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DEVELOPMENT AND VALIDATION OF RP-HPLC AND TLC METHODS FOR SIMULTANEOUS ESTIMATION OF PARACETAMOL, THIOCOLCHICOSIDE AND ACECLOFENAC IN BULK AND COMBINED PHARMACEUTICAL DOSAGE FORM

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ABSTRACT

Two accurate, precise, and sensitive methods were developed for the simultaneous determination of ternary mixtures containing paracetamol (PAR), thiocolchicoside (THIO) and aceclofenac (ACE) in bulk powder and combined dosage form without prior separation. The first method is RP-HPLC which depended on isocratic elution using an agilent C18 column and mobile phase consisting of ammonium acetate buffer (pH 7) – methanol – acetonitrile--triethylamine (50:30:20:0.1, v/v) pumped at flow rate of 1 ml/min with UV detection at 258 nm. The second method is based on TLC separation of the three drugs followed by densitometric measurements of their spots at 248, 380 and 275 nm for PAR, THIO and ACE, respectively. The separation was carried out on silica gel 60 F254 using ethyl acetate-toluene-methanol –glacial acetic acid (2:2:1:20.25 v/v/v/v) as a developing system. The suggested methods were tested using laboratory-prepared mixtures and were successfully applied for the analysis of pharmaceutical preparations. The methods retained their accuracy and precision when the standard addition technique was applied. HPLC method was applied over the concentration range (1-120 µg/ml) for Paracetamol, (0.8-60 µg/ml) for Thiocolchicoside and (10-80 µg/ml) for Aceclofenac while densitometric method was linear over the concentration range (200-10000ng/spot) Paracetamol, (10-1000ng/spot) for Thiocolchicoside and (100-10000ng/spot) for Aceclofenac, respectively. The developed RP-HPLC and TLC densitometric methods are rapid, sensitive and accurate and can be applied in quality control laboratories for routine determination of Paracetamol, Thiocolchicoside and Aceclofenac simultaneously in their ternary mixture without prior separation.

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INTRODUCTION

Paracetamol (PAR) is chemically designed as N-acetyl-p-aminophenol, Fig1a. It inhibits prostaglandin synthesis in the Central Nervous System (CNS). This explains its antipyretic and analgesic properties. It has a weak anti-inflammatory activity due to its peripheral inactivation [1].

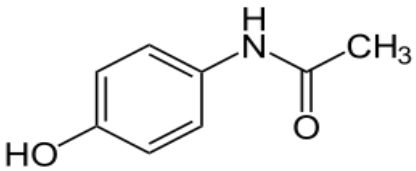

Fig 1a: Paracetamol.

Thiocolchicoside (THIO) is chemically designed as N-[3-(B-D-glucopyranoxyloxy)-5,6,7,9-tetrahydro-1,2-dimethoxy-10-(methylthio)-9-oxobenzo[a] heptalen-7yl] acetamide, Fig 1b. It acts on the muscular contracture by activating the GABA-inhibitory pathways thereby acting as a potent muscle relaxant [2].

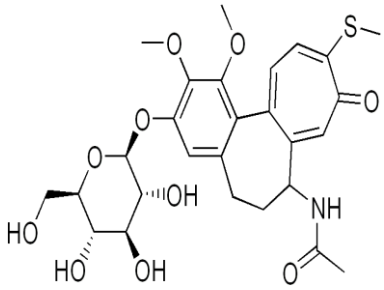

Fig 1b: Thiocolchicoside.

Aceclofenac (ACE) is chemically designed as [*o*-(2,6- Dichloroanilino)phenyl]acetate glycolic acid ester; 2-(2,6-Dichloroanalino) phenylacetoxyacetic acid, Fig 1c. It is a non-steroidal anti-inflammatory drug (NSAID) analog of Diclofenac, it inhibits both cyclo-oxygenase-1 (COX-1) and cyclo-oxygenase-2 (COX-2), it is used in the management of osteoarthritis, rheumatoid arthritis, and ankylosing spondylitis [3].

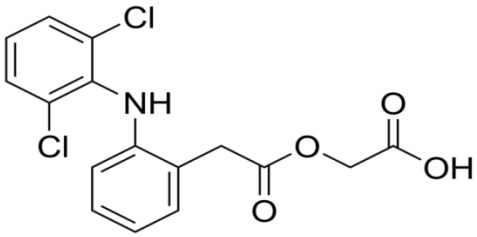

Fig 1c: Aceclofenac.

Tablets formulations containing mixture of the three drugs are used as analgesic and anti-inflammatory combination recommended to relieve some painful rheumatic symptoms such as: osteoarthritis, rheumatoid arthritis and ankylosing spondylitis.
The literature review revealed that many analytical methods were developed for the determination of paracetamol, alone or in combined mixtures. These methods include HPLC [4-7], HPTLC [8-10], spectrophotometry [11,12], and electrochemical [13].

Few methods were reported for the determination of Thiocolchicoside alone or in combined pharmaceutical formulations mixtures; they include HPLC [14-16], HTPLC [17,18] and spectrophotometric [19-22]. For aceclofenac many methods were available for its analysis, individually or in combined pharmaceutical formulations mixtures, they include: HPLC [23-25], HPTLC [26], spectrophotometric [27-32] and electrochemical [33,34].

For the simultaneous determination of aceclofenac with either thiocolchicoside or paracetamol, few methods were reported, including HPLC [35,36] and spectrophotometric [37-40] .

A thorough literature survey revealed that very few studies were available for the simultaneous determination of the three drugs in their ternary combination mixture. Only two HPTLC [41, 42], two HPLC [43, 44] and one spectrophotometric [45] methods were reported. The purpose of the present study is the development of new simple and sensitive HPLC and TLC- densitometric methods for the assay of paracetamol, thiocolchicoside and aceclofenac in ternary pharmaceutical combination. The HPLC method depends on simple isocratic separation and the TLC method aims to detect all the three drugs in very low concentrations, especially for thiocolchicoside (which is present in low concentration in the pharmaceutical formulation)

EXPERIMENTAL

Samples

Pure samples

Paracetamol was kindly supplied by Eva Company – Egypt and its purity was 100%, thiocolchicoside was supplied by Memphis Company – Egypt, its purity was 100.9% while aceclofenac was supplied by Amoun Company-Egypt and its purity was 99.36%.

