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Determination of thiocolchicoside in its binary mixtures (thiocolchicoside–glafenine and thiocolchicoside–floctafenine) by TLC–densitometry

N.A. El-Ragehy *, M.M. Ellaithy, M.A. El-Ghobashy

Analytical Chemistry Department, Faculty of Pharmacy, Cairo University, Kasr El-Aini St., Cairo 11562, Egypt

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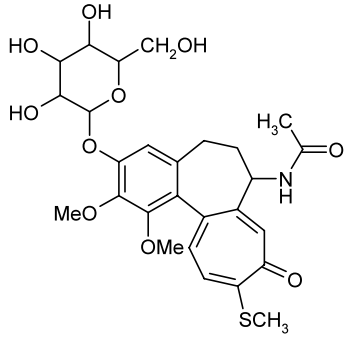
Abstract

The proposed method is based on TLC separation of thiocolchicoside from its binary mixtures (thiocolchicoside–glafenine and thiocolchicoside–floctafenine) followed by densitometric measurement at 375 nm. Separation was carried out on silica gel plates GF_{254} using ethyl acetate:methanol:acetic acid (84:13:3%, v/v/v). Various conditions affecting separation and measurement were studied and optimized. Calibration was performed using third-order polynomial equation. It was found superior to first-order with respect to quantification range (0.25–25 μg per spot), correlation coefficient and standard error of estimation. The proposed method was successfully applied for the determination of thiocolchicoside in its synthetic binary mixtures and commercial tablets. Results were compared with those obtained by reference methods and non-significant difference was obtained regarding accuracy and precision. Assay precision using two-way ANOVA was performed on results of inter- and intra-day applications of the method.

Keywords: TLC–densitometry; Polynomial regression; Two-way ANOVA; Thiocolchicoside; Floctafenine and Glafenine

1. Introduction

Thiocolchicoside is (*s*)-*N*-[3-(*B*-D-glucopyranoxy-loxy)-5,6,7,9-tetrahydro-1,2-dimethoxy-10-(methylthio)-9-oxobenzo[*a*]heptalen-7yl]acetamide. Its structural formula is given below. It occurs as yellow powder soluble in water, slightly soluble in alcohol and practically insoluble in ether and acetone. It melts with decomposition at 275 °C [1,2].



Mol. Wt 563.6

Several attempts have been made to determine thiocolchicoside in its dosage forms, but corner stone was to find a reliable method which can determine minor component in (1:100) mixture with NSAIDs. These methods include RP-HPLC [3], HPLC [1] and radioimmunoassay [4]. Highly specific and sensitive liquid chromatographic separation with tandem mass spectrometric detection was developed for determination of thiocolchicoside primary metabolite in human plasma (3-desmethylthiocolchicine) [5].

Thin-layer chromatography (TLC) is a subdivision of liquid chromatography, in which the mobile phase is a liquid and the stationary phase is situated as a thin layer on the surface of a flat plate [6]. TLC is sometimes grouped under the term ''planar chromatography'' due to its flat geometry. Methods for the quantitative evaluation of thin-layer chromatograms can be divided into two categories. In the first one, solutes are assayed directly on the layer either by visual comparison, area

* Corresponding author.
E-mail address: n.a.ragehy@mailcity.com (N.A. El-Ragehy).

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measurement or densitometry. In the second, solutes are eluted from the sorbent before being measured [6].

Reflectance and transmission measurements are particularly sensitive to small changes in experimental technique. Variation in layer thickness, layer quality (particle size and particle size distribution), spot properties (shape, size and linearity of spotting line) and uniformity of sample development (time and distance) will affect the accuracy and precision of quantitative measurements [7].

Several factors were reported to affect the reproducibility of the results obtained by TLC–densitometry. These factors include chamber saturation (time, temperature, chamber volume and vapor pressure of solvents). Furthermore, reproducibility is also affected by plate drying method, time delay between separation and measurement, scanner positioning and slit width [8].

