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ORIGINAL PAPER

Validated HPLC and stability-indicating densitometric chromatographic methods for simultaneous determination of camylofin dihydrochloride and paracetamol in their binary mixture

Sawsan A. Abdel Razeq[1] · Israa A. Khalil[1] · Samah A. Mohammd[2]

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Abstract
Two accurate, sensitive, precise and selective HPLC and stability-indicating TLC methods were developed for the simultaneous determination of camylofin-2HCl and paracetamol. Forced acid, alkali and oxidative degradation of camylofin-2HCl were tried where complete degradation was achieved using 5 N HCl. HPLC method was developed to determine the mixture of the two drugs using Zorbax NH_2 column and a mobile phase of 0.5% triethylamine and pH 3.0 adjusted with 0.1% phosphoric acid and methanol (70:30 v/v) over concentration ranges of 3–90 and 10–95 µg/mL for camylofin-2HCl and paracetamol, respectively.TLC method was used for the separation of camylofin from its acid degradate and paracetamol using chloroform–methanol–acetone–conc. ammonia (8:2:2:0.1, by volume) as developing system and band scanning at 254 nm over concentration ranges of 5–40 µg/band for camylofin-2HCl and 0.1–0.5 µg/band for paracetamol. The validation of two methods was carried out according to ICH guideline. Accuracy ranged between 98.47 and 100.67% for the two methods with acceptable precision RSD% ranging between 0.66 and 1.47%.

Keywords Camylofin · Paracetamol · Acidic degradate · HPLC · TLC densitometry

Introduction

Camylofin-2 HCl (3-methyl butyl 2-(2-diethyl amino ethyl amino)-2-phenyl acetate hydrochloride) is a part of anticholinergic, spasmolytic and gastrointestinal sedative group [1, 2]. It is found in market as single or combined formulation with paracetamol, nimesulide, diclofenac and analgin. The literature revealed many analytical techniques for the quantification of camylofin-2HCl such as spectrophotometry [3, 4], HPLC [5–9] TLC densitometry [6, 7, 10–12] and GC [13, 14].

Paracetamol (N-acetyl-para-amino-phenol) has antipyretic and analgesic action and weak anti-inflammatory effect [1]. Many techniques have been used for its determination, including spectrophotometry [15–17], HPLC [18–21] TLC [22–25], GC [26–29], fluorometry [30–32] and electrochemistry [33–36].

Literature review of the mixture analysis revealed that there are only two reported HPLC methods [37, 38] and one spectrophotometric method [39] which were developed for the determination of both drugs simultaneously. But all the reported methods lack a sensitivity, and no reported TLC densitometric method for the simultaneous determination of camylofin-2HCl and paracetamol was developed up till now. The novelty of this work is the first development of TLC densitometric method for the determination of paracetamol and camylofin-2HCl in the presence of its acidic degradate. The value of this work over all the published methods is the complete degradation of camylofin and the identification of degradate by IR and ^{1}HNMR with the suggestion of degradation pathway which matched that reported uncompleted degradation [8]. Also the proposed HPLC method has an advantage of high sensitivity over the two reported ones [37, 38] (Fig. 1).

✉ Israa A. Khalil
p_isr@yahoo.com

[1] Analytical Chemistry Department, Faculty of Pharmacy (Girls), Al-Azhar University, Cairo, Egypt

[2] National Organization for Drug Control and Research (NODCAR), Giza, Egypt

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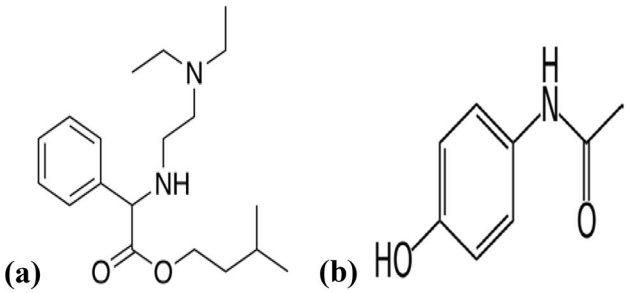

Fig. 1 Chemical structure of **a** camylofin and **b** paracetamol

Experimental

Instruments

- HPLC (Agilent 1100 series, Waldbronn, Germany) consists of an Agilent pump, equipped with autosampler and variable wavelength detector.
- An Agilent Zorbax NH_2 column (250×4.6 mm, 5 µm particle).
- TLC scanner with WINCATS software (Camag, Switzerland).
- TLC plates precoated with Silica Gel 60 F254, 10×20 cm (Merck, Germany).
- An automatic sample applicator provided with 100 µL syringe.
- Five-digit electronic balance (Radwag, Germany).
- Sonicator (Crest, USA).

Materials and reagents

Pure camylofin-2HCl and paracetamol were supplied by Kahira Pharmaceutical Co. (Cairo, Egypt) and Chemical Industries Co, (Cairo, Egypt) with purity of 99.64% and 99.85% [38]. Anafortan® tablet which contains 25 mg of camylofin-2HCl and 300 mg paracetamol and is the product of Abbot, (India) was purchased from a local pharmacy in India.

