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Sensitive and Cost-Effective TLC-Densitometric Method for Determination of Metronidazole and Tinidazole in Tablets

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Abstract: A sensitive, easy-to-use, fast, and cost-effective TLC-densitometric method was developed for the separation of metronidazole, secnidazole, ornidazole, tinidazole, and 2-methyl-5-nitroimidazole and for the determination of metronidazole and tinidazole in *Metronidazole Polpharma* and *Tinidazolum Polpharma* tablets. Analyses were performed on chromatographic plates precoated with silica gel $60F_{254}$ using chloroform + methanol + diethylamine in a volume ratio of 9:1:1 as the optimal mobile phase. The method has been validated. The intraday and interday precision values for the three different concentrations ranged from 0.99% to 1.48% and 0.89% to 1.76%, and the precision values ranged from 1.13% to 2.48% and 0.95% to 2.49% for metronidazole and tinidazole, respectively. The limit of quantification (LOQ) was 0.036 and 0.066 µg/spot for metronidazole and tinidazole, respectively. The mean recovery was 103.1% and 100.6% for metronidazole and tinidazole, respectively. The content of metronidazole and tinidazole in tablets in relation to the content declared by the manufacturer was 101.3% and 99.8%, respectively. The obtained results were verified using the pharmacopeial method. The presented method is fast, sensitive, precise, selective, accurate, and robust. It allows for the analysis of several samples on one chromatography plate at the same time.

Keywords: metronidazole; tinidazole; pharmaceutical preparation; TLC; densitometry

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1. Introduction

Medicinal products on the market must be of appropriate quality to guarantee their safe application and effectiveness. Identity, purity, active substance content, and suitability are quality criteria describing a medicinal substance or product. The most commonly used methods for examining the identity of drugs are infrared absorption spectrophotometry, high-performance liquid chromatography (HPLC), thin-layer chromatography (TLC), and gas chromatography (GC) [1,2]. TLC combined with densitometry is also a great tool for investigating the content of biologically active substances in a drug and any impurities present in the drug, as well as for investigating many physicochemical properties, including the lipophilicity of biologically active substances [3–13].

The counterfeiting and illegal trade of medicine is a global problem. The scale of this phenomenon is becoming more and more common. This poses a threat to the safety and lives of patients. Falsified pharmaceuticals do not meet the quality requirements established for given medicinal products. They usually contain ingredients of lower quality, inappropriate proportions, impurities, or other unapproved active substances with unknown safety of use—these substances are dangerous to health and life. Quite often, their composition is completely different from what is declared on the packaging [14,15]. Hence, there is a need to constantly establish new analytical methods for the qualitative and quantitative testing of drugs. One such method could be TLC coupled with densitometry. Metronidazole (M), secnidazole (S), ornidazole (O), and tinidazole (T) are drugs commonly used to treat protozoan infestations and infections caused by anaerobic bacteria. The scientific literature describes using thin-layer chromatography methods for the determination of selected 5-nitoimidazole in the presence of another drug, e.g., metronidazole and di-iodohydroxychinoline [16], di-iodohydroxyquine [17], spiramycin [18], furazolidone [19,20], loperamide [19], tetracycline

hydrochloride [21], ciprofloxacin [22], diloxamide furoate [23], and clotrimazole [24] as well as tinidazole and clotrimazole [25,26], omeprazole [27], clarithromycin [27], fluconazole [28,29], norfloxacin [30–32], and ciprofloxacin [33]. TLC methods presented in the scientific literature are often not fully validated. Most often, the chromatographic systems used are not optimized, i.e., the separation of the determined 5-nitroimidazole from related substances is not verified. Sometimes, the authors do not determine the LOD and LOQ of the studied 5-ntroimidazoles. Previous publications have not presented details concerning checking the robustness of the used TLC method, nor have M and T been determined simultaneously in previous pharmaceutical preparations.

So far, the scientific literature has not described simultaneous chromatographic separation of M, S, O, T, and 2-methyl-5-nitroimidazole (IMP) using the TLC technique. Therefore, the aim of this work was to develop a fast and cheap TLC method allowing the chromatographic separation of M, S, O, T, and IMP as potential sources of contamination. For this reason, the chromatographic system used in this study is optimal. Therefore, the developed chromatographic conditions can be used in the pharmaceutical industry to determine the presence of M, S, O, and T in simple and combined drugs containing the mentioned 5-nitroimidazoles. It is also possible to check whether the tested pharmaceutical preparation is contaminated with IMP. Only pharmaceutical preparations containing M and T are available on the Polish pharmaceutical market. Therefore, the chromatographic conditions established here were only applied to determine the presence of M and T in tablets. The proposed method has been fully validated. The development of this method was also motivated by the need to obtain a quick and effective method of determination with a low detection limit and low cost. The low cost of the proposed TLC-densitometric method results from the use of TLC plates, which are much cheaper than the HPTLC plates used in numerous publications in which M or T were determined. As it has been shown, the TLC plates used provide similar or better sensitivity than those using HPTLC plates.

2. Materials and Methods

2.1. Chemicals and Reference Standards

Silica gel $60F_{254}$ (E. Merck, #1.05554, and #1.05570) plates were used in the investigation. The following solvents were applied: benzene (Bz), diethyl ether (DEET) methanol (MeOH), toluene (TLN), acetone (Ace), chloroform (TCM), ammonia 25% (AM), n-hexane (Hx), ethyl acetate (EA), ethanol 99.8% (EtOH), isopropanol (IP), acetic acid 80% (AcOH), glacial acetic acid (GAcOH), diethylamine (DEA), triethylamine (TEA), methylene chloride (MCL), acetonitrile (ACN). These solvents were of analytical purity and produced by POCh (Gliwice, Poland), Chempur (Piekary Śląskie, Poland), or Merck (Darmstad, Germany) and used as components of the mobile phases. MeOH was also used to extract the M and T present in the tablets and to prepare the standard solutions. M, S, O, and T as well as IMP were supplied by Sigma-Aldrich (St. Louis, MI, USA). M and T were pharmaceutical primary standards with purities >99% according to the United States Pharmacopeia and European Pharmacopoeia, respectively. IMP was of British Pharmacopoeia (BP) reference standard. S and O were analytical standards with quality levels equal to 100. *Metronidazole* and *Tinidazolum* tablets (Polpharma, Starogard Szczeciński, Poland) contained 500 mg of M and 500 mg of T, respectively. Anhydrous AcOH (analytical purity, Chempur, Poland) and chloric acid (VII) (analytical purity, POCh, Poland) were used to determine M and T using the pharmacopeia method.

2.2. Standard Solutions of Active Pharmaceutical Ingredients (APIs)

Standard solutions of M and T have were made by dissolving their standard substances in MeOH. The following M and T solutions were obtained: 0.60, 0.55, 0.50, 0.45, 0.40, 0.35, 0.30, 0.25, 0.20, 0.15, 0.10, 0.08, 0.06, 0.04, 0.02, 0.01 $mg \cdot mL^{-1}$. MeOH solutions with M, S, O, T, and IMP were made at concentrations of 0.20 $mg \cdot mL^{-1}$.

2.3. Solutions of Metronidazole and Tinidazolum

After weighing, ten tablets of *Metronidazole Polpharma* and *Tinidazolum Polpharma* were crushed for 25 min using a three-ball mill at 6000 rpm. Then, the equivalent of 100 mg of M and 100 mg of T was weighed from the obtained powdered tablet masses. Extraction of M and T from tablet masses was carried out using 15 mL of MeOH using a three-ball mill at 6000 rpm for 20 min. The drug extracts obtained in this way were filtered through paper filters into volumetric flasks and supplemented with MeOH to a volume of 50 mL, obtaining solutions with a concentration of 100 mg/50 mL. In the next step, a series of dilutions were made to obtain solutions with the following concentrations of M and T: 0.3 mg/5 mL, 1.0 mg/5 mL, 1.75 mg/5 mL.

