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Determination of the Active Pharmaceutical Ingredients in Saridon Tablets Using an Economical and Sensitive Thin Layer Chromatography Method Combined with Densitometry

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Abstract: A new TLC–densitometric method has been developed for the identification and quantification of paracetamol (PA), propyphenazone (PP) and caffeine (C) in Saridon tablets using the NP-TLC technique combined with densitometry. This method allows for the simultaneous determination of PA, PP, and C in the same sample. Among all the tested chromatographic conditions, the mixture consisting of chloroform + toluene + ethyl acetate + ethanol + acetic acid (18:18:7.5:5.0:0.3, *v*/*v*/*v*/*v*/*v*) and a silica gel 60F_{254} plate proved to be the most effective for the separation of the three tested active pharmaceutical ingredients (APIs) and substances related to paracetamol. The full validation of the proposed NP-TLC method proved that it is specific, precise, accurate, robust and sensitive. The percentage content in relation to the content declared by the manufacturer was for propyphenazone 99.8%, paracetamol 101.6% and caffeine 100.8%, which was in accordance with pharmacopoeial requirements. The results presented indicate the possibility of using the developed method in the routine control of pharmaceutical preparations containing these APIs. The proposed method is economical and more sensitive compared to the previously proposed planar methods for the simultaneous determination of APIs. What is more, the presented method may be an excellent economical alternative when the HPLC method is unavailable for such a determination.

Keywords: APIs; Saridon; normal-phase thin layer chromatography; densitometry

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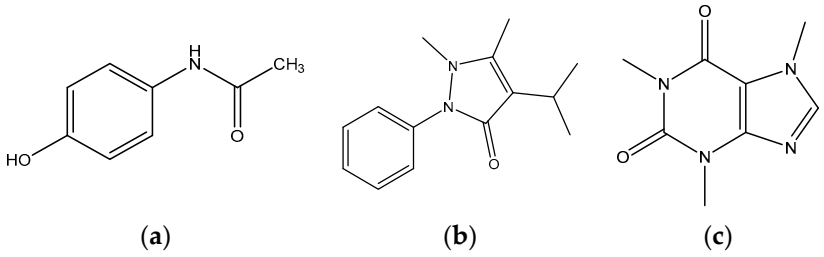
1. Introduction

Combined pharmaceutical preparations with analgesic, antipyretic and anti-inflammatory properties often also contain caffeine. Caffeine is a methylxanthine, which is chemically similar to theophylline and theobromine. Caffeine naturally occurs in plant materials such as kola nuts, tea leaves, guarana seeds, and coffee beans. It has a stimulating effect on the central nervous system. It is used in states of fatigue, drowsiness and in situations requiring arousal [1,2]. Caffeine has been proved to enhance the effects of many non-steroidal anti-inflammatory drugs (NSAIDs) and paracetamol [3]. Many of these drugs are being sold as over-the-counter (OTC) drugs. Another relevant issue is the growing availability of counterfeit drugs. Not only is it observed in Asia and Africa but also in European countries [4,5]. About 50% of the drugs bought online are counterfeit, including painkillers. As a result, many counterfeit, contaminated pharmaceutical preparations may appear on the market. Hence, there is a need for constant control of their quality and purity.

Saridon is a pharmaceutical preparation with the combined action of ingredients. It contains paracetamol and propyphenazone with analgesic and antipyretic properties, as well as a small dose of caffeine, which enhances the analgesic effect of the paracetamol. Thanks to the presence of propyphenazone, it also has weak anti-inflammatory properties. The indications for the use of Saridon are headache, toothache, menstrual pain, postoperative pain, rheumatic pain and ailments, like fever, associated with colds and flu.

Various analytical methods for the determination of paracetamol, propyphenazone and caffeine have been described in the scientific literature [6–32]. High-performance

liquid chromatography (HPLC) [6], reversed-phase high-performance liquid chromatography (RP-HPLC) [7], reversed-phase high-performance thin layer chromatography (RP-HPTLC) [8,9], adsorption high-performance thin layer chromatography (HP-TLC) [9–11] and adsorption TLC [12,13] were used for the simultaneous determination of paracetamol and caffeine in pharmaceutical preparations. Propyphenazone and caffeine in drugs were determined using Fourier-transform infrared spectrometry [14], liquid chromatography (LC) [15] and liquid chromatography-electrospray tandem mass spectrometry (LC-ESI-MS) [16]. Propyphenazone and caffeine were also determined in the presence of other biologically active substances using liquid chromatography with ultraviolet detection (LC/UV) [17], HPLC [18] and LC-ESI-MS [16]. Paracetamol, propyphenazone and caffeine (Scheme 1) were determined side by side using the spectrophotometric method [19,20], HPLC [20–23], RP-HPLC [22,24,25], spectrophotometric [20,26,27], liquid chromatography (LC) [20], square-wave voltametric [28], high-performance liquid chromatography with diode-array detection (HPLC-DAD) [29], normal-phase thin layer chromatography (NP-TLC) [24], pressurized planar electrochromatography [30] and RP-HPTLC [30]. Micellar electrokinetic capillary chromatography (MECC) has also been used to separate paracetamol, propyphenazone and caffeine in the presence of diclofenac [31]. Paracetamol, propyphenazone and caffeine have also been determined in the presence of other biologically active substances (phenobarbital, codeine phosphate, and domperidone, ergotamine tartrate, drotaverine) using the RP-HPLC and HPLC techniques, respectively [23,32]. Only a few studies have demonstrated the specificity of the developed methods by separating paracetamol, propyphenazone and caffeine from selected potential impurities [21,22,24,29].



(**a**) (**b**) (**c**)

Scheme 1. Structural formulas of paracetamol (**a**), propyphenazone (**b**), and caffeine (**c**).

Few scientific studies have described whether the proposed methods demonstrate the chromatographic conditions that allow for the separation of propyphenazone, paracetamol and caffeine from the potential impurities of the drug with 4-nitrophenol and 4-aminophenol. The separation of yet another potential impurity, namely 4-chloroacetanilide, has not been investigated in any study. The crucial aim of this work was to develop an economical and sensitive TLC method combined with densitometry for the determination of the active pharmaceutical ingredients (APIs) in the Saridon preparation. Chromatographic conditions that allow for the simultaneous quantitative determination of propyphenazone, paracetamol and caffeine and their separation from potential impurities of the drug with 4-chloroacetanilide, 4-aminophenol and 4-nitrophenol via normal-phase thin layer chromatography (NP-TLC) combined with densitometry have been developed. The proposed method for the quantitative determination of the above-mentioned APIs in a combined pharmaceutical preparation was also validated. Spectrodensitometric analysis of the listed APIs was used to determine their identity.

