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Determination of Dipyridamole in the presence of its degradation products and in the presence of Aspirin

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ABSTRACT

A stability-indicating thin-layer chromatographic method (TLC) was developed for the determination of Dipyridamole in presence of its acid, alkaline, oxidative and thermal induced degradates. This TLC method and a double divisor ratio spectra derivative method were developed for determination of Dipyridamole in presence of Aspirin and Salicylic acid, as the combination of Dipyridamole and Aspirin is widely used to reduce thrombosis in patients with thrombotic diseases. Dipyridamole was separated by TLC densitometic method from its degradates on silica gel plates using acetonitrile:ammonia 33% (4:1, v/v) as a developing system. This method depends on quantitative densitometric evaluation of thin layer chromatogram of Dipyridamole at 230 nm over a concentration range of 0.30-15.00 µg/spot with mean percentage recovery 99.87 ± 0.62. The double divisor ratio spectra derivative method (DDRD) depends on the measurement of the amplitude at 252nm using Aspirin and Salicylic acid as double divisor. Calibration graph of (DDRD) method for Dipyridamole is linear in the concentration range 1–10 µg /ml with mean percentage recovery 99.47 ± 1.41. The proposed methods have been successfully applied for the analysis of Dipyridamole in pharmaceutical dosage form without interference from other dosage form additives and the results were statistically analyzed and compared with those obtained by certain validated published method. Moreover, The TLC-densitometric method was successfully applied for the determination of Dipyridamole in spiked human plasma with mean percentage recovery 101.96± 4.28.

KEYWORDS

Stability;
Acid;
Plasma;
Divisor.

INTRODUCTION

Dipyridamole is widely used as a coronary vasodilator in patients with high blood pressure, a prophylactic agent in patients with angina pectoris and an inhibitor of platelet aggregation in various thromboembolic conditions[1]. The known pharmacological approach for the prevention of thrombotic accidents is to use Dipyridamole in combination with Aspirin (acetylsalicylic acid). The mechanism of action is to inhibit the cellular uptake and metabolism of adenosine with resulting vasodilatory and anti-aggregatory effects. Aspirin inhibits platelet aggregation by irreversible inhibition of platelet cyclooxygenase and thus inhibiting the generation of Thromboxane A2. Dipyridamole inhibits the uptake of adenosine into platelets and

endothelial cells, thus decreasing the adhesion of platelets to thrombogenic surfaces[2,3]. Analytical methods such as high performance liquid chromatography HPLC[4-10], electrochemical analysis[11-14], spectrophotometric methods[15] and phosphorimetric methods were reported for the determination of Dipyridamole[16,17]. A few analytical procedures were also proposed for the determination of Dipyridamole in dosage forms in human plasma, serum, urine and feces[18-20]. Although the combinational use of Aspirin and Dipyridamole is continuously increasing, few methods were reported for the simultaneous determination of Aspirin and Dipyridamole including combination of liquid chromatographic and mass spectrometric detection[21], second-order derivative spectrophometry[22] and by spectrofluorimetric method[23]. The focus of the our study is to develop and validate a simple stability-indicating method for the determination of Dipyridamole in presence of its acid, alkaline, oxidative and thermal degradates for the quality control of Dipyridamole as well as in presence of Aspirin and Salicylic acid without any interference that can be used for quality control and routine analysis.

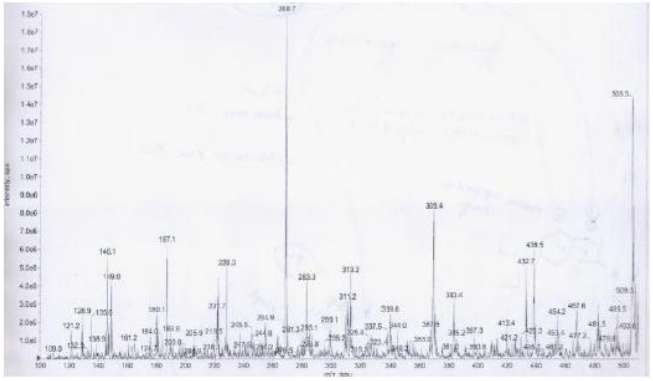
MATERIALS AND METHODS

Instruments

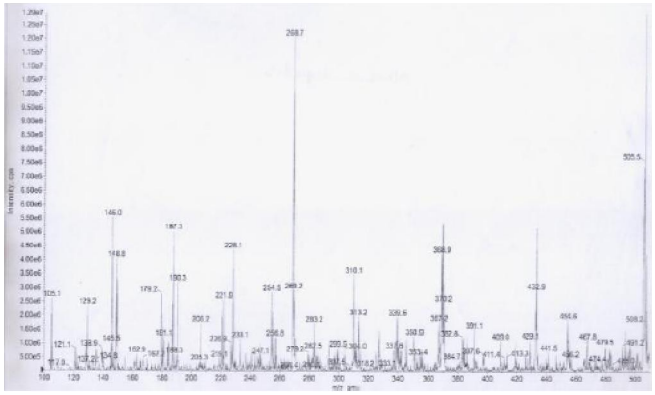
TLC scanner (Desaga Densitometer model CD60 (Germany) with the following requirements are taken into consideration:

- Photo mode : Reflectance.
- Scan mode : Linear slit scanning.
- Scanning speed : 20 mm/ sec.
- Slit width = 0.4 mm.
- Slit height = 0.02 mm
- Result output : Densitogram and peak list.

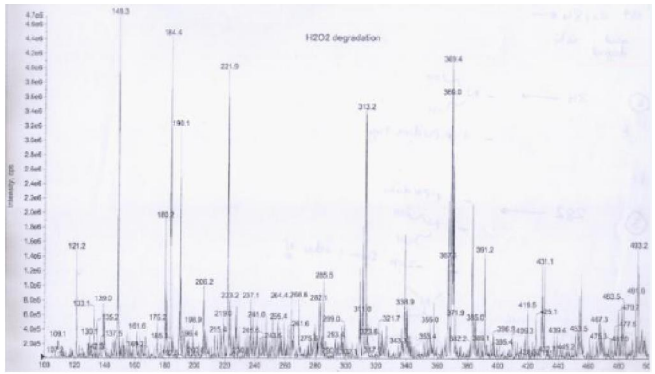
TLC plates (20cmX10cm) coated with silica gel 60 F254(Merck, Germany). A 30 Desaga Applicator. A Desaga chromatographic tank 20× 21× 9 cm. A Desaga UV lamp (254 nm), (USA). Oven, TEQ, Model D4MV (Portugal). Jenway PH meter (Germany). Thermostatic multiple water bath, model BT- 15 (Spain). Double beam UV-VIS spectrophotometer (Shimadzu, Japan) model1601 Pc with quartz cell of 1 cm pathlength, connected to IBM compatible computer. The software was UVPC personal spectroscopy software version 3.7. The spectral band width was 2 nm and the wavelength scanning speed 2800nm /min. High Performance Liquid Chromatograph (HPLC) consists of a solvent delivery system (SHIMADZU LC-20AT), which is a stand-alone modular unit that features a reciprocating single piston isocratic pump. The pump is coupled with an efficient pulse damping hardware and delivers flow in



(a)



(b)



(c)

Figure 1 : LC-MS spectra of stressed Dipyridamole samples (a- acid degradates, b- alkaline degradates and c- oxidative degradates.