All the reagents were of HPLC grade; methanol and triethylamine were supplied from Fisher scientific, USA, and chloroform and acetone were supplied from Scharlau Chemie, Spain, while orthophosphoric acid and ammonia were supplied from Sigma, USA. Water for HPLC was prepared by double distillation and then filtration using 0.2 µm membrane filter.

Standard solutions

Stock solutions of camylofin-2HCl (10.00 mg/mL) and paracetamol (1.00 mg/mL) were prepared in water for HPLC method and in methanol for TLC method.

Working standard solutions for both drugs (100.00 µg/mL) for HPLC and (5.0 mg/mL) camylofin-2-HCl, (0.05 mg/mL) paracetamol for TLC were prepared from the stock solutions by dilution with water for HPLC and by methanol for TLC method.

Degraded sample

Acid and basic degradation

Acidic and basic degradates of camylofin-2HCl were prepared by refluxing 0.5 g pure drug with either 100 mL 5 N HCl or 5 N NaOH for about 6 h. The solution was neutralized with either 5 N NaOH or 5 N HCl to pH about 7 and evaporated till dryness under vacuum. Residue was extracted three times, each with 30 mL methanol, and then filtered into a 100-mL volumetric flask followed by dilution with the same solvent. Portion of the prepared methanolic degradate was then evaporated to dryness, and the structure of the degradate was confirmed by IR and ^{1}H NMR.

Oxidative degradation

The above steps for acidic and basic degradation were followed using 30% H_2O_2 for oxidative degradation without refluxing.

Chromatographic conditions

HPLC method

Chromatographic separation was performed on Zorbax NH_2 column (250×4.6 mm, 5 µm particle) using water containing 0.5% triethylamine, and pH was adjusted to 3 by 0.1% phosphoric acid/methanol (70:30 v/v) as mobile phase pumped with flow rate of 1 mL/min and UV detection at 220 nm.

TLC method

Camylofin-2HCl, its acidic degradate and paracetamol solutions were applied to a Silica Gel 60 F254 plates (20×10 cm) using automatic applicator and a developing system of chloroform/methanol/acetone/conc. ammonia (8:2:2:0.1, by volume). The plates were scanned at 254 nm under the following conditions;

- Silt dimensions: 6.0×0.2 mm.
- Scanning speed: 20 mm/s.
- Data resolution: 100 µm/step.
- Measurement mode: absorption.
- Result output: chromatogram and integrated peak area.

Validation

(a) Linearity and calibration curves

*HPLC method*Hundred microliters from solutions containing 3.00–90.00 µg/mL or 10.00–95.00 µg/mL of camylofin or paracetamol was injected on the column and chromatographed under the previously described conditions under "*Chromatographic conditions*."

TLC method Aliquots of 2.5–20 mg from camylofin-2HCL working standard solution (5.0 mg/mL) or 0.05–0.25 mg from paracetamol working standard solution (0.05 mg/mL) were transferred into two separate sets of 10-mL volumetric flasks, and the volume was completed to the mark with methanol. Twenty microliters of each solution was applied to Silica Gel 60 F254 plates. The procedure under "*Chromatographic conditions*" was followed.

The calibration curves were constructed by plotting the peak area against the corresponding drug concentration. Regression equations were computed.

(b) Accuracy

The accuracy of the suggested methods was determined by the standard addition technique in which known concentrations of standard camylofin and paracetamol (3,20, 85 µg/mL and 10, 20, 30 µg/mL and 5, 10, 30 µg/band and 0.1,0.2, 0.3 µg/band) drugs for HPLC and TLC methods, respectively, were added to fixed amount of pharmaceutical formulation. The concentrations of the added standard were then calculated from their corresponding regression equation.

(c) Precision

Triplicate analyses at three concentration levels of 5, 50 and 90 µg/mL and 5, 30 and 40 µg/band for camylofin-2HCl and 10, 50 and 95 µg/mL and 0.1, 0.3 and 0.5 µg/band for paracetamol were performed in the same day to study the intraday precision of HPLC and TLC methods, respectively. The interday precision was confirmed by repeating the analysis in three successive days using the same drug concentration levels.

(d) Selectivity

Selectivity was evaluated by simultaneous determination of camylofin and paracetamol for HPLC and in the presence of camylofin acid degradate for TLC method.

(e) Application to pharmaceutical formulations

HPLC method

An amount of powdered tablet equivalent to 25 mg of camylofin-2HCl and 300 mg of paracetamol was transferred into a 100-mL volumetric flask and dissolved in 70 ml of water and sonicated for 15 min, and the volume was completed with water and filtered. The filtrate was labeled to contain 250 µg/mL of camylofin-2HCl and 3 mg/mL of paracetamol.

TLC method

Camylofin-2HCl An amount of fine powder equivalent to 50 mg camylofin-2HCl and 600 mg of paracetamol was transferred into a 50-mL volumetric flask, dissolved in 30 ml of water and sonicated for 15 min, and the volume was completed with methanol and filter. The filtrate was labeled to contain 1.00 mg/mL of camylofin-2HCl and 12.00 mg/mL of paracetamol

Paracetamol An amount of fine powder equivalent to 50 mg of paracetamol was transferred into 100-mL volumetric flask, dissolved in 30 ml of water and sonicated for 15 min, and the volume was completed with methanol and filter. Filtrate was labeled to contain 0.50 mg/mL of paracetamol.

