Trade Science Inc.

September 2009 Volume 8 Issue 3

Analytical CHEMISTRY

An Indian Journal

Review

ACAIJ, 8(3) 2009 [391-399]

Determination of lacidipine by thin layer chromatographic-densitometric, spectrophotometric methods and modified Vierordt's method in presence of its photodegradates

Mohammed F.EL-Tarras[1], Lories.I.Bebawy[2,*], Samah Abd Elsabour Mohammed[2]

[1]Analytical department, Faculty of pharmacy, Cairo university, (EGYPT)

[2]National Organization of Drug Control and Research, (EGYPT)

E-mail : bebawy_lories@yahoo.com

Received: 5th July, 2009 ; Accepted: 15th July, 2009

ABSTRACT

Four methods are presented for the determination of lacidipine in presence of its photodegradates. The first method was based on the determination of lacidipine in presence of its photodegradates by densitometric thin layer chromatography (TLC) measurement at 284 nm using toluene: acetone: methanol: ammonia 25 % (60:20:6:2 by volume) as mobile phase. The second method was based on the determination of the drug at the zero crossing point of its photodegradate, where first derivative (D_1)measurement is carried out at 345 nm. The third method was based on the resolution of the drug and its degradate by first derivative ratio spectra (DD_1)at 250 nm. The fourth method was the determination of lacidipine and its photodegradate by modified Vierordt's method at 274 nm and 376 nm. The proposed methods were successfully applied for the determination of lacidipine in bulk powder, in pharmaceutical formulation with good accuracy and precision. The methods have been validated by analyzing laboratory prepared mixtures containing lacidipine and its degradates without any separation steps. The results obtained were statistically compared with that of the reported method.

KEYWORDS

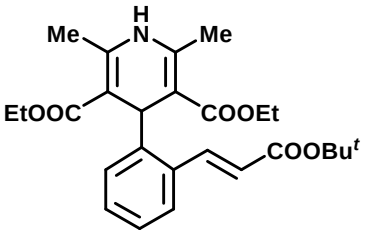
Lacidipine;
TLC densitometry;
First derivative;
First derivative of ratio spectra;
Modified Vierordt's method.

1. INTRODUCTION

Lacidipine is dihydropyridine calcium channel blocker with actions similar to those of nifidipine, it was developed for oral administration, for use in mild to moderate hypertension, and is widely used in therapy since early 90s[1,2].

Lacidipine belongs to dihydropyridine class and its structure is characterized by the presence of cinnamate moiety; the active trans form is used in therapy, while the cis isomer is inactive. The drug is sensitive to light, in line with well-known photosensitivity of dihydropyridine compound class[3,4] to give the inactive cis isomer. Lacidipine is Diethyl- 4- (2-[ter-butoxycarbonyl)vinyl]phenyl) – 1,4 – dihydro-2,6-dimethylpyridine-3,5-dicarboxylate[5]. It is white to pale yellow crystalline powder, partial insoluble in water, soluble in dehydrated alcohol, acetone and dichloromethane[6]. It has the following structural formula[6].

Few procedures have been published for the determination of lacidipine UV spectrophotometry[7], polarography[8], partial least square method[7], Factorial Wavelet method[9], HPTLC[10] and HPLC[11-18] and



Lacidipine

Molecular weight: 455.5[6].

micellar electrokinitic chromatography[19].

Degradation was enhanced by the exposure of 1mg/ml ethanolic solution of the drug to UV- lamp at 254 nm wave length for about 16 hours.

Elucidation of the structure of the degradation product was carried out using mass spectroscopy.

The aim task of this work was the development of simple, selective and accurate methods for the determination of lacidipine in the presence of its photodegradates without the need of the prior separation step. The developed methods to determine the content of the cited drug in bulk powder and in pharmaceutical formulations are also demonstrated.

2. EXPERIMENTAL

2.1. Instruments

1-A double- beam Shimadzu (Japan) 1601 PC UV-Visable spectrophotometer connected to computer fitted with UVPC personal spectroscopy software version 3.7 (Shimadzu) was used, the spectral band width was 2 nm, the absorbance spectra of the test and reference solution was recorded in 1 cm quartz cells over the rang 200 - 400 nm. The first derivative was obtained using the accompanying soft ware with $\Delta\lambda = 4$ and scaling factor= 10.