2. Materials and Methods

2.1. Chemicals and Reference Standards

Merck chromatography plates on aluminum foil precoated with silica gel $60F_{254}$ (#1.05554) and RP18W (#114296) were used for the study. The solvents used were acetone, chloroform, ammonia 25%, n-hexane, toluene, ethyl acetate, methanol, acetic acid (80%), glacial acetic acid, ethanol (99.8%), acetonitrile and buffer pH = 5 (citric acid 0.97 mmol/L + disodium hydrogen phosphate 2.06 mmol/L). They were components of the mobile

phases used. Ethanol 99.8% was used to extract the APIs present in the Saridon drug and to dissolve the standards. The solvents mentioned were produced by POCh Gliwice and showed analytical purity. The paracetamol and propyphenazone with USP purity and 4-aminophenol and 4-nitrophenol with >99% purity were supplied by Sigma-Aldrich (St.Louis, US). The USP anhydrous caffeine and 4-chloroacetanilide with >98% purity were purchased from Fluka. The Saridon tablets (Bayer, Leverkusen, Germany) contained 250 mg of paracetamol, 150 mg of propyphenazone and 50 mg of caffeine.

2.2. Preparation of Standard Solutions of Propyphenazone, Paracetamol and Caffeine as Well as Potential Impurities

Standard solutions of propyphenazone (PP), paracetamol (PA) and caffeine (C) were prepared by dissolving their standard substances in 99.8% ethyl alcohol. The following PP, PA, and C solutions were obtained, respectively: 6.0 mg/5 mL, 5.6 mg/5 mL, 5.2 mg/5 mL, 4.8 mg/5 mL, 4.4 mg/5 mL, 4.0 mg/5 mL, 3.6 mg/5 mL, 3.2 mg/5 mL, 2.8 mg/5 mL, 2.4 mg/5 mL, 2.0 mg/5 mL, 1.6 mg/5 mL, 1.2 mg/5 mL, 0.8 mg/5 mL, 0.4 mg/5 mL, 0.3 mg/5 mL, 0.2 mg/5 mL, and 0.1 mg/5 mL. Ethanol solutions of 4-chloroacetanilide (CA), 4-aminophenol (AF) and 4-nitrophenol (NF) were prepared at a concentration of 1 mg/5 mL.

A mixture solution of PP, PA, C, CA, AF and NF in ethanol was also prepared. The concentrations of PP, PA, C in this mixture were 5 mg/5 mL, and 1 mg/5 mL for CA, AF, and NF.

Five μL of standard solutions prepared in this way were taken and applied to the chromatographic plates.

2.3. Preparation of Saridon Drug Solutions

After weighing five tablets of Saridon, they were crushed for 30 min using a four-ball mill at 4000 rpm. From the obtained powder mass, the following quantities were weighed out:

- 30 mg (A1), 20 mg (A2), 10 mg (A3) of propyphenazone,
- 30 mg (A4), 20 mg (A5), 10 mg (A6) of paracetamol,
- 30 mg (A7), 20 mg (A8), 10 mg (A9) of caffeine.

The aliquots containing the stated amounts of active substances were extracted for 30 min at 4000 rpm after being quantitatively transferred to a 4-ball mill and 15 mL of 99.8% ethanol added. The next step was to filter the solution into a 50 mL flask and make up to the mark with 99.8% ethyl alcohol. Nine solutions of Saridon samples were obtained. Samples from A1 to A9 were used to investigate the precision of the proposed TLC method. However, for the quantitative determination of PA, PP and C in the drug, sample A4 was used, in which the content of the individual components was within the range of the developed calibration curves. Sample A4 at 5 μL contains 3 μg PA, 1.8 μg PP, and 0.6 μg C.

Five μL of the above-mentioned solutions were taken and applied to the chromatographic plates.

2.4. Description of the Conditions of TLC Combined with Densitometry

The TLC analysis was performed using 10 cm × 20 cm aluminum plates coated with silica gel 60 F_{254} (#1.05554). The first stage of the analysis was a 30 min activation of the plates in an incubator set at 120 °C. Then, standard solutions of PP, PA, C, solutions of potential impurities (CA, AF, NF) and Saridon drug extracts were applied using 5 μL micropipettes. The tests were performed using the mobile phase: chloroform + toluene + ethyl acetate + ethanol + acetic acid (80%) at a volume ratio of 18:18:7.5:5:0.3. It was selected experimentally from among the 22 mobile phases tested (Table S1). The chromatography chamber was saturated for 15 min. The plates were developed in a chromatographic chamber to a height of approx. 7.5 cm and then dried in a fume hood for 24 h.

Using the Camag TLC 3 densitometer, in which the radiation source is a deuterium lamp, spectrodensitometric and densitometric analysis were performed. The parameters

for the first of these analyzes were: wavelength 200÷400 nm, slit size 12.00 × 0.40 mm, Macro, scanning speed—20 nm/s, resolution 1 nm/step. The densitometric scanning parameters were: range from 250 to 350 nm with a change in wavelength every 25 nm, slit size 12.00 × 0.40 mm, macro, resolution 100 µm/step and scanning speed 20 mm/s. Quantitative studies were conducted using following wavelengths: PP and C at λ = 272 nm and PA at λ = 248 nm.

2.5. Thin Layer Chromatography (TLC) Method Validation

The range, linearity, precision, accuracy, specificity, robustness, limit of detection and quantification were determined according to the validation guides and previous papers [33–37], which allowed for validating the TLC method for the determination of paracetamol, propyphenazone and caffeine.

The specificity of the normal-phase thin layer chromatography (NP-TLC) method was determined by selecting the appropriate chromatographic sorbent and mobile phase, with the use of which it is possible to separate propyphenazone, paracetamol and caffeine as well as paracetamol related substances, i.e., 4-chloroacetanilide, 4-aminophenol and 4-nitrophenol.

The linearity and range of the TLC method was evaluated by analyzing 15 standard solutions of paracetamol, propyphenazone, and caffeine applied to chromatographic plates at a volume of 5 µL. The analyses were repeated three times.

The intra-day and inter-day precisions of the method were determined based on the analysis of the surface area of the chromatographic bands of the tested samples. Test solutions of PA, PP, and C (A1–A9) were used. Densitometric measurement of the resulting spots was performed and the relative standard deviation CV (%) was calculated.

The accuracy of the method was determined by measuring the recovery of standard substances added to drug samples. The accuracy of the method was also assessed by comparing the results with the literature method [30]. The analyses of the sample (A6) described in Section 3.3 of the manuscript were analyzed according to the conditions given by Hałka-Grysińska et al. [30] (method B) via the RP-HPTLC technique on RP18W plates using mobile phase acetonitrile + buffer pH = 5.0 (citric acid 0.97 mol/L and disodium hydrogen phosphate 2.06 mmol/L) at a volume ratio of 22.5:77.5.

The limit of detection (LOD) and limit of quantification were determined using the calibration curve [33–36].

The robustness of the method was tested according to guidelines described in the papers by Nagy-Turák et al. and Ferenczi-Fodor et al. [34–37]. The robustness of the method was checked by spotting sample solutions on the plate and developing the plate after altering the conditions (Table S2). The method's conditions and the selected factors for which the values of their (+) and (-) levels are summarized in Table S2. A high level is represented by "+" and a low level by "-". The main effects of seven factors were tested on two levels in eight experiments [35]. The levels of the factors investigated and the experimental design matrix (2^3) are shown in Table S3. The ways of calculation of the effects (E) characterizing the particular individual factors and rank probabilities [37] were presented in previous research [35,36]. The calculated effects (E) were then evaluated using a semi-normal probability plot [37].

