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SIMULTANEOUS DETERMINATION OF ETODOLAC AND PARACETAMOL IN BULK DRUGS AND PHARMACEUTICAL FORMULATION BY HPTLC-DENSITOMETRIC AND UV-DERIVATIVE SPECTROPHOTOMETRY METHODS

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Keywords:

High Performance Thin Layer Chromatography, Derivative Spectrophotometry, Paracetamol, Etodolac, p-Aminophenol, Impurity

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ABSTRACT: In current research work two methods have been developed for simultaneous determination of etodolac and paracetamol in binary mixtures. HPTLC-densitometry was employed for the determination of the mixture for etodolac 50-400 ng band^{-1} and for paracetamol 50-300 ng band^{-1}. Separation was carried out on a silica gel 60 F_{254} HPTLC plates, using toluene: acetone: methanol: glacial acetic acid (6:2:1:0.5 v/v) as mobile phase. Etodolac, paracetamol and the toxic impurity para-aminophenol were well resolved with R_f values of 0.61±0.04, 0.41±0.04 and 0.20±0.03. Determination has been carried out at 254 nm with a mean percentage recovery of 99.77±1.30 for etodolac and 99.86 ± 0.97 for paracetamol. First derivative (D1) spectrophotometry was employed for simultaneous determination of etodolac (217nm) and paracetamol (236 nm). Linearity ranges of both the compounds were found to be 2.5-12.5µg mL^{-1} with a mean percentage recovery of 98.07±1.62 for etodolac and100.65±0.84 for paracetamol respectively. Methods were validated according to ICH guidelines and successfully implemented for the analysis of bulk powder and pharmaceutical formulations.

INTRODUCTION: Etodolac (ETO) (R, S)-2-[1, 8-diethyl-1, 3, 4, 9-tetrahydro-pyrano-(3, 4-b)-indole-1-yl] acetic acid **(Fig. 1a),** is a cyclo-oxygenase inhibitor used as an analgesic to reduce pain in arthritis [1]. ETO also used in the therapy of postoperative pain and rheumatic diseases[2]. Paracetamol (PA), N-(4–hydroxyphenyl) acetamide **(Fig. 1b),** is a widely used analgesic and antipyretic agent throughout the world. However, the indications for PA are limited, especially in the post-operative setting, because of its poor solubility which prevents its use in a soluble dosage form for parenteral administration[3]; it is official in USP, BP and IP [4-6].

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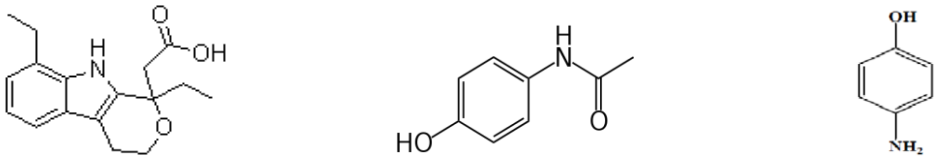
p-aminophenol (PAP) (**Fig. 1c**), is an analogue and metabolite of common household analgesics, such as acetaminophen. It is well-known that acetaminophen in overdose can cause severe hepatic centrilobular necrosis in humans and experimental animals [7]. Like acetaminophen, PAP-induced hepatotoxicity may also involve a chemically reactive intermediate and GSH may play an important role in its toxicity. This possibility has not yet been explored [8]. p-aminophenol is a nephrotoxic metabolite of acetaminophen. It is 5 times more potent than acetaminophen as nephrotoxicant in F344 rats.

Inhibition of acetaminophen deacetylation to PAP diminished renal toxicity, suggesting that acetaminophen renal toxicity is partly mediated by formation of PAP. The major toxicity of acetaminophen or PA is mainly due to the presence of toxic impurity called PAP, so one needs to have an analytical method that separates the PA with PAP.

Limits of PAP in USP are (NMT 0.005%) in bulk drug. The limit varies among different dosage forms. Limits of PAP in BP are (NMT 0.005%) in bulk drug, (NMT 0.1%) in PA tablet.



1(a) **1(b)** **1(c)**

FIG. 1: CHEMICAL STRUCTURES OF 1(a) ETO, 1(b) PA, 1(c) PAP.