2.4. TLC-Densitometry

The chromatographic plates (#1.05554) were activated at 120 °C for 30 min. All solutions described in Sections 2.2 and 2.3 were placed on chromatographic plates in 5 µL samples. The analyses were performed using TCM + MeOH + DEA as the mobile phase in a volume composition of 9:1:1. This composition was determined experimentally from among the 19 mobile phases analyzed (Table S1). The chromatographic chamber was saturated for 30 min. The development distance was 7.5 cm. Then, the plates were dried under a fume hood for 2 h.

Spectrodensitometric and densitometric analyses were performed using the Camag TLC 3 densitometer with a deuterium lamp. The parameters of the spectrodensitometric analyses were as follows: wavelength, 200 ÷ 400 nm; slit size, 12.00 × 0.40 mm; macro; scanning speed, 20 nm/s; resolution, 1 nm/step. Densitometric scanning parameters were as follows: λ_{max} = 313 nm; slit size, 12.00 × 0.40 mm; macro; resolution, 100 µm/step; and scanning speed, 20 mm/s.

2.5. TLC Method Validation

Range and linearity, precision, accuracy, specificity, robustness, and limits of detection and quantification were determined according to validation guides [34,35], which allowed the validation of the TLC method for use in the determination of M and T. The accuracy of the method was additionally checked by comparison with the pharmacopeial method, already recognized as accurate [1]. Validation details are provided in Tables S2 and S3.

2.6. Quantitative Analysis of Metronidazole and Tinidazole in Tablets and Comparison with Pharmacopeial Method

The proposed TLC-densitometric method (method A) was compared with the pharmacopeia method (method B) to determine M and T in tablets. The comparison was studied by using ten independently repeated different analyses. The samples with concentrations of about 1 mg/mL described in the experimental section were investigated by method A. Method B involves the potentiometric titration of samples [1]. Powdered tablet samples containing 150 mg of M and T, respectively, were dissolved in 50 mL of anhydrous AcOH. The samples were titrated with chloric acid (VII) at a concentration of 0.1 mol/L, and the end point of the titration was determined using combined pH electrodes of type EPS (Elmetron, Zabrze, Poland). The differences between the two analytical methods were evaluated using Student's *t*-test and the F-Snedecor value.

2.7. Statistical Analysis

The Statistica v. 13 PL program (StatSoft, Kraków, Poland) was used for statistical evaluation of the analytical results. Microsoft Office Excel 2016 was used to construct charts.

The arithmetic means, standard deviation (SD), and then a confidence interval with a confidence level equal to 95% were calculated for the series of measurements (n = 10) of the retardation factor (R_F). Arithmetic means were calculated for a series (n = 5) of determined resolution factors R_s.

Intraday and interday precision (n = 3) were assessed by calculating the coefficient of variance (CV, %). The coefficient of variation was determined by calculating the arithmetic mean and standard deviation of the area of chromatographic bands for a given drug concentration.

The arithmetic means of series of measurements (n = 3) of the area of chromatographic bands for each concentration of metronidazole and tinidazole standard solutions were calculated and used to construct calibration curves. The areas of the chromatographic bands for each concentration were also characterized by calculating the standard deviation and the coefficient of variation. The determined calibration curves were characterized by calculating the correlation coefficient (r), the Fischer–Snedecor value (F), the value of statistical significance (p), and the standard error of the estimate (s). The correctness of the determined correlation equations was checked by calculating the differences in the area of the chromatographic bands calculated from the correlation equations and measured values. The standard deviation was also calculated for these differences.

Variance coefficients were used to assess the accuracy of the method. The average contents (previously calculated from calibration curves) of metronidazole and tinidazole in samples in terms of the internal standard, standard deviations, and then coefficients of variance were calculated.

Arithmetic means (n = 3) and standard deviations were also calculated for the determined effects when testing the robustness of the method and for the determined values of the detection limit and quantification limit of metronidazole and tinidazole.

The contents of metronidazole and tinidazole in tablets were calculated as the arithmetic mean of 10 independent measurements. The results of these determinations were characterized by calculating the amount of metronidazole and tinidazole (%) in relation to the label claim, standard deviation (SD), coefficient of variation (CV, %), and the confidence interval of the arithmetic mean with a confidence level equal to 95%. The results of the content of metronidazole and tinidazole in tablets obtained by the proposed TLC-densitometric method and the pharmacopeial method were compared using the Student's t-test and Fischer–Snedecor test with a significance level of $\alpha = 0.05$.

3. Results and Discussion

3.1. Validation

A TLC-densitometric method was developed for the separation of M, S, O, T, and IMP; i.e., the TLC-chromatogram present five chromatographic bands coming from the mentioned 5-nitroimidazoles. The elaborated chromatographic conditions were applied to determine the content of M and T in *Metronidazole Polpharma* and *Tinidazolum Polpharma* tablets. The method has been fully validated (Table 1, Table 2, Table 3, Table 4, Table 5 and Table S4, Figure 1, Figure 2, Figure 3, Figure 4, Figure 5, Figure 6, Figure 7, Figure 8 and Figures S1–S10).

Table 1. R_F and R_s values for the best separations of M, S, O, T, and IMP.

No of Mobile Phase [a]	Average R_F and R_s Values
2	R_F $R_{F(M)} = 0.35 \pm 0.02$, $R_{F(O)} = 0.43 \pm 0.02$, $R_{F(T)} = 0.50 \pm 0.02$, $R_{F(S)} = 0.57 \pm 0.02$, $R_{F(IMP)} = 0.64 \pm 0.03$;
	R_S $R_{S(M/O)} = 0.78$, $R_{S(O/T)} = 0.77$, $R_{S(T/S)} = 0.83$, $R_{S(S/IMP)} = 0.80$
8	R_F $R_{F(IMP)} = 0.36 \pm 0.02$, $R_{F(M)} = 0.41 \pm 0.02$, $R_{F(O)} = 0.46 \pm 0.03$, $R_{F(S)} = 0.51 \pm 0.03$, $R_{F(T)} = 0.59 \pm 0.03$;
	R_S $R_{S(IMP/M)} = 0.75$, $R_{S(M/O)} = 1.07$, $R_{S(O/S)} = 1.05$, $R_{S(S/T)} = 1.29$
9	R_F $R_{F(IMP)} = 0.31 \pm 0.02$, $R_{F(M)} = 0.34 \pm 0.02$, $R_{F(S)} = 0.39 \pm 0.02$, $R_{F(O)} = 0.47 \pm 0.03$, $R_{F(T)} = 0.59 \pm 0.03$;
	R_S $R_{S(IMP/M)} = 0.83$, $R_{S(M/S)} = 1.27$, $R_{S(S/O)} = 1.43$, $R_{S(O/T)} = 1.78$
12	R_F $R_{F(M)} = 0.45 \pm 0.02$, $R_{F(IMP)} = 0.49 \pm 0.02$, $R_{F(O)} = 0.55 \pm 0.03$, $R_{F(S)} = 0.65 \pm 0.02$, $R_{F(T)} = 0.74 \pm 0.03$;
	R_S $R_{S(M/IMP)} = 0.83$, $R_{S(IMP/O)} = 1.33$, $R_{S(O/S)} = 1.33$, $R_{S(S/T)} = 1.33$