2.6. Quantitative Determination of PA, PP and C in Saridon and Comparison with the Literature Method

Developing a new analytical method (method A) for the determination of paracetamol, propyphenazone, and caffeine in Saridon tablets required the comparison of the obtained results with other methods, e.g., the RP-HPTLC method (method B) described by Hałka-Grysińska et al. [30].

The comparison of the proposed NP-TLC–densitometric method (method A) with the RP-HPTLC method (method B) to determine PA, PP, and C in the pharmaceutical preparation was studied via the use of ten independently repeated different analyses. The

samples A4 and A6 (described in Section 2.3) were investigated using methods A and B, respectively, Students t-test and the F-Snedecor value were used to check the significance of the differences between the two analytical methods.

2.7. Statistical Analysis

Statistical studies of the analysis results were performed using the Statistica v. 13 PL program (StatSoft, Kraków, Poland), and the charts using Microsoft Office Excel 2016.

3. Results and Discussion

3.1. Validation

The proposed method for the simultaneous determination of paracetamol, propyphenazone and caffeine in Saridon tablets has been fully validated. The validation results are presented in Tables 1–4 and Figure 1, Figure 2 and Figures S1–S16, and they are described in the following subsections.

3.1.1. Selection of Chromatographic Conditions

According to the guidelines for the validation of analytical methods [33,34] used in pharmaceutical analysis, specificity (selectivity) is an important parameter proving that an analytical procedure allows us to determine the API presence in the tested pharmaceutical preparation along with related substances that may be their contaminants. In order to select the optimal mobile phase ensuring the separation of propyphenazone (PP), paracetamol (PA), caffeine (C), 4-chloroacetanilide (CA), 4-aminophenol (AF) and 4-nitrophenol (NF), 22 chromatographic conditions were tested (Table S1). The subjects of the research were mobile phases consisting of selected solvents such as acetone, chloroform, ammonia, n-hexane, toluene, ethyl acetate, methanol, ethanol, acetic acid 80%), acetonitrile, and buffer at pH = 5. Hałka-Grysińska et al. [30] determined the separation of propyphenazone (PP), paracetamol (PA) and caffeine (C) via RP-HPTLC on RP18W plates using the mobile phase acetonitrile + buffer pH = 5.0 at a volume ratio of 22.5:77.5. However, these authors did not demonstrate the specificity of this method. The use of chromatographic conditions in this manuscript did not allow for the separation of caffeine from 4-nitrophenol and 4-aminophenol from paracetamol (Figure S1). Further analyses were performed on ordinary NP-TLC plates, i.e., aluminum foil precoated with silica gel $60F_{254}$. Other selected mobile phases that have previously been used to analyze paracetamol and caffeine in simple and combined preparations were tested [12,38]. These mobile phases (acetone + chloroform + ammonia, 10:40:0.5 and n-hexane + acetone + ammonia, 25:25:0.5) failed to separate caffeine from 4-aminophenol and paracetamol from 4-nitrophenol (Figure S2) and paracetamol from 4-nitrophenol (Figure S3). The mobile phase chloroform + toluene + ethyl acetate + methanol + acetic acid 80%, 6:6:1:2:0.1 was previously used for the analysis of propyphenazone (PP), paracetamol (PA), caffeine (C), 4-nitrophenol (NF), and 4-aminophenol (AF) [24]. This mobile phase, however, failed to separate the caffeine from the 4-chloroacetanilide under these conditions (Figure S4). This mobile phase was modified in terms of changing the volume composition of individual components and the methanol was also replaced with ethanol. A total of 18 mobile phases were tested (Table S1). Studies have shown that the optimal mobile phase is: chloroform + toluene + ethyl acetate + ethanol + acetic acid (80%) in a volume ratio of 18:18:7.5:5:0.3. The densitogram of standard substances such as propyphenazone (PP), paracetamol (PA), caffeine (C), 4-chloroacetanilide (CA), 4-aminophenol (AF) and 4-nitrophenol (NF) is shown in Figure 1. The mobile phases: chloroform + toluene + ethyl acetate + methanol + acetic acid 80%, 6:6:2:2:0.1 and chloroform + toluene + ethyl acetate + ethanol + acetic acid 80%, 6:6:6:2:0.2 also allow for the separation of all the tested substances. However, using the mobile phase of chloroform + toluene + ethyl acetate + ethanol + acetic acid (80%) at a volume ratio of 18:18:7.5:5:0.3 meant better separation of C from CA is obtained than using chloroform + toluene + ethyl acetate + methanol + acetic acid 80%, 6:6:2:2:0.1 (Figure S5) and better

separation of C from PA than mobile phase chloroform + toluene + ethyl acetate + ethanol + acetic acid 80%, 6:6:6:2:0.2 (Figure S6).

