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Validated analytical method development of inosine pranobex in drug products by thin layer chromatography

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Abstract: Thin layer chromatography (TLC) with densitometry has been established for the identification and the quantification of inosine pranobex in drug substance and drug products. Inosine pranobex is a combination of inosine, acetamidobenzoic acid, and dimethylaminoisopropanol. UV densitometry was performed in absorbance mode at 260 nm. The separation was carried out on aluminum sheet of silica gel 60 f 254 [chloroform - methanol- - toluene -10% ammonia solution (6:5:1: 0.1% v/v)] as mobile phase. Linearity range was found to be 1-12, 2-12, 2-20 and 2-16 µg/ml for inosine pranobex, inosine, acetamidobenzoic acid, and dimethylaminoisopropanol with the mean percentage recoveries 99.74± 1.73%, 99.88 ± 1.75%, 99.56 ±1.08%, and 99.36 ± 0.71% respectively, (Correlation coefficient r2 = 0.9998 for inosine pranobex, r2 = 0.09999 for inosine, r2 = 0.9998 for acetamidobenzoic acid and r2= 0.9998 for dimethylaminoisopropanol). The detection and quantification limits for inosine pranobex and other components are also reported. The presented method was validated according to ICH guidelines. Statistical comparison of the results was performed using Student's t-test and F-ratio at 95% confidence level, and there was no significant difference between the reference and proposed method with regard to accuracy and precision. It could be said that the validated TLC-densitometry method is suitable for the routine analysis of inosine pranobex in quantity control laboratories.

Keywords: Inosine Pranobex, Inosine, Acetamidobenzoic Acid, TLC-Densitometry Method, Dimethylaminoisopropanol

1. Introduction

Inosine pranobex (Isoprinosine or Methisoprinol) (Molecular formula $C_{52}H_{78}N_{10}O_{17}$), 9-[(2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl]-3H-purin-6-one : 4-acetamidobenzoic acid : 1-(dimethyl amino)propan-2-ol Fig.1 [1]. The antiviral agent inosiplex is composed of inosine, p-acetaminobenzoic acid (PABA), and N, N-dimethylamino-2-propanol (DIP) in molar ratio of 1:3:3 Fig. 1. Although little is known about the precise mechanism of the action of inosiplex, numerous studies have shown its ability to potentiate certain aspects of the cellular immune response [2] both in vitro and in vivo. Inosiplex was found to have a broad spectrum of antiviral activity, including alleviating symptoms of sub-acute sclerosing pan encephalitis, symptomatic subclinical human papillomavirus infection, and cervical condylomata acuminate because of genital human papillomavirus [3-5]. Inosiplex could also delay the progression of HIV infection to overt AIDS [6]. In addition, inosiplex has been employed as an immunoregulating agent for the treatment of immunopathological disorders, such as rheumatoid arthritis and alopecia aerate [7, 8]. However, limited data was published up to now on the bioassay and pharmacokinetics of inosiplex [9]. Two analytical methods have been reported to determine Inosine pranobex in both pharmaceutical preparation and biological specimens. These included the conventional method for tablets [10], and another TLC method but in this method all component except N, N dimethylamino-2-propanol were determined by densitometry scanning [11]. Liquid chromatography and tandem MS techniques were used to investigate the pharmacokinetics of inosine and p-acetaminobenzoic acid (PABA) salt of N, N-dimethylamino-2-propanol (DIP) in plasma [12].