Table 1. *Cont.*

No of Mobile Phase [a]	Average R_F and R_s Values
13	R_F $R_{F(M)} = 0.39 \pm 0.02$, $R_{F(IMP)} = 0.42 \pm 0.02$, $R_{F(O)} = 0.50 \pm 0.03$, $R_{F(S)} = 0.61 \pm 0.02$, $R_{F(T)} = 0.67 \pm 0.02$;
	R_S $R_{S(M/IMP)} = 0.53$, $R_{S(IMP/O)} = 1.18$, $R_{S(O/S)} = 1.58$, $R_{S(S/T)} = 1.13$
14	R_F $R_{F(M)} = 0.28 \pm 0.02$, $R_{F(S)} = 0.35 \pm 0.02$, $R_{F(T)} = 0.41 \pm 0.03$, $R_{F(O)} = 0.47 \pm 0.03$, $R_{F(IMP)} = 0.56 \pm 0.03$;
	R_S $R_{S(M/S)} = 0.88$, $R_{S(S/T)} = 1.07$, $R_{S(T/O)} = 1.07$, $R_{S(O/IMP)} = 1.33$
16	R_F $R_{F(M)} = 0.35 \pm 0.02$, $R_{F(S)} = 0.41 \pm 0.02$, $R_{F(T)} = 0.51 \pm 0.02$, $R_{F(O)} = 0.54 \pm 0.02$, $R_{F(IMP)} = 0.63 \pm 0.03$
	R_S $R_{S(M/S)} = 1.05$, $R_{S(S/T)} = 1.44$, $R_{S(T/O)} = 0.30$, $R_{S(O/IMP)} = 1.20$
19	R_F $R_{F(IMP)} = 0.30 \pm 0.02$, $R_{F(M)} = 0.38 \pm 0.03$, $R_{F(S)} = 0.44 \pm 0.03$, $R_{F(O)} = 0.51 \pm 0.03$, $R_{F(T)} = 0.70 \pm 0.04$
	R_S $R_{S(IMP/M)} = 1.33$, $R_{S(M/S)} = 1.22$, $R_{S(S/O)} = 1.29$, $R_{S(O/T)} = 2.10$

[a] 2—Ace + TCM + EA (4:4:1, *v*/*v*); 8—TCM + MeOH + AM (9:1:0.06, *v*/*v*); 9—TCM + MeOH + AM (9:1:0.1, *v*/*v*); 12—TCM + MeOH + GAcOH (9:1:0.1, *v*/*v*); 13—TCM + MeOH + GAcOH (9:1:0.05, *v*/*v*); 14—Ace + TCM + EA + GAcOH (4:4:1:0.05, *v*/*v*); 16—Ace + TCM + EA + ACN (3:4:1:1, *v*/*v*); 19—TCM + MeOH + DEA (9:1:1, *v*/*v*).

Table 2. Method validation data for the quantitative determination of M and T using the TLC-densitometric method.

Method Characteristic		5-Nitroimidazole	
		Metronidazole	**Tinidazole**
Retardation factor (R_f)		0.38 ± 0.03	0.70 ± 0.04
Range ([μg/spot)]		0.2–2.0	0.2–2.0
Linearity ([μg/spot)] A = a·X + b	a	7340.4 (±133.1)	7828.0 (±114.2)
	b	3071.5 (±140.2)	5567.9 (±130.1)
	n	10	10
	r	0.9989	0.9992
	s	235.6	218.6
	F	3557	4701
	p	<0.0001	<0.0001
LOD ([μg/spot)] SD		0.012 0.001	0.022 0.001
LOQ ([μg/spot)] SD		0.036 0.003	0.066 0.003
		For tablets	
		Accuracy (*n* = 6)	
for 50% standard added		R = 103.8%; CV = 1.96%	R = 101.8%; CV = 0.95%
for 100% standard added		R = 104.3%; CV = 1.13%	R = 99.1%; CV = 2.31%
for 150% standard added		R = 101.2%; CV = 2.48%	R = 100.9%; CV = 2.49%
Average recovery		103.1%	100.6%
		Precision (CV, ([%]))	
Intraday (*n* = 3)			
for 1.75 μg/spot		1.08	0.76
for 1.00 μg/spot		1.12	0.89
for 0.30 μg/spot		0.99	1.28
Interday (*n* = 3)			
for 1.75 μg/spot		1.33	0.99
for 1.00 μg/spot		1.39	1.44
for 0.30 μg/spot		1.48	1.76
Robustness (CV, ([%]))		robust	robust

A—area of the chromatographic band (spot) M, T ([AU]); n—number of measurement points; X—micrograms M/spot or T/spot; r—correlation coefficient; SD—standard deviation.

Table 3. Comparison of LOD and LOQ of M and T obtained by other authors.

Method	Mobile Phase	LOD and LOQ ([μg/spot)]	Ref.
	Metronidazole		
HPTLC	MeOH + TCM (9:1, *v*/*v*)	LOD = 0.61 LOQ = 0.95	[18]
HPTLC	TLN + EA + MeOH + AM (3:1.5:0.5:0.1, *v*/*v*)	LOD = 0.046 LOQ = 0.116	[19]
HPTLC	Bz + EA + TLN + MeOH + GAcOH (9.5:2:5:1.5:0.5, *v*/*v*)	LOD = 0.88 LOQ = 1.93	[21]
TLC	ACN + AM + MeOH + MCL+ Hx (1.3:1.1:2:3:1, *v*/*v*)	LOD = 0.32 LOQ = 0.96	[22]
TLC	EA + Ace + Hx + AM (9.5:0.5:0.3:0.3, *v*/*v*)	LOD = 0.13 LOQ = 0.38	[23]
TLC	TCM + Ace + GAcOH (7.5:2.5:0.1, *v*/*v*)	LOD = 0.51 LOQ = 1.55	[16]
TLC	TCM + MeOH (9:1, *v*/*v*)	LOD = 0.052 LOQ = 0.159	[36]
	Tinidazole		
HPTLC	TLN + EA + MeOH + -TEA (5.5:1.0:1.0:0.1, *v*/*v*)	LOD = 0.011 LOQ = 0.037	[25]
HPTLC	Ace + EtOH + 2% watery sodium dodecyl sulfate (WSDS) (3:4:2, *v*/*v*)	LOD = 0.0067 LOQ = 0.0203	[33]
HPTLC	30% Trifluoroacetic acid (TFAA)	LOD [a] = 0.01 LOQ [a] = 0.03 LOD [b] = 0.12 LOQ [b] = 0.36	[32]
TLC	IP + butanol (BT) + AM + water (W) (25:50:5:25, *v*/*v*)	LOD = 0.1 LOQ = 0.3	[30]
TLC	MCL + IP + ACN + AM (11:1.2:5:0.2, *v*/*v*)	LOD = 0.2 LOQ = 0.6	[27]
TLC	TCM + MeOH (9:1, *v*/*v*)	LOD = 0.058 LOQ = 0.174	[36]

[a] low concentration calibration; [b] high concentration calibration.

Table 4. Experimental design matrix (2^3) for robustness test for M and T ingredients in tablets [a].

Experiment No	X_1	X_2	X_3	X_4	X_5	X_6	X_7	Content of Active Pharmaceutical Ingredient (y_i) ([mg·tablet^{-1}])	
								M	T
1	+	+	+	+	+	+	+	493.8	494.2
2	+	+	−	+	−	−	−	489.9	502.5
3	+	−	+	−	−	+	−	496.9	495.2
4	+	−	−	−	+	−	+	500.2	506.1
5	−	+	+	−	+	−	−	502.8	499.5
6	−	+	−	−	−	+	+	493.1	490.2
7	−	−	+	+	−	−	+	493.2	493.5
8	−	−	−	+	+	+	−	513.8	508.2

Table 4. *Cont.*

Experiment	No	X_1	X_2	X_3	X_4	X_5	X_6	X_7	Content of Active Pharmaceutical Ingredient (y_i) ([mg·tablet^{-1}])	
									M	T
Size of effect	M	−5.525	−6.125	−2.575	−0.575	9.375	2.875	−5.775		
	T	1.650	−4.150	−6.150	1.850	6.650	−3.450	−5.350		
The label claim ([mg)]									500	500
Average amount ([mg])									498.0	498.7
Variance									58.4	41.7
Standard deviation (SD)									7.64	6.46
Coefficient of variation (CV, %)									1.5	1.3

[a] M—metronidazole; T—tinidazole; "+"—high level of change in analysis parameter; "−" low level of change in analysis parameter.