The shape of calibration curves for absorption measurements has caused some concern since they are inherently nonlinear as mentioned in Kubelka-Munk theory [7]. In reflectance measurements, the area is plotted versus the substance quantity per spot. Slightly curved calibration graphs are obtained which pass through the origin [9]. For TLC quantitative purposes, only the second- and third-order polynomials are useful. Higher-order polynomials can introduce invalid maxima or minima into the approximated calibration curve [7].

In this paper, TLC–densitometry was applied for quantification of the skeletal muscle relaxant thiocolchicoside in the presence of NSAIDs glafenine and floctafenine using solvent system (ethyl acetate:methanol:acetic acid (84:13:3%, v/v/v)).

2. Experimental

2.1. Equipment

- SHIMADZU CS-9000 dual wavelength flying spot scanning densitometer with background correction supported with UV lamp short wavelength of 254 nm.
- Precoated TLC plates, silica gel GF_{254} (20×20 cm^2), 0.25 mm (Merck).
- Hamilton syringes (10 μl).
- Chromatographic tanks ($15 \times 20 \times 30$ cm^3).
- IBM computer loaded with Sigma plot 5 and Excel 97, and equipped with HP 600 printer was used for regression analysis and statistical treatment of data.

2.2. Pure samples

Thiocolchicoside and glafenine pure samples were kindly supplied by Memphis for pharmaceuticals and chemical industries, Egypt. Their purity was checked by m.p. determination which agrees with those reported in Refs. [1,10] (275 and 165 °C, respectively). They were assayed by direct spectrophotometry and found to contain $99.95 \pm 0.21\%$ [1] and $100.00 \pm 0.61\%$ [10], respectively.

Floctafenine pure sample was kindly supplied from Hoechst–Marion–Roussel, Egypt. Its purity was checked by m.p. determination (180 °C) which agrees with that reported in Ref. [10]. It was assayed spectrophotometrically and found to contain $99.98 \pm 0.31\%$ [10].

2.3. Stock solutions

- Thiocolchicoside stock solution: a stock solution containing 5 mg ml^{-1} in methanol was prepared by dissolving 250 mg of thiocolchicoside in sufficient amount of methanol and completing to 50 ml with the same solvent.
- Glafenine stock solution: a stock solution containing 50 mg ml^{-1} in methanol was prepared by dissolving 2.50 g of glafenine in sufficient amount of methanol and completing to 50 ml with the same solvent.
- Floctafenine stock solution: a stock solution containing 50 mg ml^{-1} in methanol was prepared by dissolving 2.50 g of floctafenine in sufficient amount of methanol and completing to 50 ml with the same solvent.

2.4. Thiocolchicoside working standard solutions

Take 0.5, 2.5 and 10 ml of thiocolchicoside stock solution (5 mg ml^{-1}) into 25-ml volumetric flasks and complete to the mark with methanol. This gives thiocolchicoside standard solutions of 0.1, 0.5 and 2 mg ml^{-1}, respectively.

2.5. Pharmaceutical formulations

Glifarilax tablet (produced by Memphis): batch number 399071, each tablet is labeled to contain 2 mg thiocolchicoside and 200 mg glafenine.

Idarilax tablet (Hoechst–Marion–Roussel): batch number 027, each tablet is labeled to contain 2 mg thiocolchicoside and 200 mg floctafenine.

2.6. Reagents

All solvents used were of analytical grade:

- methanol (Merck);
- glacial acetic acid, 99.8% (Adwic);
- ethyl acetate (Adwic); and
- chloroform (Prolabo).

2.7. Preparation of synthetic binary mixtures (thiocolchicoside–glafenine and thiocolchicoside–floctafenine)

Into 50 ml volumetric flasks prepare mixtures containing different ratios (1:125, 1:50 and 1:100 w/w) of each of (thiocolchicoside–glafenine) and (thiocolchicoside–floctafenine) using stock solutions thiocolchicoside (5 mg ml^{-1}), glafenine (50 mg ml^{-1}) and floctafenine (50 mg ml^{-1}).

2.8. Preparation of application solutions for determination of thiocolchicoside in Glifarilax tablets and Idarilax tablets

Weigh accurately and finely powder 20 tablets. Accurately weigh an amount of the powder equivalent to 2.5, 5, 25, 50 and 100 mg of thiocolchicoside and transfer into 100-ml beaker. Extract the drug in about 30 ml methanol using magnetic stirrer for 5 min. Filter into 50-ml volumetric flasks and complete to volume with methanol.

