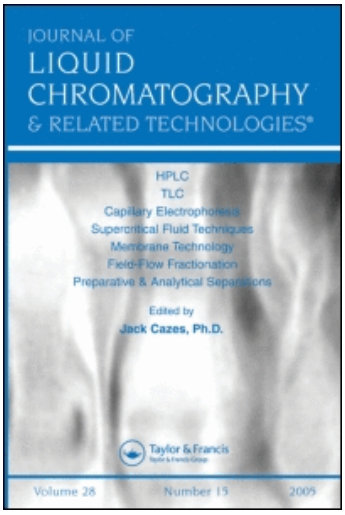

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Simultaneous Determination of Pyrathiazine Theoclate and Pyridoxine HCl by TLC-Densitometry in Commercial Tablets: Validation of the Method

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Abstract: A simple and rapid densitometric method has been developed for determination of pyrathiazine theoclate and pyridoxine HCl in commercial tablets. After extracting the samples four times with ethanol, the solutions were spotted on pre-coated silica gel TLC plates, which were eluted with a mixture of dichloromethane–methanol–acetone–acetic acid (19.5:6.0:3.0:1.5, v/v). Quantitative evaluation was performed by measuring the absorbance reflectance of the analyte spots at $\lambda = 254$ nm. The TLC-densitometric method is cheap, selective, precise, and accurate and can be used for routine analysis of pyrathiazine theoclate and pyridoxine HCl in tablet preparations at industry quality control laboratories.

Keywords: Densitometry, Parathiazine theoclate, Pyrathiazine theoclate, Pyridoxine HCl, Tablets, TLC, Validation

INTRODUCTION

A drug combination of pyrathiazine theoclate and pyridoxine HCl was recently marketed in Indonesia as an anti-vomiting tablet.[1] Pyrathiazine

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(synonym:parathiazine), which has chemical name 10-[2-(1-pyrrolidyl) ethyl]phenothiazine, was not yet described officially in Indonesian pharmacopoeia[2] and other Pharmacopoeias,[3–5] whilst the method of analysis of pyridoxine HCl were reported in some common Pharmacopoeias.[2–5]

Pyrathiazine theoclate consisted of one molecule of pyrathiazine base and theoclate (chloro-8-dimethyl-1,3 dioxo 2,6 tetrahydro-1,2,3,6 purine), respectively.[6] Some TLC methods of Pyridoxine HCl were available in Camag Bibliography Service data base.[7] To the best of our knowledge, no reports were available for the simultaneous densitometry determination of pyrathiazine theoclate and pyridoxine HCl.

The aim of this present work was to develop a simple, validated, and rapid densitometry method for routine analysis of pyrathiazine theoclate and pyridoxine HCl in tablets.

EXPERIMENTAL

Materials and Reagents

Pyrathiazine theoclate (Hy-Gro Chemicals Pharmtek, Secondarabad, India; Batch: PT-001; Assay 99.57%; Expiration date: October 2009), Pyridoxine hydrochloride (BASF South East Asia Pte. Ltd; Singapore; Batch: 0703427; Assay 99.8%;) were pharmaceutical grade substances. The substances were used as received for the standard addition method, and standard solutions. Commercial tablets (CT) which containing pyrathiazine theoclate, 40 mg and pyrodoxine HCl, 37.5 mg tablet^{-1} was bought in a local pharmacy at Surabaya in November 2007 (Batch: SZ6A003, Expire date November 2009).

Methanol, ethyl acetate, dichloromethane (Mallinckrodt Baker, Inc, Philipsburg, NJ, USA), acetone (J.T. Baker, Philipsburg, NJ, USA), and acetic acid anhydride, potassium hydroxide, potassium hydrogen phosphate, ammonium hydroxide, ethanol 96% (E. Merck, Darmstadt, Germany) were analytical grade solvents and reagents, and were used without further purification.