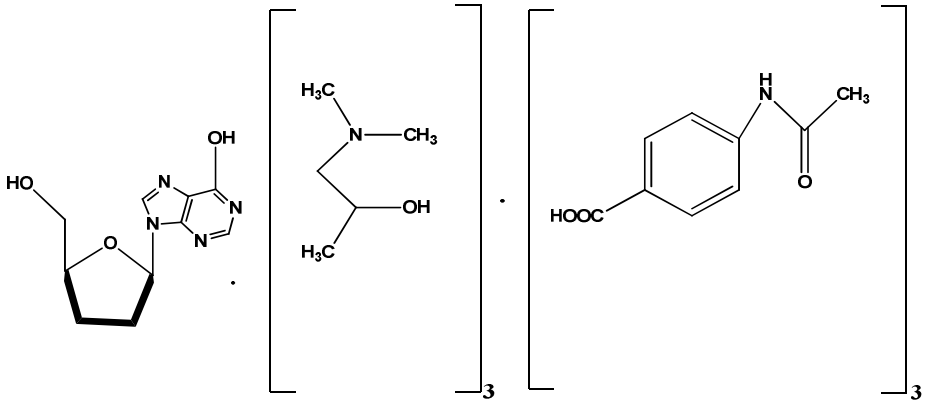


Figure 1. *Structure of inosine pranobex (Isoprinosine)*

The aims of this work were to determine all component of inosine pranobex in tablets and syrup. The method was successfully applied for determination, identification and isolation of inosine pranobex, inosine, p-acetaminobenzoic acid (PABA) and N, N-dimethylamino-2-propanol (DIP) in drug substance and in drug products. The results obtained were found in agree statistically with those obtained by reported method. The developed method was validated as per the International Conference on Harmonization (ICH) guidelines [13-14].

2. Materials and Methods

2.1. Apparatus

SHIMADZU CS-9301 PC dual wavelength flying spot scanning densitometer. TLC plated silica gel 60 F 254 (20×20 cm) E. Merck (Darmstadt- Germany).

Hamilton syringes 25µl.

TLC plated silica gel 60 F 254 (20×20 cm) E. Merck (Darmstadt- Germany).

UV Lamp (Dosage- Germany).

Heidolph Duomax shaker 1030, Germany. Chromatographic chambers: twin-trough chamber for 20 × 20 cm plates (#0.222.5255, Camag, Muttenz, Switzerland) and twin-trough chamber for 20 × 10 cm plates (#0.222.5221, Camag, Muttenz, Switzerland).

2.2. Materials and Reagents

Inosine pranobex, certified to contain 99.60%, was kindly supplied by Medical Union Pharmaceutical Co. (Ismailia, Egypt) according to HPLC manufacturer's method [15]. Isoprinosine tablets 500mg/tab., and Isoprinosine syrup 250mg/5ml were manufactured by MUP Co. (Ismailia, Egypt) under license from: Newport Pharmaceuticals Limited Dublin-Ireland. Inosine (>99.8%, ClaroChem Ireland LTD), Para-acetamido Benzoic Acid (PAcBA) (>99.9%, %, ClaroChem Ireland LTD), and N, N Dimethylamino-2-propanol (DIP) (>99.9%, %, ClaroChem Ireland LTD) were used as standards. Chloroform Methanol, Toluene, and Conc. ammonia used for TLC were analytical grade (LAB-SCAN),

2.3. Preparation of Standard Solution of Ionsine Pranobex and other Components

Stock standard solutions were prepared by dissolving inosine pranobex and other components (inosine, PAcBA, and DIP) 0.1mgml-1 in methanol. The working standard solutions within linearity range were prepared using the specified solvent.

Ten micro liters of each standard solution was applied to the TLC plate. Triplicate applications were made for each solution. The plate was developed using previously described mobile phase.

The peak area of each concentration was plotted against the corresponding concentration to obtain the calibration graph.

The general procedure for TLC method described under calibration graph was followed and the concentration of inosine pranobex and other components (inosine, PAcBA, and DIP) was calculated.

2.4. Sample Preparation

For tablets: (Isoprinosine tablets 500 mg), Ten tablets were ground for 20 min with a speed equal to 4000 rpm using an IKA Ultra-Turrax Tube Drive Workstation with a BMT-20-S tube for grinding with balls of stainless steel. An accurate weight of the mixed powder equivalent to 10mg was dissolved in 50ml methanol. After 30min. of mechanically shaking, the solution was filtered in a 100-ml volumetric flask. The solution was completed to the mark with methanol, and preceded as described under TLC method.