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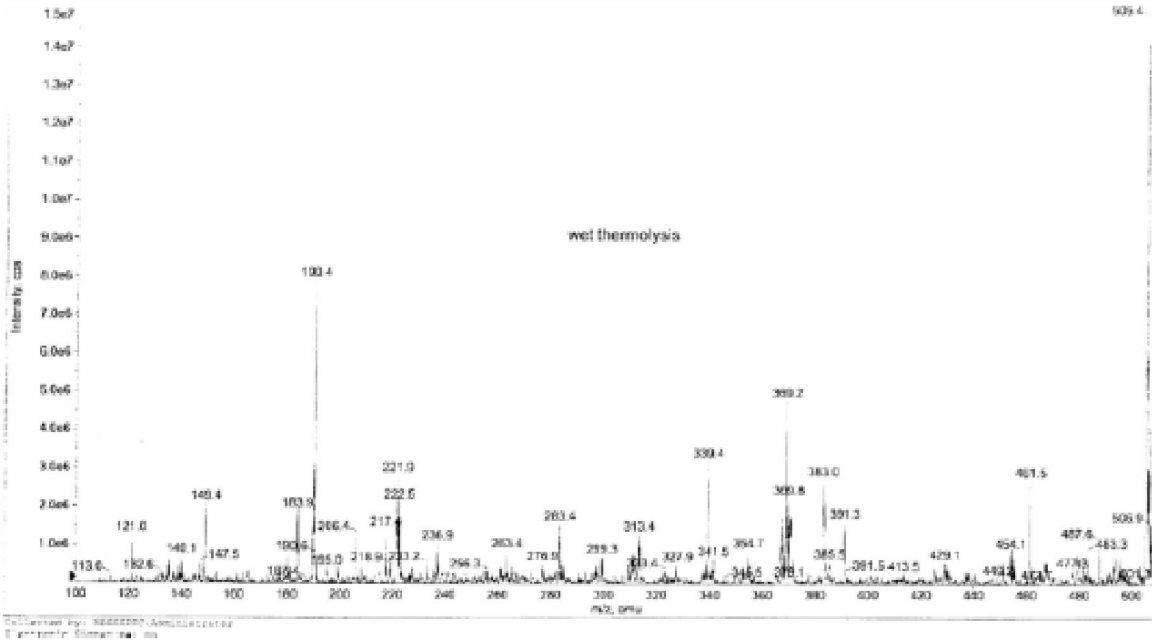

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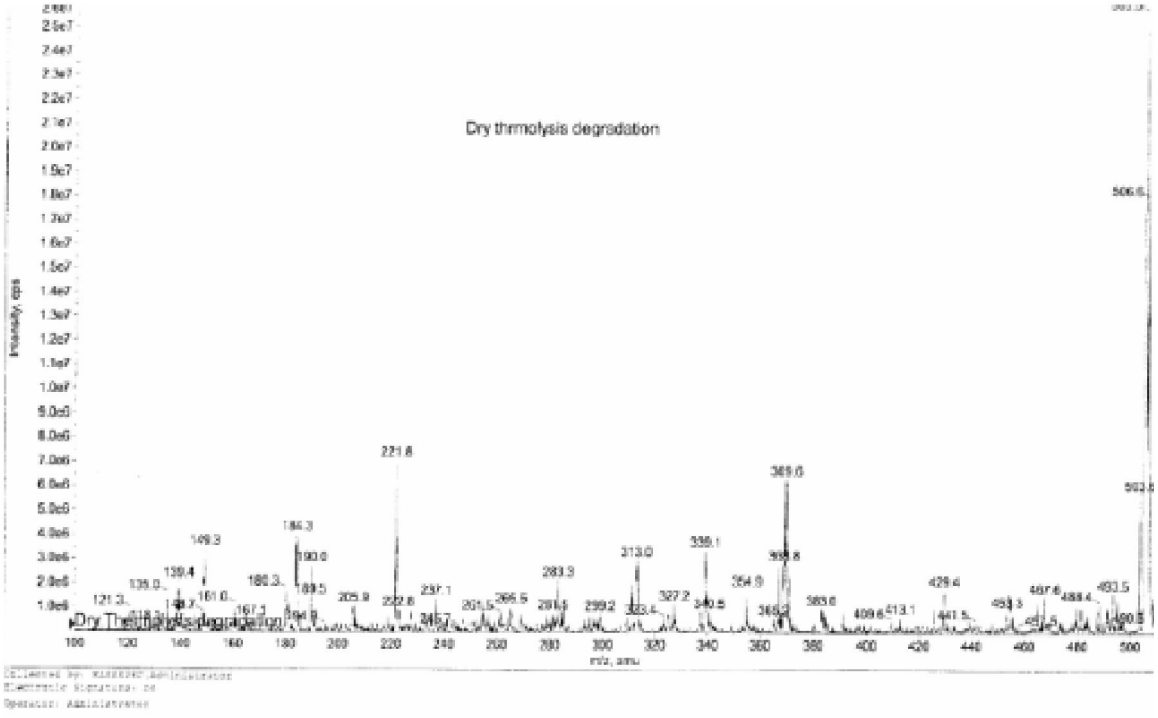


(b)

Figure 2 : LC-MS spectra of stressed Dipyridamole samples (a- wet thermolysis degradates and b- dry thermolysis degradates)

the linear dynamic range up to 10 (ml/min). SHIMADZU "chromatography data system" was employed for data collection and handling. Shimadzu Autosampler Model SIL-20A. Analyst software version 1.4.3, applied biosystems, MDS, SCIEX, Canada. API 3200 LC/ MS/MS system (mass detector) applied biosystems MDS, SCIEX, Canada.

Materials

Pure standards

Dipyridamole was supplied by Cid Pharamaceutical Company (Cairo, Egypt). Its purity was found to be 99.90% [12]. Acetyl salicylic acid was supplied by Hikma Pharma (6th October, Egypt). Its purity was found to

be 99.50% [23]. Salicylic acid (El-Nasr Pharmaceutical company, Abu-Zabal, Cairo, Egypt).

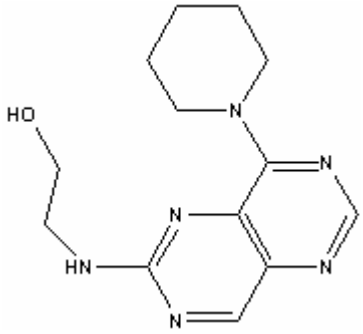
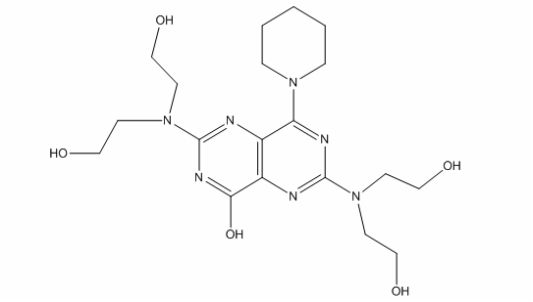
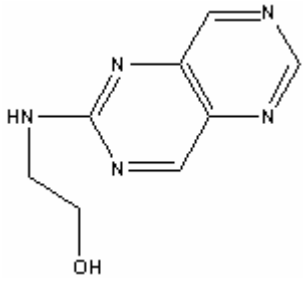
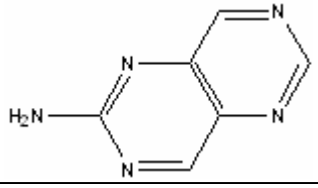
Pharmaceutical dosage forms

Persantin®-75 tablets were manufactured by Cid Pharamaceutical Company (Cairo, Egypt) under license of BoehringerIngelheim, Canada. Each tablet was labeled to contain 75 mg of Dipyridamole(batch No.100122).

Chemicals and Reagents

All chemicals and solvents used throughout this work were of analytical grade. Ethyl acetate, Sodium hydroxide, Hydrogen peroxide 50% w/v, Ammonia

TABLE 1 : Acid degradants of Dipyridamole identified by the proposed LC/MS method

Degradant	m/z	Chemical name	Structure
A	268	2-((8-(piperidin-1-yl)pyrimido[5,4-d]pyrimidin-2-yl)amino)ethanol	
B	437	2,2',2'',2'''-((4-hydroxy-8-(piperidin-1-yl)pyrimido[5,4-d]pyrimidine-2,6-diyl)bis(azanetriyl))tetraethanol	
C	190	2-(pyrimido[5, 4-d]pyrimidin-2-ylamino)ethanol	
D	147	pyrimido[5, 4-d]pyrimidin-2-amine	

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33%, Hydrochloric acid (El-Nasr Pharmaceutical company, Abu-Zabal, Cairo, Egypt). Acetonitrile, Methanol ((J.T.Baker, USA), (HPLC grade). Ortho phosphoric acid (Ridel-deHa¸n, Sigma-Aldrich, Germany).