Stock standard solutions were prepared by dissolving accurately weighed pyrathiazine theoclate (20.0, 30.0, 50.0 mg) and pyridoxine HCl (20.0, 30.0, 50.0 mg) in 25.0 mL in ethanol, 96%. Various standard solutions were prepared from the stock solution by dilution with ethanol, 96%. For basic linearity study, the solutions were prepared containing 750, 1000, 1250, 1500, 1750, 2000, 2250, 2500, and 2750 μg mL^{-1} of both substances. Of these solutions, 2 μL was spotted onto the TLC plate. The standard solutions were stable at least for 48 hours at room temperature (Mean ± RSD (%) for pyrathiazine theoclate and pyridoxine HCl were

100.3 ± 1.50 and 100.8 ± 0.63, respectively, $n = 3$, at $24 \pm 2°C$, room humidity $50 \pm 10\%$).

Phosphate buffer pH 6.8 was used as the dissolution media. The dissolution experiments were performed using the paddle method (75 rpm, 900 mL; 60 min).

Sample Preparation

Twenty commercial tablets were each weighed, and their mean was determined. After homogenizing the powder, an equivalent weight of a 0.4 tablet (equivalent to 16.0 and 15.0 mg pyratahiazine theoclate and pyridoxine HCl, respectively) was transferred into a 10.0 mL volumetric flask containing about 5 mL of ethanol 96%, ultrasonicated for 10 min, and diluted to 10.0 mL with ethanol 96%. The solution was filtered through 0.45 μm Durapore®, membrane filters (Milipore, Ireland) filters before spotting on to TLC plates (2.0 μL), together with the standard.

For accuracy studies, aliquots of pyrathiazine theoclate and pyridoxine HCl solutions (20, 40, 60, and 80% of the label claim) were added to powdered commercial drugs; after air dried, the powdered tablets were extracted with ethanol 96% as describe above. Aliquots of standard solutions (80, 100, 120% of the label claim) were added to the dissolution medium (total volume was 900 mL). 2 μL of this solution, 2 μL was spotted on the TLC plates.

For differentiating the spots of pyrathiazine base and theoclate, 45 mg of pyrathiazine theoclate was dissolved in 6.0 mL of water, than drops of NH_4OH (25%) was added until pH was 10. The solution was extracted four times using 6 mL of ethyl acetate by using a vortex mixer. The ethyl acetate fractions (F-EtOA) was collected in 25 mL volumetric flasks and diluted with ethyl acetate to volume. The 2.0 μL ethyl acetate fraction and water (F-Wt) was than spotted on the TLC plate.

Chromatography

Chromatography was performed on pre-coated silica gel F254 aluminum back sheets (E. Merck. # 1.05554, all the pre-coated plates were cut into 10×20 cm before used). The plates were used as obtained from the manufacturer without any pretreatment; a Nanomat III (Camag, Muttenz, Switzerland) equipped with a dispenser magazine containing 2.0 and 5.0 μL glass capillaries (Camag) was used for sample application (as spotted with diameter *ca.* 2 mm). The mobile phase used in this experiment is dichloromethane–methanol–acetone–acetic acid (19.5:6.0:3.0:1.5, v/v). The distance from the lower edge was 10 mm; distance from the side

was 15 mm, and track distance was 10 mm. Ascending development was performed in a Camag twin-through chamber (for 20×10 cm plates) after at least 60 min of saturation; the mobile phase migration distance in all experiments was 8.0 cm. (development time *ca.* 20 min at $24 \pm 2^{\circ}$C). The plate was air dried and than scanned in the TLC scanner.

