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Article

Stability-Indicating TLC–Densitometric Method for Simultaneous Determination of Paracetamol and Chlorzoxazone and their Toxic Impurities

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A highly sensitive, selective and accurate thin-layer chromatographic (TLC)–densitometric method has been developed and validated for the simultaneous determination of paracetamol (PAR) and chlorzoxazone (CZ) and their toxic impurities, 4-amino phenol (4AP) and 2-amino-4-chlorophenol (2ACP), respectively, which are also considered to be the hydrolytic degradation products and related substances of the studied drugs. A developing system consisting of chloroform–methanol–glacial acetic acid (9.5:0.5:0.25, by volume) was found to be sufficient for chromatographic separation among the four studied components using pre-activated silica gel 60 F254 TLC plates with ultraviolet detection at 225 nm. Calibration curves were constructed in the ranges of 0.3–3, 1–10, 0.06–3 and 0.04–3 μg/band for PAR, CZ, 4AP and 2ACP, respectively, using polynomial equations. The developed method was validated according to International Conference on Harmonization guidelines and demonstrated good accuracy and precision. Moreover, the method was successfully applied for the determination of PAR and CZ in different marketed samples and the results were statistically compared to those obtained by the reported reversed-phase high-performance liquid chromatography method using F-test and Student's-*t* test. The low detection and quantitation limits of the developed method make it suitable for quality control and stability studies of PAR and CZ in different pharmaceutical formulations.

Introduction

Paracetamol (PAR) is acetamide,*N*-(4-hydroxy phenyl) (1, 2); it is widely used as a minor analgesic, which is an alternative to aspirin without the side effects of salicylate on gastric mucosa (3). PAR is affected by hydrolytic degradation conditions, giving 4-amino phenol (3, 4). Chloroxazone (CZ) is 5-chloro-2-benzoxazolinone (1), which is a centrally acting skeletal muscle relaxant with sedative properties (5). CZ is highly unstable due to the presence of lactone and lactame functional groups, so it is hydrolyzed in alkaline media to give 2-amino-4-chlorophenol (5, 6). PAR and CZ are used together for myo-relaxation and analgesic purposes (3). 4-Amino phenol (4AP) has been reported to be the primary degradation product of PAR (3, 4), and is also considered to be a PAR impurity and related substance (1, 2). 4AP is known to cause nephro-toxicity in rats, in which it produces selective necrosis to renal proximal tubules (7), and was also reported to have a significant teratogenic effect (4). 2-Amino-4-chlorophenol (2ACP) is the starting material (8), which is produced by hydrolysis of CZ, especially in alkaline media, and hence it is considered to be the primary degradation product of CZ (5, 6). Moreover, 2ACP is an impurity and a related substance of CZ, as reported in the United States Pharmacopeia (USP) (1). 2ACP has a chemical structure similar to that of 4AP, but the addition of a chloride atom to 4AP forming 2ACP enhances its renal toxicity (9). It has been published that high levels of 2ACP can interfere with the ability of blood to carry oxygen, and higher levels may cause trouble breathing and death (10).

A review in the literature revealed that PAR and CZ have been determined together by different analytical methods, either in their binary mixtures or in mixtures with other drugs. PA and CZ have been determined by spectrophotometric (11, 12), thin-layer chromatographic (TLC)–densitometric (13), reversed-phase high-performance liquid chromatographic (RP-HPLC) (14) and capillary electrophoretic (CE) (15) methods. Additionally, ternary mixtures of PA, CZ and diclofenac sodium have been analyzed by spectrophotometric (16), RP-HPLC (17, 18) and supercritical fluid chromatographic (SCFC) (19) methods. Mixtures of PAR, CZ and aceclofenac have been determined by RP-HPLC (20), while PAR, CZ and ibuprofen ternary mixtures have been determined by different chromatographic methods, including RP-HPLC (21, 22), TLC (22, 23), gas chromatographic (GC) (22) and SCFC (24) methods. Only one journal has published an RP-HPLC method for the determination of PAR, CZ and related impurities (4AP, *P*-chlorophenol and 4-chloroacetanilide) (25). Due to the nephrotoxic and teratogenic effects of both 4AP and 2ACP, it was necessary to develop a sensitive method for the detection and determination of the lowest concentrations of these impurities in pure samples and pharmaceutical formulations of PAR and CZ. In the present study, an attempt has been made to develop and validate a sensitive and accurate stability-indicating TLC-densitometric method for the determination of PAR and CZ in the presence of their toxic impurities and degradation products, 4AP and 2ACP. The developed method has advantages over the published stability-indicating RP-HPLC method (25) in possessing lower limit of detection (LOD) and limit of quantification (LOQ); additionally, it is a cost-effective and accurate method and therefore, it is more suitable for application in developing countries.