Standard solutions

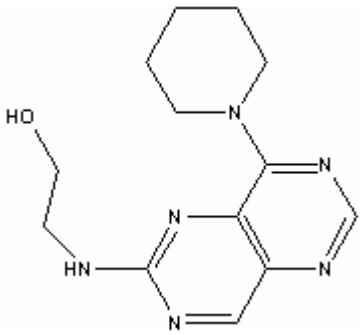
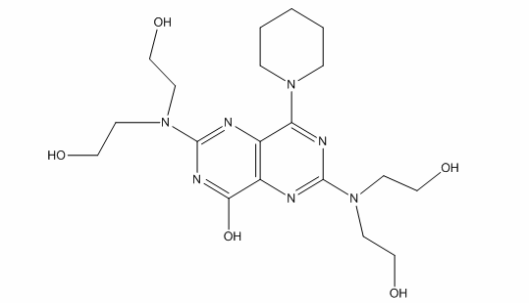
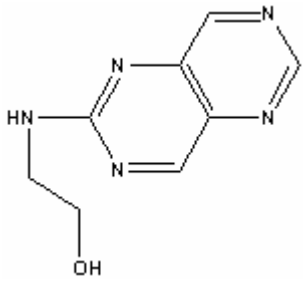
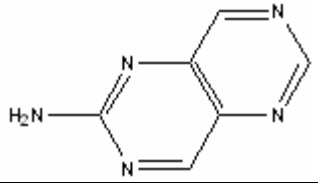
Stock standard solutions

Dipyridamole (1.00 mg/mL), Aspirin (1.00 mg/mL) and Salicylic acid (1.00 mg/mL), each was dissolved in methanol.

Working standard solutions

Dipyridamole (50 µg/mL), Aspirin (50 µg/mL) and

TABLE 2 : Alkaline degradants of Dipyridamole identified by the proposed LC/MS method

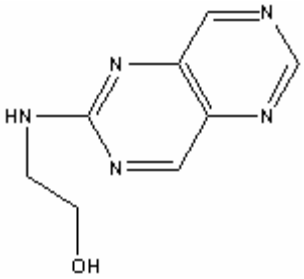
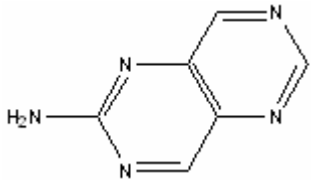
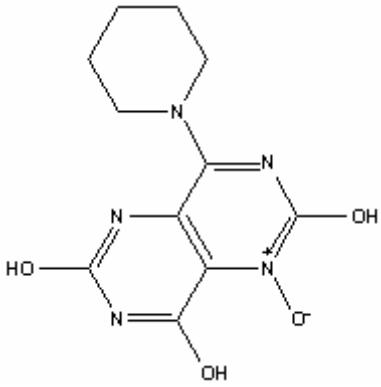
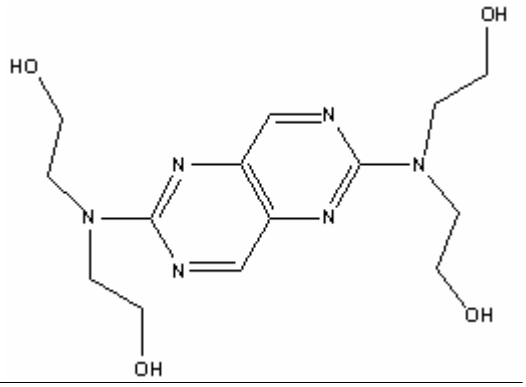
Degradant	m/z	Chemical name	Structure
A	268	2-((8-(piperidin-1-yl)pyrimido[5,4-d]pyrimidin-2-yl)amino)ethanol	
B	437	2,2',2'',2'''-((4-hydroxy-8-(piperidin-1-yl)pyrimido[5,4-d]pyrimidine-2,6-diyl)bis(azanetriyl))tetraethanol	
C	190	2-(pyrimido[5, 4-d]pyrimidin-2-ylamino)ethanol	
D	147	pyrimido[5, 4-d]pyrimidin-2-amine	

Salicylic acid (50 µg/mL). They were prepared by suitably diluting the stock standard solution with methanol.

Synthetic mixtures for DDRD method

Aliquots of (0.2- 1.2) mL of Dipyridamole working standard solution (50µg/mL) were transferred

TABLE 3 : Oxidative degradants of Dipyridamole identified by the proposed LC/MS method

Degradant	m/z	Chemical name	Structure
C	190	2-(pyrimido[5, 4-d]pyrimidin-2-ylamino)ethanol	
D	147	pyrimido[5, 4-d]pyrimidin-2-amine	
E	286	2,6,8-trihydroxy-4-(piperidin-1-yl)pyrimido[5,4-d]pyrimidine-1-oxide	
F	339	2,2',2",2'" pyrimido[5, 4-d]pyrimidine-2,6-diylbis(azanetriyl)tetraethanol'	

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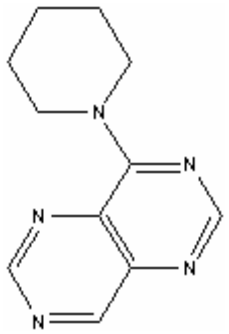
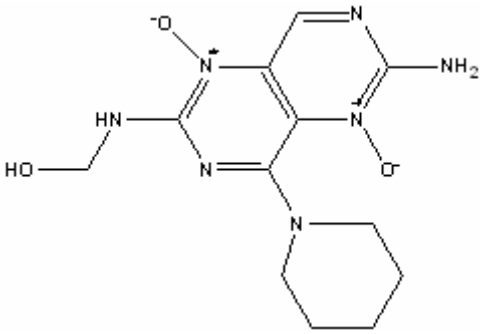
Degradant	m/z	Chemical name	Structure
G	221	4-(piperidin-1-yl) pyrimido [5, 4-d] pyrimidine	
H	313	6-amino-2-((hydroxymethyl)amino)(4-(piperidin-1-yl) pyrimido[5,4-d]pyrimidin-2-yl) azanediyl) diethanol	

separately into a series of 10-mL volumetric flasks. To the previous solutions accurately measured volumes (0.6 mL) of each of Aspirin and salicylic acid (50µg/mL) were added. The volume was completed to mark with methanol.

Using the proposed methods, the samples were analyzed for Dipyridamole contents. The concentrations were calculated from the corresponding regression equation; the mean percentage recoveries and standard deviations were then calculated.

Degraded samples

Acid and alkaline induced degradation

Two mL of Dipyridamole stock solution were transferred into a conical flask and mixed with 2.00 mL of 1 M HCl or 2.00 mL of 1 M NaOH for acid and alkaline degradation, respectively. The mixture was heated on a thermostatically controlled water bath at 90R"C for one hour while fitting air condenser. The solution was cooled and then neutralized with 2 M NaOH or 2 M HCl. PH was monitored with PH glass electrode. The solution was transferred quantitavely into 10-mL volumetric flask and the volume was then completed with methanol.

Hydrogen peroxide-induced degradation

Two mL of Dipyridamole stock solution were transferred into 10-mL volumetric flask and mixed with 2.00 mL 3% H_2O_2. The mixture was left for 30 minutes at room temperature. The volume was completed with methanol.