Densitometric scanning was performed with a Camag TLC-Scanner III. The purity and identity of the analyte spots were determined by scanning the absorbance-reflectance mode from 200 to 400 nm. Quantitative evaluation was performed by measuring the absorbance reflectance of the analyte spots at λ 254 nm. The densitometric scanning parameters were: bandwidth 10 nm, slit width 4, slit length 6, and scanning speed 4 mm s^{-1}. Calculations for identity, purity checks ($r_{S,M}$ and $r_{M,E}$ where $S =$ start, $M =$ center, $E =$ end spectrum), *sdv* (relative standard deviation) of the linear/calibration curve, and quantification of the analyte spots were performed by software winCATS version 1.4.2. (2006, Camag). Routine quantitative evaluations were performed *via* peak areas (pyrathiazine base and pyridoxine HCl) with linear regression, using 4–5 points' external calibration on each plate (80 to 120% of the targeted value). For quantitative evaluation, the concentration of pyrathiazine theoclate in samples was calculated based on the peak area of pyrathiazine base. Each of the extract aliquot samples was spotted at least in duplicate.

Validation

The method was validated for linearity, detection limit (DL), quantitation limit (QL), accuracy, precision, and robustness by the method[8,9] with modification. Accuracy study was performed by the standard addition method. Aliquots of standard solutions of pyrathiazine theoclate and pyridoxine HCl were added to powdered tablets and dissolution medium (900 mL); after air-dried the powdered tablets were extracted as described above. Design and analysis of effect of the robustness evaluation were performed and calculated by using Unscramble 9.6™ (2006) software from CAMO (Bangalore, India).

RESULTS AND DISCUSSION

After the TLC plate was eluted the densitogram at 254 nm of mixed standard solutions (Figure 1, line 8, 9) in 96% ethanol showed 4 spots of pyridoxine HCl (peak A, R_f ca. 0. 39), 2 spots of pyrathiazine theoclate (peak B, R_f 0.59 and Peak C, R_f. 0.87) and peak D, which was the mobile phase's front. The F-EtOA (Figure 1, line 6, 7) and F-Wt (Fig. 1, line 4, 5)

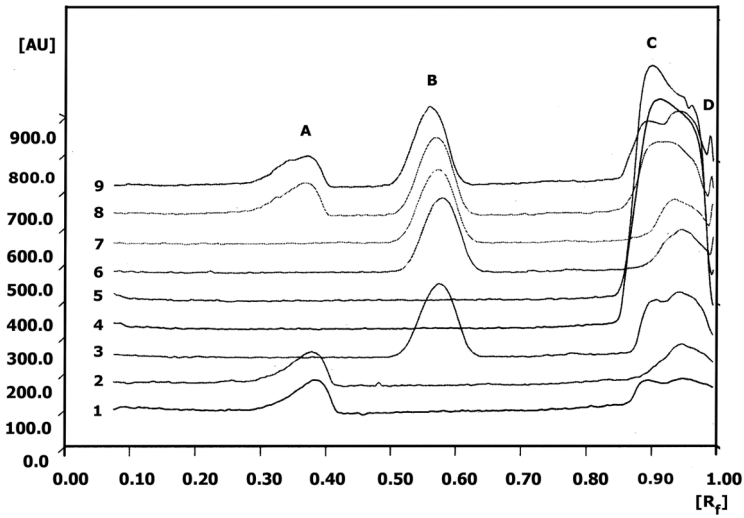

Figure 1. Typical densitograms observed at 254 nm. Solutions of standad Pyridoxine HCl in ethanol 96% (line 1,2); Solutions of standard pyrathiazine theoclate in ethanol 96% (3); Solution of F-Wt (4,5); Solution of F-EtOA (6,7); Solutions of mixed standards in ethanol 96% (8,9). Peak identity: A (Pyridoxine HCl); B (Pyrathiazine base); C (Theoclate); D (Mobile phase's front).

yielded one spot, respectively, (R_f 0.59 and 0.87). This indicated that the spots with R_f 0.59 and 0.87 were spots of pyrathiazine base and theoclate, respectively. The confirmation was performed by measuring their *in situ* UV spectra (See Figure 2 and 3). Figure 5 showed the densitograms of mixed standard solutions, extract of commercial tablets, and dissolution media. For further experiments, the peak of theoclate was ignored. This TLC-system demonstrated that all analyte spots of furnished *in situ* UV spectra, were identical with those of standards ($\mathbf{r} \geq 0.9999$). Purity check of the analyte spots using CATS software also showed that all analyte spots of the extracts were pure. The values of $r_{S,M}$ and $r_{M,E}$ were $\geq$0.9999, demonstrating that the proposed TLC method is highly selective.