Table 5. Comparison of M and T assays ([mg/tablet]) obtained from ten different repeated analyses using the proposed TLC-densitometric (A) and pharmacopeial (B) methods.

	Metronidazole		Tinidazole	
	Method			
	A	B	A	B
Number of analyses	10	10	10	10
1	510.3	528.6	489.9	492.5
2	523.0	506.4	495.2	488.9
3	520.0	521.5	492.3	512.6
4	495.6	516.2	487.6	501.8
5	503.6	508.3	502.6	481.7
6	488.4	498.2	509.9	479.6
7	520.1	501.3	487.1	509.9
8	515.3	496.3	512.3	508.1
9	491.7	517.8	507.8	505.5
10	496.7	497.5	508.8	491.8
Average	506.5	509.2	499.4	497.2
Label claimed	500	500	500	500
Amount of metronidazole and tinidazole (%) in relation to the label claim	101.3	101.8	99.8	99.4
Standard deviation (SD)	12.9	11.3	10.0	11.9
Coefficient of variation (CV, %)	2.55	2.22	2.00	2.39

Table 5. *Cont.*

	Metronidazole		Tinidazole	
	Method			
	A	**B**	**A**	**B**
Confidence interval of arithmetic mean with confidence level equal to 95%	$\mu = 506.5 \pm 9.2$	$\mu = 509.2 \pm 8.1$	$\mu = 499.4 \pm 7.2$	$\mu = 497.2 \pm 8.5$
t calculated	0.497		0.447	
$t_{(95\% \cdot 18)}$ tabulated	2.101		2.101	
F calculated	1.30		1.42	
$F_{(95\% \cdot f1 = f2 = 9)}$ tabulated	3.18		3.18	

Method A—proposed in this work: TLC-densitometric method; Method B—pharmacopeial method.

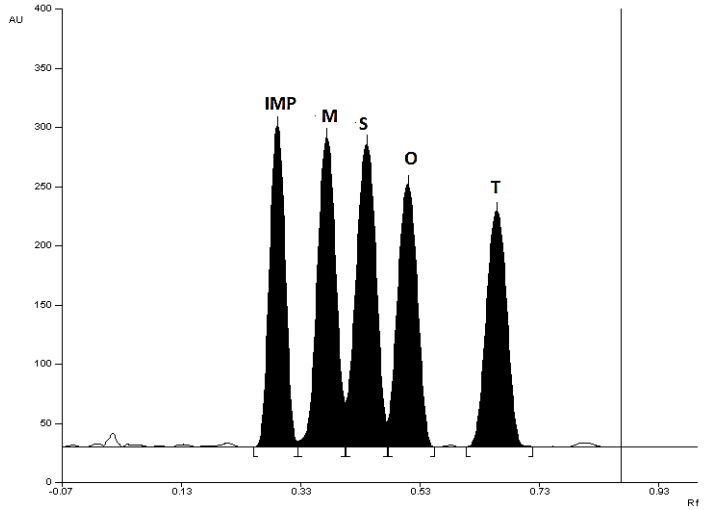

Figure 1. TLC-chromatogram of a mixture of standard substances: M, S, O, T, and IMP at 313 nm, using mobile phase of TCM + MeOH + DEA (9:1:1, *v*/*v*).

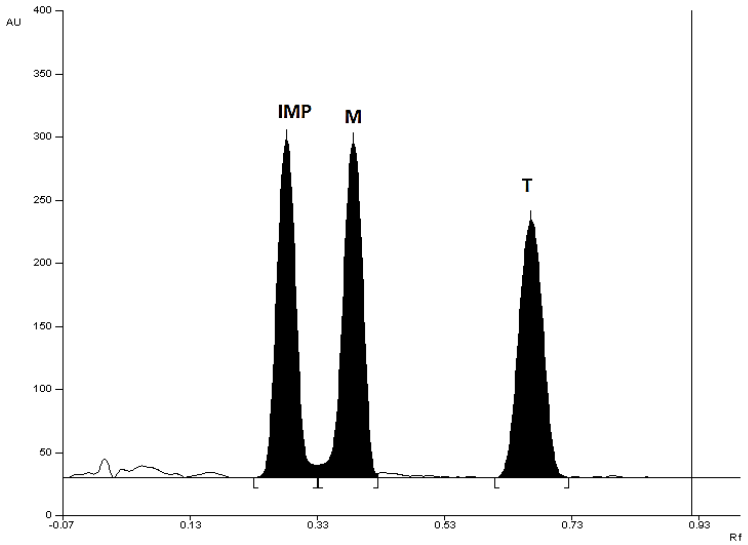

Figure 2. TLC-chromatogram of a mixture of standard substances (M, T, and IMP) captured at 313 nm, using the mobile phase TCM + MeOH + DEA (9:1:1, *v*/*v*).

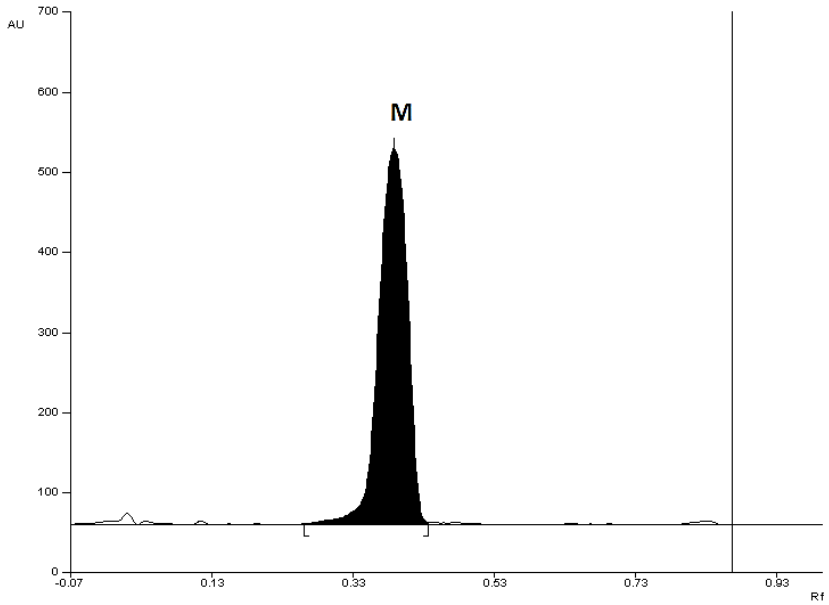

Figure 3. TLC-chromatogram of a *Metronidazole Polpharma* drug sample captured at 313 nm, using the mobile phase TCM + MeOH + DEA (9:1:1, *v*/*v*); M—metronidazole.

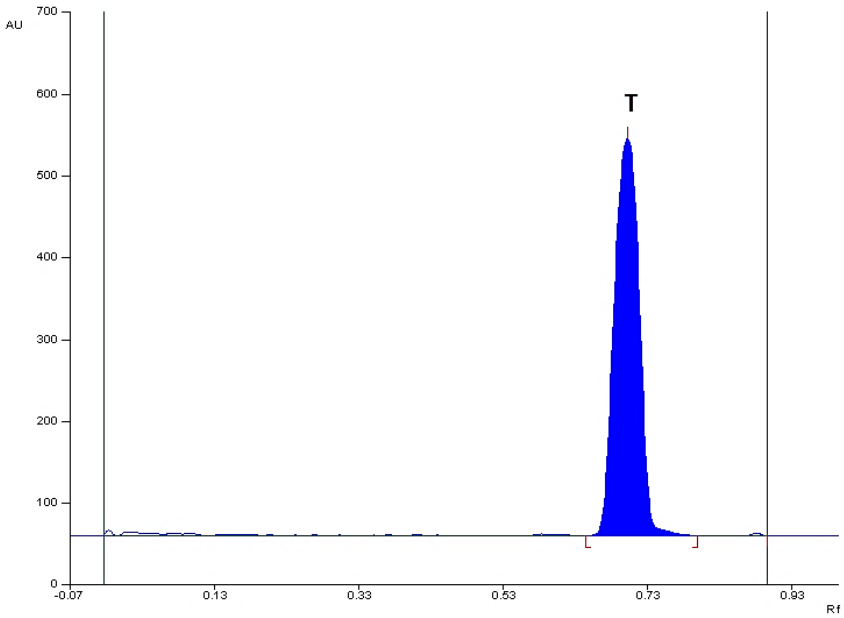

Figure 4. TLC-chromatogram of a *Tinidazolum Polpharma* drug sample captured at 313 nm, using the mobile phase TCM + MeOH + DEA (9:1:1, *v*/*v*); T—tinidazole.