2-For TLC measurement the samples were spotted using Hamilton micro- syringe onto silica gel GF_{254} precoated TLC plates of particle size of 0.25 mm (E-Merck, Darmstadt, Germany). The plates were prewashed with methanol and activated at 60^0C for 5 minutes prior to chromatography. The plates were scanned densitometrically using a Shemadzu dual wavelength flying spot densitometer Model CS 9301, the experimental conditions of the measurements were, wavelength = 284 nm, photo mode = reflection, scan mode = zigzag, swing width = 16.

3- Sonicstor, Bandelin-Sonorex TK (Germany).

2.2. Sample

2.2.1. Pure sample

Pharmaceutical grade of lacidipine was kindly supplied by Glaxo Company (Cairo, Egypt), its purity was 100.03±0.593% according to manufacturer method[20].

2.2.2. Market sample

The commercial Lacipil tablet used (Batch No. 043708A) was produced by Glaxo Company (Cairo, Egypt). Each tablet contains 2 mg lacidipine.

2.2.3. Reagents

All reagents and chemicals used were of analytical grade and solvent were of spectroscopic grade.

1-Ethyl alcohol (E. Merck).
2-Toluene (ADWIC).
3-Acetone (ADWIC).
4-Methanol (E. Merck).
5-Ammonia, 25% solution (ADWIC). Sp.gr. 0.91.

2.3. Chromatographic condition

The TLC plates were developed in toluene: acetone: methanol: ammonia 25 % (60:20:6:2 by volume) as a mobile phase. For detection and quantification, 25 µl of different concentrations of test and standard solutions within the quantification range were applied as separate compact apart 15 mm apart and 15 mm from the bottom of TLC plate using 25 µl Hamilton microsyring. The plate was developed up to the top (over a distance of 15 cm) in usual ascending way. The chromatographic tank was saturated with the mobile phase in the usual mode. After elution the plate was air dried and scanned for lacidipine at 284 nm as under the described instrumental parameters.

2.4. Preparation of photodegradates

An accurately weighed 25 mg of lacidipine bulk powder was dissolved in 20 ml methanol and transferred into 100-mL loosely stopper conical flask, and exposed to UV- lamp at 360 nm wave length for about 16 hours. The solution was applied in a band form into TLC plate and 5 µl of standard solution in ethanol (1 mg/ml) was spotted on the same plate. The plate was placed in chromatographic tank pre-

viously saturated for 1 hr with the developing solvent and then air dried. The photolytic degradates was visualized under UV lamp at 254 nm to test for the complete degradation.

The bands corresponding to the degradates were scrapped and extracted with 3 × 20 ml ethanol. The extract was filtered and evaporated just to dryness. The residue was used to prepare the laboratory prepared mixtures.

The stock solution of the degradation product was prepared in ethanol (1.00 mg / ml).

2.5. Laboratory prepared mixtures

From both standard solutions, aliquots were accurately transferred into series of 10 ml volumetric flask to prepared different mixtures containing (10-90%) of the degradates and the volume of each flask was completed with the solvent used in the proposed methods.

2.6. Standard solutions and calibration graphs

2.6.1. Thin layer chromatography (TLC) method

Standard in the concentration of 50-450.0 µg /ml were prepared in ethanol. Each standard solution of 10 µl was applied to the TLC plate. The plates were chromatographed as mentioned above and the peak areas were measured. Calibration graph was constructed by plotting the peak areas versus concentrations of lacidipine. Linear relationship was obtained and the regression equation was computed.

2.6.2.First derivative (D_1) method

Standard solution of lacdipine in the concentration range 10.00- 80.00 µg/ml were prepared in ethanol. The values of D_1 amplitudes were measured at 345 nm. The D_1 amplitudes were plotted against the concentration of lacidipine. Linear relationship was obtained and the regression equation was computed.

2.6.3. First derivative of ratio spectrophotometric (DD_1) smethod

Standard solution of lacdipine in the concentration range 10.00- 70.00 µg/ml were prepared in ethanol, the spectrum of each solution was scanned and stored. The values of DD_1 amplitudes were measured at 250 nm using 3.00 µg/ml of photodegradate as a divisor. The DD_1 amplitudes were plotted against the concentrations of lacidipine. Linear relationship was obtained and the regression equation was computed.

2.6.4. Modified Vierordt's method

Standard solution of lacidipine in concentration range of 2.50-22.00 µg/ml and standard solution of its photodegradate in concentration range of 10.00- 40.00 µg/ml were prepared in ethanol, the absorption spectrum of each solution were measured at 274 nm. The absorption values were plotted against the concentration.