The peak area was observed to be linearly dependent on the amount of pyrathiazine theoclate within the range of 1500 to 5500 ng spot^{-1}, with linear regression line $Y = 3353 + 1.945$ X (the relative process standard deviation value V_{XO}[8] was 2.08%; $n = 9$; $sdv = 1.40$; $r = 0.9987$). The calculated value of test parameter X_p (for $p = 0.05$) and r were satisfactory (376 ng spot^{-1} and $\geq$0.99, respectively).[8–10] For pyridoxine HCl, the linear range was 1500 to 5500 ng spot^{-1} ($Y = 335 + 0.953X$; $Vxo = 3.03\%$, $n = 9$; $sdv = 2.76$, $r = 0.9973$; Xp (for $p = 0.05$) = 545 ng spot^{-1}). ANOVA regression test for linearity testing of the regression line showed

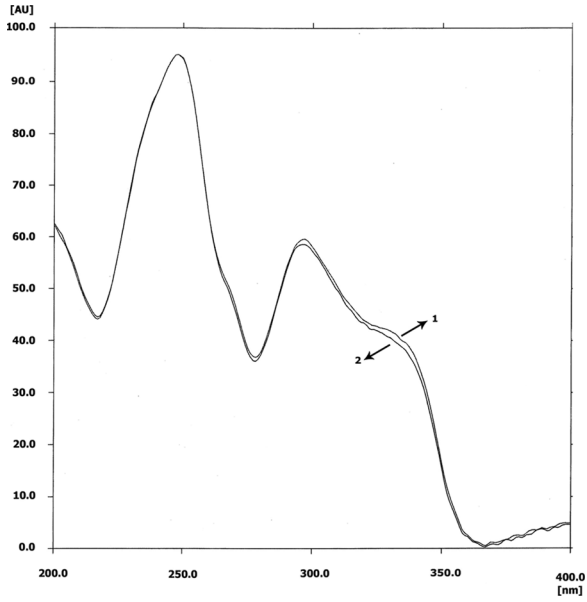

Figure 2. UV spectra of pyrathiazine base's spot; 1 (in ethanol 96%); 2 (in ethyl acetate, Ft-ETOA).

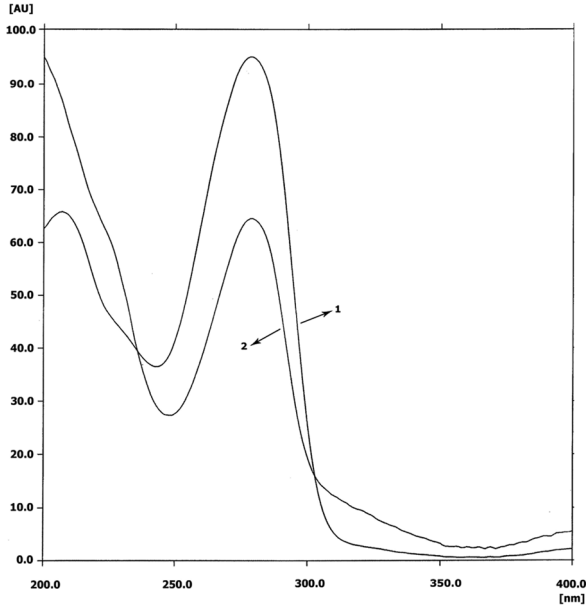

Figure 3. UV spectra of theoclate's spots; 1 (in water, F-Wt); 2 (in ethanol 96%).