ETO and PA combination is especially used for the arthritis and pain however with these combination minor side effects like nausea and headache are reported. High Performance Thin Layer Chromatographic [9-11], spectrophotometric [12-14] methods for determination of PA either in single or in combination with other drugs was reported. Analytical methods have been reported for the determination of ETO includes Spectrophotometric [15-17] and HPLC [18-19] as single component or in combinations with other drugs. ETO and PA combination was developed by using HPLC [20] and HPTLC from spiked human plasma [21], by using this method one cannot separate PAP with PA. To our knowledge there is no report for separation of ETO, PA and toxic impurity PAP. Our main piece of work is to develop simple, sensitive and accurate analytical methods for the determination of ETO, PA and toxic impurity of PAP in their binary mixture and pharmaceutical formulation with satisfactory precision for good analytical practice (GAP). HPTLC technique can be performed in a short period of time and requires less mobile phases and allows the simultaneous analysis of large number of samples, this property of the HPTLC has advantage over the techniques because the separation of either multiple samples or a single sample takes the same time.

Experimental:

Instrumentation: An online automatic sample applicator equipped with 100µL syringe (Camag Linomat 5, Switzerland) and TLC scanners 3 were employed for preparation and measurement of TLC plates, respectively. Both of the scanner and densitometer were controlled using win CATS software, version 1.4.3. A UV lamp with short wavelength 254nm was used for visualization of the TLC plates. A double beam UV–visible spectrophotometer model UV-2100 (RAYLEIGH, China) connected to PC with UV-2100 operation software. D^1 measured spectra was obtained by using accompanying software with $\Delta\lambda$= 5 and scaling factor =10 using methanol as a blank.

MATERIALS AND REAGENTS: ETO pure standard was kindly supplied by kreative organics (p) Ltd, Hyderabad. PA pure standard was kindly supplied by Sri Krishna Pharmaceuticals, Hyderabad. Toluene, methanol, acetone and glacial acetic acid were obtained from Sisco Research Laboratories (Mumbai, India). All were of analytical grade. TLC plates (10×10 cm) pre-coated with silica gel 60 F_{254} were obtained from Merck, Germany. Phenol reagent- Dilute 2.3ml of liquid phenol to 100ml with de-ionized water and stored in a brown glass bottle at 4-8^{0}C. NaOH, 1mol/L- Add 4g of NaOH to 100ml volumetric flask, dilute volume with de-ionized water.

Color reagent-Phenol reagent/NaOH reagent, 7/3 vol, freshly prepared.

Samples of etogesic-P tablets (Zydus); batch no. BP/ET/F1/00, labeled to contain 400 mg ETO and 500 mg PA per tablet were purchased from local market.

HPTLC conditions: The HPTLC plates were developed with toluene: acetone: methanol: glacial acetic acid (6:2:1:0.5 v/v) as mobile phase. For detection and quantification 0.5 to 4.0 µl of ETO and 0.5 to 3.0 µl of PA were applied as separate bands 15 mm apart and 10 mm from bottom of the plate using 100 µl syringes. Linear ascending development of the plate was carried out to a distance of 8.5 cm. After complete development, the plate was dried with blower and scanned at 254 nm.

Standard solutions and calibration: ETO and PA stock solutions (1mg mL^{-1}) were prepared by weighing accurately 10 mg of each powder into two separate 10mL volumetric flasks and makeup the volume. Working solutions (100 µg mL^{-1} in methanol) were prepared by accurately transferring 1mL of the stock solutions of ETO and PA in to two separate 10mL measuring flasks and diluting up to the mark with methanol.

The limit of PAP is not more than 0.005% (BP 2009 and USP 2009). 0.5g of standard PAP was dissolved in 10 ml methanol. 1 ml of the resulting solution was diluted to 100 ml with methanol to get a final concentration of 50µg/ml.

For HPTLC method: 50-400 ng μL^{-1}conentration of ETO and 50-300 ng μL^{-1} concentration of PA were applied to the HPTLC plates. The plate was developed using previously described mobile phase. Calibration curves were constructed by plotting the integrated peak area versus the corresponding concentrations of each drug and regression equation parameters were calculated.