3.1.1. Optimization of Chromatographic Conditions

Chromatographic analyses were performed on plates precoated with silica gel $60F_{254}$. Nineteen mobile phases (Table S1) were tested for their ability to separate five substances, namely M, S, O, T, and the potential contamination of IMP. For this purpose, there must be a separation of each component, M, O, S, T and IMP, from the standard mixture; that is, there must be five chromatographic bands on the TLC-chromatogram. The chromatographic system can be considered optimal when this is achieved. In this study, mobile phases that were previously used for studies on metronidazole and tinidazole were tested [3,16,20,21,36–38]. Attempts were also made to optimize the developing distance. It turned out that increasing the chromatogram's development distance had an adverse effect on the blurring of the chromatographic bands (the chromatographic bands of the substances were more blurred when a development distance greater than 8.0 cm was used). This is why in thin-layer

chromatography without the forced flow of the mobile phase (i.e., analysis in a regular chromatographic chamber), when ordinary or high-performance plates are used, the values of the theoretical plate height (H) increase over a longer development distance. It is known that the lower the degree of blurring of the chromatographic bands, the better the separation of the bands and the lower the value of the theoretical shelf height (H) is, i.e., the more efficient the chromatographic system is. Moreover, increasing the chromatogram's development distance also contributes to extending the analysis time. The tested mobile phase was TCM + MeOH 9:1 (phase no. 11), which proved to be effective for testing the degradation products of M, S, O, and T carried out in separate samples [36]. Using this mobile phase, four chromatographic bands were obtained on the TLC-chromatogram; no separation of metronidazole from secnidazole was achieved. On the TLC-chromatograms using mobile phase 18, only two chromatographic bands were obtained; using mobile phases 10 and 17, three chromatographic bands were obtained; and using mobile phases 1 and 3–7, four chromatographic bands were obtained. Five bands from individual tested biologically active substances were obtained using mobile phases number 2 (Figure S1), 8 (Figure S2), 9 (Figure S3), 12 (Figure S4), 13 (Figure S5), 14 (Figure S6), 16 (Figure S7), and 19 (Figure 1).

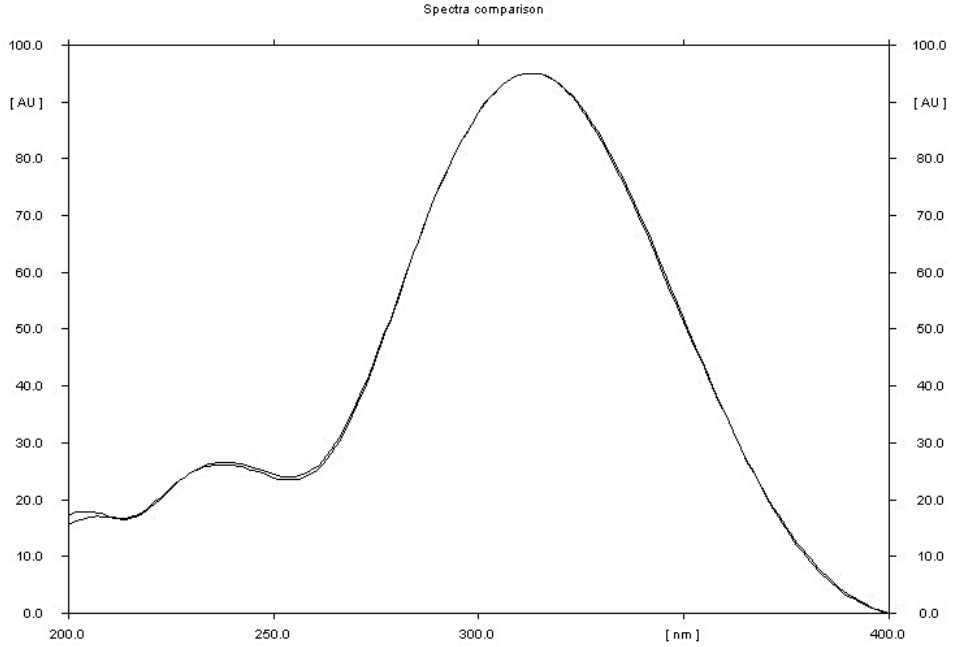

Figure 5. Comparison of the UV spectrum obtained for the standard substance M with the UV spectrum obtained for M, the source of which was a sample of *Metronidazole Polpharma* tablets.

The order of elution of the substances depends on the mobile phase used. Also, the quality of separation of chromatographic bands varies depending on the mobile phase used. The R_S separation coefficient was used to assess the quality of chromatographic separation. Table 1 lists the R_F and R_S values for the best M, S, O, T, and IMP separations. The presented comparison shows that the TCM + MeOH + DEA (9:1:1, *v*/*v*) mobile phase proposed in this work is the best. When using this mobile phase, all R_S values are greater than 1. Using this mobile phase, the following R_F values were obtained: $R_{F(IMP)} = 0.30 \pm 0.02$, $R_{F(M)} = 0.38 \pm 0.03$, $R_{F(S)} = 0.44 \pm 0.03$, $R_{F(O)} = 0.51 \pm 0.03$, $R_{F(T)} = 0.70 \pm 0.04$. The resolution factor (R_S) had the following values: $R_{S(IMP/M)} = 1.33$, $R_{S(M/S)} = 1.22$, $R_{S(S/O)} = 1.29$, $R_{S(O/T)} = 2.10$. Therefore, it is possible to check whether the drug is contaminated with other 5-nitroimidazoles. For example, if a drug sample containing M is tested, whether it is contaminated with S, T, O, or IMP should be investigated. Spectrodensitometric analysis indicates that the maximum absorption of all five investigated compounds occurs at 313 nm (Figure S8). The TLC-chromatogram of a mixture of standard substances, namely M, T, and

IMP, captured at 313 nm using TCM + MeOH + DEA (9:1:1, *v*/*v*) as the mobile phase is presented in Figure 2.

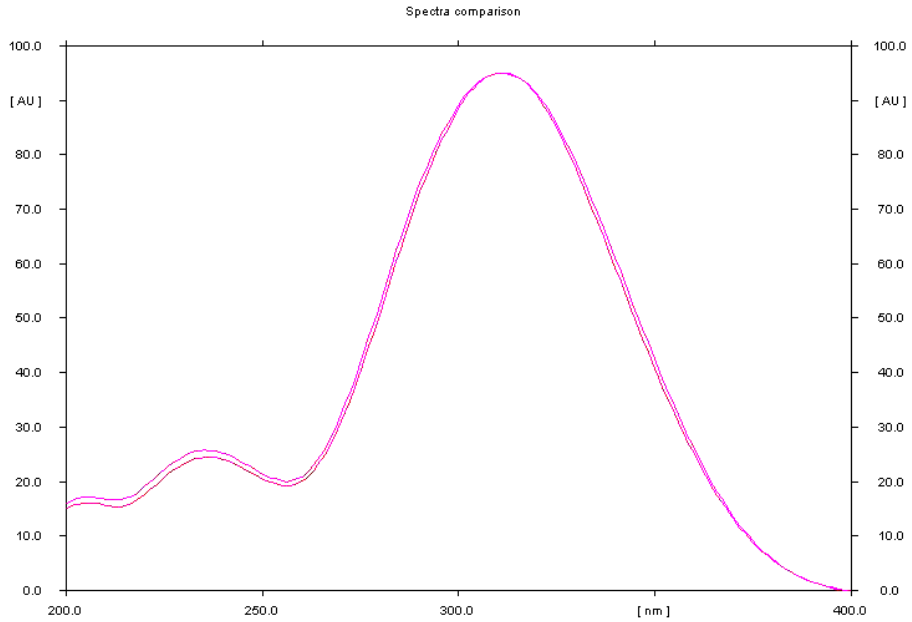

Figure 6. Comparison of the UV spectrum obtained for the standard substance T with the UV spectrum obtained for T, the source of which was a sample of *Tinidazolum Polpharma* tablets.