Standard solution of lacidipine in concentration range 10.00-80.00 µg/ml were prepared in ethanol, the absorption values at 367 nm were measured and the standard curve was plotted.

2.7. Analysis of laboratory prepared mixtures

Each laboratory prepared mixture was analyzed as described under each method. The concentration of lacidipine in the prepared mixture was determined from the corresponding regression equation.

2.8. Sample preparation

Twenty tablets of lacidipine were weighed and powdered. An accurately weighed quantity of the powdered tablet equivalent to 25 mg was extracted with ethanol 2 X 10 ml by shaking in ultrasonic bath for about 10 minutes, the solution was filtered each time into 25 – ml volumetric flask and the volume was completed to the mark with ethanol (1.00 mg/ml). Other dilution with ethanol was done to prepare (200 µg/ml) for D_1 and DD_1. Then the procedures were completed as mentioned under calibration for each method. In the first method, the concentration of lacidipine was determined either by substituting in the regression equation or by comparing to standard spotted, developed, scanned under the same conditions and in the other methods the concentration of lacidipine was determined using the corresponding regression equations

2.9. Percentage recovery study

This study was preformed by adding lacidipine standard and its photodegradate to a known concentration of commercial tablet (standard addition technique). The resulting mixtures were assayed and the results obtained for the drug were compared with expected results.

Review

3. RESULTS AND DISCUSSION

Physicochemical properities of the photodegradates of lacidipine is studied by *Fillippis et al*[11] and the degradates were elucidated. In this work lacidipine is subjected to photodegradation under UV lamp at 254 nm for 16 hours, then the solution was identified by a mass spectroscopy.

3.1. TLC method

The experimental condition for TLC method as mobile phase, scan mode and wavelength of detection were optimized to provide accurate, precise and reproducible results for the determination of lacidipine in the presence of its photodegradates. The chosen scan mode was zigzag and the wave length of the scanning was 284 nm. The best separation of the studied drug and its photodegradates was obtained using toluene: acetone: methanol: ammonia 25 % (60:20:6:2 by volume) as the developing mobile phase. The R_f values for lacidipine and its photodegradates were 0.807 and 0.926, respectively, as shown in Figure 1,2.

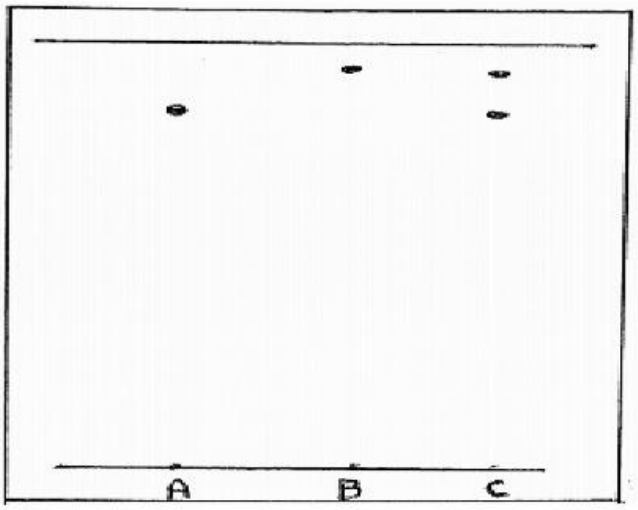


Figure 1 : TLC chromatogram of lacidipine and its photodegradate visualized under UV lamp at 254 nm. Mobile phase : Toluene: acetone: methanol: ammonia (60:20:6:2 by volume) (A) pure drug, (B) Photodegradates, (C)Laboratory prepared mixture.

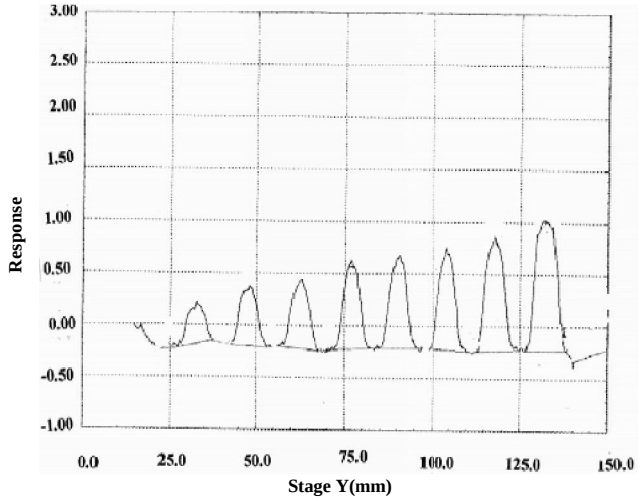


Figure 2 : Scanning profile of lacidipine (0.50-4.50 µg/spot) at 284 nm. Mobile phase: toluene: acetone: methanol: ammonia (60: 20: 6: 2 by volume).