For UV derivative spectrophotometry (D^1) method: For UV derivative spectrophotometry different dilutions equivalent to 25-125µg of ETO and 25-125µg of PA working solutions (10µg mL^{-1} in methanol) were accurately transferred into a series of 10mLvolumetric flasks then diluted to volume using methanol. Calibration curves were obtained by plotting the peak amplitude at 217 and 236 nm versus the corresponding concentration of ETO (2.5-12.5µg mL^{-1}) and PA (2.5-12.5µg mL^{-1}) respectively.

Sample preparation: A total of twenty etogesic P tablets were accurately weighed and crushed to a fine powder. An amount equivalent to one twentieth of the tablet (containing 20 mg of ETO and 25 mg of PA) was taken, extracted with 30 mL of methanol, the mixture was transferred into a 100 mL volumetric flask through a whatman No. 40 filter paper. The residue was washed repeatedly with methanol, by combining the filtrate and washings were made up to the mark with methanol to a final concentration of 200 ng $\mu L^{-1}$of ETO and 250ng μL^{-1} of PA.

Method validation: The method was validated in accordance with ICH (International Conference on Harmonization) guidelines (ICH, 1994, 1996). The following parameters were determined for validation of the developed methods.

Linearity: For HPTLC method 50-400 ng μL^{-1}conentration of ETO and 50-300 ng μL^{-1} concentration of PA were prepared and for UV derivative spectrophotometry (D^1) standard stock solutions of ETO and PA (1mg mL^{-1}) solutions were prepared and diluted to give 2.5-12.5µg mL^{-1}concentrations, calibration curves were plotted results were shown in **Table 1.**

Precision: The precision of the elaborated methods was estimated by the means of six determinations of drug in powdered tablets and sample mixtures. Intraday precision was found out by carrying out the analysis of standard drugs at three different concentrations in the linearity range for three times on the same day. Inter day precision was found out by carrying out the analysis of standard drugs at three different concentrations in the linearity range of drugs for two days for three times and the percentage RSD was calculated results were shown in **Table 2.**

Sensitivity: The sensitivity of both the methods was determined by measuring the limit of detection (LOD) and limit of Quantitation (LOQ). Various validation parameters are summarized in **Table 3.**
Specificity: The specificity of the method was estimated by analyzing standard drug and sample **(Fig. 2)**

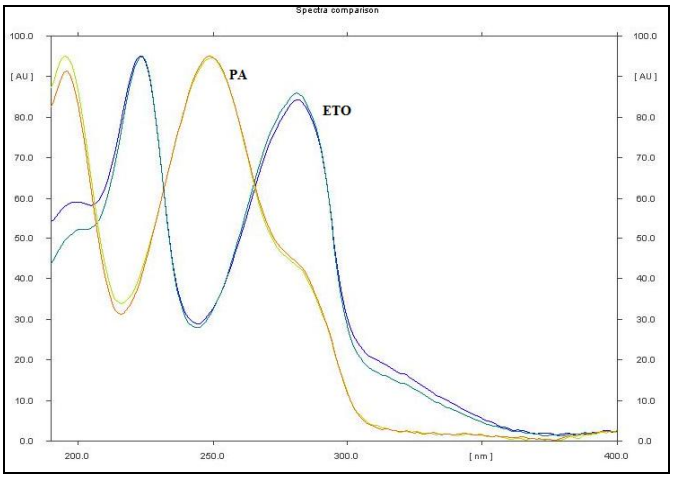


FIG. 2: OVERLAIN UV SPECTRUM OF STANDARD DRUG AND SAMPLE

Percent recovery study: This study was performed by the addition of known amounts of pure drugs to a known concentration of commercial tablets called standard addition method; results were shown in **Table 4**. Percent recoveries for various marketed formulations were calculated and results were shown in **Table 5**.

Robustness: HPTLC–densitometric method: It was determined by changing the parameters like the time from application to development and time from development to scanning and temperature& RH.