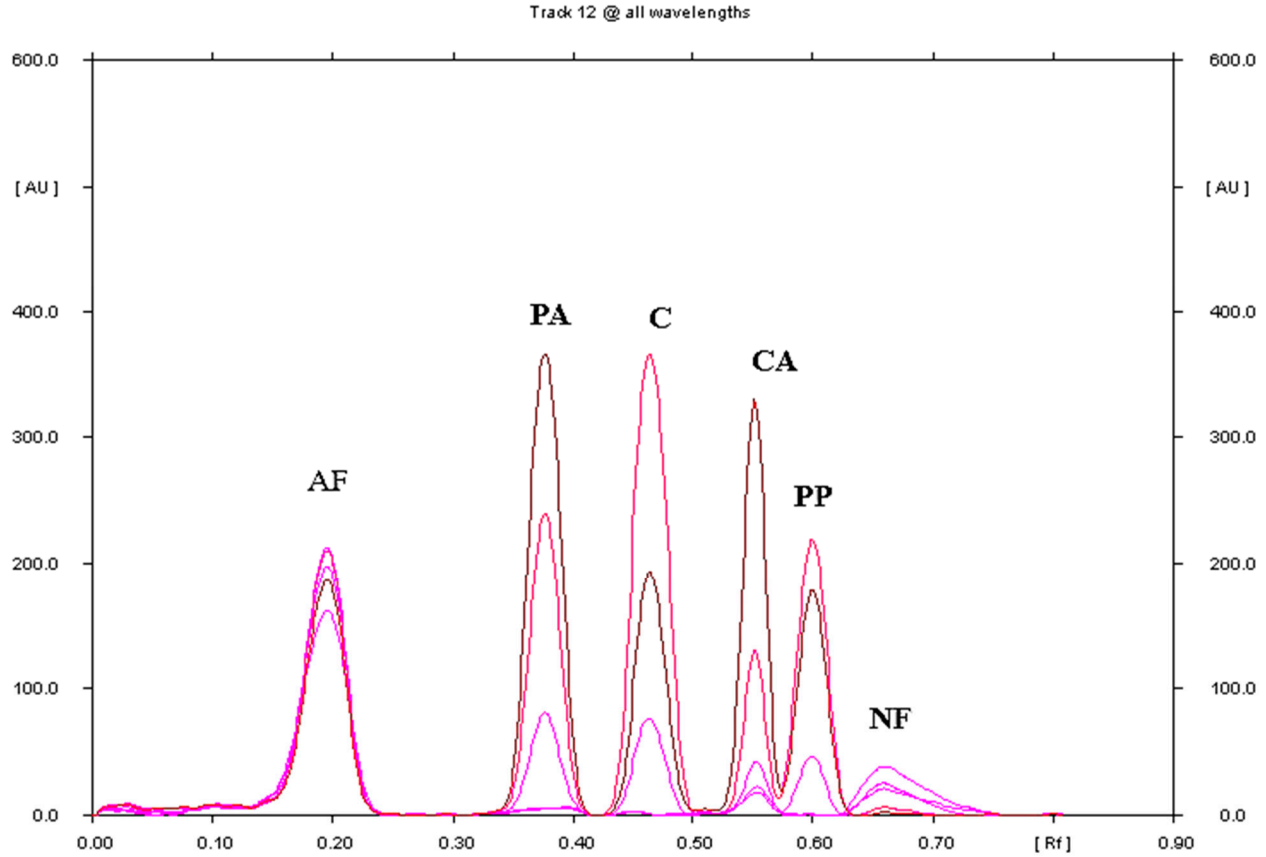


Figure 1. Densitogram of a mixture of standard substances: propyphenazone (PP), paracetamol (PA), caffeine (C), 4-chloroacetanilide (CA), 4-aminophenol (AF), and 4-nitrophenol (NF) made in the range of 250–350 nm, with a change in wavelength every 25 nm (different colors on the densitogram), using plates precoated with silica gel $60F_{254}$ and phase mobile phase: chloroform + toluene + ethyl acetate + ethanol + acetic acid (80%) in a volume composition of 18:18:7.5:5:0.3.

It follows that the best mobile phase ensuring the separation of the reference substances propyphenazone (PP), paracetamol (PA) and caffeine (C), as well as 4-chloroacetanilide (CA), 4-aminophenol (AF) and 4-nitrophenol (NF), was mobile phase with the composition of chloroform + toluene + ethyl acetate + ethanol + acetic acid 80%, and the volume ratio 18:18:7.5:5:0.3. Using this mobile phase, the following R_f values were obtained: $R_{f(AF)} = 0.20 \pm 0.05$, $R_{f(PA)} = 0.38 \pm 0.04$, $R_{f(C)} = 0.47 \pm 0.02$, $R_{f\,(CA)} = 0.54 \pm 0.03$, $R_{f(PP)} = 0.60 \pm 0.02$, $R_{f(NF)} = 0.67 \pm 0.02$. The resolution factor (R_S) calculated had the following values: $R_{S(AF/PA)} = 3.00$, $R_{S(PA/C)} = 1.62$, $R_{S(C/CA)} = 2.14$, $R_{S(CA/PP)} = 1.23$, $R_{S(PP/NF)} = 0.86$. Spectrodensitometric analysis indicated that the maximum absorption of propyphenazone and caffeine occurs at 272 nm, and that of paracetamol at 248 nm.

The densitogram obtained from the Saridon drug extract for the optimal chromatographic conditions, as shown in Figure 2, indicates that there are no additional chromatographic bands from the analyzed preparation. Only three chromatographic bands are observed on the densitogram, i.e., those corresponding to paracetamol, propyphenazone and caffeine. No matrix effect on the separation and determination of PA, PP, and C was observed.

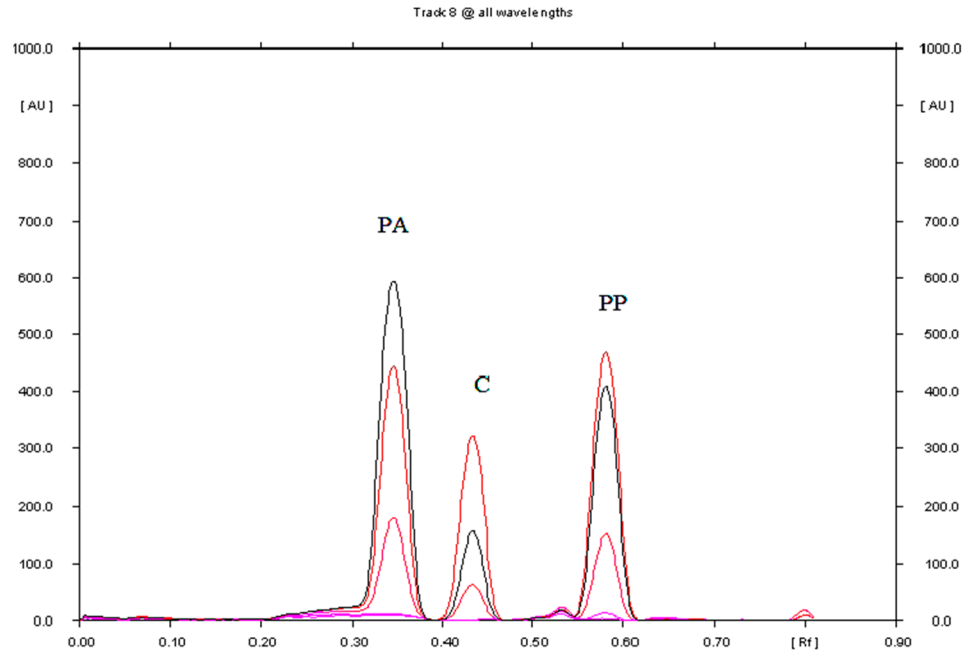


Figure 2. Densitogram of Saridon drug sample analyzed via NP-TLC at wavelengths from 200 to 350 nm, with a change in wavelength every 25 nm (different colors on the densitogram), on plates precoated with silica gel $60F_{254}$ using the mobile phase: chloroform + toluene + ethyl acetate + ethanol + acetic acid (80%) in a volume ratio of 18:18:7.5:5:0.3.

Comparisons of the spectrodensitograms of the propyphenazone (PP), paracetamol (PA) and caffeine (C) standards with the spectrodensitograms of these substances present in the Saridon samples are presented in Figures S7, S8 and S9, respectively. The absorption maximum occurs at λ = 272 nm for PP and PA, as well as at λ = 248 nm for C. Therefore, the developed chromatographic conditions can also be successfully used to confirm the identity of paracetamol, propyphenazone and caffeine in their combined Saridon preparation.

The densitogram of propyphenazone (PP), paracetamol (PA) and caffeine (C) presented in Figure 2 confirms that the TLC technique combined with densitometry used for the quantitative determination of propyphenazone (PP), paracetamol (PA) and caffeine (C) in the pharmaceutical preparation Saridon is highly selective. The mean R_f of PA, C, and PP values were 0.38, 0.47, and 0.60, respectively. The R_f values of the propyphenazone (PP), paracetamol (PA) and caffeine (C) standards are consistent with the R_f for PP, PA and C determined in Saridon. What is more, the spectrodensitograms of propyphenazone (PP), paracetamol (PA) and caffeine (C) standards are similar to the PP, PA and C spectrodensitograms from the drug samples (Figures S7–S9).