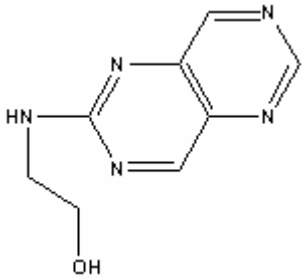
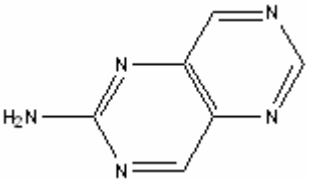
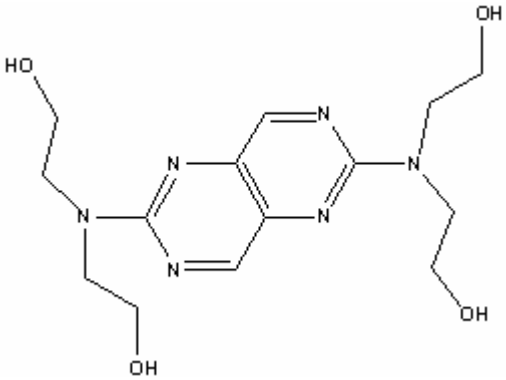
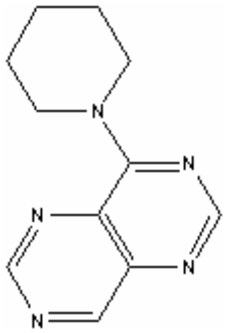
Wet themolysis

Two mL of Dipyridamole stock solution were transferred into a conical flask and mixed with 2.00 mL distilled water, the mixture was heated on a thermostatically controlled water bath at 90R"C for two hours while fitting air condenser. The solution was cooled and transferred quantitavely into 10-mL volumetric flask and the volume was then completed with methanol.

Dry thermolysis

Dipyridamole bulk powder was spread on petridish in thin film about 2 mm thickness) in an oven at 90R"C for an hour. After exposure, about 50 mg of Dipyridamole powder were accurately weighed into

TABLE 4 : Wet thermolysis degradation of Dipyridamole identified by the proposed LC/MS method

Degradant	m/z	Chemical name	Structure
C	190	2-(pyrimido[5, 4-d]pyrimidin-2-ylamino)ethanol	
D	147	pyrimido[5, 4-d]pyrimidin-2-amine	
F	339	2,2',2",2"' pyrimido[5, 4-d]pyrimidine-2,6-diylbis(azanetriyl)tetraethanol'	
G	221	4-(piperidin-1-yl) pyrimido [5, 4-d] pyrimidine	

250-mL volumetric flask, dissolved in and diluted to volume with methanol.

Procedures

Construction of calibration curve for the TLC-densitometric method

Different aliquots (0.3-15.0 µL) of stock standard solutions of Dipyridamole, Aspirin and (0.3-20.0 µL) of Salicylic acid, were spotted as bands of 5 mm width

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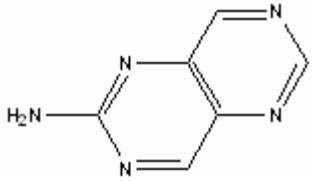
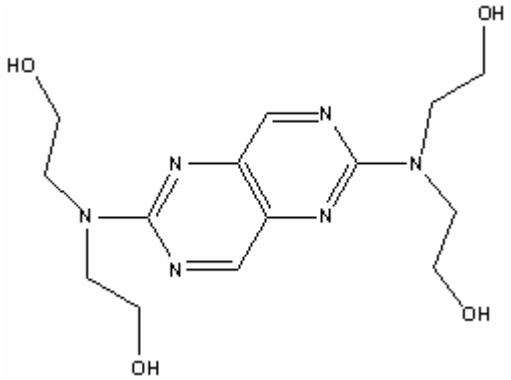
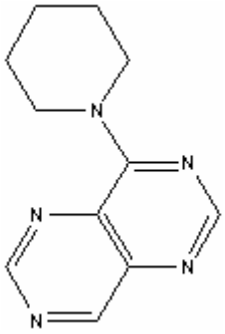
TABLE 5 : Dry thermolysis degradation of Dipyridamole identified by the proposed LC/MS method

Degradant	m/z	Chemical name	Structure
D	147	pyrimido[5, 4-d]pyrimidin-2-amine	
F	339	2,2',2",2"' pyrimido[5, 4-d]pyrimidine-2,6-diylbis(azanetriyl)tetraethanol	
G	221	4-(piperidin-1-yl) pyrimido [5, 4-d] pyrimidine	

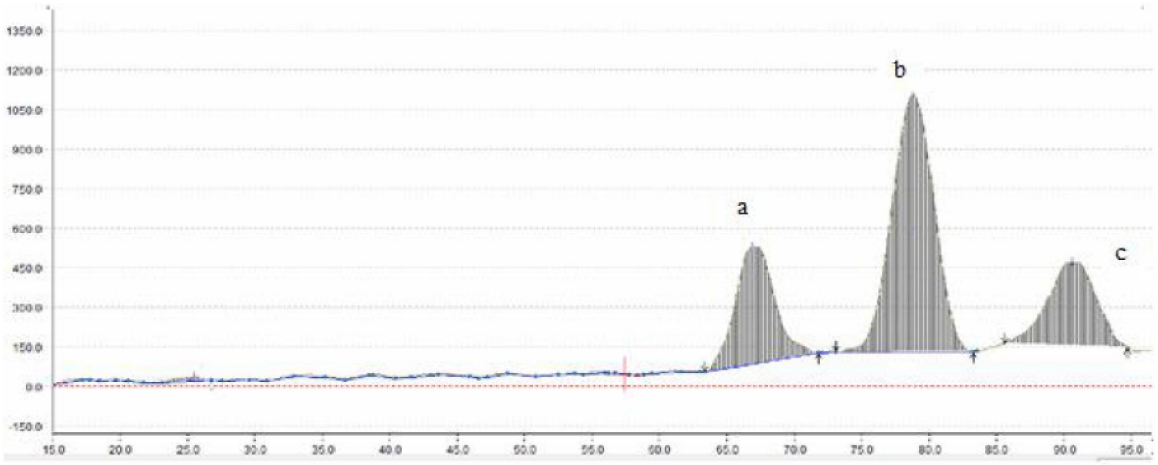


Figure 3 : TLC densitogram of a: Salicylic acid, b: Dipyridamole and c: Aspirin in pure sample

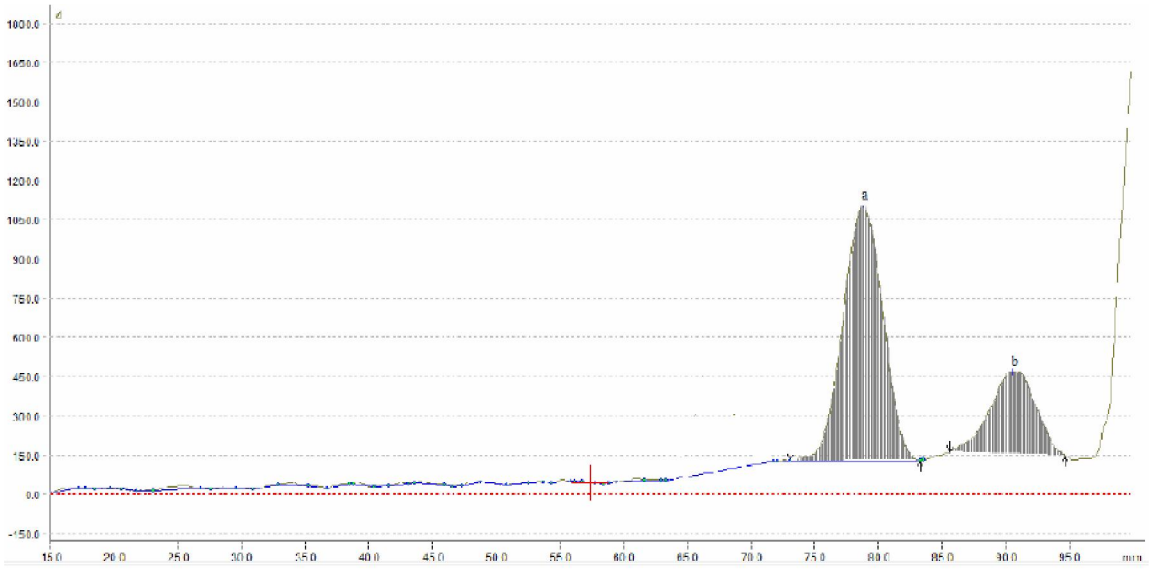


Figure 4 : TLC densitogram of a: Dipyridamole and b: Aspirin in spiked human plasma