For UV derivative spectrophotometry (D^1) method: It was determined by changing the parameters like temperature, R_H and the experiment was performed by other analyst and by using other instrument from different manufacturer. The results were shown in **Table 6.**

RESULTS AND DISCUSSION:

HPTLC method: A laboratory prepared mixture of ETO, PA and PAP is used to investigate the optimum separation conditions. Developing systems of various compositions and ratios were tried chloroform–methanol (8:2, v/v), chloroform–propanol (9:1, v/v), ethylacetae–methanol (7.5:2.5, v/v), and chloroform-acetone-formic acid (8:2:1, v/v), toluene-acetone-methanol (6:2:2, v/v). Various band dimensions were tested in order to get sharp peaks with symmetrical shape. Reported TLC-densitometric methods for the simultaneous determination of ETO/PA mixture employed different mobile phases [21]. Plates were scanned at four different wavelengths (232nm, 246nm, and 266nm, 254nm) and using different slit dimensions. Different band dimensions were tested in order to obtain sharp, symmetrical and well resolved peaks. The optimum bandwidth was chosen (6 mm) and the inter-space between bands was found to be 5 mm. Different scanning wavelengths were tried in that 254 nm was found optimum for both the compounds.

Scanned peaks were sharp, symmetrical and low noise was noticed. Selection of slit dimension is in such a way that the scanning light beam should ensure complete coverage of band dimensions on the scanned track and it should not interfere with adjacent bands. Different slit dimensions were tried, in that 5.0×0.45 mm proved to be the slit dimension of choice with high sensitivity. Finally toluene: acetone: methanol: glacial acetic acid (6:2:1:0.5 v/v) was selected as suitable mobile phase, bandwidth was chosen 6 mm and wavelength selected was 254 nm and slit dimension was 5.0×0.45 mm for the development. The above conditions were allowed good separation between the binary mixture and impurity with good R_f values without tailing of the separated bands **(Fig. 3).**

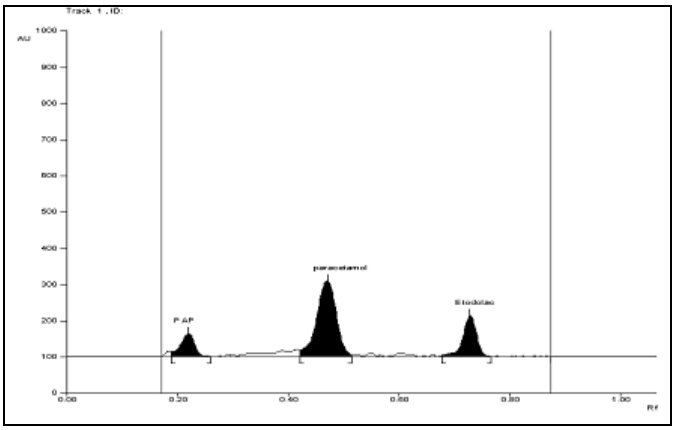


FIG. 3: THIN LAYER CHROMATOGRAM OF SEPARATED PEAKS OF PAP, PA, ETO, HAVING 300 ng BAND^{-1} CONCENTRATION USING TOLUENE: ACETONE: METHANOL: GLACIAL ACETIC ACID (6:2:1:0.5 v/v) AS A MOBILE PHASE.

BP limit for PAP impurity in tablet formulation was 0.1% but our method is able to separate 0.05% of PAP impurity in tablet formulation shown in **Fig 4.** The developed plate was sprayed with color reagent and scanned at 710 nm where the PAP shows maximum sensitivity for detection, shown in **Fig. 4**.

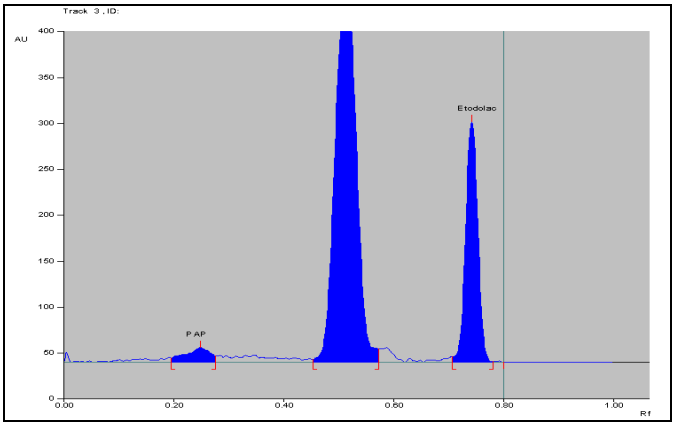


FIG. 4: SEPARATION OF PAP IMPURITY (SPIKED, 0.05%) IN TABLET FORMULATION BY USING MOBILE PHASE TOLUENE: ACETONE: METHANOL: GLACIAL ACETIC ACID- (6:2:1:0.5 v/v).