The procedure of "*linearity*" for each method was followed, and the concentration of each drug was calculated from its corresponding regression equation.

Results and discussion

Literature review revealed that no stability densitometric TLC method was reported for the determination of camylofin-2HCl and paracetamol mixture. Only two HPLC methods [37, 38] were reported for the determination of mixture without ^{1}HNMR or IR identification of degradate. This work introduces the first preparation, separation and identification of degradate of camylofin with a stability indicating TLC method for determination of camylofin-2HCl which is also simultaneous for its determination with paracetamol. Both drugs were also determined simultaneously with a new more simple and sensitive HPLC method than the reported methods.

Forced degradation of camylofin-2HCl

Degradation of camylofin-2HCl was performed by refluxing the drug using different media with different normalities and for different time intervals: 0.1 N HCl and NaOH for 10 h, 1 N HCl and NaOH for 8 h, 3 N HCl and NaOH for 8 h, 5 N HCl and NaOH for 6 h and 10%, 20%, 30% H_2O_2 for 24 h. Acidic, basic and oxidative degradates were then treated as under "degraded samples." Complete degradation was attained upon refluxing the drug with 5 N HCl for about 6 h. TLC separation of the degradate was attained after testing different developing systems for good separation, and

the most suitable system was chloroform/methanol/acetone/conc. ammonia (8: 2:2: 0.1, by volume). Upon calculating of Rf of degradate and the intact drug, acidic, basic and oxidative degradates have the same Rf values. Thus, the acidic degradate is subsequently used for the stability-indicating analysis of the drug as the alkaline and oxidative degradation is not complete. The structure of acidic degradates was confirmed by IR and ^{1}HNMR (Figs. 2a, b, 3a, b, 4a, b).

Comparing the ^{1}HNMR spectra of intact camylofin-2HCl and its acidic degradate shows that there is a signal at 7.3 for "H of the aromatic ring" which is also present in both spectra. A signal at 5.2 ppm for "H of benzylic carbon" (1) is shifted to 4 ppm in the degradate spectrum. The spectrum of camylofin-2HCl shows signals at 3.5, 1.4, 1.2 and 0.73 ppm for H of two methylene groups located between 2 amino groups (2), H of two methylene of tertiary amines (3), H of methyl groups (4) and H of ($O–CH_2–CH_2$) (5), respectively, which completely disappear in the ^{1}HMNR spectrum of the degradate.

^{1}HMNR spectrum of camylofin-2HCl shows a signal at 3.4 ppm, which completely disappears in D_2O spectrum, which represents H for secondary amino group (6), while at degradate, the spectrum shows a signal at