3.2. D_1 method

The main instrumental parameters that affect the shape of the derivative spectra such as the wavelength, scaling factor speed, the wavelength over which the derivative is obtained ($\Delta\lambda$) and the smoothing were optimized to give a well- resolved, good selectivity, large sensitivity in the determination. Generally, the noise level decreases with an increase in $\Delta\lambda$, thus decreasing the fluctuation in the derivative spectrum. However if the value of $\Delta\lambda$ is too large, the spectral resolution is very poor. Therefore, the optimum value of $\Delta\lambda$ should be determined by taking into account the noise level and the resolution of the spectrum. $\Delta\lambda = 4$ nm, scaling factor 10 were selected for D_1 as the optimal conditions to give a satisfactory signal to noise ratio.

Zero order absorption spectra of lacidipine and its degradates in ethanol showed sever overlapping which interfere with the direct determination of the drug Figure 3. By applying the first derivative technique, overlapping was eliminated and a well separated peak at 245 nm for the drug which lies at the zero crossing of its degradates was obtained Figure 4. The calibration curve was constructed by plotting peak amplitude versus concentration conforming with Beer's law was evident in the concentration range cited in TABLE 1.

3.3. DD_1 method

The ratio spectra method is one of the popular methods for simultaneous determination of the compounds in presence of interfering substance. The ratio derivative method allowed the determination of lacidipine in presence of its photodegradates without any interference. The peak amplitude was measured at 250 nm as shown in Figure 5, 6.

The main parameters that affect the shape of the

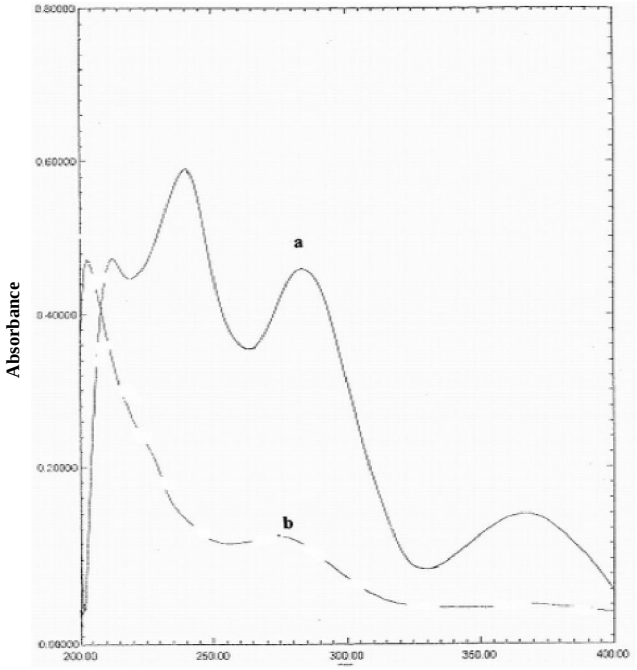


Figure 3 : Zero order absorption spectra of (a) lacidipine (70.00 µg / ml) in ethanol (___), (b) its photodegradates (30.00 µg / ml) in ethanol (-----).