Results of statistical comparison of proposed methods were shown in **Table 7,** results shows that the calculated t and F values are less than the theoretical ones, confirming accuracy and precision at 95% confidence level

Color test for PAP: Developed plate containing ETO, PA and PAP when sprayed with color reagent, the PAP spots showed blue colour which can be identified with naked eye. A blue colour (indophenol derivative) was obtained when PAP is treated with color reagent solution. A clear detectable blue colour was obtained at a concentration of 0.005% PAP in comparison to a blank.

Reaction mechanism: When PAP is treated with Phenol in the presence of base it gets converted into a blue colour indophenol complex. For the separation of PAP from PA the same conditions (i.e.) toluene: acetone: methanol: glacial acetic acid (6:2:1:0.5 v/v) was selected as suitable mobile phase, the developed plate was sprayed with color reagent and scanned at 710 nm where PAP shows maximum absorbance for detection as showed in **Fig. 5.**

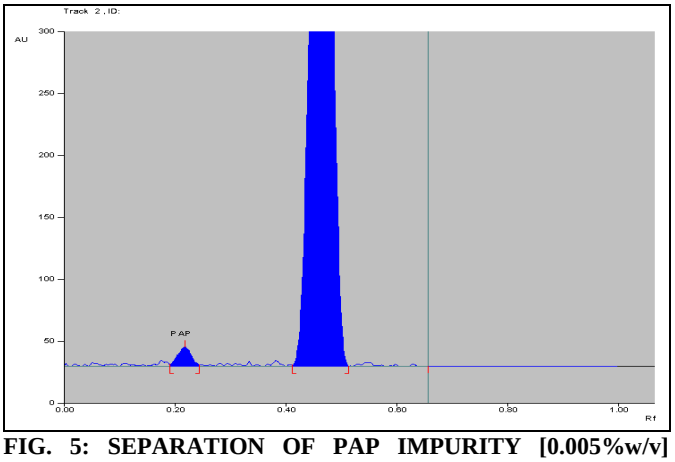


FIG. 5: SEPARATION OF PAP IMPURITY [0.005%w/v] FROM PA BY USING MOBILE PHASE TOLUENE: ACETONE: METHANOL: GLACIAL ACETIC ACID (6:2:1:0.5 v/v).

UV derivative spectrophotometry (D^1) method: The absorption spectrum of ETO and PA shows severe overlap (**Fig. 6**.) which makes their simultaneous determination was difficult with normal UV method. Therefore we developed first derivative spectrum (**Fig. 7.)** of ETO (217nm) and PA (236nm).Validation parameters were reported in **Table 3.**