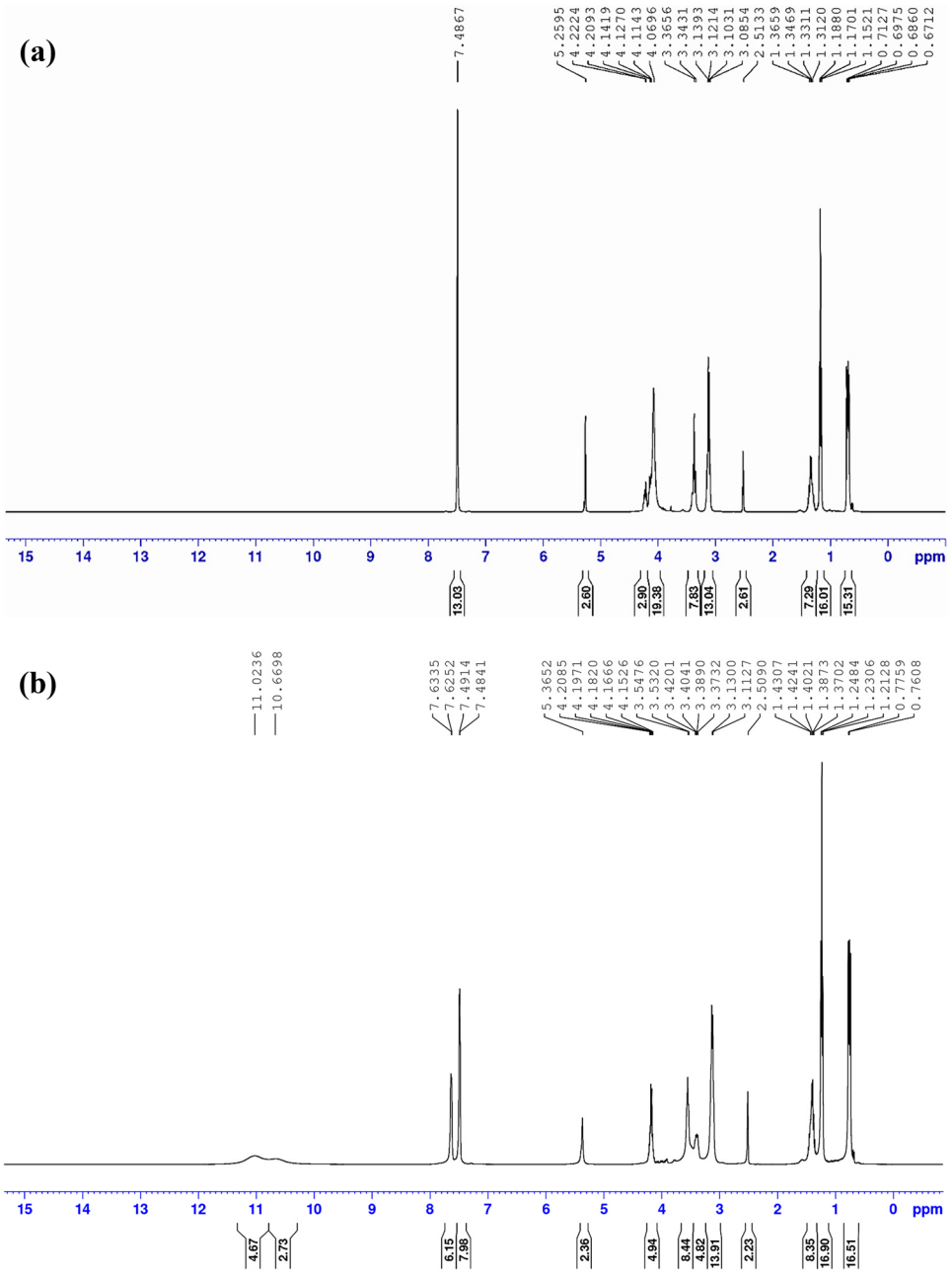

Fig. 2 **a** ^{1}HNMR D_2O spectrum of intact camylofin, **b** ^{1}HNMR spectrum of intact camylofin

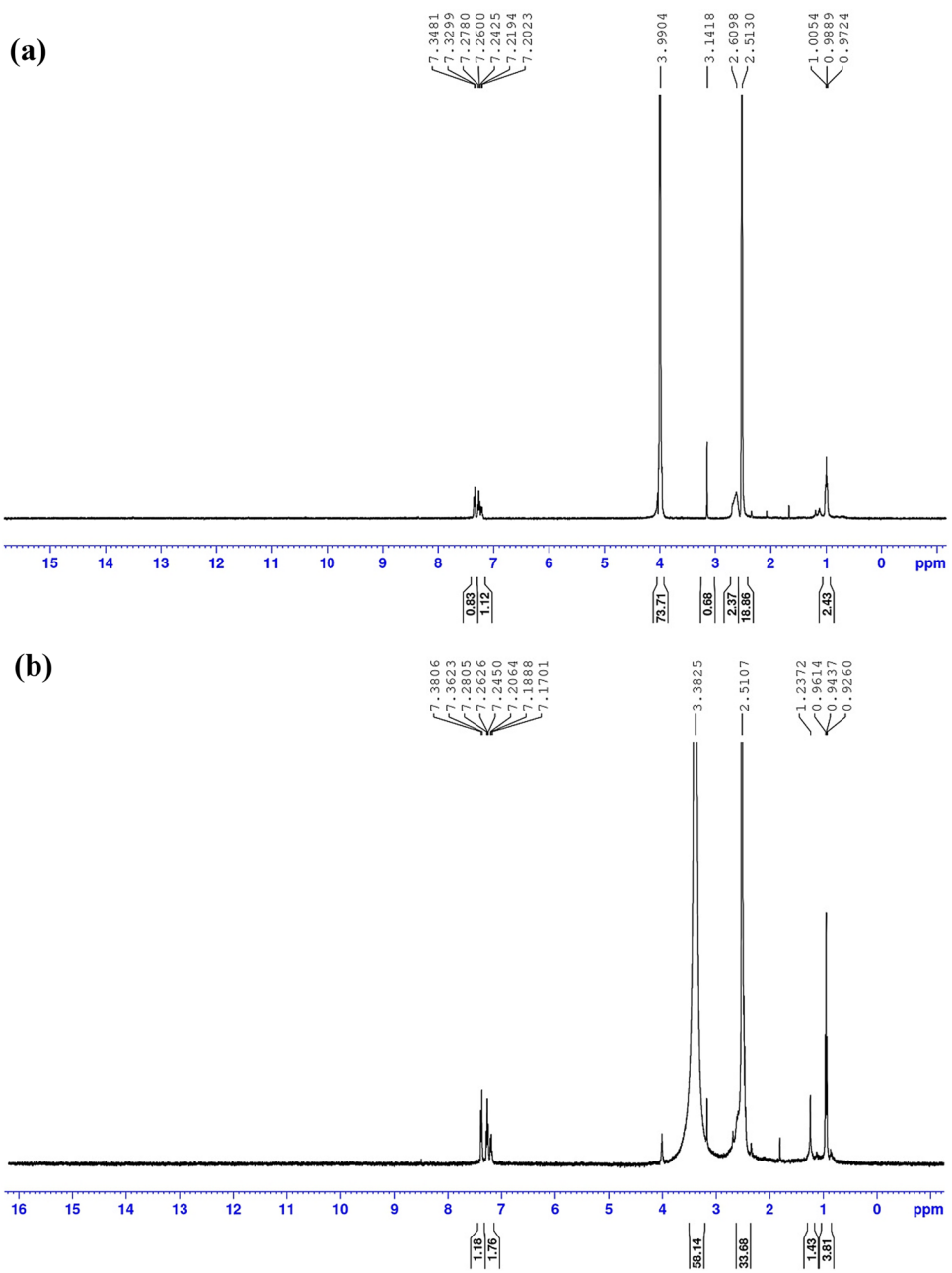

Fig. 3 **a** ^{1}HNMR D_2O spectrum of degradate, **b** ^{1}HNMR of degradate

1.2 ppm which completely disappears in D_2O spectrum which represents H of NH_2 (7).