Normal-phase thin layer chromatography (NP-TLC) with densitometry can be used for the quantitative analysis of propyphenazone (PP), paracetamol (PA) and caffeine (C) in drugs using the area values of the chromatographic bands for the calculations. Qualitative identification of the above-mentioned compounds can be performed on the basis of the values of the retardation coefficients and spectrodensitometric analysis.

3.1.2. Linearity and Range

To determine the range of linearity, the relationship between the area of the densitometric band (Table 1, Figures S10A, S11A and S12A) and the concentration of the propyphenazone (PP), paracetamol (PA) and caffeine (C) standards [μg/spot] was used. The linearity range for propyphenazone was found to be 0.8÷4.0 μg/spot, for paracetamol and caffeine 0.4 ÷ 4.0 μg/spot, as shown in Figures S10A, S11A and S12A, respectively. The differences between the real values of the chromatographic band areas and the values calculated using the appropriate calibration curves are shown in Figures S10B, S11B and S12B.

Figures S10B, S11B and S12B indicate the correct choice of linearity ranges for paracetamol, propyphenazone and caffeine.

Table 1. Method validation data for the quantitative determination of paracetamol, propyphenazone, and caffeine via thin layer chromatography with densitometry.

Method Characteristic		Results		
		Paracetamol	**Propyphenazone**	**Caffeine**
Retardation factor (R_f)		0.38 ± 0.04	0.60 ± 0.02	0.47 ± 0.03
Range [μg/spot]		0.4–4.0	0.8–4.0	0.4–4.0
Linearity [μg/spot] A = a·X + b	a	3462.8 (±26.6)	3598.2 (±36.8)	3317.4 (±30.5)
	b	3083.5 (±66.0)	33,645.6 (±96.2)	4211.3 (±75.7)
	n	10	9	10
	r	0.9918	0.9996	0.9997
LOD [(μg/spot]		0.016	0.032	0.054
LOQ [(μg/spot]		0.048	0.096	0.162
For tablets				
Accuracy				
for 50% standard added (n = 6)		R = 100.2%; CV = 1.77%	R = 99.0%; CV = 2.12%	R = 99.7%; CV = 1.60%
for 100% standard added (n = 6)		R = 100.0%; CV = 2.47%	R = 98.8%; CV = 2.68%	R = 100.2%; CV = 1.66%
for 150% standard added (n = 6)		R = 99.6%; CV = 2.11%	R = 98.4%; CV = 1.98%	R = 99.2%; CV = 1.17%
Precision (CV, [%])				
Intraday				
for 3 μg/spot (n = 3)		0.35	1.10	0.75
for 2 μg/spot (n = 3)		1.56	0.92	1.08
for 1 μg/spot (n = 3)		1.10	0.68	1.02
Inter-day				
for 3 μg/spot (n = 3)		0.79	1.26	0.82
for 2 μg/spot (n = 3)		1.23	0.53	0.93
for 1 μg/spot (n = 3)		1.54	0.73	1.06
Robustness (CV, [%])		robust	robust	robust

where: A—area of the chromatographic band (spot) of propyphenazone, paracetamol, caffeine [AU], n—number of measurement points, X—micrograms PP/spot, PA/spot or C/spot, r—correlation coefficient.

3.1.3. Precision

The measure of the precision of the developed method is the coefficient of variation (CV). The intra-day and inter-day precisions were determined by analyzing solutions of API samples with concentrations of 3.0; 2.0 and 1.0 μg per spot. The resulting chromatographic spots were evaluated via densitometric analysis and the CV was determined. The values of the coefficients CV allow the method to be considered precise, because for propyphenazone they ranged from 0.68% to 1.10% and from 0.53% to 1.26%, for paracetamol from 0.35% to 1.56% and from 0.79% to 1.54%, and for caffeine from 0.75% to 1.08% and from 0.82% to 1.06%, respectively for intra-day and inter-day precisions (Table 1).

3.1.4. Accuracy

Measurement of the recovery allowed us to assess the accuracy of the proposed NP-TLC method. The appropriate amount of the powdered tablet mass was supplemented with standard substances of propyphenazone, paracetamol and caffeine at the ratio of 50%, 100% and 150% to their content in the weighed amount of the drug. The average recovery for propyphenazone was: 99.0%, 98.8% and 98.4%, for paracetamol: 100.2%,

100.0% and 99.6% and for caffeine: 99.7%, 100.2% and 99.2%, respectively, for 50%, 100% and 150% of standard substances added to the Saridon samples (Table 1). The accuracy of the method is indicated by the low values of the coefficient of variation, which was <3% for propyphenazone and paracetamol, and <2% for caffeine. The repeatability of the developed method, which is measured using the recovery, is comparable and in some cases better than the repeatability obtained using the TLC and HPTLC techniques by other researchers for simple and combined pharmaceutical preparations containing paracetamol, propyphenazone, and caffeine [8–13,24,30,38].

3.1.5. Limit of Detection (LOD) and Limit of Quantification (LOQ) of the Investigated APIs and Comparison with the Literature Data

Based on the parameters of the special calibration curve obtained for paracetamol, propyphenazone and caffeine, respectively, the limit of detection (LOD) and the limit of quantification (LOQ) for PA, PP and C were estimated using the NP-TLC method combined with densitometry. These calibration curves were obtained on the basis of the analysis of three standard solutions containing paracetamol, propyphenazone, and caffeine with the following concentrations: 0.20; 0.30 and 0.40 mg/5 mL. An example densitogram of a mixture of PA, C, and PP standards with a concentration of each standard of 0.20 µg/spot is shown in Figure S13. The average values of the limit of detection of the tested APIs were 0.016, 0.032 and 0.054 µg/spot, respectively, for PA, PP and C. The limit of quantification of the tested APIs were 0.048, 0.096 and 0.162 µg/spot for PA, PP and C, respectively (Table 1). The proposed method is characterized by a low LOD and LOQ for the determination of PA, PP and C, which confirms the sensitivity of the proposed method.

Table 2 compares the LOD and LOQ values with the literature data for PA, PP and C. Comparing all the LOD and LOQ values presented in Table 2, it should be noted that particularly high LOD and LOQ values for PA, C and PP were obtained by Ibrahim et al. [24]. This may be due to, among others, the fact that Ibrahim et al. [24] performed densitometric measurements at 220 nm, while the maximum absorption occurs at λ = 272 nm for PP and PA and at λ = 248 nm for C. The method developed in this work is more sensitive, with lower LOD and LOQ values for PA, PP and C than the previously described TLC methods for the simultaneous determination of PA, PP and C [24,30]. Better LOD and LOQ values were obtained only when paracetamol and caffeine [9] or caffeine alone [39] were determined in pharmaceutical preparations. But these analyses were carried out with more cost-intensive techniques, namely NP-HPTLC and RP-HPTLC.