Experimental

Samples

Pure samples

PAR and CZ were supplied from the Egyptian Company for Chemicals and Pharmaceuticals (ADWIA, Ramadan City, Egypt),

and their purities were certified to be 99.84 and 98.98%, respectively. Pure standard 4AP and 2ACP were purchased from Sigma-Aldrich Co. (Cairo, Egypt) with claimed purities of 99.56 and 99%, respectively.

Pharmaceutical formulations

Pharmaceutical formulations included Myolgin capsules (batch 111028A), labeled to contain 300 and 250 mg of PAR and CZ per capsule, respectively. Myolgin capsules were manufactured by Galaxo Smithkline (GSK) (Cairo, Egypt). Relax capsules (batch 11119056) were labeled to contain 300 and 250 mg PAR and CZ per capsule, respectively, manufactured by Alpha Chem Advanced Pharmaceutical Industries (ACAPI) (Cairo, Egypt).

Chemicals and solvents

All chemicals and solvents used throughout this work were of analytical grade and used without further purification. Methanol, acetone, chloroform, glacial acetic acid and ammonia solution (30%) were purchased from El-Nasr Pharmaceutical Chemicals Co. (Cairo, Egypt).

Solutions

Stock standard solutions of PAR, CZ, 4AP and 2ACP were prepared in methanol at concentrations of 1 mg/mL. Working standard solutions of 4AP and 2ACP were prepared in methanol at concentrations of 0.2 mg/mL.

The content of 10 capsules each of Myolgin and Relax capsules were separately emptied, mixed and accurately weighed. An amount of each capsules' powder equivalent to 120 mg PAR (containing 100 mg CZ) was accurately transferred to a 100-mL volumetric flask. Seventy-five milliliters of methanol was added and the prepared solutions were sonicated for 15 min and cooled well, and then the volume was completed to prepare a 1.2-mg/mL PAR and 1-mg/mL CZ stock solution of each pharmaceutical formulation.

Instruments

The instrument used was a Camag TLC scanner 3 S/N 130319 with WinCATS software. The following requirements were taken into consideration: source of radiation, deuterium lamp; scan mode absorbance mode; slit dimension, 3×0.45 mm; scanning speed, 20 mm/s; output, chromatogram and integrated peak area. The other instruments included a Linomat 5 autosampler (Switzerland), a Camag micro syringe (100 μL), precoated silica gel aluminium plates 60 F254, Allugram SIL G/UV 254 (Machenary-Nagel, Germany), 20×20 cm with 0.1 mm thickness and a Sonix TV SS-series ultrasonicator.

Procedures

Construction of calibration curves

Accurate aliquots equivalent to 0.3–3 and 1–10 μL each of PAR and CZ, respectively, were separately transferred from their respective stock standard solutions (1 mg/mL) and separate aliquots equivalent to 0.2–15 μL each of 4AP and 2ACP were transferred from their respective working standard solutions (0.2 mg/mL) and applied in triplicate to TLC plates (20×12 cm). The plates were pre-washed with methanol and dried at 60°C for 5 min before sample application in the form of bands with a band length of 4 mm. Bands were applied 20 mm apart and 15 mm from the bottom edge. Linear ascending development was performed in a chromatographic tank previously saturated with chloroform–methanol–glacial acetic acid (9.5:0.5:0.25, by volume) mixture for 30 min. The migration distance was 100 mm from the lower edge of the plate; the developed plates were air dried and scanned at 225 nm under the specified instrumental conditions. The area under the peak was recorded and calibration curves relating the integrated area under peak versus the corresponding concentrations as μg/band were then constructed, from which the polynomial regression equations were computed.