IR spectrum of degradate shows peak at 1581 cm^{-1}, which represents carbonyl group of carboxylic acid formed after breakage of ester bond and peak at 3436 cm^{-1} for the formed primary amine (Fig. 3a, b).

Accordingly, a degradation pathway was suggested as shown in Fig. 5, which is consistent with the published finding [8].

Method development

(a) HPLC method

The main challenge in the development of HPLC method was the difference in the solubility of both drugs; camylofin is freely soluble in water, while paracetamol is not. The mobile phase should contain an appropriate amount of water

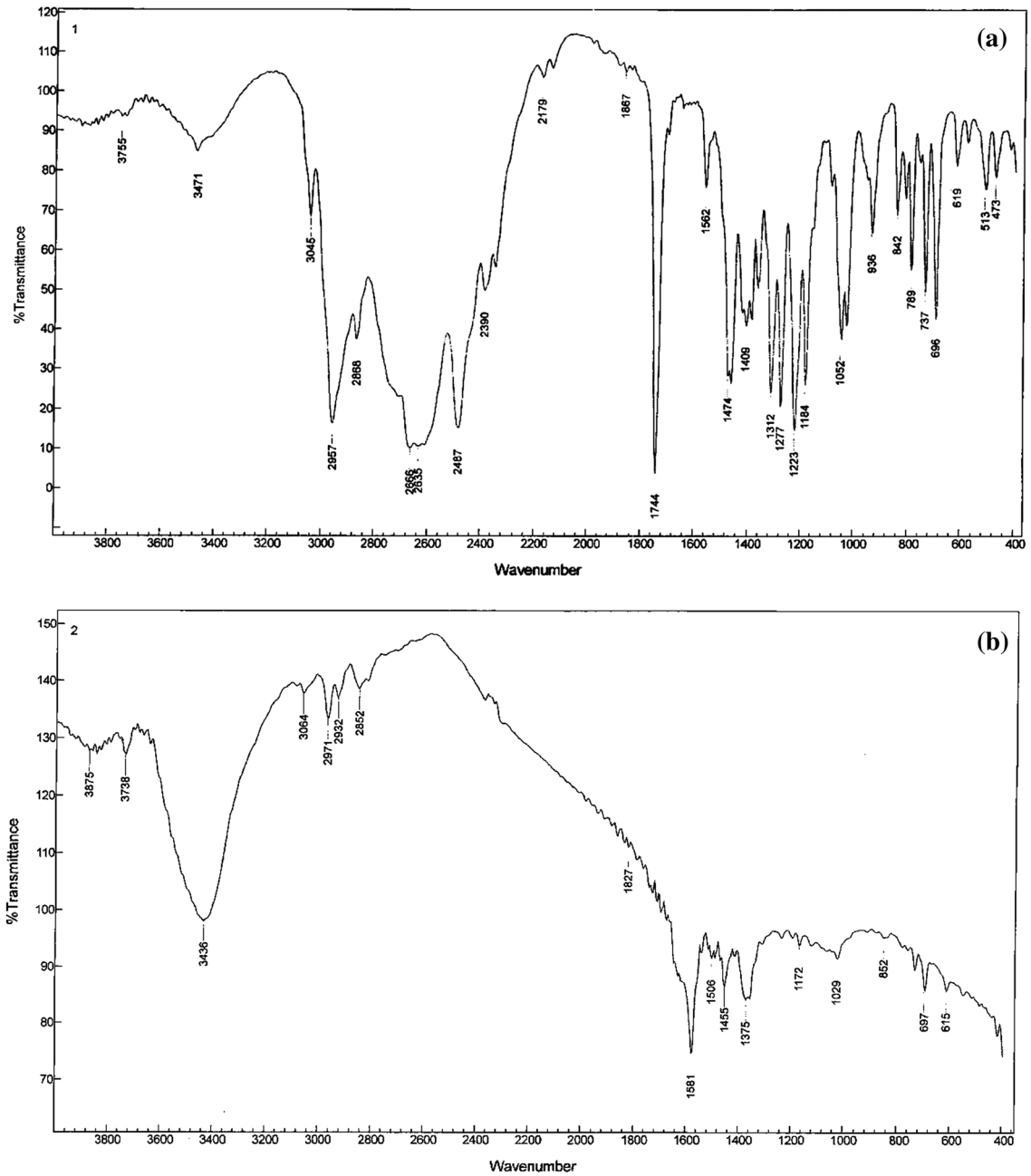

Fig. 4 **a** IR spectrum of intact camylofin, **b** IR spectrum of degradation product