For syrup: (Isoprinosine syrup 250 mg/5ml), Aliquots equivalent to 10mg Isoprinosine were transferred into 100ml volumetric flask. The solution was completed to the mark with methanol, and preceded as described under TLC method.

2.5. Thin Layer Chromatography

The plates were prewashed with methanol and dried for 24 h at room temperature. TLC-densitometry method for determination of inosine pranobex was performed on aluminum plates of silica gel 60 f 254 (E. Merck, # 1.05570). Additionally, anther aluminum plates of silica gel 60 f 254 (E.

Merck, # 1.05554) were used to test robustness. The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters.

The solutions of inosine pranobex sample and inosine, PAcBA, and DIP standards (10 µL) were spotted manually on the chromatographic plates.

The mixture of chloroform + methanol + toluene +10% ammonia solution in volume compositions 6: 5:1: 0.1 was used as mobile phase. Of the mobile phase, 50 mL was used in all cases. After saturation of the twin-trough chamber (20 cm × 20 cm) with the mobile-phase vapor for 15 min, the plates were developed vertically at room temperature (20°C) to a distance of 10 cm. After development the plates were air-dried then scanned at 260 nm by means of a Shimadzu (Tokyo, Japan) model CS-9301 PC dual-wavelength flying-spot densitometer in reflection photo mode, zigzag scan mode, and swing width = 10.

Additionally, a twin-trough chamber of 20 × 10 cm (#0.222.5221, Camag) was used to test robustness.

3. Results and Discussion

3.1. Densitometry Investigations

Thin layer chromatography is known to be one of the simplest chromatographic separation techniques. The quantitative TLC scanning allows the application of the method for micro quantitative analysis; the densitometry technique could be used for the assay of drugs in mixture, due to the high power of resolution of TLC [16, 17].

The proposed method is contribution of the analysis of the studied drug in presence of other components inosine, PAcBA, and DIP. The suitable mobile phase has been selected to achieve the best separation of the studied drug from other components inosine, PAcBA, and DIP; other necessary conditions have been established. The method was based on difference in R_f values of each drug and other components inosine, PAcBA, and DIP.

Experimental conditions such as mobile phase, wavelength of scanning and slit dimension were optimized to provide accurate, precise and reproducible results. Different solvent systems were tried, best separation of inosine pranobex and other components inosine, PAcBA, and DIP; was obtained by using chloroform + methanol + toluene +10% ammonia solution (6:5:1:0.1 v/v). R_f values were 0.48±0.0063 for intact inosine pranobex, 0.42±0.0084 for inosine, 0.31±0.0052 for PAcBA and 0.29±0.0032 for DIP (for n=10). Densitometry scanning was done Fig.2, and the scanning profiles are shown in Fig.3-6.

A linear correlation was obtained between the peak area and the concentration in the range of 1 – 12 µg/spot for intact inosine pranobex, 2 – 12 µg/spot for inosine, 2 - 20 µg/spot for PAcBA and 2 - 16 µg/spot for DIP.

