/v
5	acetonitrile: methanol: H_2O: ammonia: isopropanol (36:50:5:4: 5) v/v
6	acetonitrile: methanol: H_2O: ammonia: dichloromethane (25:42:4:9:20) v/v
7	acetonitrile: methanol: H_2O: ammonia: dichloromethane (25:42:4:12:17) v/v

1.3. Optimization of Chromatographic Conditions:

Few trials were carried to determine MIN, CIP and MET, in dosage form. The optimum condition of separation was determined.

The pre-coated TLC plates silica gel 60 F254 (20 cm × 20 cm, 250 µm thickness) were used. 5 µL on spot from each standard mixture solutions and sample solution, were applied on TLC plates. The chromatograms were run to the solvent front of 120 mm by ascending development in the chamber previously saturated for 20 min with (acetonitrile: methanol: H_2O: ammonia: dichloromethane) (25:42:4:12:17) v/v. as the mobile phase (run time 30 min). After development, the plates were removed immediately and dried in an oven at 60°C for 1 hr.

Densitometry scanning at λ = 220 nm was performed with a Shimadzu TLC Scanner in the absorbance mode. The silt dimension was kept at 4.0 mm × 0.45 mm and a scanning rate of 20 mm s^{-1} was employed. The chromatograms were integrated using the densitometer

.

RESULTS AND DISCUSSION:

The aim of this work was to develop a TLC–densitometry method for the simultaneous determination of 3 co-formulated drugs, namely, MIN, CIP and MET, in bulk powder and

in lab made mixture. To the best of our knowledge, there was no reported TLC–densitometry method for the simultaneous determination of MIN, CIP and MET. TLC–densitometry has the advantages of being simple, cost-effective (for the instrument and the solvents used), and rapid, when compared to HPLC.

Densitometry scanning at λ = 220 nm was performed for standard mixtures of MIN, CIP and MET (7.5, 10.0, 15.0, 20.0, 30.0, 40.0) µg/spot for each compound fig. (2,3).

System suitability test parameters must be checked to ensure that the system was working correctly during the analysis. Method performance data including retardation factor (R_f), resolution (R_s), Selectivity (α) and Theoretical plates Number (N) are listed in Table (2).

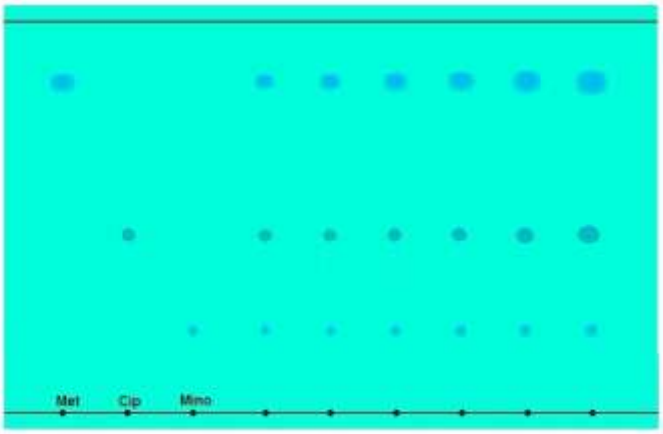


Fig (2). UV Photo of TLC silica gel 60 F_{254} used for separation of a standard mixtures of MIN, CIP, MET (7.5, 10.0, 15.0, 20.0, 30.0, 40.0) µg/spot for each compound.

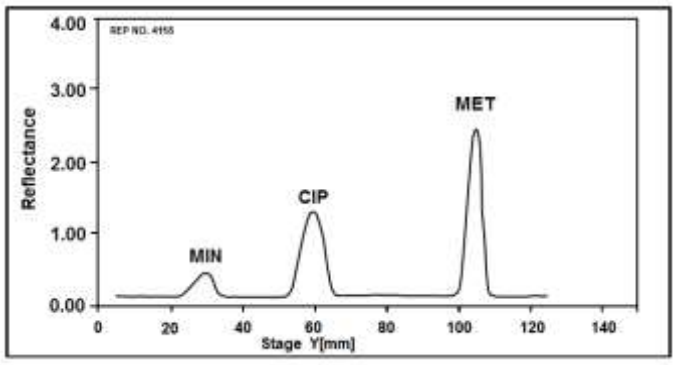


Fig (3). Densitogram of standard mixture of MIN, CIP, MET with concentration (30 µg/spot) for each compound, using Acetonitrile: methanol: H_2O: ammonia: Dichloromethane, (25:42:4:12:17) v/v as the developing system measured at 220 nm.