Application to pharmaceutical formulations

The procedure mentioned previously was separately followed on Myolgin and Relax prepared solutions (1 mg/mL). Concentrations of PAR and CZ were then calculated from the previously computed corresponding regression equations, from which the percentage recoveries were calculated.

Recovery studies

Recovery studies were conducted to establish the accuracy of the developed method by spiking pre-analyzed pharmaceutical formulation samples with known amounts of pure PAR and CZ at three different concentration levels (80, 100 and 120%). The spiked samples were then analyzed three times at each level and the percentage recoveries of the added pure drugs were calculated.

Results and Discussion

This work aims to investigate a highly sensitive, selective and accurate TLC–densitometric method for the simultaneous determination of PAR and CZ along with their nephrotoxic impurities (4AP and 2ACP). An advantage of the developed TLC–densitometric method over the published stability-indicating RP-HPLC method (25) is its ability to separate and quantify the active drugs and their toxic impurities with low LOD and LOQ (Table I); additionally, the developed method is less time-consuming and more cost-effective, hence, it can be used as an alternative to the RP-HPLC technique.

All of the reported TLC–densitometric methods for the determination of PA in association with CZ (13, 18, 22, 23) employed different mobile phases to separate the four studied components (PAR, CZ, 4AP and 2ACP). None of these developing systems has been able to achieve good resolution among the studied components, because of the similarity in the chemical structures of 4AP and 2ACP.

Method development and optimization

Method optimization was conducted to achieve satisfactory chromatographic resolution among the four components and to improve detection and quantitation limits of the studied impurities.

Table I
Regression and Analytical Parameters of the Proposed Methods for the Determination of PAR, CZ, 4AP and 2ACP*

Parameters	PA	CZ	4AP	2ACP
Calibration range	0.30–3.00 μg/band	1.00–10.00 μg/band	0.06–3.00 μg/band	0.04–3.00 μg/band
Slope				
Coefficient 1†	311.60	−16.421	41.142	135.26
Coefficient 2‡	−1,546.20	422.75	−506.20	−1,278.80
Coefficient 3§	4,712.20		2,735.80	5,483.70
Intercept (*D*)	429.62	1,229.60	360.37	307.10
Correlation coefficient	0.9998	0.9994	0.9999	0.9997
Number of points used in calibration curve	7	6	8	8
Accuracy	99.81	100.68	99.66	99.67
Precision				
Repeatability	0.981	1.002	0.789	1.334
Intermediate precision	1.220	1.312	1.159	1.161
LOD	0.10	0.4	0.03	0.03
LOQ	0.30	1.00	0.06	0.04

*Note: Following a polynomial regression, $A = bX^2 + cX + D$; $A = aX^3 + bX^2 + cX + D$; where A is the integrated peak area, X is the concentration in μg/band, a, b and c are coefficients 1, 2 and 3, respectively, and D is the intercept.
†Average of six determinations.
‡Average of three determinations.
§The values in the parentheses are the corresponding theoretical values at $p = 0.05$.

Developing system

Different developing systems were attempted to achieve good chromatographic separation with symmetrical non-tailed peaks, such as chloroform–acetone–methanol–ammonia (30%) (5:5:0.5:0.1, by volume), chloroform–acetone–methanol–glacial acetic acid (7:3:0.5:0.1, by volume) and chloroform–methanol–glacial acetic acid (7:3:0.1, by volume). When using the first system, 4AP, CZ, 2ACP had the same retardation factor (R_f) value. When using the second system, poor resolution was obtained between PAR and 4AP and between CZ and 2 ACP. Removing acetone from the second system slightly enhanced the resolution; hence, different ratios of chloroform–methanol (5:5 to 9.5:0.5) with different amounts of glacial acetic acid (0.1–0.4) were tested to enhance the resolution. Using a developing system consisting of chloroform–methanol–glacial acetic acid (9.5:0.5:0.25, by volume) gave the best resolution with symmetric untailed peaks.