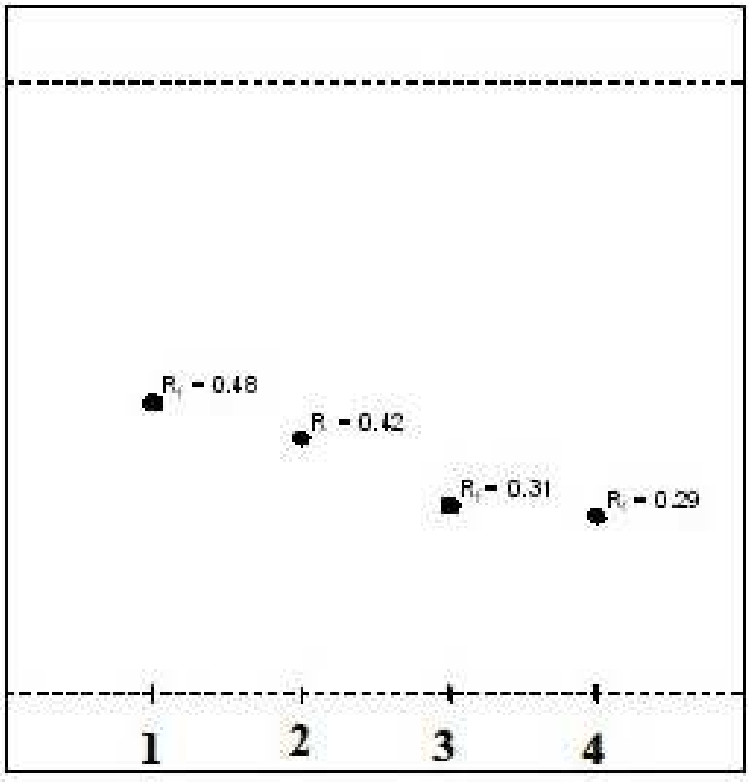
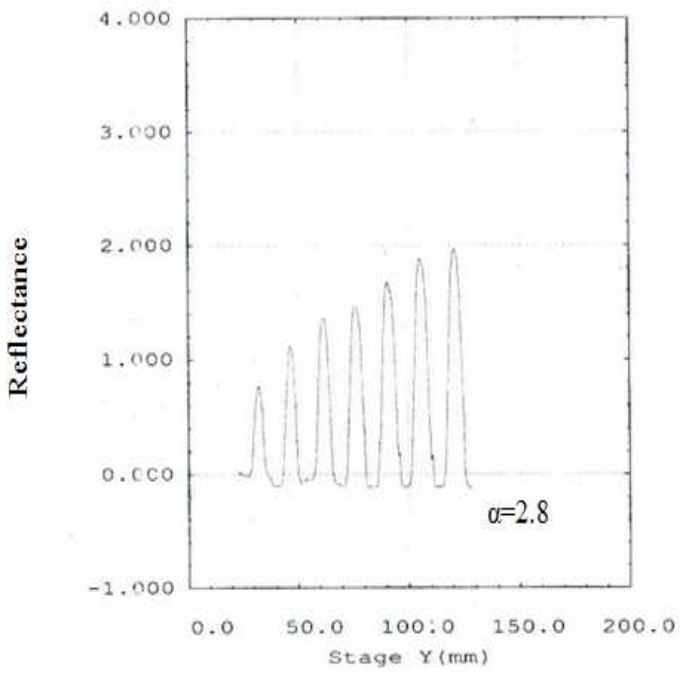
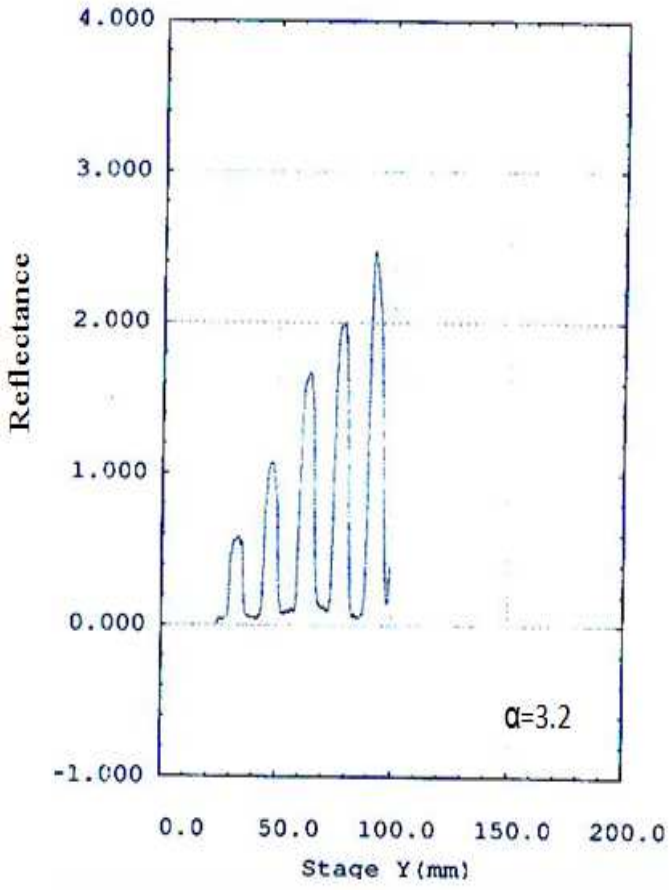