on the TLC plates, in triplicates. The bands were applied at 1 cm interval and 1.5 cm apart from the bottom edge. Linear ascending chromatographic developing to a distance of 8 cm was performed in a chromatographic tank previously saturated for 30 minutes with the developing mobile phase consisted of acetonitrile: ammonia 33% (4:1, by volume) at room temperature. Subsequent to the development, the plates were air dried, detected under UV lamp and then scanned at 230 nm. After scanning, the average peak areas were calculated, the calibration curve, relating the integrated peak area and its corresponding concentration, was constructed for each drug and the regression equations were computed.

Application of the TLC-densitometric method to laboratory prepared mixture of Dipyridamole with Aspirin and Salicylic acid

The suggested procedure was applied for the analysis of Dipyridamole in presence of Aspirin and Salicylic acid, where a spot containing (15, 5, 15 µg/ spot), respectively was applied and the specified chromatographic conditions were set.

Application of TLC- densitometric method to Pharmaceutical formulation

The suggested procedure was applied for the analysis of Dipyridamole in Persantin® tablets, where ten tablets were weighed and their mean weight was determined. The tablets were finely powdered and an accurately weighed portion of the powder equivalent

TABLE 6 : Recovery of Dipyridamole and Aspirin in spiked human plasma by the proposed TLC densitometric method

Dipyridamole			Aspirin		
Taken µg/mL	Found* µg/spot	Recovery %	Taken µg/mL	Found[a] µg/spot	Recovery %
0.50	0.49	98.00	0.50	0.487	97.40
2.50	2.55	102.00	2.50	2.35	94.00
5.00	5.24	104.80	5.00	5.30	106.00
10.00	9.56	95.60	10.00	10.23	102.30
15.00	15.45	103.00	15.00	14.90	99.33
20.00	19.75	98.75	20.00	20.32	101.60
50.00	53.80	107.60	50.00	51.20	102.40
Mean ± RSD %		101.40± 4.13	Mean ± RSD %		100.43 ±3.90

[a]Mean of three determinations

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TABLE 7 : Results of assay validation of the proposed TLC spectrodensitometric method for the analysis of Dipyridamole and Aspirin in spiked human plasma

Parameter	Dipyridamole	Aspirin
Range (µg/mL)	0.50-50.00 µg/mL	0.50-50.00 µg/mL
Intercept	203.30	19.39
Coeffecient$_1$ [a]	6.496	4.839
Coeffecient$_2$ [b]	0.246	0.199
Correlation coefficient	0.9995	0.9999
Accuracy	101.40± 4.13	100.43 ±3.90
RSD% [* c]	2.05	1.24
RSD% [* d]	3.11	2.05
Specificty	Specific	Specific
LOQ (µg/spot)[e]	0.36	0.066
LOD (µg/spot)[e]	0.12	0.20

[a, b]Regression equation= $A=a+b_1C+b_2C^2$, where A is the peak area and C is the concentration. [c] the intraday (n=3) and [d] the interday (n=3) relative standard deviations of Dipyridamole and Aspirin of concentrations 5.00, 10.00 and 15.00 (µg/mL); [e] LOD and LOQ are determined using Standard deviation of the response method

to one tablet was transferred to 100-mL volumetric flask, complete to volume with methanol and then filtered. 5 µL of the prepared solution (0.75 mg/mL) was applied to the TLC plate and the procedure stated under linearity was followed.

Standard addition technique was applied to assess the validity of the proposed method by spiking different known concentrations of pure Dipyridamole to its pharmaceutical product. The procedure described under the assay of Persantin® tablets was followed. Concentrations were calculated; the mean percentage recoveries and the relative standard deviations were then calculated.

Application of TLC- densitometric method to spiked human plasma

The suggested procedure was applied for the analysis of Dipyridamole and Aspirin in plasma. Different aliquots from working solution of Dipyridamole and Aspirin were transferred into 5 mL centrifuge tubes, separately. One mL of the frozen plasma was thawed under room temperature and transferred to each of the mentioned centrifuge tubes, two mL of methanol were added then the mixture was vortexed for 1 minute and centrifuged for 10 minutes. The upper layers were transferred to beakers for evaporation after three times

TABLE 8 : System suitability results of the TLC spectrodensitometric method for determination of Dipyridamole

Compound	Selectivity factor	Resolution
Aspirin in pure form	2.43	1.5
Aspirin in spiked human plasma	2.48	1.45
Acid degradation	2.11	2.23
Alkaline degradation	2.00	1.93
Oxidative degradation	2.19	2.08
Wet thermolysis	1.86	2.08
Dry thermolysis	1.70	2.00

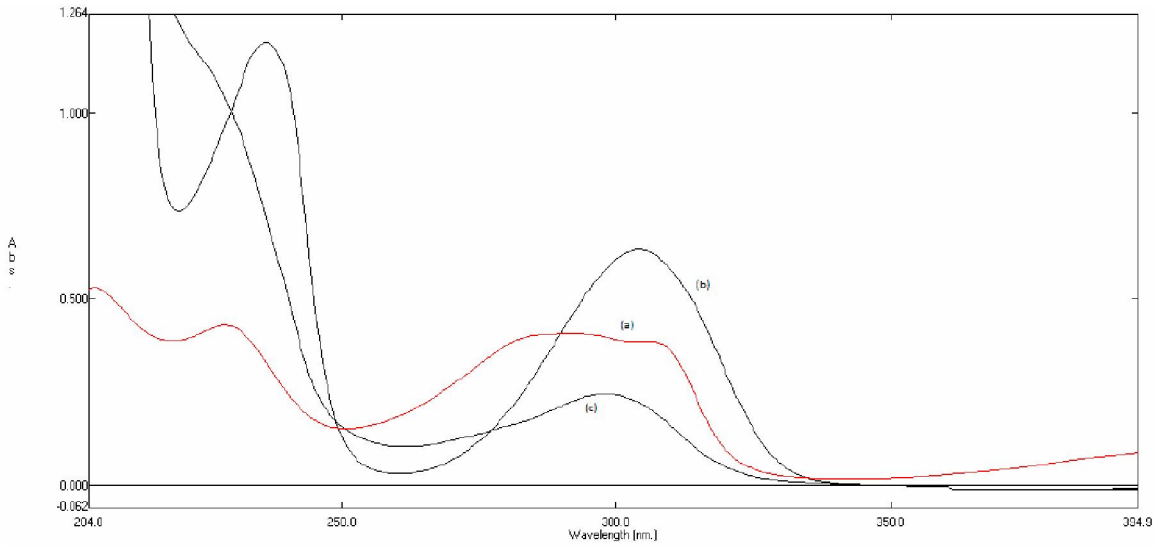

Figure 5 : Zero order absorption spectra of (a): 3 µg/mL Dipyridamole, (b): 3 µg/mL Aspirin and (c): 3 µg/mL Salicylic acid