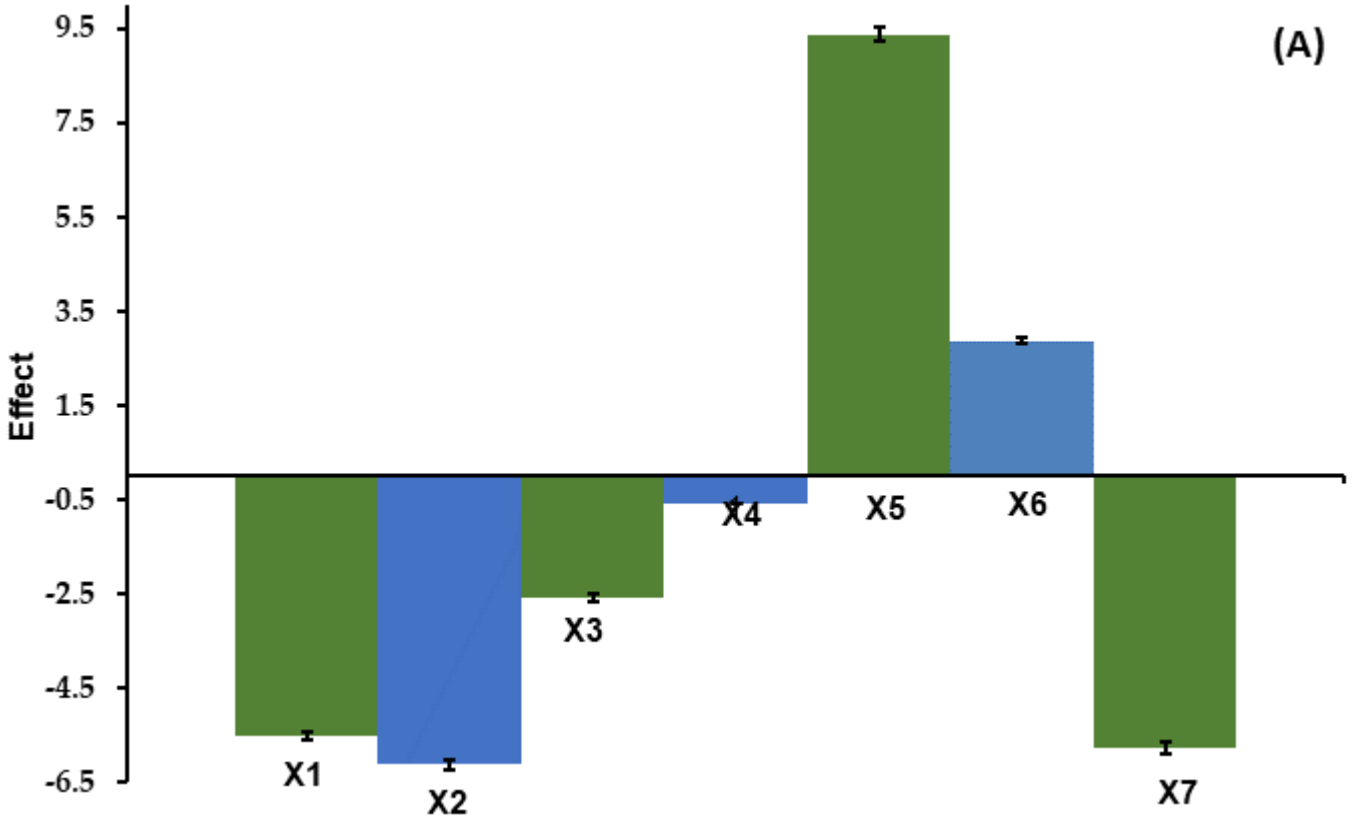

Figure 7. *Cont.*

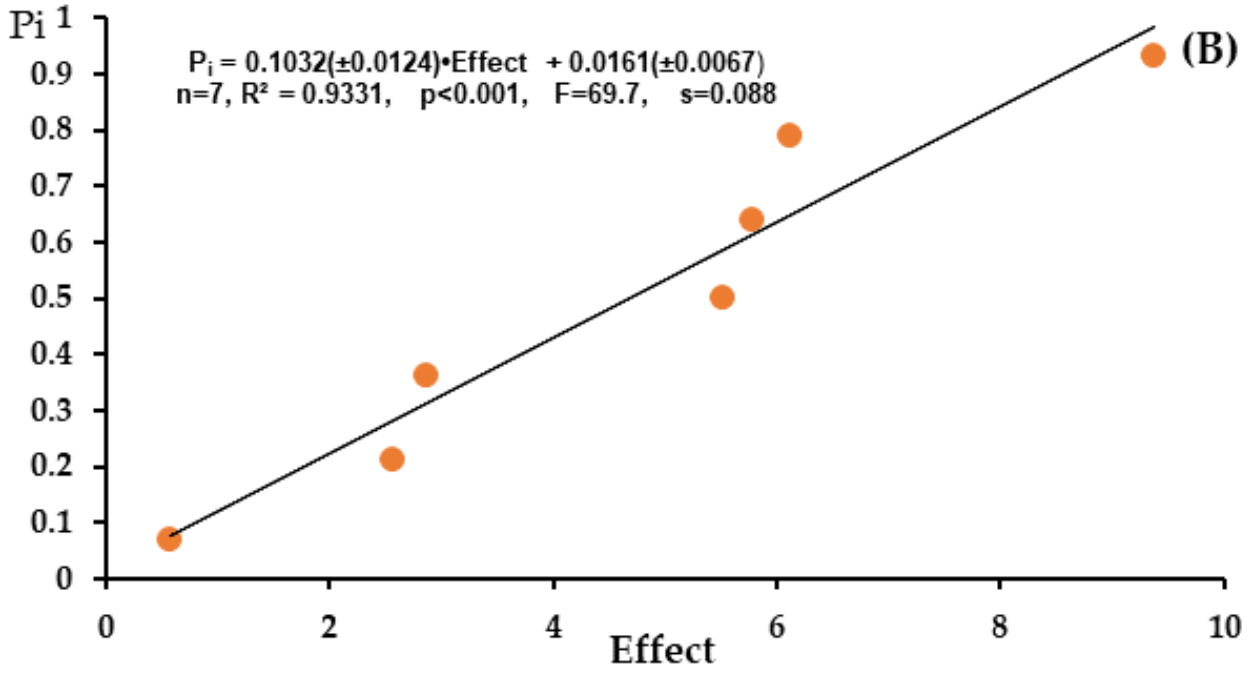

Figure 7. The effects (±SD) of factors (**A**) and relationship between the half-normal probability and the effects (**B**) for determination of metronidazole (M) in *Metronidazol Polpharma* tablets.