Marketed samples

Acenac MR 8® labeled to contain 325mg PAR, 100mg ACE and 8mg THIO per tablet was supplied by Medley Pharmaceuticals LTD. (India). Batch No. 15LGKT010

Reagents

Acetonitrile and methanol, HPLC grade (Fisher scientific, UK). All other chemicals are of analytical reagent grade: ammonium acetate (Loba chemie, India), triethylamine 99% extra pure (Loba chemie, India), absolute methanol (Sigma Aldrich, Germany), glacial acetic acid (Adwic,Egypt), ethyl acetate (El- Nasr Company, Egypt), toluene (El- Nasr Company, Egypt) and high pure water was prepared by using Milli-Q purification system.

Instrumentation and chromatographic conditions

For HPLC Method

The HPLC system, used for method development and method validation was Agilent Technologies 1200 series liquid chromatographic RRHT (Rapid Resolution High Throughput) system comprising of quaternary gradient pump, degasser, thermostat, variable wavelength UV detector and a manual injector Rheodyne valve with 20 µl fixed loop. Separation was carried out on C18 column (250 mm ×3.2 mm, 5 µm) – nucleosil® and data was analyzed using Chemstation software. A mobile phase consisting of 40 mM ammonium acetate buffer (pH 7): methanol: acetonitrile: triethylamine (50:30:20:0.1, *v/v/v*), at flow rate 1 ml/min was used, and the UV detection was carried out at 258 nm.

For TLC - Densitometric Method

Thin layer chromatographic aluminium plates pre-coated with silica gel 60 F_{254} 20×10 cm (Merck, Germany) were employed as stationary phase. Micro syringe 0.1-100 µl capacity was used. A UV lamp – short wavelength 254 nm was employed for detection of bands. Densitometric scanning was performed on Camag TLC scanner III using Camag auto sampler; the scanning speed was at 20 mm s^{-1}, baseline correction was used. Plates were developed at ambient temperature. A mobile phase composed of ethyl acetate: toluene: methanol: glacial acetic acid (2:2:1:0.25, v/v/v/v) was used with saturation time 15 min and UV detection at 380 nm, 275 nm and 248 nm for THIO, PAR and ACE, respectively.

Preparation of Standard Solutions

Standard Stock solutions

An accurately weighed 50 mg of each PAR, THIO and ACE (HPLC method) and 100 mg of each PAR, THIO and ACE, (TLC-densitometric method) were transferred separately into 50 ml volumetric flasks, sonicated and completed to volume with methanol producing a final concentration of (1 mg/ml) for each PAR, THIO and ACE (HPLC method) and (2mg /ml) for each PAR, THIO and ACE (TLC-densitometric method).

Standard working solutions

A 200 µg/ml working solutions PAR, THIO or ACE were prepared by appropriate dilution of the corresponding stock solutions in mobile phase (HPLC method) or in methanol (TLC method).

Laboratory prepared mixtures containing different ratios of PAR, THIO and ACE

HPLC method

Into a series of 10 ml volumetric flasks, different aliquots of PAR, THIO and ACE working solutions containing (500-1150 µg) of Paracetamol (20-50 µg) of Thiocolchicoside, and (150-350 µg) of Aceclofenac were transferred, mixed well then completed to volume with the mobile phase to give mixtures of the three drugs.

TLC method

Into a series of 10 ml volumetric flasks, different aliquots of PAR, THIO and ACE stock solutions containing (1.3-9 mg), (0.032-0.5 mg) and (0.04-6.5 mg), respectively, were transferred, mixed well then completed to volume with methanol to give mixtures of the three drugs.

General Procedures and Calibration curves

For HPLC Method

Accurate aliquots of standard working solutions were transferred into three series of 10 ml volumetric flasks to obtain solutions of 1-120 µg/ml PAR, 0.8-60 µg/ml THIO and 10-80 µg/ml ACE in mobile phase. A volume of 20 µl of each solution was injected in triplicates.

The chromatographic conditions including the mobile phase at a flow rate 1 ml/min and detection at 258 nm were followed. A calibration curve was obtained by plotting area under the peak (AUP) against the corresponding concentration (C) and the regression equation was computed and recorded.

For TLC-Densitometric Method

Accurate aliquots of standard working methanolic solutions of PAR, THIO and ACE were transferred into three 10 ml volumetric flasks and diluted to volume with methanol to get 1000 ng/µl, 40 ng/µl and 100 ng/µl, respectively, 0.2-100 µl solutions were spotted on TLC plates to obtain the concentration range of 200-10000 ng/ spot for PAR, 10-1000 ng/spot for THIO and 100-10000 ng/ spot for ACE following the previously mentioned chromatographic conditions. A calibration curve was obtained by plotting area under the curves (AUC) against the corresponding concentration (C) and the regression equation was computed and recorded.

Assay of Acenac MR® Tablets

HPLC method

An accurate weight of the powdered tablets equivalent to (325mg) of PAR, (8 mg) of THIO and (100 mg) of ACE was transferred into a 100 ml volumetric flask, diluted with 50 ml methanol, sonicated for about 15 min, completed to volume with methanol. This solution was filtered using 0.45 µm membrane filter and the first few milliliters were discarded to produce tablet stock solution of (3.25 mg/ml) of PAR, (0.08 mg/ml) of THIO and (1 mg/ml) of ACE.

An aliquot of (20 ml) was further diluted to 100 ml with methanol giving a working solution of (650 µg/ml) of PAR, (16 µg/ml) of THIO and (200 µg/ml) of ACE. Different aliquots of the tablet working solution in the range of (500-1000 µg) of PAR, (8-30 µg) of THIO and (100-350 µg) of ACE were transferred into a series of 10 ml volumetric flasks and completed to volume with the mobile phase.

The method was performed as mentioned under general procedure starting from "A volume of 20µL". This experiment was repeated with addition of known amount of drug standard to known concentrations of preanalyzed tablets which is called "the standard addition technique" and regression equations (1-3), were used to calculate the recovered concentrations of the labeled and the added standard of PAR, THIO and ACE.