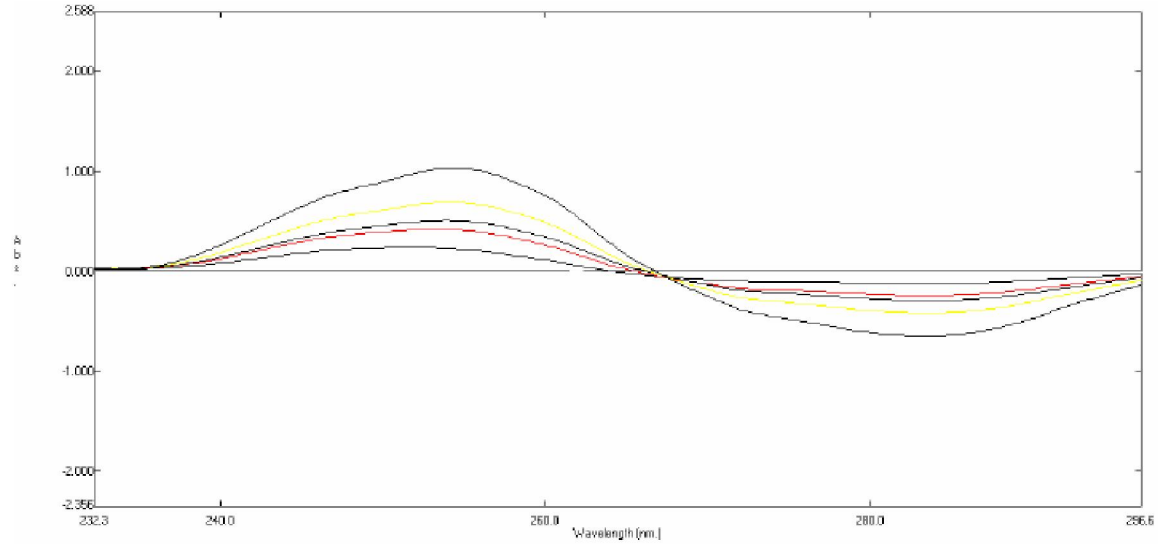

Figure 6 : First derivative of the double divisor ratio spectra of Dipyridamole [2,4,6,7&10] µg/mL using 3µg/mL Aspirin and 3µg/mL Salicylic acid as double divisor

washing with methanol, then one mL methanol was added, from each dilution spot 10 µL into TLC plate, the specified chromatographic conditions were set. After development and scanning, the average peak areas were calculated, the calibration curve, relating the integrated peak area and its corresponding concentration in plasma, was constructed for each drug and the regression equations were computed.

Double divisor-ratio spectra derivative method

The Double divisor-ratio spectra derivative method is based on the use of the coincident spectra of the derivative of the ratio spectra obtained by using a 'double divisor' (sum of two spectra) and the measurements at either the maximum or minimum wavelengths. The mathematical expression of DDRD method is shown in the following equation[24]:

$$\frac{d}{d\lambda}\left[\frac{A_{\text{ternary mix.., }\lambda_i}}{[\alpha_{X,\lambda_i} + \beta_{y,\lambda_i}]C_X^0}\right] = \frac{d}{d\lambda}\left[\frac{\gamma_{z\lambda_i}}{[\alpha_{X\lambda_i} + \beta_{y\lambda_i}]}\right]\frac{C_z}{C_X^0}$$

Where $A_{\text{ternary mixture}}$ is the absorbance of the mixture at λ_i =252 nm and $\alpha_{x,\lambda i}$, $\beta_{y,\lambda i}$ and $\lambda_{z,\lambda i}$ are the absorptivities

TABLE 9 : Application of standard addition technique on Persantin® tablets by the proposed methods

Pharma-ceutical formulation	TLC-densitometric method				
	Taken (µg/spot)	Recovery[a] %± RSD	Pure added	Recovery[b] %	Mean ± RSD
Persantin tablets®	3.75	99.74± 1.64	2.50	98.80	100.62± 1.80
			5.00	102.40	
			7.50	100.67	
	DDRD method				
	Taken (µg/ml)	Recovery[a] %± RSD	Pure added	Recovery[a] %	Mean ± RSD
Persantin tablets®	4	99.50 ±1.64	4	3.94	100.50±1.73
			2	2.03	
			1	1.015	

[a] average of three determination

of X, Y and Z, respectively. Cx, Cy and Cz represent the concentrations of compounds. Cp x and Cp y are standard solutions of x and y of equal concentrations. In this method, linearity was obtained by dividing the absorption spectra of Dipyridamole (1-10 µg/mL) by the sum of the spectra of Aspirin and Salicylic acid (3 µg/mL of each one in methanol) as double divisor. The first derivatives of the obtained ratio spectra were computed using the UVPC software at $\Delta\lambda$= 8 nm and scaling factor = 10 at 252 nm.

Application of DDRD to pharmaceutical dosage form

Ten Persantin® tablets were weighed and their mean weight was determined. The tablets were finely powdered and an accurately weighed portion of the powder transferred to 100 mL volumetric flask to obtain concentration of (40 µg/mL). The solution was diluted to required volume then filtered. The samples were analyzed using the procedures stated under linearity.

To assess the validity of the proposed methods, standard addition technique was applied. Known amounts of pure drugs were added to the corresponding prepared solution of drug dosage form. The concentrations, mean percentage recoveries and the standard deviation were calculated.

RESULTS AND DISCUSSION

TLC- densitometric method

The international conference on Harmonization(ICH) guidelines entitled " stability testing of new drug substances and products" requires the stress testing to be carried out to elucidate the inherent stability characterstics of the active substances [25]. An ideal stability indicating method is one that quantifies the standard drug alone and also resolves its degradation products. Several degradates of Dipyridamole were formed when conducting different stress testing studies and the structures of acid, alkaline, oxidative, wet and dry thermal degradates were elucidated by LC-MS. Figure 1, 2 demonstrate the LC-MS chromatograms of acid, alkaline, oxidative, dry and wet thermal degradates for Dipyridamole. The m/z values, chemical names and structures of the proposed degradates are presented in TABLES 1–5. The suggested mechanism of degradation is nucleophilic substitution either by water molecule potentiated by acid in case of acidic hydrolysis or by hydroxyl group in case of alkaline hydrolysis and formation of N oxide in oxidative degradation by hydrogen peroxide.

Development of the optimum mobile phase for TLC-densitometric method

The TLC procedure was optimized to develop a stability-indicating assay method by trialing different solvent systems. Systems like mixtures of ethyl acetate, methanol and ammonia 33% in varying ratios were used, but they show poor resolution of Dipyridamole from its degradates. Systems containing acetonitrile, methanol and ammonia 33% didn't separate many of degradates produced. Systems containing acetone, methanol and ammonia 33% with varying ratios, the drug migrate with the mobile phase to reach solvent front. Systems containing diethyl ether, acetonitrile and ammonia 33% in varying ratios were examined but produce poor resolution of Dipyridamole and Aspirin.

In our proposed method, the optimum mobile phase was acetonitrile: ammonia 33% (16: 4, by volume). This phase gave sharp and symmetric peak of Dipyridamole at R_f =0.75, together with good separation of the drug peak from Aspirin and Salicylic acid (Figure 3) and also from the degradates peaks.

The densitograms of the stressed samples of the bulk powders and the TLC plates showed well separated spots of pure Dipyridamole. Well-defined spots were obtained when the chamber was saturated with the mobile phase for 30 minutes at room temperature and development distance was adjusted to be 8 cm. The wavelength of detection was chosen to be 230 nm. All variables in the scanning procedure such as the scan mode and the slit size were optimized to obtain maximum precision, accuracy and sensitivity.