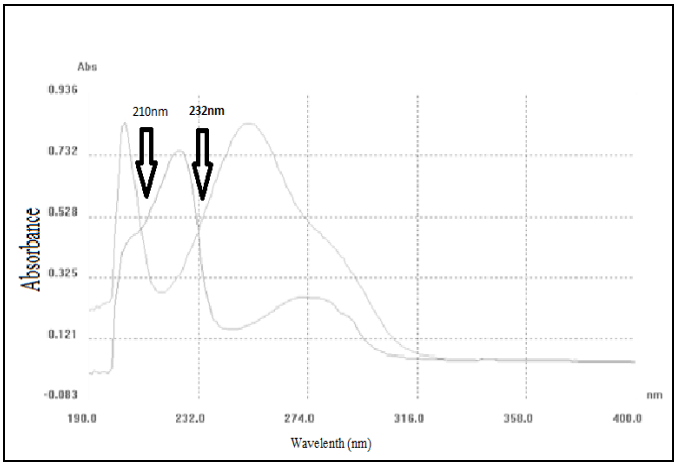


FIG. 6: ZERO ORDER ABSORPTION SPECTRA OF 5 mg mL⁻¹ OF ETO 5 mg mL⁻¹ OF PA USING METHANOL AS A BLANK

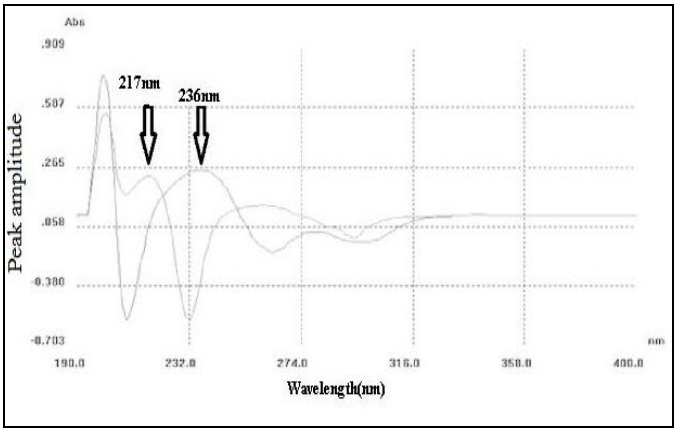


FIG. 7: FIRST DERIVATIVE ABSORPTION SPECTRA OF 5 mg mL⁻¹ OF ETO 5 mg mL⁻¹ OF PA USING METHANOL AS A BLANK.

TABLE 1: LINEAR REGRESSION DATA FOR CALIBRATION CURVES (N=6)

Parameters	TLC densitometry		D^1	
	ETO	PA	ETO	PA
Linearity range	50-400ng band^{-1}	50-300ng band^{-1}	2.5-12.5µg mL^{-1}	2.5-12.5µg mL^{-1}
Correlation coefficient	0.9989	0.9949	0.999	0.993
Slope ± S.D.	3.917 ± 0.005	10.29 ± 0.005	0.165 ± 0.001	0.143 ± 0.005
Intercept ± S.D.	215.8 ± 10.33	192.6 ± 3.90	0.322 ± 0.0059	0.562 ± 0.029
Confidence limit of slope[(a)]	3.917 ± 0.0039	10.29 ± 0.004	0.165 ± 0.0008	0.143 ± 0.004
Confidence limit of intercept[(a)]	[(a)] 215.8 ± 7.1	192.6 ± 3.12	0.322 ± 0.005	0.562 ± 0.025

[a] 95% confidence limit

TABLE 2: INTRA- AND INTER-DAY PRECISION OF ETO (a) AND PA (b) (n=6)

TLC densitometry				D[1]			
Intra-day precision		Inter-day precision		Intra-day precision		Inter-day precision	
S.D. of areas	%R.S.D.	S.D. of areas	%R.S.D.	S.D. of areas	%R.S.D.	S.D. of areas	%R.S.D.
(a) ETO (n=6)							
6.687	0.47	7.758	1.23	0.0071	0.29	0.0078	0.94
(b) PA							
3.052	0.127	3.068	0.249	0.203	0.85	0.204	2.3

(a) Average of three concentrations 100, 200, 300 ng/spot and 2.5, 7.5, 12.5 µg/ml for HPTLC and D^1 derivative method, respectively.
(b) Average of three concentrations 50, 150, 250 ng/spot and 2.5, 7.5, 12.5 µg/ml for HPTLC and D^1 derivative method, respectively.

TABLE 3: SUMMARY OF VALIDATION PARAMETERS: STATISTICAL DATA FOR THE CALIBRATION GRAPHS OF ETO AND PA BY TLC DENSITOMETRIC AND D^1 DERIVATIVE METHOD

Parameters	TLC densitometry		D[1]	
	ETO	PA	ETO	PA
Linearity range	50-400 ng spot^{-1}	50-300 ng spot^{-1}	2.5-12.5µg mL^{-1}	2.5-12.5µg mL^{-1}
Correlation coefficient	0.9989	0.9949	0.999	0.993
Limit of detection	14.24 ng spot^{-1}	12.9 ng spot^{-1}	0.691 µg mL^{-1}	0.554 µg mL^{-1}
Limit of quantitation	42.1 ng spot^{-1}	39.38 ng spot^{-1}	2.09 µg mL^{-1}	1.6 µg mL^{-1}
Recovery (n = 6)	99.77 ± 1.30	99.86 ± 0.97	98.07 ± 1.62	100.65 ± 0.84
Precision (%R.S.D.)				
Repeatability [(a)]	0.479	0.127	0.29	0.85
Intermediate precision [(b)]	1.23	0.249	0.94	2.3
Robustness	Robust	Robust	Robust	Robust

LOD= (SD of the response/slope) ×3.3; LOQ= (SD of the response/slope) ×10.
[a] The intraday (n=3), average of three concentrations repeated three times within day.
[b] The interday (n= 3), average of three different concentrations repeated three times in three successive days.