Figure 2. *TLC chromatogram of (1) inosine pranobex intact, (2) Inosine, (3), PAcBA and (4) DIP. Developing system, chloroform: methanol: toluene: 10% ammonia solution 6:5:1:0.1 by volume.*



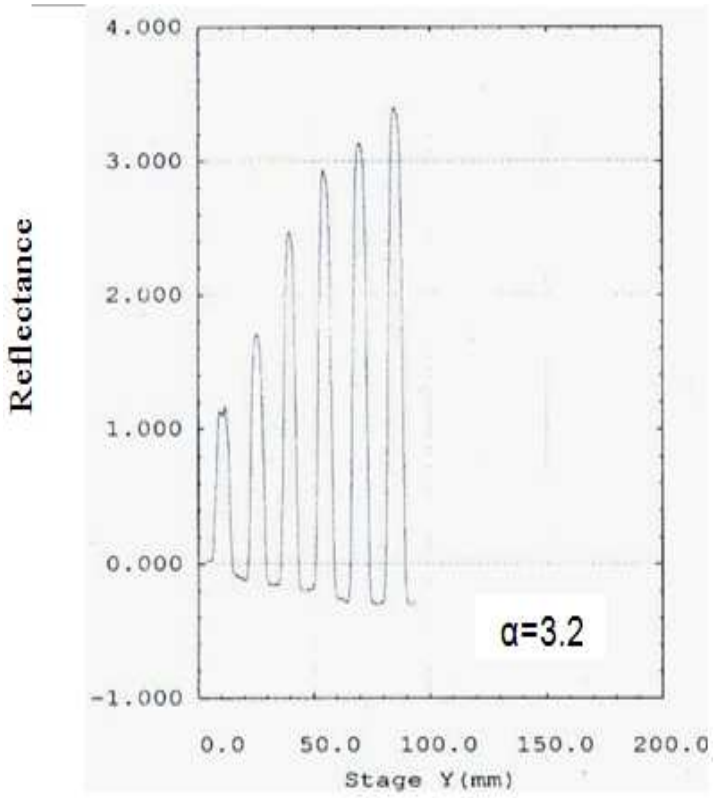
Spot position (mm)

Figure 3. *Densitometry Scanning Profile of TLC- chromatogram of different concentrations of inosine pranobex (1.0-12µg/spot) at 260nm. α= selectivity*



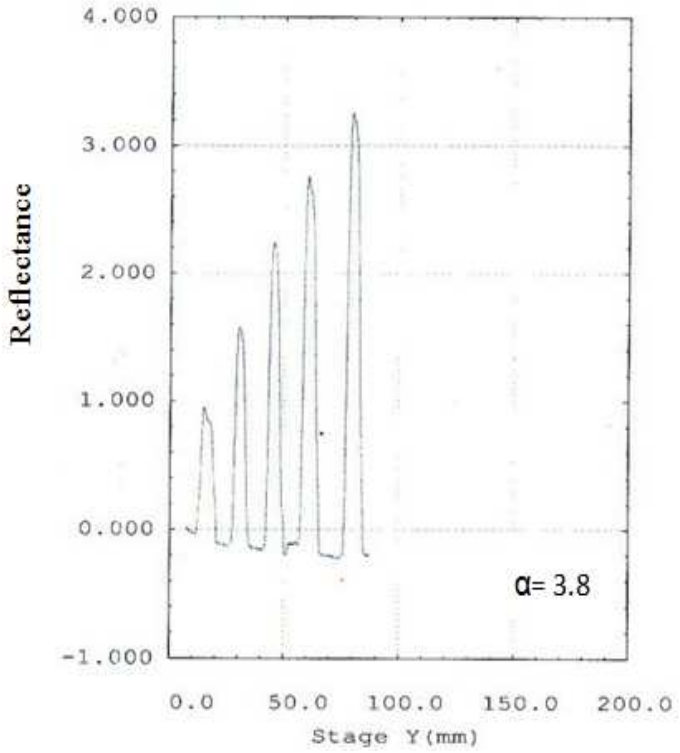
Spot position (mm)