TLC method

An accurate weight of the powdered tablets equivalent to (162.5 mg) of PAR, (4 mg) of THIO and (50 mg) of ACE was transferred into a 100 ml volumetric flask. The drug was extracted with 25 ml methanol using sonication for 15 min, completed to volume with methanol and filtered using 0.45 µm membrane filter. The first few milliliters were discarded providing tablets stock solutions of (1.625 mg/ml) of PAR, (0.04 mg/ml) of THIO and (0.5 mg/ml) of ACE, then accurate aliquots (1-6 ml) of each stock tablets solution were transferred to a series of 10 ml volumetric flasks and completed to volume with methanol. Then a volume of 10 µl of these solutions were spotted on TLC plate producing a final concentration of (1625-9750 ng/spot), (40-240 ng/spot) and (500-3000 ng/spot) of PAR, THIO and ACE, respectively. The TLC plate was developed in optimized mobile phase and the analysis was repeated in triplicate.

This experiment was repeated with addition of known amount of standard and regression equations (4-6), were used to calculate the recovered concentrations of both the labeled and the added standard of PAR, THIO and ACE.

Statistical analysis

Statistical comparison between the results obtained by the developed HPLC and TLC- Densitometric methods and the reported one [41] was performed. At 95% confidence limit (P=0.05), T and F values were calculated and compared to tabulated values.

RESULT AND DISCUSSION

The aim of this work was to develop new sensitive, selective, accurate and precise RP-HPLC and TLC-Densitometric methods for the determination of PAR, THIO and ACE in their ternary mixture in pure form and pharmaceutical preparations.

Method Development

For HPLC Method

Optimization of chromatographic conditions

The optimization process of the proposed method was carried out; many trials have been performed to achieve the best separation between PAR, THIO and ACE. Different mobile phase compositions containing different ratios of aqueous and organic phases were attempted in an isocratic mode.

Initial efforts to develop a separation method using an isocratic elution system with acetonitrile-based mobile phase with a ratios of buffer: acetonitrile [(20: 80), (30: 70), (40: 60), (50: 50) and (60: 40), *v/v*].

All those trials failed; the peaks of components obtained couldn't be separated efficiently and led to early elution of all the mixture components which resulted in overlapping of the PAR, THIO and ACE. Good resolution between peaks was obtained with addition of methanol to mobile phase at lower acetonitrile ratios. The presence of methanol and few triethyl amine in the mobile phase led to better separation of the compounds peaks with reasonable retention times. Separation of the three drugs was best obtained at a ratio of buffer: methanol: acetonitrile: triethylamine (50: 30:20:0.1, *v/v/v*) at 40°C. Changing the pH of the mobile phase had an effect on the resolution between PAR, THIO and ACE. A mobile phase of (40 mM) ammonium acetate buffer pH 7 was the most appropriate one as it resolved the overlapping between PAR and THIO. Different flow rates were tested and a flow rate of 1 ml/min was optimal for good separation. Good chromatographic separation of the examined drugs in their ternary mixtures could thus be achieved by using C18 column (250 mm ×3.2 mm, 5 µm) – nucleosil® with a mobile phase consisting of 40 mM Ammonium acetate buffer (pH 7): methanol: acetonitrile: triethylamine (50:30:20:0.1, v/v), respectively, followed by UV detection at 258 nm with flow rate 1 ml/min, The retention times were 3.57, 4.55 and 9.81 min for PAR, THIO and ACE, respectively (Fig. 2a).

System suitability tests were used to verify that the conditions of the chromatographic system were adequate for the resolution and analysis [46]. The parameters of these tests are tailing of chromatographic peak (T), column efficiency (number of theoretical plates) (N), capacity factor (K'), peak resolution factor (R), selectivity factor (α) and repeatability as % R.S.D of retention times (t_R) and peak areas. All the measured parameters are within the recommended limits (Table 1).

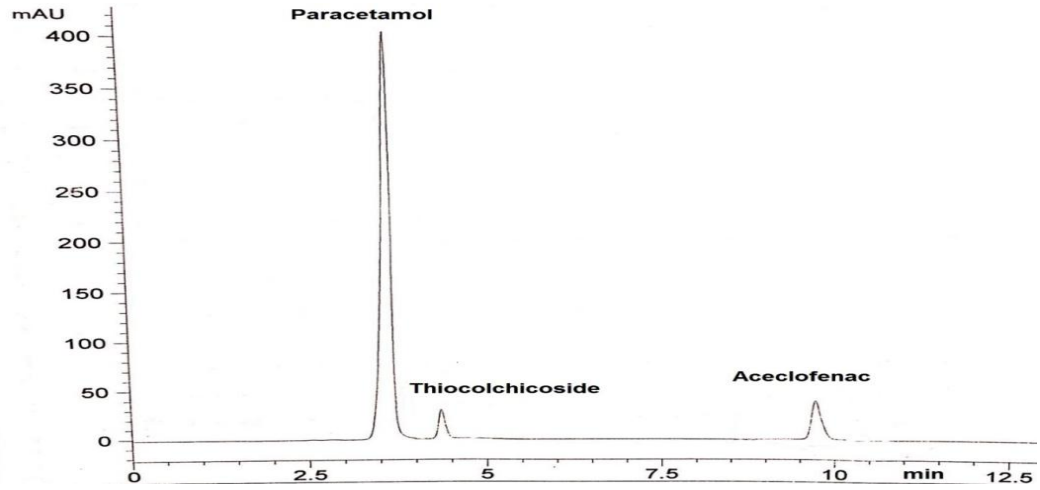

Fig.2a HPLC chromatogram of 20 µl injector of ternary mixture of PAR (97.5 µg/ml) (R_t = 3.57 min), THIO (2.4 µg/ml) (R_t = 4.55 min) and ACE (30 µg/ml) (R_t = 9.81 min).

Table 1: System suitability results of the proposed HPLC method.