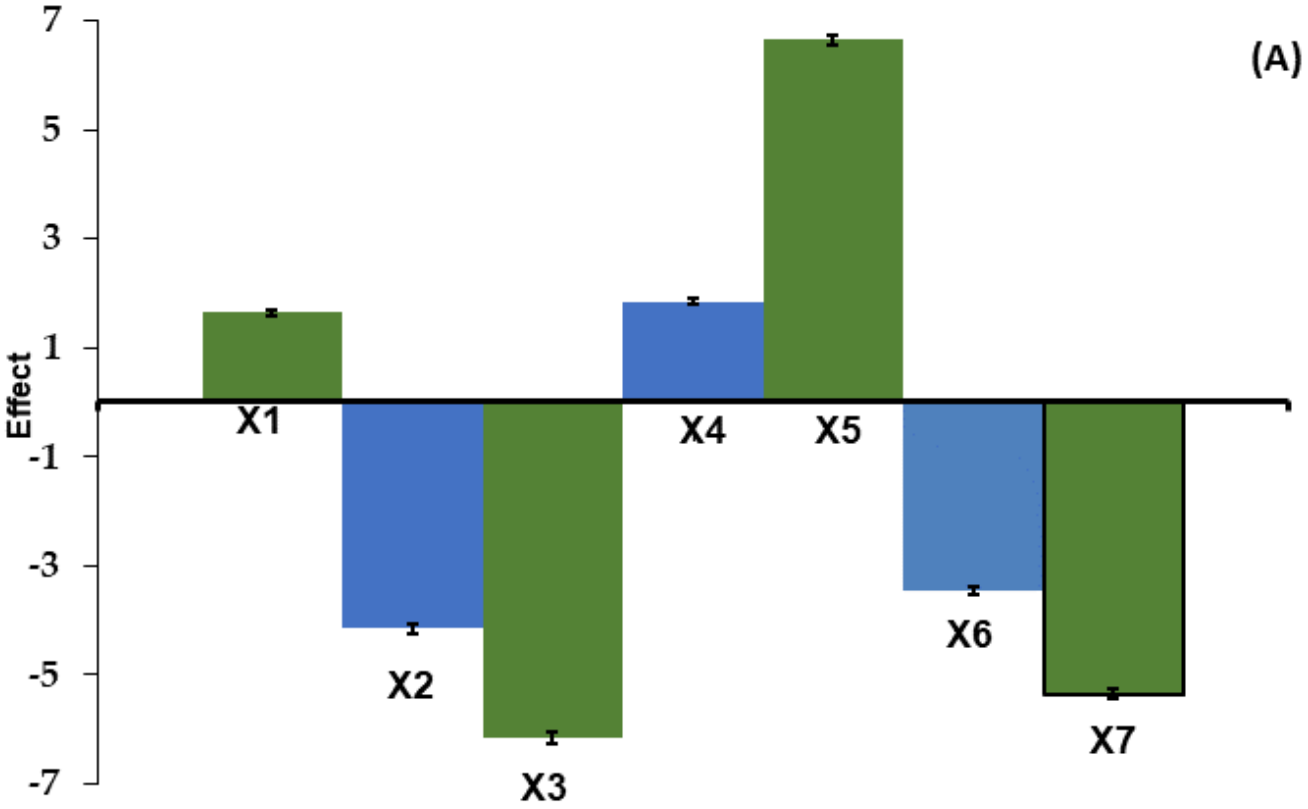

Figure 8. *Cont.*

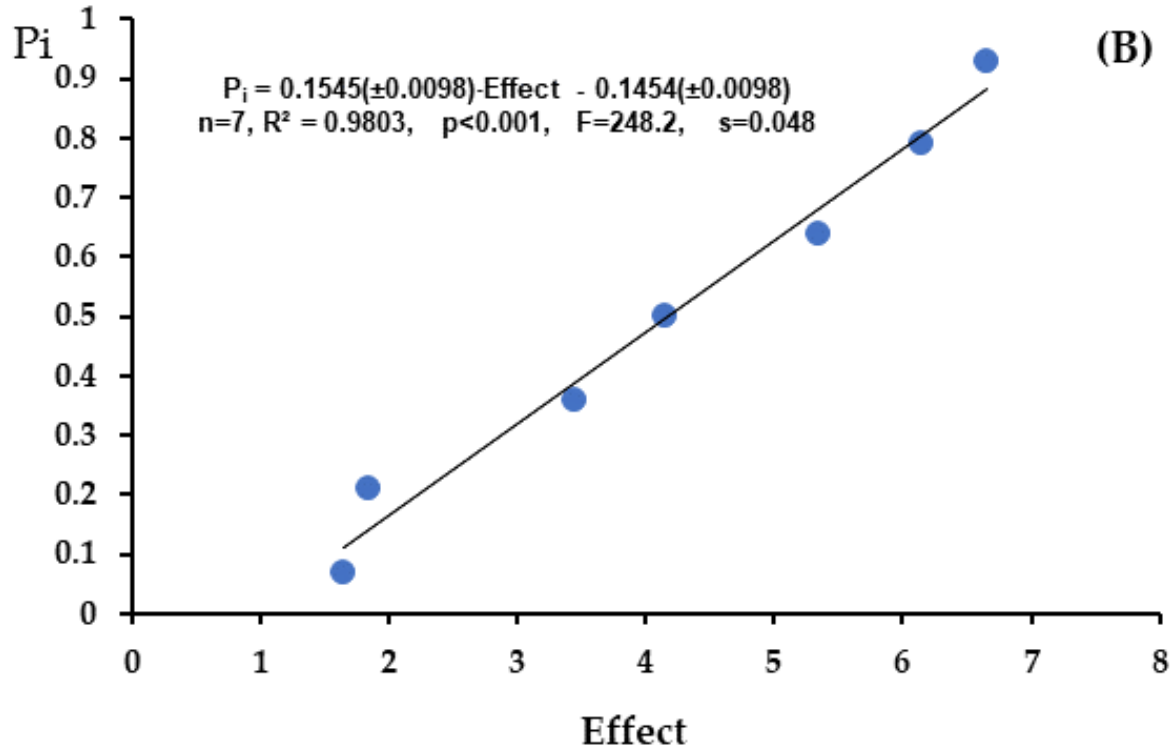

Figure 8. The effects (±SD) of factors (**A**), and relationship between the half-normal probability and the effects (**B**) for determination of tinidazole (T) in *Tinidazolum Polpharma* tablets.

The TLC-chromatograms obtained from the *Metronidazole Polpharma* tablet and *Tinidazolum Polpharma* tablet extracts using the optimal chromatographic conditions are shown in Figures 3 and 4, respectively, and indicate that there are no additional chromatographic bands detected in the analyzed tablets. This means that no impurities, including 2-methyl-5-nitroimidazole, were found in the drug samples. The R_F values of the reference substances metronidazole and tinidazole are consistent with the R_F values of metronidazole and tinidazole from the tablet samples. The spectrodensitograms of metronidazole and tinidazole standards were also found to be consistent with the spectrodensitograms of metronidazole and tinidazole from tablet samples (Figures 5 and 6).

3.1.2. Linearity and Range

It was found that the linear range of M and T determined was from 0.20 to 2.00 µg/spot. The determined linear equations describing the relationship of the area of the chromatographic band (Table S4) to the amount of micrograms/spot of M and T are presented in Table 2 and in Figures S9A and S10A. The correlation coefficients (r) of the fitted models are equal to 0.9989 and 0.9992 for M and T, respectively, and indicate a relatively strong relationship between variables. The significance level (p) of less than 0.0001 shows that there is a statistically significant correlation between the peak area registered from the chromatogram and the amount of M and T at each level. The differences between the real chromatographic band area values and those calculated from the correlation equations are presented in Figures S9B and S10B. It can be observed that the residuals (±SD) were distributed above and below the zero residuals line, thus confirming the linearity of the proposed TLC method. This indicates that the construction of the correlation equations is correct.

3.1.3. Precision

The intra- and interday precision were described using the coefficient of variation (CV, %) by measuring the area of the chromatographic spots of M and T samples with concentrations of 0.30, 1.00, and 1.75 µg/spot. The CV values ranged from 0.99% to 1.12% and from 1.33% to 1.48% for M and from 0.76% to 1.28% and from 0.99% to 1.76% for T,

respectively, for intraday and interday precision (Table 2). These results indicate that the proposed method is precise.