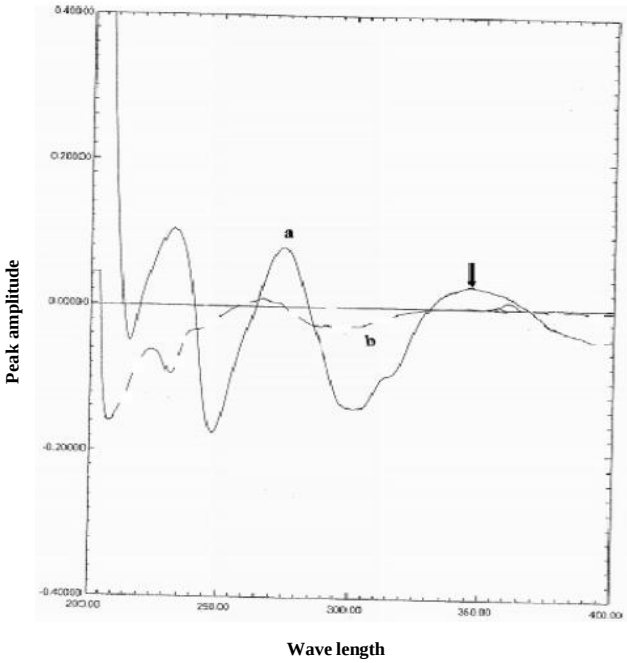


Figure 4 : First derivative of: (a) Lacidipine (70.00 µg/ml) in ethanol at 345 nm (___), (b) Its photodegradates (30.00 µg/ml) in ethanol (-----).

TABLE 1 : Validation parameters for the regression equations of TLC, D_1, DD_1 and modified Vierordt's methods for the determination of lacidipine.

Parameters	TLC	D_1	DD_1	Modified Vierordt's method		
				Lacidipine at 274 nm	Lacidipin at 367 nm	Degradates at 274 nm
- Wave length	284	345	250	274	367	274
-Specificity (SD)						
R_f intra-day[a]	0.0050					
R_f inter-day[a]	0.03008					
P.A or P. Am. intra- day[a]	0.0052	0.0035	0.0455			
P.A. or P. Am. inter-day[a]	0.0200	0.0016	0.0228			
-Linearity	µg/spot 0.50-4.50	µg/ml 10.00-80.00	µg/ml 10.00-70.00	µg /ml 5.00-20.00	µg/ml 10.00-80.00	µg/ml 10.00-40.00
-Regression equation[b]						
Slope (b)	1379.5	2.7×10^{-3}	34.10×10^{-3}	0.0445	0.0136	0.0265
Intercept (a)	- 59.576	-1.4×10^{-3}	0.0209	0.0194	-0.0074	0.48
Correlation coefficient (r)	0.999	0.999	0.999	1.000	0.999	1.000
-SD of the slope[c]	0.0202	0.0212	0.0094	0.0245	0.0021	0.0035
-SD of the intercept[c]	0.2827	0.1465	0.4045	0.0021	0.0005	0.0360
-SD of r	0.0031	00000	0.0010	0.0014	0.0002	0.0006
Precision (mean ± RSD%)						
Intra-day[d]	100.12±0.471	100.296±1.043	100.17±1.057	99.56±1.02	98.172±1.207	101.54±0.945
Inter-day[d]	99.67±0.245	98.23±1.259	100.89±0.357	100.24±0.642	100.48±0.814	100.46±0.147

[a]Average of n=5, [b]Y= a+ b C, where C is the concentration of lacidipine and Y is the peak area (P.A.) for TLC or peak amplitude (P.Am.) for D_1 and DD_1 or absorbance for modified Vierordt's method, [c]Average of n = 3, [d]Average of n = 9.

Review

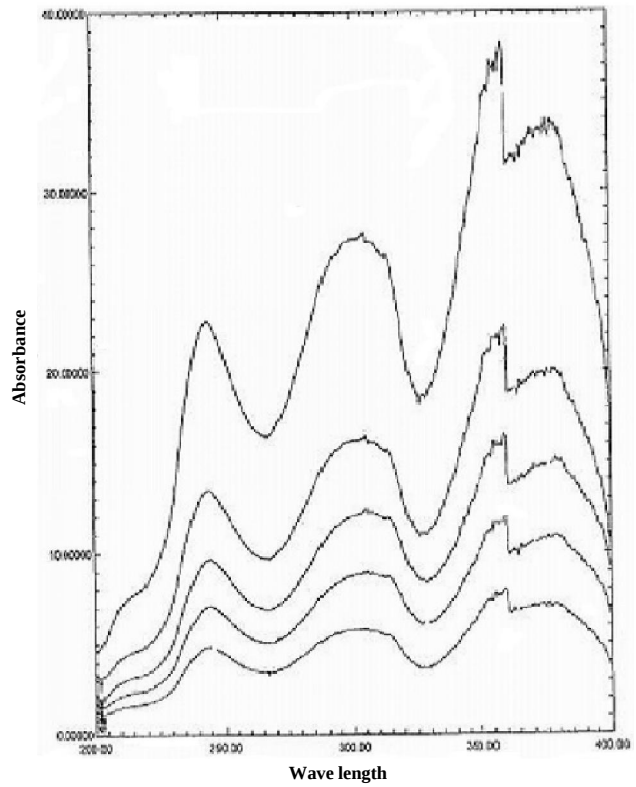


Figure 5 : Ratio spectra of lacidipine (10.00-70.00 µg / ml) in ethanol using (30.00 µg/ml) photodegradates as divisor.