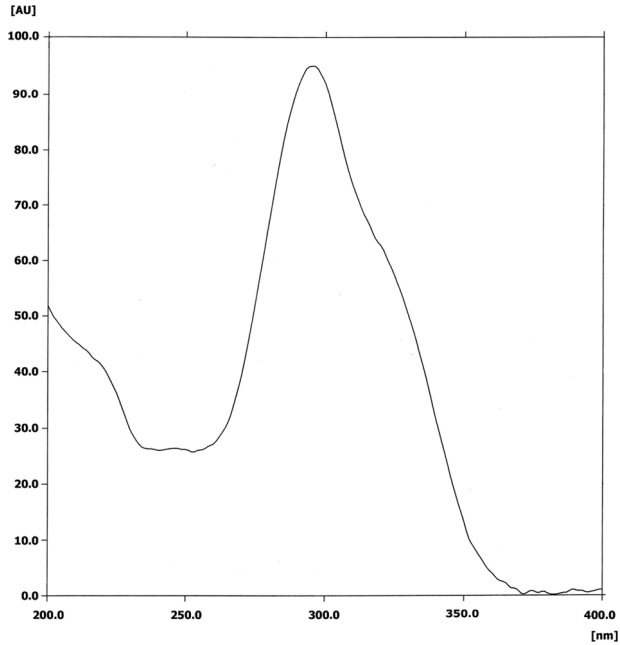

Figure 4. UV spectrum of pyridoxine HCl's spot (in ethanol 96%).

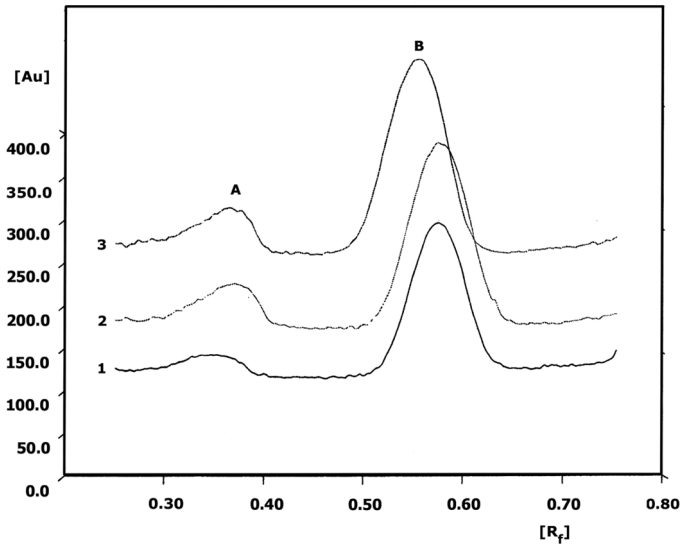

Figure 5. Typical densitograms observed at 254 nm. Solutions of dissolution media (1); Extracts of CT in 96% ethanol (2); Solution of mixed standards in ethanol 96% (3); Peak identity: A (pyridoxine HCl); B (Pyrathiazine base).

significant calculated F-value ($p < 0.0001$; $F_{calculated}$ was 2820 for pyrathiazine theoclate; and 1328 for pyridoxine HCl). The linearity of the basic calibration curve was also proved by the Mandel's fitting test.[8] The plots of the residuals against the quantities of the analyte confirmed the linearity of the basic calibration graph (data not shown). The residuals were distributed at random around the regression lines; neither trend nor unidirectional tendency was found. The basic linear calibration curve showed variance homogeneity over the whole range. The calculated test values PW[8] was 4.227 (pyrathiazine) and 2.597 (pyridoxine HCl); the PW-values less than the F_{table}-value (6.03 for $f_1 = 8$, $f_2 = 8$; $p = 0.01$).