Table 2. Literature values of LOD and LOQ of PA, PP, and C investigated via planar techniques.

Method	Stationary Phase	Mobile Phase	LOD and LOQ [µg/spot] of			Ref
			PA	PP	C	
NP-TLC	Silica gel $60F_{254}$	Chloroform + toluene+ ethyl acetate + methanol+ acetic acid (6:6:1:2:0.1 *v*/*v*)	LOD: 1.50 LOQ: 4.54	LOD = 1.59 LOQ = 4.83	LOD: 1.21 LOQ: 3.67	[24]
NP-TLC	Silica gel GF_{254}	Dichloromethane + methanol + acetone + glacial acetic acid (9:1:0.5:0.3, *v*/*v*)	LOD: 0.3 LOQ: 1.0	-	LOD: 0.15 LOQ: 0.5	[14]
NP-TLC	Silica gel $60F_{254}$	Chloroform + acetone + ammonia 25%, 39.6 + 9.9 + 0.5, *v*/*v*	LOD: 0.070 LOQ: 0.231	-	LOD: 0.064 LOQ: 0.194	[12]
NP-TLC	Silica gel $60F_{254}$	Chloroform + acetone + ammonia 25%, 8 + 2 + 0.1, *v*/*v*	LOD: 0.09 LOQ: 0.27	-		[38]
RP-HPTLC	RP18W	Methanol+ glacial acetic acid + water, 25:4.3:70.7, *v*/*v*	LOD: 0.100 LOQ: 0.191	-	LOD: 0.040 LOQ: 0.076	[8]
HP-TLC	Silica gel $60F_{254}$	Toluene + ethyl acetate + methanol + formic acid, 16:2:4:0.8, *v*/*v*	LOD: 0.039 LOQ: 0.118	-	LOD: 0.041 LOQ: 0.124	[10]

Table 2. *Cont.*

Method	Stationary Phase	Mobile Phase	LOD and LOQ [µg/spot] of			Ref
			PA	PP	C	
HP-TLC	Silica gel	Ethyl acetate + ethanol + ammonia, 9:1:0.1, *v*/*v*	LOD: 0.262 LOQ: 0.793	-	LOD: 0.265 LOQ: 0.802	[11]
NP-HPTLC	Silica gel 60F_{254}	Ethyl acetate + ethanol, 85:15, *v*/*v*	LOD: 0.017 LOQ: 0.051	-	LOD: 0.017 LOQ: 0.050	[9]
RP-HPTLC	Silica gel	Ethanol + water, 50:50, *v*/*v*	LOD: 0.0087 LOQ: 0.0256	-	LOD: 0.0085 LOQ: 0.0256	[9]
RP-HPTLC	RP18W	Acetonitrile + buffer pH = 5.0	LOD: 0.12 LOQ: 0.36	LOD: 0.06 LOQ: 0.19	LOD: 0.09 LOQ: 0.28	[30]
PPEC	RP18W	Acetonitrile + buffer pH = 5.0	LOD: 0.08 LOQ: 0.26	LOD: 0.04 LOQ: 0.13	LOD: 0.10 LOQ: 0.36	[30]
RP-HPTLC	Silica gel 60F_{254s}	Ethanol-water, 55:45, *v*/*v*	-	-	LOD: 0.017 LOQ: 0.051	[39]
NP.-TLC	Silica gel 60F_{254}	Chloroform + toluene + ethyl acetate + ethanol + acetic acid (80%) (18:18:7.5:5.0:0.3, *v*/*v*)	LOD: 0.016 LOQ: 0.048	LOD: 0.032 LOQ: 0.096	LOD: 0.052 LOQ: 0.162	in this work

3.1.6. Robustness

The rules for testing robustness were described in detail in the reference publications [35–37].

Seven factors were changed, namely the activation temperature of the chromatographic plate, the time of tablet extraction, the time of saturation of the chromatographic chamber and slight changes in the volume of the mobile phase components, i.e., chloroform, toluene, ethyl acetate and ethanol (Table S2). These factors were tested on two levels in eight experiments (Table S2). Table 3 shows the results obtained for the PA, PP, and C content (y_i) in Saridon tablets. The main effects (E) of the factors calculated from these results (yi) are also presented in Table 3. The calculated statistical data presented in Table 3 indicate that the changed analysis parameters have no impact on the analysis results, because the coefficient of variations is less than 2%. These results show that no factor has a significant effect on the results. To evaluate whether the proposed NP-TLC method combined with densitometry is robust, these results were evaluated via the half-normal probability plotting of the rank probabilities (p_i) as a function of the absolute values of the main effects. The effects of the factors and the half-normal probability plot of the effects for the determination of PA, PP, and C in Saridon tablets are presented in Figures S14, S15 and S16, respectively. The points of all the factors lie near the straight line, which indicates that their effect is negligible ($R^2 \geq 0.9424$). Therefore, the presented NP-TLC–densitometric method can be regarded as robust. The standard deviation of the paracetamol, propyphenazone, and caffeine content (y_i) in the Saridon tablets for the seven parameters that have been changed in the conducted experiment in order to check the robustness of applied method is placed at 1.5%, 1.2% and 1.8% for PA, PP, and C, respectively. The value of CV in percent (<2) indicates the reliability of the proposed NP-TLC–densitometric method during its normal use. The only criterion to fulfill the robustness of this method is the content of the acetic acid (80%) in the mobile phase must be constant.

Table 3. Experimental design matrix (2^3) for robustness test for active pharmaceutical ingredients in *Saridon* tablets.

Experiment No		X_1	X_2	X_3	X_4	X_5	X_6	X_7	Active Pharmaceutical Ingredient [a] Content (y_i) [mg·tablet^{-1}]		
									PA	PP	C
1		+	+	+	+	+	+	+	245.6	149.1	49.2
2		+	+	−	+	−	−	−	243.9	148.7	49.1
3		+	−	+	−	−	+	−	247.6	150.9	49.8
4		+	−	−	−	+	−	+	253.2	152.4	50.9
5		−	+	+	−	+	−	−	249.9	152.4	50.3
6		−	+	−	−	−	+	+	246.9	150.5	49.2
7		−	−	+	+	−	−	+	246.3	150.1	49.1
8		−	−	−	+	+	+	−	254.6	153.8	51.5
Size of effect (E)	PA	−1.850	−3.850	−2.300	−1.800	4.650	0.350	−1.000			
	PP	−1.425	−1.625	−0.725	−1.125	1.875	0.175	−0.925			
	C	−0.275	−0.875	−0.575	−0.325	1.175	0.075	−0.575			
The label claim [mg]									250	150	50
Average amount [mg]									248.5	151.0	49.9
Variance									14.1	3.1	0.9
Standard deviation (SD)									3.76	1.76	0.92
Coefficient of variation [CV, %]									1.5	1.2	1.8

[a] PA—paracetamol, PP—propyphenazone, C—caffeine.