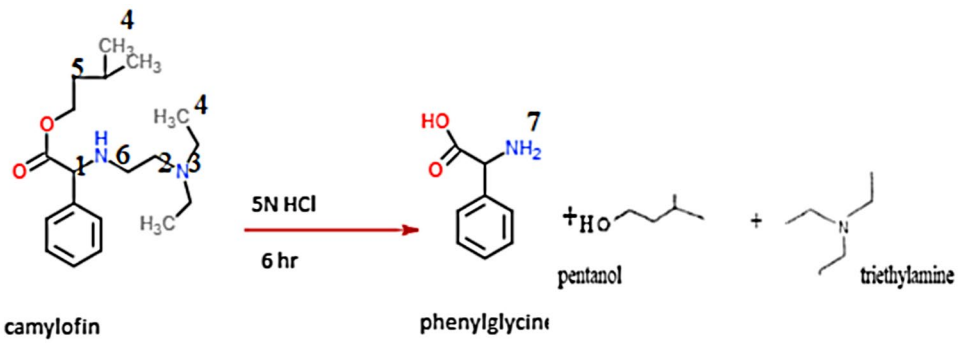

Fig. 5 Suggested degradation pathway camylofin-2HCl

to ensure separation and to decrease retention time. Different mobile phase systems were tried for separation of two drugs with different organic ratios such as acetonitrile/methanol, acetonitrile/phosphate buffer, acetonitrile/methanol/water, acetonitrile/phosphate buffer, methanol/water, and the best separation was obtained by using a mixture of water containing 0.5% triethylamine with pH of 3.0 adjusted by 0.1% phosphoric acid/methanol (70:30 v/v). Good resolution and acceptable symmetric peaks were achieved by using Zorbax—NH_2 column (250 mm × 4.6 mm, 5 µm particle) after testing a variety of column types. The detection at 220 nm was chosen to increase the sensitivity of the measurement. Also the best resolution between peaks was obtained by pumping the mobile phase with a flow rate of 1.0 mL/min. The retention times of two peaks were 2.047 and 3.015 for camylofin-2HCl and paracetamol, respectively (Fig. 6). RT of camylofin is shorter than that reported in previously HPLC methods [37, 38], while that of paracetamol is longer than the reported value in Ref. [37] and shorter than that reported in Ref. [38].

(b) TLC method

Difference in the affinity of the two drugs to TLC mobile phases causes a difficulty in their separation by TLC. That is why there is no published method developed for the mixture separation till now. In this work, many developing systems were tested for the separation of three compounds (Table 1). First, a mobile phase composed of acetone/methanol/ammonia (6:4:0.1, by volume) was tried; both drugs moved with eluting system but there was over-peak tailing. To increase unipolarity of the system, Systems IV, VI, VII, VII were tried but the problem of tailing was still present. Complete separation of the three compounds with suitable

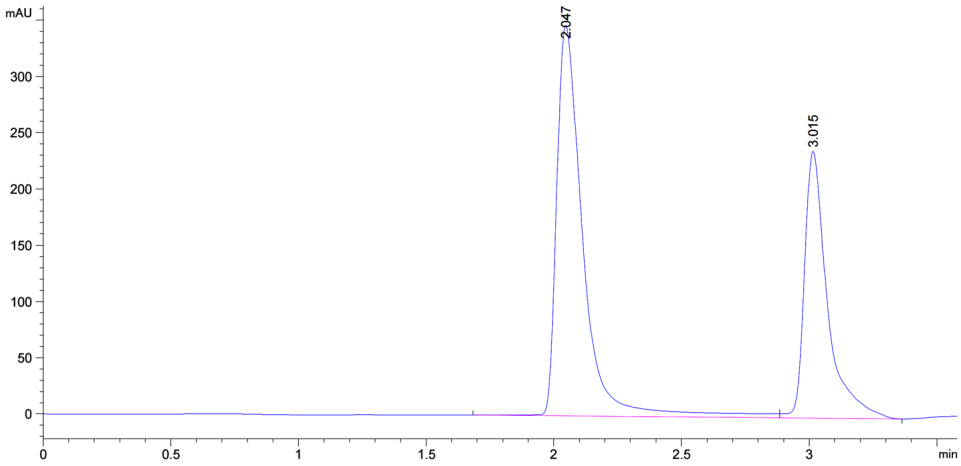

Fig. 6 HPLC chromatogram of mixture of camylofin-2HCl (90 µg/mL) and paracetamol (10 µg/mL)

Table 1 Different development systems tested for the separation of camylofin-2HCl, its acidic degradate and paracetamol