All the linear regression calibration curve parameters of those used in this present work showed satisfactory results (data not shown). All values of the correlation coefficient r in this present work are >0.99; and the values of other parameters such as, Xp (less than lower limit in the calibration range), *sdv* (<5), Vxo (<5%), and *p* (<0.05) for ANOVA linear-test are also showed satisfactory results.[8–10]

DL was determined by making a linear regression of relatively lower concentration of pyrathiazine theoclate (100 to 900 ng $spot^{-1}$) according to the method of Funk et al.[8] The calculated equation of the regression line was $Y = -72 + 5.1\ X$ ($n = 9$; $V_{XO} = 2.11\%$; $r = 0.9994$; $sdv = 2.17$). The calculated value of test parameter X_p(for $p = 0.05$)[8] was 49 ng $spot^{-1}$. In this case, the value of $DL = X_p$.[8] According to Carr and Wahlich,[11] the value of the QL could be estimated as 3 times of the DL-value (147 ng $spot^{-1}$). With the same method, DL and QL for pyridoxine HCl were 26 and 78 ng $spot^{-1}$, respectively, ($Y = -461 + 1.76\ X$; $n = 6$; 400 to 900 ng $spot^{-1}$, $Vxo = 0.450\%$; $r = 0.9999$; $sdv = 0.83$).

Table 1 demonstrated good accuracy as revealed by the percentage of mean recovery data of the assay of the CT and dissolution media. To prove whether systematic errors did not occur, linear regression of the recovery curve of $\mathbf{X_f}$ (concentration of the analyte measured by the propose method) against $\mathbf{X_c}$ (nominal concentration of the analyte) was constructed. The confidence interval data ($p = 0.05$) of the intercept {$VB(a_f)$} and slope {$VB(b_f)$} from the recovery curves did not reveal the occurrence of constant- and proportional-systematic errors.[8–10]

All the relative standard deviation (RSD) of the repeatability and intermediate precession evaluations have values less than 2% (see Table 2), and the calculation by using David-, Dixon-, and Neumann–Test[12] showed satisfactory results (data not shown). All the standard deviation (SD) (data not shown) of the precision studies yielded values below the permitted maximum standard deviation as reported by Ermer (2.43 for specification range 95–105%, basic lower limit 99%, $n = 6$).[13] The measurements were performed in one laboratory by different analysts, on different plates and days for CT. For dissolution media, the studies were performed on three different concentrations on the same day

Table 1. Results of the accuracy evaluation of Commercial Tablets (CT) and dissolution media

Sample (Analyte)	Amount found[a] (Mean ± SD)[d]	Amount added[a]	% Recovery (Mean ± SD)	Recovery curve[b]	$V_{B(af)}$[c]	$V_{B(bf)}$[c]
CT						
(Pyrathiazine theoclate)	100.42 ± 1.16	20, 40, 60, 80[e]	99.73 ± 0.58	$X_f = 2.73 + 0.978\ X_c$	2.73 ± 5.24	0.978 ± 0.584
(Pyridoxine HCl)	100.63 ± 0.75	20, 40, 60, 80[e]	99.75 ± 0.75	$X_f = -3.34 + 1.02\ X_c$	−3.34 ± 6.39	1.02 ± 0.04
Dissolution media						
(Pyrathiazine theoclate)		80, 100, 120[f]	101.54 ± 1.34	$X_f = 13.01 + 1.00\ X_c$	13.01 ± 77.53	1.00 ± 0.07
(Pyridoxine HCl)		80, 100, 120[f]	100.92 ± 1.54	$X_f = -16.13 + 1.02\ X_c$	−16.13 ± 85.25	1.02 ± 0.08

[a]% of label claim.
[b]X_f and X_c are, respectively, the measured and theoretical nominal amount of the analyte spotted (ng spot^{-1}).
[c]For $p = 0.05$.
[d]$n = 3$.
[e]$n = 2 \times 4$ levels $= 8$.
[f]$n = 3 \times 3$ levels $= 9$.

Table 2. Results from evaluation of precision of CT and dissolution media

	RSD % (n = 6)					
	CT[a]			Dissolution media[b,c]		
Analyte	1[b,c]	2[b,c]	3[b,c]	80%[d]	100%[d]	120%[d]
Pyrathiazine theoclate	1.30	0.65	1.16	0.34	0.55	1.25
Pyridoxine HCl	0.77	1.88	0.76	0.87	1.48	1.47

[a]Intermediate Precision Evaluation.
[b]Each measurement was performed by a different analyst on the different days, and plates within one laboratory.
[c]Repeatability Evaluation.
[d]% of Label Claim.