3.2. Quantification of APIs in Saridon Tablets by Proposed NP-TLC–Densitometric Method and Comparison with the Literature Method

Table 4 presents the results of the actual content of the tested paracetamol, propyphenazone and caffeine in Saridon tablets obtained using the developed NP-TLC method in combination with densitometry. The statistical parameters listed in Table 4 made it possible to evaluate the obtained results. According to Table 4, the actual API contents are 254.1 mg/tablet, 149.7 mg/tablet, and 50.4 mg/tablet for PA, PP and C, respectively. Compared to the values declared by the manufacturer of Saridon, the presented method indicates values of 101.6%, 99.8% and 100.8% for PA, PP and C, respectively. These values are in accordance with the pharmacopoeial guidelines, i.e., they should be in the range of 95–105% for paracetamol and caffeine and in the range of 90–110% for propyphenazone. All these results meet the recommendations of the Polish and USP pharmacopoeias [40,41]. The proposed method allows for the simultaneous determination of PA, PP, and C in the same sample.

To verify the results obtained using the proposed NP-TLC–densitometric method (method A), a comparison was performed with a previous report using the method (method B). The method described by Hałka-Grysińska et al. [30] was used as an accurate method (method B). The comparison of the results obtained with both methods is presented in Table 4. The PA, PP and C contents in Saridon tablets obtained using both methods A and B were similar. The coefficients of variance were smaller than 3% in each case. High reproducibility and insignificant differences between the two compared methods were obtained at the 95% probability level for the t-test and F-test of significance of $0.364 < 2.101$ and $1.11 < 3.18$; $1.756 < 2.101$ and $2.04 < 3.18$; and $0.923 < 2.101$ and $2.94 < 3.18$, respectively, for paracetamol (PA), propyphenazone (PP) and caffeine (C). These results statistically confirmed the TLC–densitometric method is accurate and can be used as a substitute method. The calculated values of the *t* and *F* tests indicate that the proposed in this work NP-TLC method is accurate.

Table 4. Comparison of the paracetamol, propyphenazone, and caffeine assays [mg/tablet] obtained from ten repeated different analysis of by proposed NP-TLC–densitometric (A) and the literature RP-HPTLC RP18W (B) methods.

	Active Pharmaceutical Ingredients (APIs)					
	Paracetamol		**Propyphenazone**		**Caffeine**	
	Determined by Methods					
	A	**B**	**A**	**B**	**A**	**B**
Number of analysis	10	10	10	10	10	10
1	258.8	248.7	151.0	148.2	50.0	49.1
2	250.4	251.5	151.8	153.1	51.1	48.2
3	263.4	257.2	148.5	146.9	49.5	50.9
4	253.5	249.4	147.3	151.8	50.4	51.8
5	249.1	262.5	149.4	152.1	51.2	48.2
6	260.9	258.7	149.7	148.7	49.9	50.2
7	251.2	252.6	151.2	149.8	50.3	49.8
8	253.1	253.5	150.2	151.6	49.3	50.3
9	249.8	251.3	148.7	152.2	50.7	49.5
10	250.5	247.2	149.6	151.0	51.4	50.8
Average	254.1	253.3	149.7	150.5	50.4	49.9
Label claimed	250	250	150	150	50	50
Amount of API (%) in relations to the label claim	101.6	101.3	99.8	100.3	100.8	99.8
Standard deviation (SD)	5.1	4.8	1.4	2.0	0.7	1.2
Coefficient of variation [CV, %]	2.01	1.89	0.94	1.33	1.39	2.40
Confidence interval of arithmetic mean with confidence level equal 95%	$\mu = 254.1 \pm 3.2$	$\mu = 253.3 \pm 3.0$	$\mu = 149.7 \pm 0.9$	$\mu = 150.5 \pm 1.2$	$\mu = 50.4 \pm 0.4$	$\mu = 49.9 \pm 0.7$
Comparison of the results using methods A and B						
t calculated $t_{(95\%.18)}$ tabulated	0.364 2.101		1.756 2.101		0.923 2.101	
F calculated $F_{(95\% \cdot f1 = f2 = 9)}$ tabulated	1.11 3.18		2.04 3.18		2.94 3.18	

Method A—proposed in this work: NP-TLC on silica gel $60F_{254}$ plates using chloroform + toluene + ethyl acetate + ethanol + acetic acid (80%) in volume composition 18:18:7.5:5:0.3. Method B—RP-HPTLC on RP18W plates using acetonitrile + buffer pH = 5.0 in volume composition 22.5:77.5.

3.3. Comparison of TLC and HPLC for the Separation and Determination of Paracetamol, Propyphenazone and Caffeine

Liquid chromatography, and more precisely HPLC and TLC in accordance with pharmacopeial recommendations, are important techniques for testing drugs. Important parameters characterizing the analytical method are the limit of detection (LOD) and the limit of quantification (LOQ) of the tested sample component. Table 2 and Table S4 present

the literature LOD and LOQ data for paracetamol (PA), propyphenazone (PP) and caffeine (C) analyzed by TLC and HPLC, respectively.

In thin layer chromatography, the rule is that the LOD and LOQ are given in μg/spot. However, in HPLC, the LOD and LOQ are given in μg/mL.

The LODs obtained using the developed in this work TLC method combined with densitometry for PA, PP and C were 0.016 μg/spot, 0.032 μg/spot, and 0.052 μg/spot, respectively, which, when converted to μg/mL, are 3.2, 6.4, and 10.4 for PA, PP and C, respectively. The developed in this work TLC method combined with densitometry for the quantitative determination of PA, PP and C is more sensitive than previously described TLC methods [8–12,14,24,30,38,39] (Table 2). However, this method is less sensitive than the developed HPLC methods for the determination of PA, PP, and C [6,7,13,15,17,20,22–25,31,42] (Table S4). In the case of determining PA, PP and C in drugs, the sensitivity of the developed TLC method combined with densitometry is sufficient due to the significant amounts of APIs present in the drug.

Substances for pharmaceutical purposes and medicinal products that are found on the market must ensure suitable quality that guarantees safe use and efficacy. The substances and medical products must fulfill all quality criteria regarding identity, purity, active substance content and suitability. These properties are contained in specified documents, including pharmacopoeial monographs forming the pharmacopoeia. To confirm the identity of therapeutic substances or excipients, tests are used that allow us to clearly confirm their identity. The preferred method for testing the identity of organic compounds is infrared absorption spectrophotometry and chromatographic methods. The high-performance liquid chromatography (HPLC), thin layer chromatography (TLC) and gas chromatography (GC) methods are the most commonly used chromatographic methods. Identity confirmation is obtained by comparing the retention times or retardation factors (TLC) of the examined substance and the reference substance using chromatographic methods. Therefore, the developed NP-TLC method combined with densitometry can be used to test the purity of drugs containing paracetamol, propyphenazone and caffeine. This method can be successfully used to test the identity and quantification of APIs in a combined drug. The elaborated NP-TLC–densitometric method is sensitive and economic. Thin layer chromatography (TLC) is a complementary method to high-performance liquid chromatography (HPLC) and can be used to determine PA, PP, C in drugs when HPLC is not available in the laboratory. Thin layer chromatography (TLC) has various features that contribute to its popularity and wide applications. Here are some important advantages of TLC: lower solvent consumption than column chromatography; several different samples can be separated simultaneously on the chromatographic layer; the separation process may be stopped at any time; stepwise or two-way elution can be used; and separated samples do not need to be pre-cleaned. Compared to other analytical methods, this method is not very labor-intensive. TLC is an inexpensive chromatographic method. TLC equipment is generally inexpensive, and consumables such as TLC plates and solvent solutions are widely available and inexpensive. As a result, TLC is a viable option for regular analysis and large-scale applications. The much lower consumption of mobile phases in TLC and the possibility of simultaneous analysis of up to a dozen samples on a chromatographic plate contribute to the fact that TLC is a more ecological method than HPLC. When using HPLC, only one sample is analyzed at a time and large amounts of eluent are used.