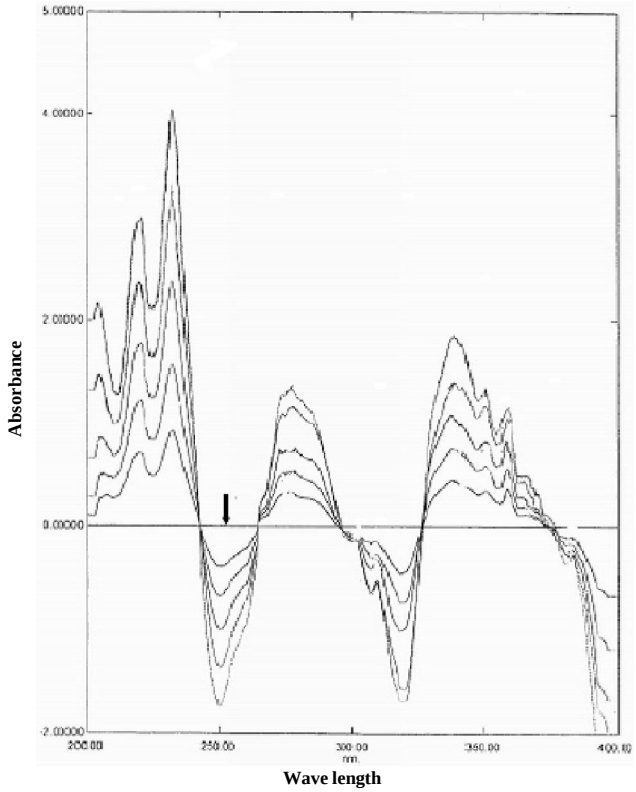


Figure 6 : First derivative of ratio spectra of lacidipine (10.00-70.00 µg/ml) in ethanol using (30.00µg/ml) photodegradates as divisor at 250.

ratio derivative spectra are the concentration of the degradates solution used as divisor, scaling factor and the wavelength increment over which the derivative was obtained $\Delta\lambda$. Different concentrations of the degradates were studied as constant divisor (10, 30 and 80 µg /ml), the best one was (30.00 µg /ml) as it produces minimum noise and give better results in accordance with selectivity and sensitivity. The concentration range is shown in TABLE 1.

3.4. Modified Vierordt's method

Modified Vierordt's was applied for determination of components in mixtures (intact lacidipine and its photodegradates) it involves absorbance measurements at two suitable wave lengths and determination of the concentration of two components x and y from the two simultaneous equations.

$$A_1 = \alpha_1 C_x + \beta_1 C_Y \quad (1)$$

$$A_2 = \alpha_2 C_x + \beta_2 C_y \quad (2)$$

Where the subscripts 1 and 2 refer to the wavelengths, A_1, A_2 denote the absorbance of mixtures solution of the two substances, C_x and C_y designate thier concentration, α and β represent the A_1 (1%, 1 cm) values for lacidipine and its photodegradates, respectively.

Substituting in equations (1) and (2) by the following expressions.

$A_2/A_1 = m$ and therefore $A_2 = m A_1$

$\alpha_2/\alpha_1 = a$ and therefore $\alpha_2 = a \alpha_1$

$\beta_2/\beta_1 = b$ and therefore $\beta_2 = b \beta_1$

These equations can be written:

$$A_1 = \alpha_1 C_x + \beta_1 C_y \quad (3)$$

$$A_2 = mA_1 = a \alpha_1 C_x + b \beta_1 C_y \quad (4)$$

and can be solved to give:

$$C_x = \frac{A}{\beta_2} \cdot \frac{b-m}{m(b-a)} \qquad C_y = \frac{A_2}{\alpha_1} \cdot \frac{b(m-a)}{b-a}$$

The ratio "m" must be calculated for each determination and the ratios "a" and "b" are constant for the determination of C_x and C_y.

The use of α and β factors minimized most of the error attributed to changes in the instrumental parameters of the spectrophotometer, which inevitably occur with time.

Once "a" and "b" are determined, there is no need to re-measure them until the analyst judges that the instrumental parameters have changed.

The method was applied for the determination of lacidipine in presence of its photodegradates without any interferences.