Item	Paracetamol	Thiocolchicoside	Aceclofenac
N	4247	4216	4298
R		2.32	9.56
T	1.21	1.10	1.16
α		1.28	2.2
K'	1.34	2.88	5.58
R.S.D.%			
Retention time	0.532	0.205	0.085
Peak area	0.156	0.135	0.285

For TLC-Densitometric Method

Several trials were done to select a developing system which can separate the examined drugs. Initially, a mixture of ethyl acetate: toluene: methanol: glacial acetic acid in the ratio of (1: 1: 1: 0.5, v/v) was tried for the examined drugs simultaneously. The spots of components obtained couldn't be separated efficiently resulted in overlapping of the PAR, THIO and ACE, so decreasing the polarity of the system by decreasing the volume of methanol used in comparison to volumes of toluene and ethyl acetate was needed. The optimum separation (Fig. 2b) was obtained using developing system ethyl acetate: toluene: methanol: glacial acetic acid (2:2:1:0.25, v/v/v/v). Linear ascending development was carried out in a 20 cm × 20 cm glass chamber saturated with the developing system. The saturation time was 15 min at room temperature. Separation was carried out on silica gel plates 20 cm×10 cm followed by densitometric measurement at 248 nm, 380 nm and 275 nm.

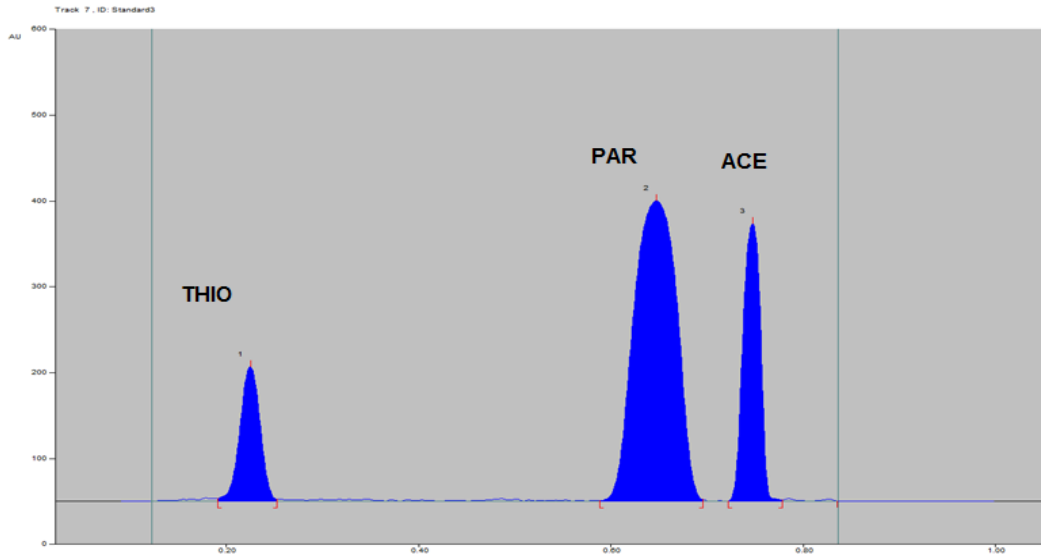

Fig. 2b ***TLC chromatogram of THIO (40 ng /spot) (R_f = 0.22), PAR (1625 ng/spot) (R_f = 0.65) and ACE (500 ng/spot) (R_f = 0.76) at 380 nm, 248 nm and 275 nm, respectively, developing system*** **ethyl acetate: toluene: methanol: glacial acetic acid (2:2:1:0.25, v/v/v/v).**

Method Validation

Both methods were validated according to ICH guidelines [47].

Linearity

HPLC method

A linear relationship was obtained between area under the curve (AUC) and the corresponding concentrations (C) over the concentration range (1-120 µg/ml) for PAR, (0.8-60 µg/ml) for THIO and (10-80µg/ml) for ACE, respectively. The following regression equations were computed and found to be:

$$AUP_{258\ nm} = 82.46\ C_{paracetamol} + 16.72 \qquad r^2 = 1 \qquad \text{-------} \qquad (1)$$

$$AUP_{258\ nm} = 61.44\ C_{Thiocolchicoside} + 17.62 \qquad r^2 = 0.9998 \qquad \text{-------} \qquad (2)$$

$$AUP_{258\ nm} = 24.82\ C_{Aceclofenac} + 2.925 \qquad r^2 = 0.9996 \qquad \text{-------} \qquad (3)$$

Where:

$AUP_{258\ nm}$: is the area under the peak at 258 nm.

C: is the concentration of drug (µg/ml).

r^2: is the regression coefficient.

TLC method

A linear relationship was obtained between area under the curve (AUC) and the corresponding concentrations (C) over the concentration range (200-10000ng/spot) PAR, (10-1000ng/spot) for THIO and (100-10000ng/spot) for ACE, respectively, and the following regression equations were computed and found to be:

$$A_{248\ nm} = 1.044\ C_{Paracetamol} + 804 \qquad r^2 = 0.9996 \qquad \text{----------} \qquad (4)$$

$$A_{380\ nm} = 16.83\ C_{Thiocolchicoside} - 55.10 \qquad r^2 = 0.9995 \qquad \text{----------} \qquad (5)$$

$$A_{275\ nm} = 2.128\ C_{Aceclofenac} + 755.6 \qquad r^2 = 0.9997 \qquad \text{----------} \qquad (6)$$

Where:

$A_{248\ nm}$: is the Area under the peak at 248 nm.
$A_{380\ nm}$: is is the Area under the peak at 380 nm.
$A_{275\ nm}$: is the Area under the peak at 275 nm.
C: is the concentration of drug (ng/spot).
r^2: is the regression coefficient.
For all the proposed methods, the intermediate precision and repeatability, the assay parameters of the regression equations and the concentration ranges are shown in Tables 2a &b.