3. Procedures

3.1. Application of TLC–densitometry for determination of thiocolchicoside in single form and in its mixtures with glafenine and floctafenine

3.1.1. Linearity of densitometric method

Apply separately 2.5, 5 and 7.5 μl of each of the three working standard solutions of thiocolchicoside to thin-layer chromatographic plates (pre-washed with chloroform:methanol (1:1 v/v)). Spots are spaced 1.5 cm apart from each other and 1.5 cm from the bottom edge of the plate. Dry the plates using air dryer and develop by ascending chromatography in a chromatographic tank previously saturated for 1 h with 60 ml of the developing mobile phase (ethyl acetate:methanol:acetic acid (84:13:3%, v/v/v)). Developing distance of 8 cm was found sufficient for separation at room temperature.

Detect the position of thiocolchicoside spots under UV lamp (254 nm). Scan each spot from three directions (right to left, left to right and against solvent stream) using the following instrumental parameters:

photo mode: reflection;
scan mode: zigzag;
result output: chromatogram and area under the peak (AUP);
swing width: 8 mm; and
wavelength: 375 nm.

Record AUP and construct calibration curve representing the relationship between the recorded AUP and the corresponding concentration of thiocolchicoside.

Linear relationship was obtained over thiocolchicoside concentration range 2.5–15 μg per spot, as shown in Fig. 1. Regression equation parameters were computed and tabulated in Table 1.

Pseudolinear relationship following third-order polynomial was obtained over concentration range 0.25–15 μg per spot, as shown in Fig. 2. The regression equation parameters were computed and tabulated in Table 2.

3.1.2. Application of the proposed densitometric method for determination of thiocolchicoside in laboratory prepared mixtures

Apply 5 μl of the prepared binary mixtures (thiocolchicoside–glafenine and thiocolchicoside–floctafenine) to silica gel plates and proceed as under linearity. Calculate concentrations of thiocolchicoside from its regression equations. Results obtained are shown in Table 3.

3.1.3. Application of the proposed densitometric procedure for thiocolchicoside determination in Glifarilax tablets and Idarilax tablets

The optimized procedure was used for determination of thiocolchicoside concentration in its application mixtures. Results obtained are summarized in Table 4.

4. Results and discussion

TLC–densitometry was applied for determination of thiocolchicoside in presence of either glafenine or floctafenine in their laboratory prepared mixtures and pharmaceutical dosage forms. Thiocolchicoside is present as minor component with glafenine and floctafenine (1:100). Direct spectrophotometry failed in determination of thiocolchicoside in its mixtures. This can be

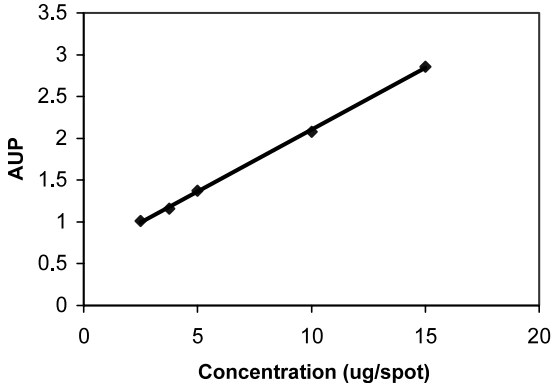

Fig. 1. Linear calibration curve for thiocolchicoside determination using TLC–densitometric method over concentration range 2.5–15 μg per spot.