Figure 4. *Densitometry Scanning Profile of TLC- chromatogram of different concentrations of inosine (2.0-12µg/spot) at 260nm. α= selectivity*



Spot position (mm)

Figure 5. *Densitometry Scanning Profile of TLC- chromatogram of different concentrations of PAcBA (2.0-20.0 µg/spot) at 260nm. α= selectivity*



Spot position (mm)

Figure 6. *Densitometry Scanning Profile of TLC- chromatogram of different concentrations of DIP (2.0-16.0µg/spot) at 260nm. α= selectivity*

Table 1. *Results from validation of the TLC method for analysis of inosine pranobex, inosine, PAcBA and DIP in drug substance.*

	inosine pranobex	inosine	PAcBA	DIP
Linear range µg/spot	1 -12	2 -12	2 -20	2 -16
Accuracy a				
Mean ± RSD% b	99.74± 1.73	99.88 ± 1.75	99.56 ±1.08	99.36 ± 0.71
Mean ± RSD% c	99.35±1.55	99.87±1.72	99.97±1.03	99.45±0.79
Precision				
Intra-day RSD (%)d	0.45	0.65	0.34	1.15
Inter-day RSD (%)d	0.20	0.64	0.28	0.46
LOD µg/spot	0.85	1.53	1.11	1.68
LOQ µg/spot	2.57	4.63	3.36	5.10
Regression equatione				
Slope	886.617	1129.651	1008.419	1039.890
SE of the slope	6.079	7.468	6.934	7.781
Confidence limit of slopef	870.990 - 902.244	1105.418 – 1135.428	989.166 – 1027.672	1015.126 – 1064.654
Intercept	3315.921	546.511	5384.668	3326.122
SE of intercept	43.898	60.487	84.170	76.559
Confidence limit of interceptf	3203.077 – 3428.765	354.013 – 739.001	5150.975 – 5618.362	3082.474 – 3569.770
Correlation coefficient (r)	0.9998	0.9999	0.9998	0.9998
SE of (r)	61.052	61.945	108.170	89.131

[a]n = 6 , [b]Drug substance, [c]Drug product, [d]n = 6
[e]Y = a + bC, where C is the concentration in µg per spot for TLC method, and Y is the peak area, [f]95% confidence limit.

3.2. Validation of the TLC Method

The methods were validated in accordance with ICH guidelines by documenting their linearity, accuracy, precision, and limits of detection and quantification Table 1. Analysis of different concentrations of the drug and other components showed the linearity of the methods was good. Accuracy was based on mean measured concentrations (n = 6) as a percentage of the actual concentration Table 2. Precision was assessed by determining RSD (%) values for intra-day and inter-day analysis (n = 6) over three days. RSD values were <2 %Table 3. The limits of detection and quantification were estimated as the amounts for which signal-to-noise ratios were 3 and 10, respectively.

The results obtained were compared statistically with those obtained from other reported methods and no significant difference was found Table 4.

3.2.1. Linearity Range

Under the experimental conditions, Beer's plots for the drug and other components using the suggested method show linear relationship with regression equation shown in Table 1.

3.2.2. Accuracy

The accuracy of the proposed method was determined by investigating the percentage recovery at six levels each three times in the concentration range 3.0 -11.0µg/spot as shown in Table 2. The percentage relative standard deviation (% RSD) revealed high accuracy.