Table 2a: Validation parameters and results obtained by the proposed RP-HPLC method for the determination of Paracetamol, Thiocolchicoside and Aceclofenac:

Item	Paracetamol	Thiocolchicoside	Aceclofenac
Retention time (R_t) (min)	3.57	4.55	9.81
λ of detection (nm)	258	258	258
Range of linearity	1-120 µg/ml	0.8-60 µg/ml	10-80 µg/ml
Regression equation	82.46 C_{PAR} + 16.72	61.44C_{THIO} + 17.62	24.82C_{ACE} + 2.925
Regression coefficient (r^2)	1	0.9998	0.9996
LOD (µg/ml)	0.199	0.187	2.315
LOQ (µg/ml)	0.607	0.560	7.149
*Intra-day % R.S.D.	0.04-0.09	0.64-0.94	0.17-0.20
**Inter-day % R.S.D.	0.05-0.07	0.58-1.02	0.05-0.51
mean recovery% ± RSD			
Drug in lab prepared mix	99.46 ± 0.90	100.63 ± 0.69	100.00 ±0.61
Drug added to dosage form	99.92 ± 0.72	101.15 ± 0.25	99.78 ± 0.89
Drug in dosage form	99.25 ± 0.39	101.54 ± 0.81	99.64 ± 0.96

*The intra-day (*n* = 3), average of three concentrations (48, 60 and 72µg/ml) of Paracetamol, (20, 25, 30µg/ml) of Thiocolchicoside and (36,45and 54 µg/ml) of aceclofenac and repeated three times within the day.
** The inter-day (*n* = 3), average of three concentrations (48, 60 and 72µg/ml) of Paracetamol, (20, 25 and 30µg/ml) of Thiocolchicoside and (36, 45and 54 µg/ml) of aceclofenac and repeated three times in three successive days.

Table 2b: Validation parameters and results obtained by the proposed TLC-Densitometric method for the simultaneous determination of Paracetamol, Thiocolchicoside and Aceclofenac:

Item	Paracetamol	Thiocolchicoside	Aceclofenac
Rf (retardation factor)	0.65	0.22	0.76
λ of detection (nm)	248	382	275
Range of linearity	200-10000 ng/spot	10-1000 ng/spot	100-10000 ng/spot
Regression equation	1.044 C_{PARl}+ 804	16.83C_{THIO} – 55.10	2.128C_{ACE}+755.60
Regression coefficient (r^2)	0.9996	0.9995	0.9997
LOD (ng/spot)	35.882	0.756	17.285
LOQ (ng/spot)	108.732	2.291	51.872
*Intra-day % R.S.D.	0.15-0.57	0.37-0.85	0.05-0.54
**Inter-day % R.S.D.	0.46-0.70	0.38-0.70	0.60-0.77
Recovery ± RSD %			
Drug in lab prepared mix	99.94 ± 0.46	100.92 ± 0.76	99.31 ± 0.75
Drug added to dosage form	100.29 ± 0.50	100.90 ± 0.55	99.37 ± 0.58
Drug in dosage form	100.38 ± 0.42	101.39 ± 0.84	99.34± 0.55

*The intra-day (*n* = 3), average of three concentrations (4000, 5000 and 6000 ng/spot) of Paracetamol, (400, 500 and 600 ng/spot) of Thiocolchicoside and (4000, 5000 and 6000 ng/spot) of Aceclofenac and repeated three times within the day.
** The inter-day (*n* = 3), average of three concentrations (4000, 5000 and 6000 ng/spot) of Paracetamol, (400, 500 and 600 ng/spot) of Thiocolchicoside and (4000, 5000 and 6000 ng/spot) of Aceclofenac and repeated three times in three successive days.

Accuracy and methods applications
(Laboratory prepared mixtures and standard addition technique)
HPLC Method

The accuracy was estimated by applying the regression equations obtained for the determination of each PAR, THIO and ACE in pure form (in lab prepared mixtures) on the response of concentrations of (500-1150 µg/ml) for PAR, (20-50 µg/ml) THIO and (150-350 µg/ml) for ACE. The mean percentage recoveries ± RSD% were calculated and found to be 99.46 ± 0.90, 100.63 ± 0.69 and 100.00 ± 0.61 for PAR, THIO and ACE respectively, as shown in Table (3a).

The accuracy of the method was also confirmed by recovery studies from Acenac MR8® tablet at different levels of standard additions; the percentage recoveries ± RSD% of added standard were 99.92 ± 0.72, 101.15 ± 0.25 and 99.78 ± 0.89 for PAR, THIO and ACE, respectively, Table (4a,b,c).

TLC Method

The accuracy was estimated by applying the regression equations obtained for the determination of each PAR, THIO and ACE in pure form (in lab mixtures) on the response of concentrations of (200-10000 ng/spot) for PAR (10-1000 ng/spot) for THIO and (100-10000 ng/spot) for ACE. The mean percentage recoveries ± RSD% were calculated and found to be 99.94 ± 0.46, 100.92 ± 0.76 and 99.31 ± 0.75 for PAR, THIO and ACE respectively, as shown in Table (3b).

The accuracy of the method was also confirmed by recovery studies from Acenac MR8® tablet at different levels of standard additions; the percentage recoveries ± RSD% of added standard were 100.29 ± 0.50, 100.90 ± 0.55 and 99.37 ± 0.58 for PAR, THIO and ACE, respectively (Table 5 a,b,c).

Table 3a: Determination of Paracetamol, Thiocolchicoside and Aceclofenac in laboratory prepared mixtures using the proposed RP-HPLC method:

Mix	Taken (µg/ml)			AUP			Found (µg/ml)			Recovery %		
No.	PAR	THIO	ACE	PAR	THIO	ACE	PAR	THIO	ACE	PAR	THIO	ACE
1	50	2	15	4138.84	139.16	376.57	49.99	1.98	15.05	99.98	99.00	100.33
2	65	3	20	5384.35	199.86	498.15	65.09	2.97	19.95	100.14	99.00	99.75
3	90	4	25	7300.36	262.93	628.38	88.33	4.01	25.20	98.14	100.25	100.80
4	97.5	2.4	30	8067.36	165.46	756.87	97.63	2.41	30.38	100.13	100.42	101.27
5	115	5	35	9397.38	324.55	880.84	113.76	5	35.37	98.92	100.00	101.06
								Mean		99.46	99.73	100.64
								*Mean x *potency/100*		99.46	100.63	100.00
								S.D±		0.90	0.69	0.61
								RSD%		0.90	0.69	0.61
								S.E±		0.40	0.31	0.27

*Potency of pure Paracetamol, Thiocolchicoside and Aceclofenac = 100%, 100.90% and 99.36%, respectively.