TABLE 4: STANDARD ADDITION TECHNIQUE FOR DETERMINATION OF ETO (a) AND PA (b) BY TLC DENSITOMETRY AND D^1 DERIVATIVE METHOD (n=6)

Amount taken	Amount added	Total amount of drug analyzed	% recovery ± S.D
(a) ETO 50 ng/spot	80	128.09 ng/spot	99.77 ± 1.30
(b) PA50 ng/spot D^1	80	129.03 ng/spot	99.86 ± 0.97
(a) ETO 5 µg/ml	10	14.2 µg/ml	98.07 ± 1.62
(b) PA 5 µg/ml	10	15.02 µg/ml	100.65 ± 0.84

TABLE 5: RECOVERY (%) FOR VARIOUS MARKETED FORMULATIONS MANUFACTURED BY DIFFERENT COMPANIES

S.no	Quantity	Brand name		Recovery (%)			
				TLC densitometry		D[1]	
	ETO	PA		ETO	PA	ETO	PA
1	200 mg	500 mg	A	98.77±0.99	100.5±0.05	97.98±1.02	99.01±0.45
2	200mg	500 mg	B	97.98±2.02	99.05±1.02	99.08±1.42	100.25±0.08
3	400 mg	500 mg	C	99.89 ±0.67	100.22±0.82	99.98±0.90	100.12±0.18

TABLE 6: ROBUSTNESS (a) TESTING OF HPTLC–DENSITOMETRIC METHOD AND (b) D^1 DERIVATIVE METHOD (n=3)

Condition	RSD of peak area (%)	
	ETO	PA
(a) TLC densitometry (n=3)		
Time from application to development	1.71	0.081
Time from development to scanning	0.593	0.104
Temperature	1.09	0.16
RH	1.23	0.17
(b) D^1		
Temperature	0.89	1.32
RH	1.12	0.998
Performed by other analyst	0.11	0.431
Using other instrument	2.10	1.98

TABLE 7: STATISTICAL COMPARISON BETWEEN THE RESULTS OF THE PROPOSED METHODS

	TLC densitometry		D^1 derivative method	
	ETO (pure)	**PA (pure)**	**ETO (pure)**	**PA (pure)**
Mean ± S.D	99.77 ±1.30	99.86 ± 0.97	98.07 ± 1.62	100.65 ± 0.84
Students				
t test	0.0015 (2.14)	0.209 (2.306)	0.49 (2.306)	0.994 (2.30)
F	0.9964 (3.78)	0.957 (5.05)	1.02 (6.38)	0.996 (6.38)
n	8	6	5	5
Formulations from different manufacturers				
A				
Mean ± S.D	98.77±0.99			
Students				
t test	0.143 (2.14)	0.112 (2.306)	0.923 (2.306)	0.947 (2.30)
F	1.23 (3.78)	1.021 (5.05)	1.62 (6.38)	0.926 (6.38)
n	8	6	5	5
B				
Mean ± S.D	97.98 ±2.02	99.05±1.02	99.08±1.42	100.25±0.08
Students				
t test	0.839 (2.14)	0.102 (2.306)	0.423 (2.306)	0.674 (2.30)
F	1.528 (3.78)	0.989 (5.05)	0.81 (6.38)	0.415 (6.38)
n	8	6	5	5
C				
Mean ± S.D	99.89 ±0.67	100.22±0.82	99.98±0.90	100.12±0.18
Students				
t test	0.539 (2.14)	0.12 (2.306)	0.323 (2.306)	0.174 (2.30)
F	1.92 (3.78)	0.29 (5.05)	0.28 (6.38)	0.099 (6.38)
n	8	6	5	5

CONCLUSION: The major toxicity of PA is mainly due to the presence of impurity called PAP.The developed HPTLC method was clearly differentiates the PA and PAP impurity, so this method is suitable for the routine quality control analysis of pure PA formulations and ETO and PA in pharmaceutical preparations. Statistical comparison of proposed methods showed that the calculated t, F values are smaller than the theoretical ones indicate that test passes the null hypothesis. Apart from HPTLC method we were also reported color test for the identification of toxic impurity PAP [0.005%w/v]. Results showed that the developed methods were lack of interference from dosage form additives.

CONFLICT OF INTEREST: Declare None.

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