TABLE 10 : Statistical comparison of the results obtained by applying the proposed methods and the published method for the analysis of Dipyridamole

Parameter	Average recovery of Dipyridamole		
	TLC method	DDRD method	Reported method[11]
Mean	99.746	99.11	99.694
SD	0.698	0.65	0.386
Variance	0.487	0.42	0.149
n	17	6	5
t	0.217 (2.16)[a]	1.83 (2.31)*	--
F	3.27 (5.84)[a]	2.85 (6.26)*	--

[a] The values between parentheses are the corresponding theortical values of t and F at the 95% confidence level

Application of TLC-spectrodensitometric method in spiked human plasma

The proposed method was suggested for the simultaneous determination of Dipyridamole and Aspirin in spiked human plasma in concentration range of (0.50-50.00 μg/mL) for each drug. We have tried several extracting solvents acetonitrile, methanol, each one separately and in combination and diethyl ether. The best one was methanol, which was optimal for

TABLE 11 : Results of assay validation of the proposed methods

	TLC densitometric method			DDRD method
Parameter	**Dipyridamole**	**Aspirin**	**Sal.**	**Dipyridamole**
Range	0.30-15.00(µg/spot)	0.30-15.00(µg/spot)	0.30-20.00(µg/spot)	1-10 (µg/ml)
Intercept	99.30	647.42	38.381	0.037
slope	--	--	--	0.093
Coeffecient$_1$ [a]	311.20	34.388	114.61	--
Coeffecient$_2$ [b]	-7.934	0.6493	-1.627	--
Correlation coefficient	0.99979	0.9995	0.9995	0.9995
Accuracy	101.31± 1.94	100.45± 1.96	100.37± 2.14	99.11 ± 0.65
RSD% [c]	0.80	0.96	1.05	0.54
RSD% [d]	1.22	1.15	1.34	0.75
Specificty	Specific	Specific	Specific	98.71±1.53
LOQ (µg/spot) [e]	0.36	0.20	0.306	0.335
LOD (µg/spot) [e]	0.12	0.066	0.101	1.015
Temperature (25?C ± 2) RSD% [f]	1.00-1.04	1.50-1.76	0.97-1.05	--
Measurement wavelength (230 ± 2 nm) RSD% [f]	2.00-2.08	1.80-2.11	1.85-2.10	--

[a,b] Regression equation= $A=a+b_1C+b_2C^2$, where A is the peak area and C is the concentration. [c] the intraday (n=3) and [d] the interday (n=3) relative standard deviations of Dipyridamole of concentrations 5.00, 10.00 and 15.00 (µg/spot); [f] the robustness factors (n=3) relative standard deviations of (1.00 µg/spot) Dipyridamole; [e] LOD and LOQ are determined using Standard deviation of the response method

precipitation of plasma proteins and extraction of the drugs.

A typical TLC densitogram of human plasma spiked with Dipyridamole and Aspirin is presented in Figure 4.

The accuracy calculated as percentage recovery was 101.40% for Dipyridamole and100.43% for Aspirin with low values of % RSD demonstrating an excellent accuracy of the method (TABLE 6).

All the assay validation parameters of the proposed method in spiked human plasma are summarized in TABLE 7. The LOD values for Dipyridamole and Aspirin were found to be 0.12 and 0.101µg/mL and LOQ values were found to be 0.36 and 0.306 µg/mL, respectively. These values indicate that the method is sensitive enough for Dipyridamole monitoring and pharmacokinetic studies as peak plasma concentration of Dipyridamole at steady state is approximately1.98 µg/mL[26].

The densitogram of Dipyridamole was well separated from Aspirin. The results of system suitability tests (TABLE 8) assured the adequacy of the proposed TLC method for the analysis of Dipyridamole and Aspirin in human plasma.

The proposed method was tested for specificity by comparing chromatograms of 6 different sources of blank human plasma. The chromatograms were free from any interfering peaks at R_f values of the drugs. Thus, the proposed method can be used for determination of Dipyridamole and Aspirin without interference by endogenous plasma components.

The spiked human plasma freeze and thaw stability[27] was assisted by storing three aliquots at each of the low and high concentrations at -20p C for 24 hours and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 12 to 24 hours under the same conditions. The freeze–thaw cycle repeated two more times, then analyzed on the third cycle, also, the spiked human plasma samples stored at over a period of one month. In both cases samples didn't suffer any appreciable changes in the assay values. In addition, Dipyridamole was found to be stable in human plasma and the stability was maintained at room temperature for more than 12 hours.

DDRD method

It's apparent from (Figure 5) that the absorption spectra of Dipyridamole, Aspirin and Salicylic acid exhibit certain degree of spectral overlap, conventional UV spectrophotometry can't be used for determination of Dipyridamole in presence of Aspirin and Salicylic acid. However, the proposed spectrophotometric method permits a selective determination of Dipyridamole in presence of Aspirin and Salicylic acid as it resolves band overlapping without physical separation.

The absorption spectra of the solutions of Dipyridamole were recorded in the range 232–296 nm

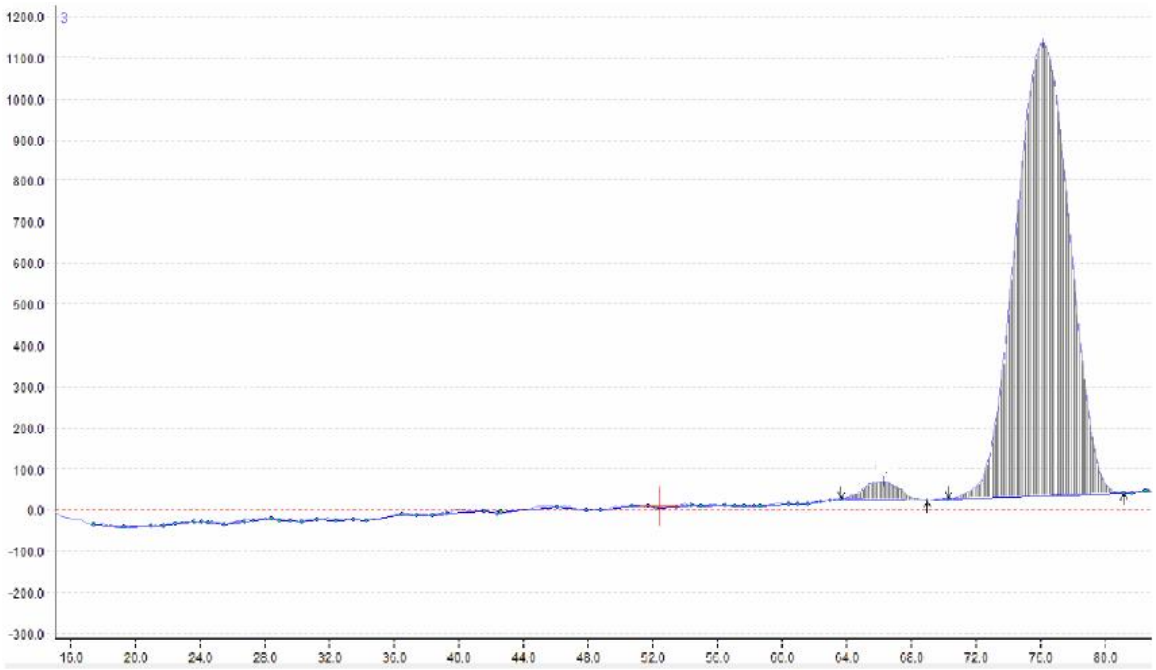

Figure 7 : TLC densitogram of Dipyridamole in Persantin® tablet

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TABLE 12 : Specificity results for the determination of Dipyridamole in the laboratory prepared mixtures by the proposed DDRD method

Ternary mixtures	Dipyridamole		
Dip: Aspirin:sal.	Taken µg/mL	Found[a] µg/mL	Recovery%
8 :1 :0.5	8.00	7.80	97.50
1 :1 :1	3.00	3.02	100.67
2 :1 :1	6.00	5.95	99.17
2 :3 :3	2.00	1.95	97.50
1.5 :1 :1	1.50	1.49	99.33
Mean ± SD			98.83±1.35

[a]Mean of three determinations

and divided by the double divisor (3µg/ml Aspirin and 3µg/ml Salicylic acid) to obtain their corresponding ratio spectra. First derivatives of the ratio spectra thus obtained were calculated with interval of $\Delta\lambda$= 8 nm and scaling factor = 10 as shown in (Figure 6). The concentration of Dipyridamole was determined by measuring the amplitude at 252.0 nm corresponding to a maximum point.