Table (2). System suitability parameters of the developed TLC–densitometry method.

Parameter	MIN	CIP	MET
Retardation factor, R_f	0.208 ±0.003	0.454 ±0.004	0.841 ±0.004
Resolution (R_S)	-	3.370	5.165
Selectivity (α)	-	3.165	6.363
Theoretical plates Number (N)	178	475	2545

1.2. Method Validation

1.2.1. Linearity and range

Five concentrations were chosen in the ranges of corresponding of to the analytical concentration of Min, CIP and MET. The linearity of peak area responses versus

concentrations was demonstrated by linear least square regression analysis. The linear regression equations were as shown in Table (3).

Table (3): Linear regression data for analysis standard mixtures of Min, CIP and MET by the developed TLC method (n = 3).

Item	MIN	CIP	MET
Linear range, µg/spot	7.5 – 40.0	7.5 – 40.0	7.5 – 40.0
Detection limit , µg/spot	0.60	0.55	0.50
Quantitation limit , µg/spot	1.80	1.65	1.50
Regression Data:			
Slope (**a**)	75.215	243.66	280.92
Intercept (**b**)	22.85	120.4	206.98
Correlation Coefficient **, r^2**	0.9956	0.9957	0.9971

y = a C+b where a is the slope, b is the intercept point, C is the concentration of the compound (µg/spot) and Y is the drug peak area.

1.2.1. Accuracy

Accuracy was assessed using 9 determinations over 3 concentration levels covering the specified range (75,100 and 125%). Accuracy was reported in Table 3 as percent recovery by the assay of known added amount of analyte in the sample, Table (4).

Fixed dose of lab made combination of Min, CIP and MET was prepared. The ratio is maintained at 250, 250, and 250 mg in tablet respectively. The resultant sample solution was used for chromatographic development and scanning followed by analysis. The analysis was repeated in triplicate, Table (5).

Table (4). Accuracy (Recovery%) of drugs in sample.

Concentration	(AV ± SD)%		
	MIN	CIP	MET
75%	101.14±0.19	99.56±0.22	98.76±0.15
100%	99.58±0.27	99.83±0.26	99.58±0.23
125%	100.42±0.28	100.33±0.41	100.02±0.40

Table (5). Assay of MIN, CIP and MET in Lab made combination (n=3).

Drug	Label claim (mg/tab)	Amount found (mg/tab)	Recovery%	RSD%
MIN	250	253.16	101.26	1.47
CIP	250	250.30	100.12	1.32
MET	250	252.48	100.99	1.41

CONCLUSIONS AND RECOMMENDATIONS:

The present work described the successful simultaneous quantitative analysis of MIN, CIP and MET in their laboratory-prepared mixture used for dental treatment. The studied drugs were quantitatively separated on Merck aluminum TLC plates covered with silica gel 60 F254 plates (20 × 20 cm) as stationary Phase, using mobile phase consists of (acetonitrile:methanol:H_2O:ammonia:dichloromethane) (25:42:4:12:17) v/v. Densitometric scanning of the separated spots was done at 220 nm. This chromatographic system results in symmetric, compact peaks at R_f values of 0.208 ±0.003, 0.454 ±0.004 and 0.841 ±0.004 for MIN, CIP and MET, respectively. using TLC–densitometry as per ICH guidelines for the simultaneous estimation of MIN, CIP and MET. The results showed that the developed TLC–densitometry had the advantages of being simpler than the HPLC, as it used simple mobile phase with no pH adjustment, sensitive, and economic, as it saves cost (inexpensive

apparatus and solvents) and time. The developed method can be successfully used in routine quality control testing, allowing qualitative and quantitative determination with high accuracy and precision.

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