Table 3b: Determination of Paracetamol, Thiocolchicosideand Aceclofenac in laboratory prepared mixtures using the proposed TLC-Densitometric method:

Mix	Taken (ng/spot)			AUP			Found (ng/spot)			Recovery %		
No.	PAR	THIO	ACE	PAR	THIO	ACE	PAR	THIO	ACE	PAR	THIO	ACE
1	1300	32	40	2158.06	486.51	840.03	1296.99	32.18	39.68	99.77	100.56	99.20
2	2000	100	600	2892.02	1642.08	2021.32	2000.02	100.84	594.79	100.00	100.84	99.13
3	3200	320	2000	4127.49	5284.25	5036.11	3183.42	317.25	2011.52	99.48	99.14	100.58
4	4000	50	1200	4964.89	778.16	3298.69	3985.53	49.51	1195.06	99.64	99.02	99.59
5	7000	500	6500	8168.99	8399.06	14712.15	7054.59	502.33	6558.53	100.78	100.47	100.90
6	9000	150	3000	10196.35	2471.66	7157.28	8996.50	150.13	3008.31	99.96	100.09	100.28
								Mean		99.94	100.02	99.95
								Mean x potency/100		99.94	100.92	99.31
								S.D. ±		0.46	0.77	0.74
								RSD%		0.46	0.76	0.75
								S.E. ±		0.19	0.31	0.30

*Potency of pure Paracetamol, Thiocolchicoside and A ceclofenac = 100%, 100.90% and 99.36%, respectively.

Table 4a: Determination of Paracetamol in Acenac MR8 ® tablets applying standard addition technique using the proposed RP-HPLC method:

Paracetamol								
Taken (µg/ml)		AUP		Found (µg/ml)			Recovery %	
Tablet	Added	Tablet	Tablet & Added	Tablet	Tablet & Added	Added	Tablet	Added
50	30	4115.96	6564.72	49.71	79.41	29.70	99.42	99.00
60	25	4913.08	6961.35	59.38	84.22	24.84	98.97	99.36
70	20	5732.92	7386.88	69.32	89.38	20.06	99.03	100.30
80	15	6531.39	7780.99	79.00	94.16	15.16	98.75	101.06
90	10	7414.09	8237.48	89.71	99.69	9.98	99.68	99.80
100	5	8235.07	8647.55	99.67	104.67	5.00	99.67	100.00
					Mean		99.25	99.92
					Mean x potency/100		99.25	99.92
					S.D. ±		0.39	0.72
					RSD%		0.39	0.72
					S.E. ±		0.16	0.31

Table 4b: Determination of Thiocolchicoside in Acenac MR ® tablets applying standard addition technique using the proposed RP-HPLC method:

Thiocolchicoside								
Taken (µg/ml)		AUP		Found (µg/ml)			Recovery %	
Tablet	Added	Tablet	Tablet & Added	Tablet	Tablet & Added	Added	Tablet	Added
0.8	10	66.18	678.8	0.79	10.76	9.97	99.64	100.60
1	8	79.57	566.01	1.01	8.93	7.92	101.91	99.89
1.5	5	109.91	414.42	1.50	6.46	4.96	100.90	100.09
2	7	139.46	566.33	1.98	8.93	6.95	99.89	100.18
2.5	3	170.46	351.35	2.49	5.43	2.94	100.51	100.33
3	4	202.12	446.35	3.00	6.98	3.98	100.90	100.40
					Mean		100.63	100.25
					Mean x potency/100		101.54	101.15
					S.D. ±		0.82	0.25
					RSD%		0.81	0.25
					S.E. ±		0.33	0.10

Table 4c: Determination of Aceclofenac in Acenac MR8 ® tablets applying standard addition technique using the proposed RP-HPLC method:

Aceclofenac								
Taken (µg/ml)		AUP		Found (µg/ml)			Recovery %	
Tablet	Added	Tablet	Tablet & Added	Tablet	Tablet & Added	Added	Tablet	Added
10	8	253.55	451.65	10.10	18.08	7.98	101.00	99.75
15	5	372.73	497.13	14.90	19.91	5.01	99.33	100.20
20	10	498.51	750.71	19.97	30.13	10.16	99.85	101.60
25	15	632.76	1004.21	25.38	40.34	14.96	101.52	99.73
30	20	753.6	1257.3	30.24	50.54	20.30	100.80	101.50
35	25	864.56	1483.72	34.72	59.66	24.94	99.20	99.76
					Mean		100.28	100.42
					Mean x potency/100		99.64	99.78
					S.D. ±		0.96	0.89
					RSD%		0.96	0.89
					S.E. ±		0.39	0.36

Table 5a: Determination of Paracetamol in Acenac MR8® tablets applying standard addition technique using the proposed TLC-Densitometric method:

Paracetamol								
Taken (ng/spot)		**AUP**		**Found (ng/spot)**			**Recovery %**	
Tablet	**Added**	**Tablet**	**Tablet & Added**	**Tablet**	**Tablet & Added**	**Added**	**Tablet**	**Added**
1625	1000	2499.68	3543.17	1624.21	2623.73	999.52	99.95	99.95
3250	2000	4193.5	6300.12	3246.65	5264.48	2017.83	99.90	100.89
4875	3000	5942.93	9101.95	4922.35	7948.23	3025.88	100.97	100.86
6500	1500	7629.71	9192.13	6538.04	8034.61	1496.57	100.59	99.77
8125	500	9338.34	9862.42	8174.66	8676.65	501.99	100.61	100.40
9750	250	11008.17	11268.88	9774.11	10023.83	249.72	100.25	99.89
					Mean		100.38	100.29
					Mean x potency%		100.38	100.29
					S.D. ±		0.42	0.50
					RSD%		0.42	0.50
					S.E. ±		0.17	0.20

Table 5b: Determination of Thiocolchicoside in Acenac MR8® tablets applying standard addition technique using the proposed TLC-Densitometric method:

Thiocolchicoside								
Taken (ng/spot)		**AUP**		**Found (ng/spot)**			**Recovery %**	
Tablet	**Added**	**Tablet**	**Tablet & Added**	**Tablet**	**Tablet & Added**	**Added**	**Tablet**	**Added**
40	10	624.4	792.53	40.37	50.36	9.99	100.93	99.90
80	20	1300.51	1638.21	80.55	100.61	20.06	100.69	100.30
120	80	1959.35	3298.7	119.69	199.28	79.59	99.74	99.49
160	40	2679.74	3352.63	162.5	202.48	39.98	101.56	99.95
200	100	3285.06	4958.66	198.46	297.91	99.45	99.23	99.45
240	160	4013.96	6731.75	241.77	403.26	161.49	100.74	100.93
					Mean		100.48	100.00
					Mean x potency%		101.39	100.90
					S.D. ±		0.85	0.55
					RSD%		0.84	0.55
					S.E. ±		0.35	0.23