	Mobile phase component	Ratio of component, by volume
I	Acetone/glacial acetic acid	5:5
II	Acetone/methanol/ammonia	8:2:0.1, 6:4:0.1
III	Acetone/methanol/acetic	6.5:3.5:2.5
IV	Hexane/methanol	5:2,6:2,7:1.5
V	Hexane/methanol/ammonia	8:4:0.2, 5:1:0.1,7:1.5:0.1
VI	Hexane/methanol/glacial acetic acid	10:2:0.1
VII	Hexane/ethyl acetate	5:1
VIII	Methanol/ethyl acetate/toluene	1:2:2
IX	hexane/propanol	8:1.5
X	Acetone/ethyl acetate	6:2
XI	Acetone/ethyl acetate/methanol	6:2:2
XII	Chloroform/methanol/ammonia	6:2:0.1,6:4:0.1, 8:4:0.1
XIII	Chloroform/methanol/ammonia/hexane.	8:4:0.1:1
XIV	Chloroform/methanol/ammonia/toluene	8:4:0.1:1
XV	Chloroform/acetone/ammonia	8:2:0.1
XVI	Chloroform/methanol/acetone/ammonia	8:2:2:0.1

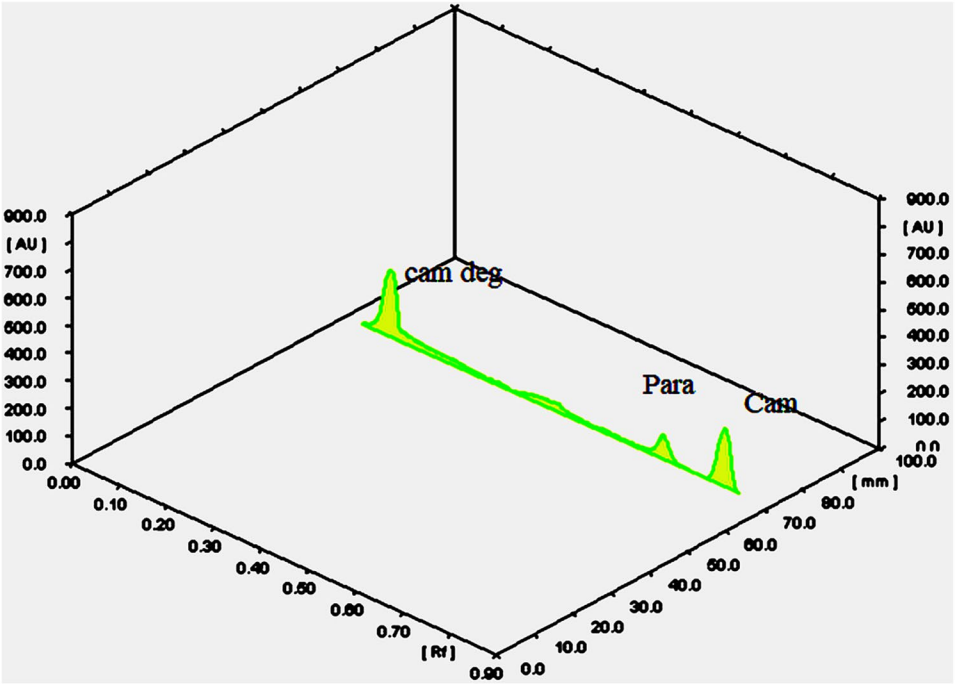

Fig. 7 Densitometric chromatogram of mixture of camylofin-2HCl (20 µg/band), the acid degradate of camylofin (20 µg/band) and paracetamol (0.1 µg/band) at 254 nm

peak characteristics was achieved using a mobile phase of chloroform/methanol/acetone/conc. ammonia (8:2:2:0.1, by volume). The Rf values were 0.84, 0.13 and 0.72 for camylofin-2HCl, its degradate and paracetamol, respectively, by detection at 254 nm (Fig. 7). TLC method was considered stability-indicating method with respect to camylofin-2HCl.

System suitability

The results are shown in Tables 2, 3 indicating that both HPLC and TLC systems are suitable.

Table 2 System suitability data for HPLC for the determination of camylofin-2HCl and paracetamol

Parameters	Obtained value		Reference value [40]
	Camylofin	Paracetamol	
	Rt = 2.047	Rt = 3.015	
Capacity factor (K')	0.9	1.7	0.5–10 is acceptable
Selectivity factor (α)	1.47		>1
Resolution factor (R)	6.13		$R>1.5$
Tailing factor (T)	1.704	1.872	$T=1$ for typical peaks
Number of plates (N)	2283	6942	The higher the value, the more efficient the column

Method validation

The two methods were validated according to ICH guideline [42].

(a) Linearity

Under the above-optimized experimental conditions, linear relationship was found between the peak areas and the corresponding drug concentration over the ranges of 3.0–90.0 and 10.0–95.0 µg/mL for HPLC method and 5.0–40.0, 0.10–0.50 µg/band for TLC method for camylofin-2HCl and paracetamol, respectively. The high r^2 values (0.9993–0.9999) indicate good linearities. Regression parameters are summarized in Table 4. The proposed method is more sensitive than the two reported ones, which measured camylofin in the concentration ranges of 20.0–80.0 µg/mL [37] and 25–75 mg/mL [38].

(b) Accuracy and precision

Accuracy was found to be 100.67% and 100.59% for camylofin-2HCl and 98.47% and 99.08% for paracetamol for HPLC and TLC methods, respectively. The results are summarized in Table 4.