4. Conclusions

The mixture of chloroform + toluene + ethyl acetate + ethanol + acetic acid (80%) 18:18:7.5:5.0:0.3, *v*/*v*, and TLC plates (#1.05554) precoated with silica gel $60F_{254}$ are optimal chromatographic conditions for the identity testing and quantification paracetamol, propyphenazone and caffeine in their combined pharmaceutical preparation Saridon. Under these chromatographic conditions, PA, PP and C separate from 4-chloroacetanilide, 4-aminophenol and 4-nitrophenol. The proposed method allows for the simultaneous determination of PA, PP, and C in the same sample. The obtained validation parameters of

the developed method confirm that the proposed NP-TLC method in combination with densitometry is precise, accurate, robust and sensitive. The sensitivity of the developed method is better than the previously described TLC methods for the determination of PA, PP and C. It allows you to determine paracetamol, propyphenazone and caffeine at the same level. The LOD and LOQ values of the proposed method are about 50–100 times lower than the LOD and LOQ values of the method described by Ibrahim H. et al. [24]. The developed NP-TLC method in combination with densitometry is economical, as it does not require the use of plates for high-performance thin-layer chromatography, which are several times more expensive than the plates for classic thin-layer chromatography. In addition, the proposed method is easy to use and can be used in the routine control of combined pharmaceutical preparations containing simultaneously paracetamol, propyphenazone and caffeine. The proposed method will be an excellent economical and ecological alternative when the HPLC method is unavailable in laboratory.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/analytica5010001/s1, Table S1: Chromatographic conditions tested. Table S2: The factors and their levels investigated in the robustness test. Table S3: Experimental design matrix (2^3) for the robustness test. Figure S1: Densitogram of a mixture of standard substances: propyphenazone (PP), paracetamol (PA), caffeine (C), 4-chloroacetanilide (CA), 4-aminophenol (AF), and 4-nitrophenol (NF) made in the range of 250–350 nm, using an RP18W plate and mobile phase: acetonitrile + buffer pH = 5.0 (22.5:77.5, v/v). Figure S2: Densitogram of a mixture of standard substances: propyphenazone (PP), paracetamol (PA), caffeine (C), 4-chloroacetanilide (CA), 4-aminophenol (AF), and 4-nitrophenol (NF) made in the range of 250–350 nm, using a silica gel $60F_{254}$ plate and mobile phase: acetone + chloroform + ammonia (10:40:0.5, v/v). Figure S3: Densitogram of a mixture of standard substances: propyphenazone (PP), paracetamol (PA), caffeine (C), 4-chloroacetanilide (CA), 4-aminophenol (AF), and 4-nitrophenol (NF) made in the range of 250–350 nm, using a silica gel $60F_{254}$ plate and mobile phase: *n*-hexane + acetone + ammonia (25:25:0.5, v/v). Figure S4: Densitogram of a mixture of standard substances: propyphenazone (PP), paracetamol (PA), caffeine (C), 4-chloroacetanilide (CA), 4-aminophenol (AF), and 4-nitrophenol (NF) made in the range of 250–350 nm, using a silica gel $60F_{254}$ plate and mobile phase: chloroform + toluene + ethyl acetate + methanol + acetic acid 80% (6:6:1:2:0.1, v/v). Figure S5: Densitogram of a mixture of standard substances: propyphenazone (PP), paracetamol (PA), caffeine (C), 4-chloroacetanilide (CA), 4-aminophenol (AF), and 4-nitrophenol (NF) made in the range of 250–350 nm, using a silica gel $60F_{254}$ plate and mobile phase: chloroform + toluene + ethyl acetate + methanol + acetic acid 80% (6:6:2:2:0.1, v/v). Figure S6: Densitogram of a mixture of standard substances: propyphenazone (PP), paracetamol (PA), caffeine (C), 4-chloroacetanilide (CA), 4-aminophenol (AF), and 4-nitrophenol (NF) made in the range of 250–350 nm, using a silica gel $60F_{254}$ plate and mobile phase: chloroform + toluene + ethyl acetate + ethanol + acetic acid 80% (6:6:6:2:0.2, v/v). Figure S7: Comparison of the spectrodensitogram obtained for the standard substance propyphenazone with the spectrodensitogram obtained for propyphenazone, the source of which was a sample of Saridon. Figure S8: Comparison of the spectrodensitogram obtained for the standard substance paracetamol with the spectrodensitogram obtained for paracetamol, the source of which was a sample of Saridon. Figure S9: Comparison of the spectrodensitogram obtained for the standard substance caffeine with the spectrodensitogram obtained for caffeine, the source of which was a sample of Saridon. Figure S10: Calibration plot (A) and plot of the residuals (B) for paracetamol (PA) in the linear working range mobile phase: chloroform + toluene + ethyl acetate + ethanol + acetic acid (80%) at a volume ratio of 18:18:7.5:5.0:0.3. Figure S11: Calibration plot (A) and plot of the residuals (B) for propyphenazone (PP) in the linear working range mobile phase: chloroform + toluene + ethyl acetate + ethanol + acetic acid (80%) at a volume ratio of 18:18:7.5:5.0:0.3. Figure S12: Calibration plot (A) and plot of the residuals (B) for caffeine (C) in the linear working range mobile phase: chloroform + toluene + ethyl acetate + ethanol + acetic acid (80%) at a volume ratio of 18:18:7.5:5.0:0.3. Figure S13: Densitogram of the standard mixture of PA, C, PP (each standard about concentration 0.20 μg/spot). Figure S14: Robustness test: The effects of factors (A) and half-normal probability plot of effects (B) for the determination of paracetamol (PA) in Saridon tablets. Figure S15: Robustness test: The effects of factors (A) and half-normal probability plot of effects (B) for the determination of propyphenazone (PP) in Saridon tablets. Figure S16: Robustness test: The effects of factors (A) and half-

normal probability plot of effects (B) for the determination of caffeine (C) in Saridon tablets. Table S4: Literature values of the LOD and LOQ of PA, PP, and C investigated via HPLC and micellar liquid chromatography techniques.

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