Table 5c: Determination of Aceclofenac in Acenac MR8® tablets applying standard addition technique using the proposed TLC-Densitometric method:

Aceclofenac								
Taken (ng/spot)		**AUP**		**Found (ng/spot)**			**Recovery %**	
Tablet	**Added**	**Tablet**	**Tablet & Added**	**Tablet**	**Tablet & Added**	**Added**	**Tablet**	**Added**
500	300	1810.29	2449.33	495.63	795.93	300.30	99.13	100.10
1000	700	2880.49	4380.52	998.54	1703.44	704.90	99.85	100.70
1500	500	3973.16	5044.03	1512.01	2015.24	503.23	100.80	100.65
2000	1500	5019.29	8189.13	2003.61	3493.2	1489.59	100.18	99.31
2500	1000	6081.2	8199.63	2502.63	3498.13	995.50	100.11	99.55
3000	2000	7128.73	11373.83	2994.89	4989.77	1994.88	99.83	99.74
					Mean		99.98	100.01
					Mean x potency%		99.34	99.37
					S.D. ±		0.55	0.58
					RSD%		0.55	0.58
					S.E. ±		0.22	0.24

Precision

Repeatability is a measurement tool to assess the precision of the developed method during the day, three concentrations (48, 60 and 72 µg/ml) of PAR, (4.8, 6 and 7.2 µg/ml) of THIO and (36, 45 and 54 µg/ml) of ACE for HPLC method and (4000, 5000 and 6000 ng/spot) of PAR, (160, 200 and 240 ng/spot) of THIO and (4000, 5000 and 6000 ng/spot) of ACE for TLC-Densitometric method were analyzed three times during the day (intra-day) and in three consecutive days for the inter-day precision (intermediate precision). Using the developed methods, good RSD% values were obtained ensuring the precision of the proposed method as shown in Table (2a,b).

Specificity

The specificity of the method was investigated by observing any interference from the common tablet excipients by comparing the chromatogram, Fig. 3a, (or densitogram for TLC method, Fig. 3b), of PAR, THIO and ACE in Acenac MR8 ® tablets with the chromatogram Fig. 2a, (or densitogram for TLC method, Fig. 2b) of PAR, THIO and ACE in pure substance. No additional peaks were observed, thus confirming the specificity of the method.

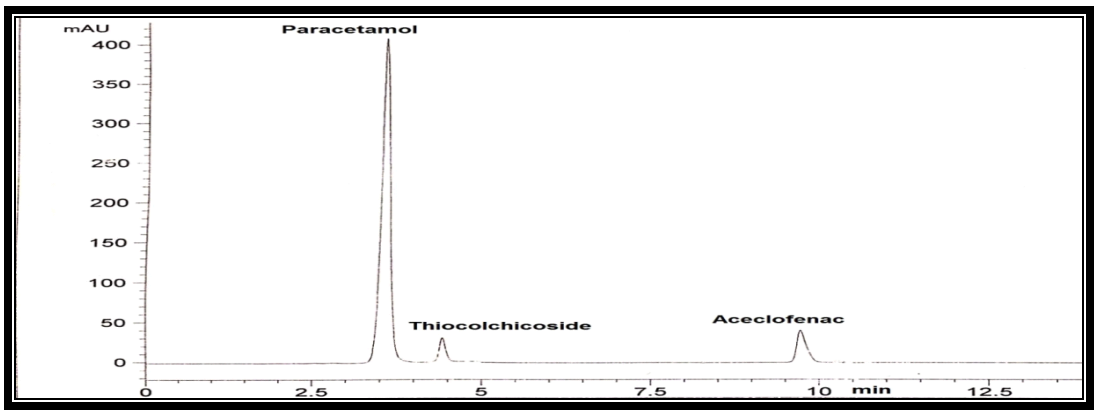

Fig.3a chromatogram of PAR (97.5 µg /ml), THIO (2.4µg/ml) and ACE (30 µg /ml) in Acenac MR ® HPLC tablets.

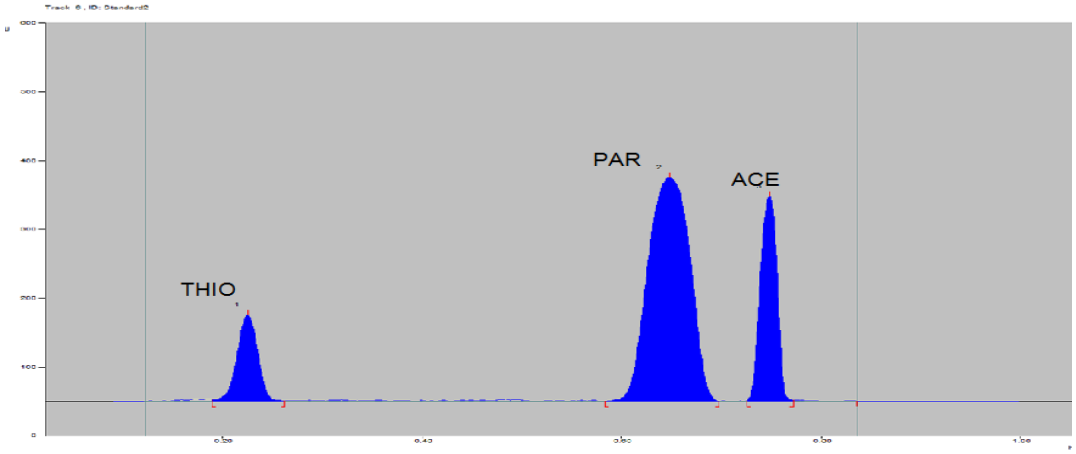

Fig.3b ***TLC chromatogram of Thiocolchicoside (40 ng /spot), Paracetamol (1625 ng/spot) and Aceclofenac (500 ng/spot) at 380 nm, 248 nm and 275 nm, respectively, in Acenac MR8® tablet.***

Robustness

Robustness of an analytical procedure is the capacity of the method to remain unaffected with small variations in method parameters and provides an indication of its reliability during normal usage [47].