Table 1
Statistical analysis of the first-order linear calibration curve for thiocolchicoside determination using TLC–densitometry

Regression equation, $Y=aX+b$	$Y=0.148X+0.6232$
Correlation coefficient	0.9970
Concentration range (μg per spot)	2.5–15.00 ($n=6$)
SE of estimation	0.0914
Slope	0.148
SE of slope	0.0020
Intercept	0.6232
SE of intercept	0.0200

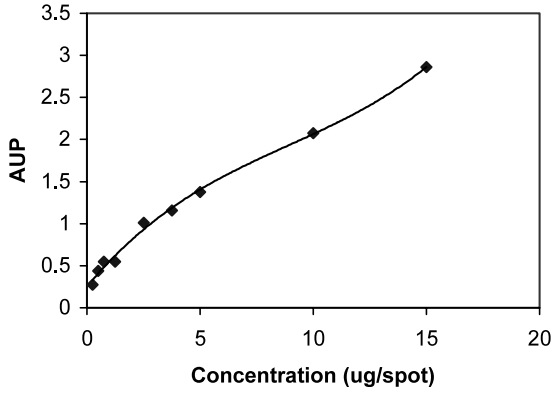

Fig. 2. Pseudolinear calibration curve for thiocolchicoside determination using TLC–densitometric method over concentration range 0.25–15 μg per spot.

Table 2
Statistical analysis of the third-order pseudolinear calibration curve for thiocolchicoside determination using TLC–densitometry

Regression equation, $Y=aX+bX^2+cX^3$	$Y=0.2523+0.3217X-0.0224X^2+0.0008X^3$	
Correlation coefficient	0.9991	
Concentration range (μg per spot)	0.25–15.00 ($n=9$)	
SE of estimation	0.0680	
Regression parameters	Coefficient	SE
Y^0	0.2523	0.0529
a	0.3217	0.0440
b	−0.0224	0.0080
c	0.0008	0.0004

attributed to the out-of-scale absorbance reading of mixtures containing either glafenine or floctafenine in such large amount even if thiocolchicoside is present in minor amount. Developing system of ethyl acetate:-methanol:acetic acid (84:13:3%, v/v/v) was found suitable for complete separation of thiocolchicoside, floctafenine and glafenine ($R_f=0.19$, 0.81 and 0.40, respectively).

Several solvent systems were tried to permit good separation without tailing. Decreasing glacial acetic acid percent gives no separation. Replacement of glacial acetic acid with ammonia results in severe tailing.

Table 3
Determination of thiocolchicoside in laboratory prepared mixtures upon the application of TLC–densitometry

Mixture number	Thiocolchicoside (μg per spot)		
	Taken	Found	Recovery (%)
1 (1:25) [a]	5.00	5.07	101.40
2 (1:25) [b]	5.00	5.00	100.00
3 (1:50) [a]	0.25	0.25	100.00
4 (1:50) [b]	0.25	0.25	100.00
5 (1:100) [a]	2.00	1.99	99.50
6 (1:100) [b]	2.00	2.03	101.50
Mean recovery (%)			100.40
SD			0.84
RSD			0.84

Each result is an average of three determinations.
[a] Mixtures of thiocolchicoside and glafenine.
[b] Mixtures of thiocolchicoside and floctafenine.

Reproducibility of separation was assessed by making runs using several prepared mixtures of thiocolchicoside with floctafenine or glafenine against their pure samples. Results revealed reproducible R_f values for thiocolchicoside, glafenine and glafenine (0.19, 0.4 and 0.81), respectively.

Exactly, strict conditions should be adhered to during TLC–densitometric applications. Plates are pre-washed with $CHCl_3$:CH_3OH (1:1 v/v) overnight before spotting 60 ml of the developing solvent and 1 h saturation time were fixed for each run. Spotting line was chosen to be 1.5 cm from plate base and 8 cm developing distance was found sufficient for good separation of thiocolchicoside from glafenine and floctafenine. Developing time was maintained constant by adjusting plate position to be always the same in the chromatographic tank and using a tank with same dimensions for all runs. Drying was carried out using cold air dryer and measurements were done immediately after drying. Each spot was scanned from three directions (right to left, left to right and against solvent stream) directly on the same plate and average AUP was taken.

First-order polynomial was found to fit concentration range 2.5–15 μg per spot. Third-order polynomial was found to fit concentration range 0.25–15 μg per spot with better correlation and standard error of estimation as shown in Tables 1 and 2.

Application of third-order polynomial equation led to better results than first-order equation with respect to calibration range (0.25–15 μg ml^{-1}), correlation (0.9991) and standard error of estimation (0.0680).