(c) Selectivity

HPLC selectivity was determined by analyzing laboratory-prepared mixtures of camylofin-2HCl and paracetamol

Table 3 System suitability data for the proposed TLC-densitometric method for the determination of camylofin-2HCl and paracetamol

Parameter	Camylofin-2HCl	Paracetamol	Ref value [41]
Retardation factor (RF)	0.84	0.72	
Capacity factor (K)	0.19	0.39	The higher the value, the longer the retention factor
Tailing factor	1	1	= 1 for typical symmetric peak
Number of theoretical plates (*N*)	864.36	501.76	Increase with efficiency of separation
Height HETP(H)	0.008	0.014	The smaller the value, the higher the efficiency

in different ratios, and it was valid for simultaneous determination of both drugs with mean recoveries of 100.38% ± 1.29 and 99.97% ± 1.59 for camylofin-2HCl and paracetamol, respectively (Table 5). Densitometric method was stability indicating for camylofin-2HCl so its selectivity was assessed by preparing mixtures containing different ratios of the camylofin-2HCl, its degradate and paracetamol with mean recoveries of 100.85 ± 1.44 and 99.82 ± 1.68 for both drugs, respectively (Table 5).

(d) Application to pharmaceutical formulation

The proposed methods were successfully applied for the determination of camylofin-2HCl and paracetamol in Anafortan® tablet indicating no interference by excipients or additives (Table 6). The results obtained by the two proposed methods were statistically compared with those obtained by a reported method [38] which involves HPLC determination of both drugs. No significant difference was observed

Table 4 Regression and assay validation parameters by the proposed methods

	Camylofin-2HCl		Paracetamol	
	HPLC	TLC*	HPLC	TLC
λ max (nm)	220	254	220	254
Linearity range	3.0–90.0 (µg/mL)	5.0–40.0 (µg/band)	10.0–95.0 (µg/mL)	0.1–0.5 (µg/band)
Slope	27.49	98.48	74.97	11,248
Intercept	15.63	222.45	706.8	650.8
Correlation coefficient (r^2)	0.9999	0.9993	0.9996	0.9994
*Accuracy (mean% ± SD)	100.67 ± 1.04	100.59 ± 0.92	98.47 ± 0.60	99.08 ± 2.05
**Precision (RSD%)*				
Intraday	0.66	0.85	0.67	0.65
Interday	1.46	1.16	1.47	1.34
Standard addition	100.03 ± 1.98	100.28 ± 1.69	98.69 ± 1.25	100.44 ± 0.72

*Average of nine determinations

Table 5 Determination of camylofin-2HCl and paracetamol in their laboratory-prepared mixtures using the proposed methods

HPLC			TLC			
camylofin/paracetamol (µg/mL)	*R* % of camylofin	*R* % of paracetamol	(A:B:C) (µg/band)	% of acidic degradate	*R* % of camylofin	*R* % of paracetamol
50:50	100.48	98.22	35:5:0.1	12.5	101.63	99.57
5:95	98.92	100.26	30:10:0.3	25	99.9	98.69
90:10	99.23	98.46	25:15:0.4	37.5	98.05	100.81
30:70	101.46	101.84	20:20:0.1	50	101.53	101.96
70:30	101.82	101.06	15:25:0.3	62.5	101.73	101.64
–	–	–	10:30:0.5	75	102.23	98.32
–	–	–	5:35:0.5	87.5	100.88	97.72
Mean% ± SD	100.38 ± 1.29	99.97 ± 1.59	Mean % ± SD		100.85 ± 1.44	99.82 ± 1.68

R % is the recovery %

(A:B:C) is camylofin/acidic degradates/paracetamol

Table 6 Determination of camylofin-2HCl and paracetamol in pharmaceutical formulation by the proposed methods and in comparison with the reported method [38]

	Camylofin-2HCl			Paracetamol		
	HPLC	TLC	Reported [38]	HPLC	TLC	Reported [38]
Mean% ± SD	101.42 ± 1.32	100.46 ± 1.55	99.55 ± 1.28	100.10 ± 0.86	100.53 ± 1.63	99.52 ± 1.13
Variance	1.73	2.41	1.65	0.74	2.67	1.28
Number	5	5	5	5	5	5
t test	2.28	1.02	–	0.91	1.13	–
f-test	1.05	1.46	–	0.58	2.08	–

The theoretical *t*- and *F*-values at $P = 0.05$ were 2.31 and 6.3, respectively

Reported [38]—HPLC method for simultaneous determination of camylofin and paracetamol solution using water C18 column with UV detection at 220 nm and a mobile phase consisting of 0.05% trifluoroacetic acid in water and 0.05% trifluoroacetic acid in acetonitrile (50:50 v/v), at a flow rate of 1.0 mL/min

between the two methods as indicated by t test and f ratio results (Table 6).

Conclusion

The first development of stability-indicating TLC densitometric method for the determination of paracetamol and camylofin-2HCl in the presence of camylofin acidic degradate is produced in this work with full identification of camylofin acid degradate by IR and ^{1}HNMR and a suggestion of degradation pathway which is considered as an advantage over all the published stability methods. Also this work introduces a simple, sensitive and accurate HPLC method for the determination of camylofin and paracetamol mixture with high sensitivity over all published HPLC methods [37, 38].

Compliance with ethical standards

Conflicts of interest The authors declare no conflict of interest.

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