3.1.4. Accuracy

The recovery was used to test the accuracy of the method. The average recovery for metronidazole was 103.8%, 104.3%, and 101.2% and 101.8%, 99.1%, and 100.9% for tinidazole for 50%, 100%, and 150% of standard substances added to the *Metronidazole Polpharma* and *Tinidazolum Polpharma* samples, respectively (Table 2). The low CV values, which are less than 3% for both M and T, indicate that the elaborated method is accurate.

3.1.5. Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD values calculated are equal 0.012 and 0.022 μg/spot for M and T, respectively. The average values of the LOQ are equal 0.036 and 0.066 μg/spot for M and T, respectively. The low LOD and LOQ values indicate that the proposed TLC-densitometric method is sensitive.

After reviewing the publicly available literature on the determination of M and T using TLC methods, the obtained LOD and LOQ results were compared with those obtained by the exemplary authors of other studies (Table 3). The presented comparison shows that the elaborated TLC combined with densitometry method provides lower LOD and LOQ values for M and comparable or lower LOD and LOQ values for T in relation to those previously described in the scientific literature. The obtained LOD and LOQ values of M and T are influenced by the chromatographic conditions used (e.g., chromatographic plates used and the qualitative and quantitative composition of the applied mobile phase).

A very important element in determining the LOD is checking whether solutions of appropriately selected concentrations were used for testing. The LOD results obtained must meet the following criteria [39]: $10 \times LOD > C$ and $LOD < C$, where C represents the concentrations of metronidazole or tinidazole used.

3.1.6. Robustness

The robustness of the method was checked in accordance with previously described guidelines [35,40,41]. Analyses were performed in accordance with the information presented in Table 4 and Table S3, with the following conditions being changed: the sorbent type, the development distance, the plate activation temperature, the extraction time, the saturation time of the chamber, the wavelength in densitometric analysis at λ, and the volume of TCM in the mobile phase. The effects (E) characterizing the particular individual factors and rank probabilities were calculated. Table 4 shows the results regarding the determination of the content of metronidazole and tinidazole in *Metronidazole Polpharma* and *Tinidazolum Polpharma* tablets under the changed analysis conditions. The results of the analyses were interpreted using the coefficient of variance. The effects of factors (A) and half-normal probability plot of effects (B) for the determination of metronidazole (M) in Metronidazole Polpharma tablets and for the determination of tinidazole (T) in *Tinidazolum Polpharma* tablets are presented in Figures 7 and 8. The strong correlations between P_i and the calculated effects ($R^2 > 0.93$) and low p values < 0.001 (Figures 7B and 8B) confirm that the method is robust. The standard deviation of M and T contents (yi) in commercial tablets with the seven parameters which have been changed in the conducted experiments in order to check the robustness of the applied method was 1.5% and 1.3% for M and T, respectively. A CV value < 2% indicates the reliability of the proposed TLC-densitometric method.

3.2. Quantitative Analysis of M and T in Tablets and Comparison with Pharmacopeial Method

Table 5 shows the results regarding the determination of M and T in *Metronidazole Polpharma* and *Tinidazolum Polpharma* tablets. The content of M and T in tablets determined by the TLC-densitometric method was 506.5 and 499.4 mg, respectively. The content of M and T in tablets in relation to the content declared by the manufacturer was 101.3% and 99.8%, respectively. These results are consistent with pharmacopeial requirements, as they

range from 95% to 105% [1,2]. The obtained results were verified using the pharmacopeial method. The comparison of both methods is summarized in Table 5. The results obtained with both methods are similar. This is confirmed by the calculated statistical parameters *t* and *F*. The calculated *t* and *F* values also confirm that the proposed TLC-densitometric method is accurate.

4. Conclusions

A sensitive and cost-effective TLC-densitometric method was developed for the chromatographic separation of M, T, S, O, and IMP, as well as for the determination of M and T in tablets. The developed chromatographic conditions can be used in the pharmaceutical industry to determine the presence of M, S, O, T in simple and combined drugs containing the mentioned 5-nitroimidazoles. It is also possible to check whether the tested pharmaceutical preparation is contaminated with IMP using this method. The method has been validated. The intraday and interday precision values for the three different concentrations ranged from 0.99% to 1.48% and 0.89% to 1.76%, and the precision values ranged from 1.13% to 2.48% and 0.95% to 2.49% for M and T, respectively. The limit of quantification (LOQ) was 0.036 and 0.066 μg/spot for M and T, respectively. The mean recovery was 103.1% and 100.6% for M and T, respectively. The content of M and T in the tablets in relation to the content declared by the manufacturer was 101.3% and 99.8%, respectively. These results are consistent with pharmacopeial requirements, as they range from 95% to 105%. The presented method turned out to be fast, sensitive, selective, accurate, and robust. The obtained results were verified using the pharmacopeial method. Comparison of both the proposed and pharmacopeial methods shows that the proposed method is accurate. The low cost of the proposed TLC-densitometric method results from the use of TLC plates, which are much cheaper than the HPTLC plates used in numerous publications in which the presence of M or T was determined. The TLC plates used provide similar or better sensitivity than HPTLC plates. The developed TLC-densitometric method can be also used to test the purity of M, S, T, and O as well as drugs containing the mentioned 5-nitroimidazoles. Our method allows for the analysis of several samples on one chromatography plate at the same time. This method is suitable for quick and routine testing of the contents of pharmaceutical preparations and for routine quality control checks of products in the pharmaceutical industry.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pr12040643/s1, Table S1: Mobile phases tested, Table S2: Details of the validation of the proposed TLC-densitometric method, Table S3: The factors and their levels investigated in robustness test, Figure S1: TLC-chromatogram of a mixture of standard substances: M, S, O, T, and IMP made at 313 nm, using mobile phase: Ace + TCM + EA (4:4:1, *v*/*v*), Figure S2: TLC-chromatogram of a mixture of standard substances: M, S, O, T, and IMP made at 313 nm, using mobile phase: TCM + MeOH + AM (9:1:0.06, *v*/*v*), Figure S3: TLC-chromatogram of a mixture of standard substances: M, S, O, T, and IMP made at 313 nm, using mobile phase: TCM + MeOH + AM (9:1:0.1, *v*/*v*), Figure S4: TLC-chromatogram of a mixture of standard substances: M, S, O, T, and IMP made at 313 nm, using mobile phase: TCM + MeOH + GAcOH (9:1:0.1, *v*/*v*), Figure S5: TLC-chromatogram of a mixture of standard substances: M, S, O, T, IMP made at 313 nm, using mobile phase: TCM + MeOH + GAcOH (9:1:0.05, *v*/*v*), Figure S6: TLC-chromatogram of a mixture of standard substances: M, S, O, T, and IMP made at 313 nm, using mobile phase: Ace + TCM + EA + GAcOH (4:4:1:0.05, *v*/*v*), Figure S7: TLC-chromatogram of a mixture of standard substances: M, S, O, T, and IMP made at 313 nm, using mobile phase: Ace + TCM + EA + ACN (3:4:1:1, *v*/*v*), Figure S8: Comparison of UV spectra of M, S, O, T, and IMP, Table S4. Average area of the chromatographic bands, standard deviations, coefficient of variations for solution series of chromatographed M and T. Figure S9: Calibration plot (A) and plot of residuals (±SD) (B) for metronidazole (M) in the linear working range mobile phase: TCM + MeOH + DEA in a volume ratio of 9:1:1, Figure S10: Calibration plot (A) and plot of residuals (±SD) (B) for tinidazole (T) in the linear working range mobile phase: TCM + MeOH + DEA in a volume ratio of 9:1:1.

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Data Availability Statement: Data are contained within the article and the Supplementary Materials.

Conflicts of Interest: The author declares no conflicts of interest.

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