DDRD Method optimization

The main instrumental parameter conditions were optimized for reliable determination of Dipyridamole. For selecting the standard binary mixture as double divisor at appropriate concentrations some different double divisor concentrations were tried. The sum of the spectra of (3µg/ml Aspirin and 3µg/ml Salicylic acid) as a double divisor was found suitable for the determination of Dipyridamole in different laboratory prepared mixtures. Furthermore, the influence of $\Delta\lambda$ for the first derivative of the ratio spectra was studied. It was found appropriate to use the values of $\Delta\lambda$= 8 that gives optimum recovery for the determination of Dipyridamole.

Analysis of pharmaceutical formulation by the proposed methods

A single spot at R_f = 0.75 was observed in the densitogram of Dipyridamole sample extracted from Persantin® tablet (Figure 7). There was no interference from the excipients commonly present in the tablet formulation. The proposed methods were successfully applied for the determination of Dipyridamole in pharmaceutical formulation and the validity of the methods was further assessed by applying standard addition technique (TABLE 9).

The results obtained by applying the proposed methods were statistically compared with those obtained by applying the reported method[11] (TABLE 10).

These results show that on using probability of 95%, the calculated t and F values are less than the theoretical ones indicating that there is no significant difference between the proposed methods and the reported one for Dipyridamole with respect to accuracy and precision. The suggested methods proved to be accurate, precise and specific over the specified range.

Methods validation

Method validation was performed for the proposed methods. TABLE 11 shows the results of accuracy, repeatability and intermediate precision of the methods. Other regression equation parameters of calibration curves are also presented, which show good linear relationship for the methods as revealed by the correlation coefficient.

In order to assess the specificity of the proposed DDRD method, several synthetic mixtures, within the linearity ranges with different concentration ratios of Dipyridamole, Aspirin and Salicylic acid were analyzed. The percentage recoveries obtained for each mixture are given in (TABLE 12). It is obvious that the proposed method could be applied to resolve Dipyridamole in presence of Aspirin and Salicylic acid with good selectivity.

CONCLUSION

The developed and validated TLC densitometric

method is a simple, precise, accurate and stability-indicating for the determination of Dipyridamol. Statistical analysis proves that the method is reproducible and specific so represents a very useful aid for determination of Dipyridamole in presence of Aspirin and its degradation product. The high sensitivity of the method allows simple and low cost application in human plasma. On the other hand, it's apparent that the absorption spectra of Dipyridamole, Aspirin and Salicylic acid exhibit certain degree of spectral overlap, conventional UV spectrophotometry can't be used for determination of Dipyridamole in presence of Aspirin and Salicylic acid. However, the proposed spectrophotometric method permits a selective determination of Dipyridamole in presence of Aspirin and Salicylic acid as it resolves band overlapping without physical separation, also the proposed DDRD spectrophotometric method provides a minimum sample preparation, low cost, fast response and simple procedures so it offers a distinct advantage over other sophisticated techniques for separation and confirms the suitability for routine analysis of Dipyridamole in presence of Aspirin and its degradation product in pure forms or in the pharmaceutical preparation.

REFERENCES

[1] B.B.Davood, P.H.Morteza, A.Mohsen, S.Abbas; High performance liquid chromatographic method for the determination of dipyridamole in human plasma, Daru, **7(2)**, 14-17 **(1999)**.

[2] N.P.Stafford, A.E.Pink, A.E.White, J.R.Glenn, S.Heptinstall; Thromb. Vasc. Biol., **23**, 1928–33 **(2003)**.

[3] Y.B.J.Aldenhoff, L.H.Koole; Platelet adhesion studies on dipyridamole coated polyurethane Surfaces, Eu Cells and Materials, **3**, 61-7 **(2003)**.

[4] B.V.Subbaiah, K.K.S.Ganesh, G.V.Krishna, K.Vyas, R.V.Dev, K.S.Reddy; Isolation and characterisation of degradant impurities in Dipyridamole formulation, J. Pharma. and Biomed. Anal., **61**, 256–64 **(2012)**.

[5] K.Prakash, R.R.Kalakuntla, J.R.Sama; Rapid and simultaneous determination of aspirin and dipyridamole in pharmaceutical formulations by reversed-phase high performance liquid chromatography method, African J. Pharmacy and Pharmacology, **5(2)**, 244-251 **(2011)**.

[6] W.Shu-jun, M.Fan-hao, S.Jin, L.Xiao, C.Ji-min, B.Dian-zhou; Chinese Pharmaceutical Journal, **9**, **(2004)**.

[7] C.Ri-lai, L.Heng-mei; China pharmacy, 17, **(2008)**.

[8] T.Qin, F.Qin, N.Li, S.Lu, W.Liu, F.Li; Biomed Chromatogr, **24(3)**, 268-73 **(2010)**.

[9] J.Zhang, R.B.Miller, R.Jacobus; Chromatogr., **44**, **(1997)**.

[10] J.H.Bridle, M.T.Brimble; Drug development and industrial pharmacy, **19**, 371-81 **(1993)**.

[11] Y.M.Issa, M.S.Rizk, A.F.Shoukry, R.M.El-Nashar; Electroanalysis, **9**, 74-8 **(1997)**.

[12] C.Li; Colloids Surf B Biointerfaces, **55(1)**, 77-83 **(2007)**.

[13] W.Zhenhui, Z.Hongzhong, Z.Shuping; Talanta, **44**, 621-26 **(1997)**.

[14] R.A.de Toledo, M.Castilho, L.H.Mazo; J.Pharm. and Biomed. Analysis, **36**, 1113-7 **(2005)**.

[15] A.F.Shoukry, N.T.Abdel Ghani, Y.M.Issa, O.A.Wahdan; Anal. letters, **34(10)**, 1689-701 **(2001)**.

[16] J.A.M.Pulgarín, A.A.Molina, P.F.López; Analyst, **122(3)**, 253-8 **(1997)**.

[17] A.M.de la Peña, A.E.Mansilla, J.A.M.Pulgarín, A.A.Molina, P.F.López; Talanta, **48(5)**, 1061-73 **(1999)**.

[18] J.Zhang, R.B.Miller, R.Jacobus; Chromatogr., **44(5/6)**, **(1997)**.

[19] T.Qin, F.Qin, N.Li, S.Lu, W.Liu, F.Li; Biomed. Chromatogr, **24(3)**, 268-273 **(2010)**.

[20] P.J.A.Murillo, M.A.Alañón, L.P.Fernández; Anal. Biochem., **245(1)**, 8-16 **(1997)**.

[21] N.Wang, F.Xu, Z.Zhang, C.Yang, X.Sun, J.Li; Biomed. Chromatogr., **22(2)**, 149-156 **(2008)**.

[22] U.Periasamy; Int. J. Pharm., **108(1)**, 11-19 **(1994)**.

[23] H.H.Hassan, A.E.Y.Fawzy, E.M.Mohamad, M.S.Ghassan, M.S.Nada; Open Spectrosc. J., **2**, 19-28 **(2008)**.

[24] E.Dinc; Talanta., **48**, 1145–1157 **(1999)**.

[25] ICH Q1A(2R), Stability Testing of New Drug Substances and Products, International Conference on Harmonization, IFPMA, Genva, **(2003)**.

[26] Lawrence R.Borgsdorf et al.; Drug facts and Comparisons, 10 th Edition, Wolters Kluwer health, 60 **(2006)**.

[27] S.Braggio, R.J.Barnaby, P.Grossi, M.Cugola; J. Pharm. and Biomed. Analysis, **14**, 375-88 **(1996)**.