Saturation time

The equilibrium time required before development is important to achieve homogeneity of the atmosphere, which minimizes the evaporation of the solvent from the TLC plate during the development (26). The saturation time of the developing system was optimized and found to be 30 min.

Scanning wavelength

Due to the nephro-toxic and teratogenic effects of both 4AP and 2ACP, it is important to detect and quantify the lowest concentration, which is important for the quality control analysis of PAR and CZ. Different scanning wavelengths (215, 225, 254 and 285 nm) were attempted to improve the LOD and LOQ of the proposed method. Scanning at 225 nm gave the highest signal-to-noise ratio, and hence, the optimum sensitivity for all the studied components. R_f values of the separated components were 0.06, 0.24, 0.45 and 0.67 for 4AP, PAR, 2ACP and CZ, respectively, obtained by a system containing chloroform–methanol–glacial acetic acid (9.5:0.5:0.25, by volume) as a developing system (Figure 1). As shown in the TLC–densitograms, the separated peaks are symmetrical and well separated.

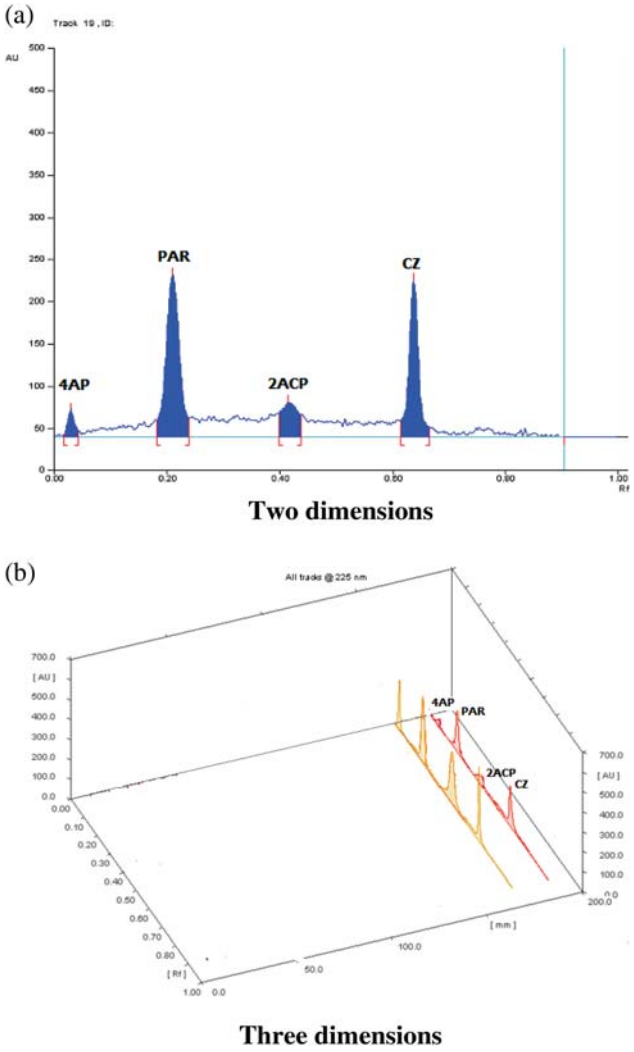

Figure 1. TLC–densitogram of a resolved mixture of standard 4AP ($R_f = 0.06$), PAR ($R_f = 0.24$), 2ACP ($R_f = 0.45$) and CZ ($R_f = 0.67$) using chloroform–methanol–glacial acetic acid (9.5:0.5:0.25, by volume) as developing system.

Method validation

Method validation was conducted according to International Conference on Harmonization (ICH) guidelines (27).

Linearity

Under optimum chromatographic conditions, linearity of the developed method was evaluated by measuring the integrated area under the peak area of different concentrations each of PAR, CZ, 4AP and 2ACP (Supplementary Figure 1), and then plotting calibration graphs relating the peak area against the corresponding concentration. The obtained regression equations and linearity ranges are listed in Table I.