The proposed method was successfully applied for determination of thiocolchicoside in synthetic mixtures with either glafenine or floctafenine. Average recovery percent was found to be 100.40 ± 0.84. Results are shown in Table 3. Furthermore, it was applied for determination of thiocolchicoside in Glifarilax tablets

Table 4
Determination of thiocolchicoside in Glifarilax tablets and Idarilax tablets using TLC–densitometry

	Glifarilax tablets (batch number 399071)				Idarilax tablets (batch number 027)			
	Taken	Found	Recovery (%)	Reference method [a]	Taken	Found	Recovery (%)	Reference method [b]
	0.25	0.25	100.00		0.25	0.25	100.00	
	0.50	0.50	100.00		0.50	0.50	100.00	
	2.50	2.52	100.80		2.50	2.52	100.80	
	5.00	4.97	99.40		5.00	4.99	99.80	
	10.00	9.88	98.80		10.00	10.02	100.20	
n			5	5			5	5
Mean recovery (%)			99.80	100.15			100.16	99.92
Variance			0.57	0.21			0.14	0.44
SD			0.75	0.46			0.38	0.66
RSD			0.75	0.46			0.38	0.66
t			0.89 (2.31)				0.70 (2.31)	
F			2.71 (6.39)				3.14 (6.39)	

Figures in parentheses correspond to theoretical t and F values ($P = 0.05$) [13]. Each result is an average of three determinations.
[a] Colorimetric method at 400 nm [11].
[b] Direct spectrophotometric method at 375 nm [12].

and Idarilax tablets. Average recovery percentages were found to be 99.80 ± 0.75 and 100.16 ± 0.38, respectively.

Results were compared with those obtained by reference methods [11,12]. Table 4 shows that the calculated t and F values are less than the theoretical ones, indicating non-significant differences between the proposed method and the reference methods regarding accuracy and precision.

Lowest analyte concentration that produces a response detectable above the noise level of the instrument is typically three times the noise level. Detection limit was measured to be 0.125 μg per spot. Since the densitometer undergoes noise and baseline correction automatically and there is no available numerical estimate of the area cut-off of the instrument, we considered the smallest area the instrument can measure as the detection limit. Lowest level of the analyte (LOQ) that can be accurately and precisely measured was found to be 0.25 μg per spot using third-order polynomial regression.

Two-way ANOVA [13] was applied to judge the inter- and intra-day precisions of the method. The proposed densitometric method was used for determination of three analyte levels (2.50, 3.75 and 5 μg per spot) performed in three different days. Non-significant difference between days mean square and residual mean square indicates good repeatability and reproducibility of the method. Also non-significant difference between interaction mean square and residual mean square indicates that the only factor which determines instrument response is sample concentration. Results are summarized in Table 5.

The validity of the proposed method was assessed by applying the standard addition technique on the two dosage forms. Mean percentage recoveries of thiocolchicoside were found to be (101.28 ± 0.90) and (99.89 ± 0.81) upon application on Glifarelax and Idarelax tablets, respectively, proving no interference from tablets additives.

Table 5
Two-way ANOVA applied on results of thiocolchicoside determination of three different concentrations (2.50, 3.75 and 5.00 μg per spot) on three different days

Source of variation	SS	d.f.	MS	Calculated *F*
Between days	11.554	2	5.777	1.177 (3.55)
Between levels	9.989	2	4.995	
Interaction	30.745	4	7.686	1.566 (2.930)
Residual	88.363	18	4.909	1.000
Total	140.652	26		

SS, sum of squares; d.f., degree of freedom; MS, mean square. Figures in parenthesis correspond to the theoretical values ($P = 0.05$) [13].

5. Conclusion

The proposed TLC–densitometric method was found suitable for thiocolchicoside determination in presence of glafenine and floctafenine in their pharmaceutical formulations. Controlling factors that may affect variability of TLC separation and measurement result in improved accuracy and precision of the method. Slightly curved calibration curve, which can be represented by third-order polynomial, was found superior to first-order calibration curve with respect to quantification range, correlation and standard error of estimation.

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