Table 2. *Accuracy of the TLC method for analysis of inosine pranobex, inosine, PAcBA and DIP in drug substance and drug products.*

Conc. Taken (µg per spot)	inosine pranobex			inosine			PAcBA			DIP		
	Drug sub.	Drug products		Drug sub.	Drug products		Drug sub.	Drug products		Drug sub.	Drug products	
		Tab.	Syr.		Tab.	Syr.		Tab.	Syr.		Tab.	Syr.
	Recovery (%)a			Recovery (%)a			Recovery (%)a			Recovery (%)a		
3	98.23	99.23	99.56	98.66	99.36	98.67	99.34	99.61	98.65	99.56	98.96	99.23
5	98.56	99.36	98.65	98.55	99.21	99.31	98.68	98.78	98.23	99.55	98.99	99.56
7	99.21	99.87	99.23	98.42	98.34	99.45	100.3	98.97	98.46	98.28	98.85	99.46
9	98.75	101.0	98.87	99.25	99.21	99.87	101.0	100.2	99.56	100.5	100.7	100.2
11	100.0	99.92	98.45	101.0	99.88	99.73	99.24	100.6	98.44	100.4	100.9	100.6
Mean±	98.95	99.88	98.95	99.18	99.20	99.41	99.71±	99.63	98.67	99.66	99.68	99.81
RSD%	± 0.692	±0.698	±0.451	±1.06	±0.558	±0.469	0.928	±0.781	±0.527	±0.894	±1.03	±0.571

aAverage from four different experiments. For drug product, recovery is given as a percentage of the amount claimed table.

3.2.3. Precision

The intraday precision was evaluated by assaying freshly prepared solutions in triplicate in the concentration range 2.0 -16.0µg/spot as shown in Table 3. The percentage relative standard deviations (%RSD) were calculated.

While interday precision was calculated by assaying freshly prepared solutions in triplicate for three days. The percentage relative standard deviations (%RSD) were calculated as shown in Table 3.

Table 3. *The results of intermediate precision study.*

Concentration (in µg / spot)	Inter-day repeatability % RSD (N = 6)			Intra-day repeatability (N = 6) Mean ±% RSD	Inter- aluminum plates* Repeatability (N = 6) Mean ± % RSD
	Day 1	Day 2	Day 3		
Inosine pranobex					
2	0.1783	0.1566	0.1134	100.11 ± 0.439%	100.06 ± 0.653
6	0.5992	0.3214	0.3188	100.08 ± 0.306 %	100.08 ± 0.257
10	0.9778	0.7210	0.5379	100.23 ± 0.601%	100.04 ± 0.206
inosine					
4	0.009	0.005	0.001	100.21 ± 0.619%	100.10± 0.276%
8	0.064	0.051	0.035	100.16 ± 0.421%	99.98 ± 0.383%
10	0.120	0.110	0.009	100.44 ± 0.472%	99.99 ± 0.299%
PAcBA					
4	0.500	0.328	0.155	100.00 ± 0.228%	100.14 ± 0.229%
10	1.850	1.365	0.998	100.15 ± 0.507%	100.12 ± 0.310%
16	3.184	2.367	1.581	100.08 ± 0.285%	100.20 ± 0.227%
DIP					
4	0.612	0.321	0.246	100.03 ± 0.214%	99.69 ± 0.125
6	0.725	0.654	1.025	99.54 ± 0.532%	101.00 ± 0.354
12	0.569	0.541	0.987	99.95 ± 0.145%	100.01 ± 0.752

Aluminum plates of silica gel 60 f 254 (E.Merck, # 1.05570).
*Aluminum plates of silica gel 60 f 254 (E.Merck, # 1.05554)

3.2.4. Detection and Quantitation Limits

According to the ICH recommendation the approach based on the S.D. of the response and the slope was used for determination of the detection and quantitation limits. The theoretical values were assessed practically and given in Table 1.

3.2.5. Robustness

The robustness of a method is its ability to remain unaffected by small change in parameters. To determine robustness of the proposed methods, experimental conditions such as variation of the volume of the constituents of TLC mobile phase by ±0.1ml did not have a significant effect on TLC chromatographic resolution. Anther twin-trough chamber for 20 × 10 cm plates (#0.222.5221, Camag, Muttenz, Switzerland) and additionally, anther aluminum plates of silica gel 60 f 254 (E.Merck, # 1.05554) were used to test robustness as shown in Table 4. The results indicated the method was not affected by small changes in the conditions used, indicating the reliability of the methods during routine work.