Accuracy and intermediate precision

Accuracy was calculated as the percentage recoveries of blind pure drugs. The accuracy was further assured by performing recovery studies at three levels (80, 100 and 120% addition) and the average percent recovery was then calculated. Good percentage recoveries were obtained, as shown in Table I.

Precision

Precision was studied with respect to both repeatability and intermediate precision. Repeatability was calculated by the analysis of three different concentrations of pure drugs (0.5, 0.8 and 1.5 μg/band for PAR; 3, 7 and 8 μg/band for CZ; 0.08, 0.6 and 1.5 μg/band for 4AP; and 0.06, 0.5 and 1 μg/band for 2ACP) in triplicate on the same day. The experiment was repeated with the same concentrations seven times on four consecutive days to determine the intermediate precision. Good results and acceptable relative standard deviations (RSDs) (Table I) were obtained.

Specificity

The specificity of the method was tested by how accurately and specifically the analytes of interest were determined in the presence of other components (impurities, degradates or excipients) (28). The TLC–densitograms (Figure 1) verified the specificity. Furthermore, good results obtained by applying the method to Myolgin and Relax capsules (Table II) proved that the additives in the capsules [glucose, cellulose, lactose or starch (12)] did not interfere with any of the four separated components.

Table II

Determination of the Studied Drugs in Myolgin and Relax Capsules by the Proposed TLC–Densitometric Method and Statistical Comparison with the Reported HPLC Method

Parameters		TLC–densitometric method	
		PAC	CZ
Myolgin capsules (batch 111028A)	% Recovery ± SD	99.83 ± 1.745	100.04 ± 2.075
	Standard addition*	98.57 ± 1.831	99.47 ± 1.227
	Degree of freedom	(10)	(10)
	F-test (5.050)†	1.525	1.843
	Degree of freedom	(10)	(10)
	Student's *t*-test (2.228)†	0.498	1.731
Relax capsules (batch 11119056)	% Recovery ± SD	101.58 ± 1.826	99.90 ± 1.745
	Standard addition*	100.07 ± 1.101	99.17 ± 1.732
	Degree of freedom	(10)	(10)
	F-test (5.050)†	1.393	1.305
	Degree of freedom	(10)	(10)
	Student's *t*-test (2.228)†	2.006	2.076

*Average of three determinations.

†The values in the parentheses are the corresponding theoretical values at $p = 0.05$.

Table III

System Suitability Testing Parameters of the Developed TLC–Densitometric Method

Parameters	4AP	PAR	2ACP	CZ
Symmetry factor	1.00	1.00	0.95	1.11
Resolution (R_s)	5.13		3.47	3.73
Selectivity (α)	6.5		2.01	1.45

LOD and LOQ

ICH recommendations (27) were followed using a visual noninstrumental method to calculate the LOD and LOQ values of 4AP and 2 ACP (impurities). Low LOD and LOQ values indicate the high sensitivity of method (Table I).

Robustness

Deliberate small changes in the studied chromatographic conditions (e.g., change in the glacial acetic acid amount ± 0.02, methanol ± 0.05; change in saturation time ± 5 min) led to no significant change in R_f values, peak area or symmetry of the peaks.

System suitability

System suitability was checked by calculating different parameters, e.g., selectivity, resolution and symmetry factors. The obtained values were in the acceptable ranges, as shown in Table III.

After optimization and validation of the developed TLC–densitometric method, it was successfully applied for determination of PAR and CZ in Myolgin and Relax capsules. Good results were obtained and presented in Table II. The accuracy of the method was further assessed by application of standard addition technique, as shown in Table II.

The results of the analysis of the studied drugs by the developed TLC–densitometric method were compared statistically with those obtained by a reported RP-HPLC method (25) using the Student's *t*-test and variance ratio F-test at a 95% confidence level, and no significant difference were found between the results.

Conclusion

The primary task of this work was to develop and validate a TLC–densitometric method that has advantages over the reported HPLC method; it offers detection and quantitation of both impurities with high sensitivity. Moreover, it is more selective, simple and feasible than other published chromatographic methods.

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