The absorbance values of the mixture were measured at 274 nm and 376 nm.

This method was used for the determination of the cited drug in presence of its photodegradates in concentration range shown in TABLE 2. The calibration curve was constructed by plotting the absorbance versus concentration.

TABLE 2 : Determination of lacidipine in laboratory prepared mixtures and commercial tablets using the proposed methods and manufacturer HPLC method.

	Mean found ± RSD%[a]				
	TLC	D_1	DD_1	Modified Vierordt's	Manufacturer method
-Laboratory prepared mixtures	99.38±1.37	100.804 ±1.643	100.31± 1.495	100.72±0.978	
-Commercial tablets mean[a] ± RSD%	99.93±1.200	100.20±1.129	99.37±0.810	99.29±0.250	98.53±0.550
t (2.306)[b]	1.837	2.303	2.265	2.179	
F (5.19)[b]	4.760	4.36	2.169	4.84	
Mean [c]± RSD%	100.60 ± 1.50	99.87±1.804	99.89±1.641	99.09±1.190	

[a]Average of five determinations, percentage recovery from the label amount., [b]Theoretical values for t and F at 95 % confidence level., [c]For standard addition of 50 % of nominal content (n= 5).

The accuracy of the proposed methods was checked by analyzing different laboratory prepared mixtures of the cited drug in presence of its degradates in different ratios, TABLE 2. Results obtained showed satisfactory recoveries with small relative standard deviation (RSD) which indicate high repeatability and accuracy of the proposed methods. The proposed methods were also successfully applied for the analysis of lacidipine in pharmaceutical dosage form, the results are shown in TABLE 2. The validity of the methods was assessed by applying the standard addition technique and the results obtained were reproducible with low RSD as shown in TABLE 2. The mean recovery of tablets was compared with that obtained by the reported method, there was no evidence of interference from excipients. TABLE 2 shows the statistical comparison of results for determination of lacidipine by the proposed methods and the manufacturer one. Results of t- test for accuracy and F- ratios for the precision measurement did not exceed the corresponding theoretical values, indicating insignificant differences between the results, supporting the robustness of the proposed methods. Also statistical comparison of results of analysis of bulk powder was done and results are shown in TABLE 3.

TABLE 3 : Determination of lacidipine in bulk powder using the proposed methods and manufacture HPLC method.

	TLC	D_1	DD_1	Modified Vierordt's	Manufacturer method
mean [a] ± RSD%	100.12±0.419	100.29±1.043	100.17±1.05720.8	100.72±0.978	99.70±0.698
t (2.306) [b]	1.153	1.051	298	1.899	
F (5.19) [b]	2.767	2.234	2.294	2.008	

[a]Average of five determinations, percentage recovery from the label amount., [b]Theoretical values for t and F at 95 % confidence level.

3.6. Method's validation

3.6.1. Specificity

TLC, D_1 and DD_1 methods showed suitable specificity for drug identification. Chromatographic specificity was investigated by R_f and peak area (PA) of the drug using standard solutions. The derivative and ratio derivative methods are specific for the determination of lacidipine in presence of its photodegradates at the selected wave lengths. The inter- day and intra – day variations (SD) of R_f and peak area for TLC method, peak amplitude for D_1 and DD_1 are shown in TABLE 1.

The assay results are unaffected by the presence of extraneous materials (degradates, excipients). The good results of laboratory prepared mixtures prove specificity of the proposed methods.

Review

3.6.2. Linearity

Three independent calibration equations were obtained. Calibration curves were prepared and analyzed daily. Linear regression analysis was used to calculate the slope, intercept and correlation coefficient (r) of each calibration. The standard deviation was calculated for each parameter.

3.6.3. Precision

Methods precision was assessed by repeatability and reproducibility of TLC, D_1 and DD_1 methods. The inter and intra- day variations expressed by mean ± RSD %, TABLE 1 were determined by assaying three samples in triplicate over a period of 3 days using concentration represented the entire range of the calibration curves (0.50, 2.00, 4.50 µg/spot for TLC), (10.00, 50.00, 80.00 µg / ml for D_1) and (10.00, 50.00, 70.00 µg/ ml) for DD_1.

3.6.4. Accuracy

The recovery method was studied where a known amount of standard drug was added to pharmaceutical formulation and recovered standard was calculated.