3.2.6. Ruggedness

Five sets of experiments for this drug were carried out using two different laboratories, different analysts, no significant difference was obtained between the results in this study as shown in Table 4.

Table 4. *The results of Robustness and Ruggedness study.*

	inosine pranobex	inosine	PAcBA	DIP
Robustness (mean% recovery ±RSD)*				
The constituents of TLC mobile phase	100.98±0.001	100.41±0.004	101.86±0.001	101.20±0.002
Anther twin-trough chamber	99.75±0.011	99.90±0.052	99.58±0.008	99.67±0.007
Anther aluminum plates	101.20±0.015	100.25±0.011	101.28±0.012	100.38±0.003
Ruggedness (mean% recovery ± RSD)*				
Different laboratories	99.95±0.042	99.71±0.053	100.21±0.130	99.71±0.033
Different analysts	100.23±0.067	100.00±0.045	100.61±0.019	100.82±

* Average of six different experiments.

3.2.7. Stability of Solutions

The stability of solutions was evaluated using TLC method. The solutions were stored in tightly capped volumetric flask, protected from light, on a laboratory bench and in the refrigerator. Recovery of these solutions was checked for 10 hrs in interval of 1hrs against freshly prepared solutions. After which decrease in concentration of drug was calculated and it was observed that the solutions kept on the laboratory bench up to 6 hrs; and in the refrigerator were to be stable up to 24hrs.

3.2.8. Method Validation for Drug Products

The method was used for analysis of the drug in its drug products. Satisfactory results were obtained Table 2. Statistical comparison of results from the proposed and from a reported method indicated there was no significant difference Table 5

Table 5. *Statistical comparison of results obtained by use of the proposed TLC method and the reported method for analysis of inosine pranobex, inosine, PAcBA and DIP in drug substance in drug products.*

	Isoprinosine Tablet				
	inosine pranobex	**inosine**	**PAcBA**	**DIP**	**Reported HPLC(15)**
Mean*	97.23	96.76	95.84	100.3	94.50
± RSD%	0.551	0.611	0.264	0.521	0.388
Variance	0.302	0.373	0.069	0.273	0.151
SE	0.224	0.249	0.107	0.213	0.092
t-test (2.228)a	1.23	1.56	1.99	1.450	
F-test (5.1)a	2.00	2.47	2.19	1.257	
	Isoprinosine Syrup				
Mean*	99.43	99.23	99.05	100.2	98.82
± RSD%	1.11	0.48	0.521	0.785	0.609
Variance	1.23	0.23	0.272	0.619	0.372
SE	0.453	0.272	0.212	0.321	0.248
t-test (2.228)a	2.1	1.91	1.23	1.12	
F-test (5.1)a					

* Average of six different experiments. a Theoretical values

4. Conclusion

The Inosine pranobex is old drug but is the main product of Newport Pharmaceuticals of Costa Rica, and still not official and still used as antiviral infection. It acts as an immunostimulant, an analog of thymus hormones. It is a non-toxic immune system stimulant prescribed in the treatment of numerous viral illnesses including herpes simplex, hepatitis, eruptive diseases of infancy and other viral afflictions related with immune-The TLC method proposed enable simple, accurate, and reproducible quantitative analysis of inosine pranobex and other components inosine, PAcBA, and DIP in this drug substance and drug products without interference from excipients. TLC is a simple, quick, and inexpensive procedure that gives the chemist a quick answer as to how many components are in a mixture. TLC is also used to support the identity of a compound in a mixture when the R_f of a compound is compared with the R_f of a known compound (preferably both run on the same TLC plate). The TLC method complied with the validation guidelines of the International Conference on Harmonization and could be used for purity testing, stability studies, quality control, and routine analysis of inosine pranobex and other components inosine, PAcBA, and DIP.

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