For HPLC Method

Robustness was examined by evaluating the effect of small variations in different conditions. Variation of the pH of the mobile phase by ±0.2 units, flow rate of the mobile phase by ±0.2 ml/min and organic strength of the mobile phase by ±2% were evaluated. The most important parameter to be studied was the resolution factor between the three peaks of PAR, THIO and ACE. As can be seen from the results (Table 6) no significant change in resolution factors were obtained for all these variations indicating good robustness of the proposed LC method.

For TLC-Densitometric Method

Different parameters were studied to assess robustness including chamber saturation time and developing solvent composition. Minor change in the ratios of the developing system components by ± 1% and in the chamber saturation time by ± 5 min did not lead to significant changes in Rf values or peak areas of the examined drugs and the method was found to be robust.

Table (6): Robustness results of the proposed RP-HPLC method for the simultaneous determination of Paracetamol, Thiocolchicoside and Aceclofenac in ternary mixtures:

Parameter studied		Resolution factors		
		Paracetamol	Thiocolchicoside	Aceclofenac
pH of buffer	7.2	2.25	9.55	
	6.8	2.33	9.71	
Flow rate (ml/min)	1.2	1.95	8.31	
	0.8	2.42	10.21	
Acetonitrile %	22%	1.81	8.52	
	18%	2.35	10.12	
Resolution factors of the applied conditions		2.32	9.56	

Limit of Detection and Limit of Quantification

Limit of detection (LOD) is the concentration at which the signal to noise ratio is equal to 3:1 while limit of quantification (LOQ) is the concentration at which the signal to noise ratio is equal to 10:1, both were calculated based on standard deviation of the response and slope. Low values of both LOD and LOQ indicate a high sensitivity of the HPLC and TLC analytical methods (Tables 2 a&b).

Statistical analysis (application on pharmaceutical formulation)

The proposed HPLC and TLC techniques were successfully applied for analysis of paracetamol, thiocolchicoside and aceclofenac in Acenac tablets with good mean % recoveries (Tables 4a,b,c and 5a,b,c). Statistical comparison between the results obtained by the developed HPLC and TLC- Densitometric methods and the reported one [41], showed that the calculated t- and F-values at 95% confidence limit (P=0.05), were less than the tabulated ones, confirming that there is no significant difference between them concerning precision and accuracy. The results of t- test and F ratio are listed in Tables 7 a&b.

Table 7a: Statistical comparison between the proposed RP-HPLC method for the simultaneous determination of Paracetamol, Thiocolchicoside and Aceclofenac and the reported methods:

Statistical term	Reported method for PAR [41]	PAR by RP-HPLC	Reported method for THIO [41]	THIO by RP-HPLC	Reported method for ACE [41]	ACE by RP-HPLC
Mean	99.58	99.25	100.70	101.54	100.09	99.64
S.D.±	0.76	0.39	0.88	0.82	0.45	0.96
S.E. ±	0.31	0.16	0.36	0.33	0.18	0.39
% R.S.D.	0.76	0.39	0.89	0.81	0.45	0.96
n	6	6	6	6	6	6
V	0.58	0.15	0.77	0.67	0.20	0.92
t (*2.23)		1.02	t (*2.23)	0.52	t (*2.23)	1.58
F(*5.05)		3.87	F(*5.05)	1.15	F(*5.05)	4.60

*Figures in parentheses are the theoretical t and F values at (p=0.05).

[41]The reported method determined the Paracetamol, Thiocolchicoside and Aceclofenac in their ternary mixture by TLC-densitometric method using toluene: acetone: methanol: formic acid 8:2:2:1 (v/v/v/v) as mobile phase on aluminum plates precoated with silica gel. Densitometric scanning was performed at 263 nm.

Table 7b: Statistical comparison between the proposed TLC method for the simultaneous determination of Paracetamol, Thiocolchicoside and Aceclofenac and the reported method:

Statistical term	Reported method for PAR [41]	PAR by TLC	Reported method for THIO [41]	THIO by TLC	Reported method for ACE [41]	ACE by TLC
Mean	99.58	100.38	100.70	101.39	100.09	99.34
S.D.±	0.76	0.42	0.88	0.85	0.45	0.55
S.E. ±	0.31	0.17	0.36	0.35	0.18	0.22
% R.S.D.	0.76	0.42	0.89	0.84	0.45	0.55
n	6	6	6	6	6	6
V	0.58	0.18	0.77	0.72	0.20	0.30
t (*2.23)		1.84	t (*2.23)	0.40	t (*2.23)	0.42
F(*5.05)		3.22	F(*5.05)	1.07	F(*5.05)	1.50

*Figures in parentheses are the theoretical t and F values at (p=0.05).

[41]The reported method determined the Paracetamol, Thiocolchicoside and Aceclofenac in their ternary mixture by TLC-densitometric method using toluene: acetone: methanol: formic acid 8:2:2:1 (v/v/v/v) as mobile phase on aluminum plates precoated with silica gel. Densitometric scanning was performed at 263 nm.

CONCLUSION

The developed HPLC and TLC-Densitometric methods provide simple, sensitive, accurate, precise and reproducible quantitative analysis for the simultaneous determination of paracetamol, thiocolchicoside and aceclofenac in bulk and pharmaceutical formulation. The two methods have advantages over the other reported methods, as the HPLC method permits simple isocratic separation of the three drugs in bulk and formulation with low LOD and LOQ values and wide ranges of linear concentrations. The TLC densitometric method is very sensitive, it permits the simultaneous determination of the three drugs in very low concentrations (especially for thiocolchicoside) and wide ranges of linearity, with good accuracy and precision. The two methods can be applied in quality control laboratories for routine determination of Paracetamol, Thiocolchicoside and Aceclofenac simultaneously in their ternary mixtures without prior separation. This study can be useful for the determination of the three drugs simultaneously in biological fluids; more studies in this field are thus recommended.

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CONFLICT OF INTEREST:

None

ABBREVIATIONS

RP-HPLC : Reversed Phase-High Performance Liquid Chromatography
TLC : Thin Layer Chromatography
PAR : Paracetamol,
THIO : Thiocolchicoside
ACE : Aceclofenac
LOD : Limit of detection
LOQ : Limit of quantification
ICH : International Conference Of Harmonization

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