3.6.5. Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small variations in method conditions. With respect to composition of mobile phase of TLC, no significant influence in R_f and peak area was found when the composition of the mobile phase toluene: acetone: methanol: ammonia (60: 20: 6: 2 by volume) was tested in different ratios. Therefore the proposed method is robust and remains unaffected by small variations in the mobile phase compositions and overtime of method performance with RSD less than 2 %, TABLE 1.

4. CONCLUSION

The proposed TLC, D_1, DD_1 and Modified Vierordt's methods provide simple, accurate and reproducible quantitative analysis for the determination of lacidipine in pharmaceutical tablets and in the presence of its photodegradates. The TLC method was found to be more specific and selective than spectrophotometric methods, while the derivative spectrophotometric methods have the advantage of low cost, rapid, and environmental protection. The TLC method is simple and uses a minimal volume of solvents. Modified Vierordt's method can also determine the drug and its degradates, the proposed methods complied with USP validation guidelines.

The advantages of the suggested methods were the ease of performance, reproducibility, the lack of complicated pretreatments before analysis. In addition the methods have a potential for application in quality control laboratories.

REFERENCE

[1] J.E.F.Renold, A.Martinale; "The Extra Pharmacopiea", 35th, The Pharmaceutical Press, London, pp. 1231 **(2007)**.

[2] P.Tcherdakoff, J.M.Mallion, K.H.J.Rahn; Cardiovasc.Pharmacol., **25**, 27 **(1995)**.

[3] J.V.Greenhil; Is the photodecomposition of drug predictable, in H.H., H.H.Tonnesen, The photostability of Drugs and Drug Formulations, Taylor-Francis, London, 93 **(1996)**.

[4] A.Albini, E.Fasani; Photochemistry of drugs: an overview and practical problems. in: A.Albini, E.Fasani (Eds). Drugs Photochemistry and Photostability. The Royal Society of Chemistry, Cambridge, 1 **(1998)**.

[5] S.Baraggio, S.Sartori, F.Angeri, M.Pellegatti; J.Chromatogr.B.Biomed.Appl., **669(2)**, 383 **(1995)**.

[6] The Merck index; 14th ed., White house station, Merck research Laboratories, pp. 1033 **(2006)**.

[7] G.Ragno, G.Ioele, M.De Luca, A.Garofalo, F.Grande, A.Risoli; J.Pharm.Biom.Anal., **42(1)**, 39 **(2005)**.

[8] S.A.Ozkan; Pharmazie., **57(7)**, 503 **(2002)**.

[9] E.Dinc, G.Ragno, G.Ioele, D.Baleanu; JAOAC, **89**, 1538 **(2006)**.

[10] V.R.Kharat, K.K.Verma, J.D.Dhake, J.Pharm.Biomed.Anal., **(3-4)**, 789 **(2002)**.

[11] P.De Filippis, E.Bovina, L.Da Ros, J.Fiori, V.Cavrini; J.Pharm.Biomed.Anal., **27**, 803 **(2002)**.

[12] M.J.Nozal, J.L.Bernal, J.J.Jiménez, M.T.Martín, F.J.Diez; J.Chromatography A, **1024**, 115 **(2004)**.

[13] J.A.Lopez, V.Martinez, R.M.Alonso, R.M.Jimenez; J.Chromatogr.A, **870(1-2)**, 105 **(2000)**.

[14] A.B.Baranda, R.M.Jimenez, R.M.Alonso; J.Chromatogr.A, **1031(1-2)**, 275 **(2004)**.

[15] N.V.Ramakrishna, K.N.Vishwottam, S.Puran, S.Manoj, M.Santosh, M.Koteshwara; J.Mass Spectrom., **39(7)**, 824 **(2004)**.
[16] A.B.Baranda, C.A.Mueller, R.M.Alonso, R.M.Jimenez, W.Weinmann; Ther.Drug Monit., **27(1)**, 44 **(2005)**.
[17] M.Pellegatti, S.Braggi, S.Sartori, F.Franceschetti, G.F.Bolelli; J.Chromatogr., **573(1)**, 105 **(1992)**.
[18] M.Pellegatti, S.Braggio, S.Sartori; J.Chromatography, **573**, 105 **(1992)**.
[19] V.Martinez, J.A.Lopez, R.M.Alonso, R.M.Jimenez; J.Chromatogr.A, **836**, 189 **(1999)**.
[20] Glaxo company; by self communication.
[21] The United State Pharmacopeia; 27, Asian Edition. United States Pharmacopial Convention, Inc., Twinbook Parkway, Rockville. MD., P. 1703, 2149, 2152 **(2008)**.