(repeatability studies). These results demonstrated that the accuracy and precision of the proposed method were satisfactory.

In order to evaluate the robustness of the proposed method, the influence of small variation on the composition of the mobile phase on the values of R_f, and % label claim of CT were evaluated. The data were presented in Table 3. Analysis of the effect of the data was performed by using Unscrambler 9.6^{TM} software. Higher order interaction effect (HOIE) method showed that the % label claim for both analytes values were significantly not affected by these small variations (Table 4; $p > 0.05$).

Table 3. Effect of the Mobile Phase compositions[a] on the R_f-values and % Analyte content of CT[b] (% Label Claim)

				Pyrathiazine theoclate		Pyridoxine HCl	
Dichloro-methane	Methanol	Acetone	Acetic acid	R_f	Label Claim (%)	R_f	Label Claim (%)
20.5	6.3	3.2	1.4	0.65	100.60	0.43	101.04
18.5	6.3	3.2	1.6	0.72	101.15	0.48	101.80
18.5	5.7	3.2	1.6	0.65	101.60	0.43	101.13
20.5	5.7	2.8	1.6	0.76	100.83	0.41	102.46
18.5	6.3	2.8	1.4	0.80	101.23	0.51	101.00
20.5	5.7	3.2	1.4	0.63	101.91	0.37	101.97
20.5	6.3	2.8	1.6	0.75	101.55	0.45	101.28
18.5	5.7	2.8	1.4	0.71	101.19	0.42	99.28
19.5	6.0	3.0	1.5	0.71	100.35	0.45	99.58

[a]Mobile phase composition presented in v/v.
[b]Mean value of triplicate determinations.

Table 4. Analysis of Effect of the Robustness data (HOIE method)[a,b]

Variable (Mobile phase)	R_f		Label claim (%)	
	Pyrathiazine theoclate	Pyridoxine HCl	Pyrathiazine theoclate	Pyridoxine HCl
Dichloromethane	NS($p^c = 0.222$)	S($p = 0.030$)	NS($p = 0.884$)	NS($p = 0.228$)
Methanol	NS($p = 0.095$)	S($p = 0.011$)	NS($p = 0.600$)	NS($p = 0.923$)
Acetone	S($p = -0.015$)	NS($p = 0.288$)	NS($p = 0.814$)	NS($p = 0.532$)
Acetic acid	NS($p = 0.241$)	NS($p = 0.464$)	NS($p = 0.911$)	NS($p = 0.305$)

[a]Calculated from data presented on Table 3.
[b]Calculation was performed by using Unscrambler 9.6 software (CAMO).
[c]Probability value.
NS means Not Significant and S Means Significant (for $p = 0.05$).

Table 5. Results of forced degradation studies of CT

Storage condition	Incubation Time	Label Claim[a] (%) (Mean ± SD, n = 3)	
		Pyrathiazine theoclate	Pyridoxine HCL
3 drops of 1 N HCL	16 hours at 80°C	92.51 ± 1.42	83.91 ± 2.31
3 drops of 1 N NaOH	16 hours at 80°C	93.77 ± 2.42	86.42 ± 1.27
3 drops of 1% H_2O_2	16 hours at 80°C	89.73 ± 0.26	84.49 ± 1.61

[a]Purity and Identity checks of analyte spots using CATS software yielded very good values (r > 0.999).

Table 5 showed that the recovery of the analytes was reduced, *ca.* 7 to 16% in stressed CT samples. The purity and identity check of the analyte spots using CATS software yielded good values (>0.999), this showed that all the analyte spots were still pure and identical with the standard. This proved that the analyte peaks were not interfered by possible degradation products.

The present work showed that the proposed TLC- densitometric method is suitable for the routine analysis of products of similar composition in the pharmaceutical industry quality control laboratories, especially for developing countries like Indonesia.

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