

Stability indicating chromatographic techniques for the determination of pipoxolan HCl

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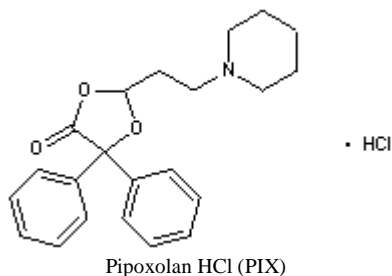
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ABSTRACT

Two simple, accurate and sensitive methods were developed for the determination of pipoxolan HCl in presence of its degradation product. HPLC method (A), performed on C18 column using acetonitrile: 1mM ammonium acetate (80:20 v/v) as a mobile phase with a flow rate of 1.8ml/min. Detection was performed at 210 nm. TLC-densitometric method (B), using silica gel 60 F254 plates; the optimized mobile phase was chloroform: toluene: methanol: 10% ammonia (6:5:3:0.1 v/v). Quantitatively the spots were scanned densitometrically at 210 nm. Linearity ranges were 1 – 10 µg/ml for method A and 2-20 µg/band for method B, with mean percentage recoveries 99.38±0.672% and 99.32±0.97% for methods A and B, respectively. The proposed methods were found to be specific for pipoxolan HCl in presence of up to 90% of its degradation product. Statistical comparison between the results obtained by these methods and the manufacturer's method was done, and it was found that there was no significant differences between them.

INTRODUCTION

Pipoxolan, 5,5-Diphenyl-2-[2-(1-piperidiny)ethyl]-1,3-dioxolan-4-one, is a smooth muscle relaxant in kidney, urinary tract and migraine headache. It's a white crystalline powder, M.P. 207° to 209° (Sweetman, 2009).



Pipoxolan is not official in any pharmacopoeia. The literature survey reveals only one HPLC method for determination of PIX in presence of its alkaline degradation products (Hassib et al, 2011). The International Conference on Harmonization (ICH)

guidelines entitled 'stability testing of new drug substances and products' requires that stress testing helps to determine the intrinsic stability of the molecule by establishing degradation pathways in order to identify the likely degradation products and to validate the stability indicating power of the analytical procedures used (ICH, 1996). Therefore, it was thought necessary to study the stability of PIX under different stress condition. The goal of the present study was to develop and validate simple stability-indicating methods to be used for quality control of PIX in drug substance and pharmaceutical formulations as no previous systematic studies focused on PIX degradation have been performed except the published LC method (Hassib et al, 2011) but it is important to remark that this literature lack the identification of the produced degradation product which was elucidated in this work. Furthermore the proposed method is more sensitive and less retention time.

EXPERIMENTAL

Instrumentation

HPLC system consists of an Agilent 1200 HPLC instrument was equipped with a model series G1354A quaternary pump, G1322A vacuum degasser, injector with a 20 µl loop and Variable wavelength detector, separation and quantization were made on 100x2.1mm ODS Hypersil column (5µm particle size).

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The detector was set at $\lambda = 210$ nm. Data acquisition was performed on Agilent 1200 software. The instrument was connected to HP compatible personal computer (PC) and an HP disk jet 5652 printer.

- Densitometer-dual wavelength flying spot (Shimadzu CS-9301, Tokyo, Japan).
- UV lamp with short wavelength 254 nm (Desaga-Germany).
- Thin layer chromatographic plates precoated with silica gel 60 F254 10×20 cm (Fluka Switzerland and Merck Germany)
- Hamilton syringes 25 μ l.
- IR spectra were recorded on a Bruker Vector 22 spectrometer (Bruker Instruments Ltd, Rheinstetten/Karlsruhe, Germany) using KBr pellets in the range (4000-400 cm^{-1}).
- Teflon membrane filter, pore size 0.45 μ m and 47mm diameter.
- Ultrasonic bath J.P- Selecta, (Barcelona, Spain).

Chemicals and Reagents

- Acetonitrile, methanol (HPLC grade ,absolute; Lab. Scan, Ireland).
- Ammonium acetate, toluene, ammonium hydroxide aqueous solution 33%, chloroform, sodium hydroxide and hydrochloric acid (Adwic El- Nasr pharmaceutical Chemicals Co., Egypt).

Samples

Reference sample

Pharmaceutical grade Pipoxolan HCl was kindly supplied by Amoun Co. (Cairo, Egypt). Its purity was found to be 99.90% according to the manufacturer's method (Manufacturer's procedure).

Pharmaceutical formulation:

Commercial Rowaprxin[®] tablets (Amoun Co., Cairo, Egypt) Batch number (23671), each tablet is claimed to contain 10 mg pipoxolan HCl.

Degraded sample

Acid or alkaline stress degradation product

Accurately weighed 100 mg of pipoxolan HCl was dissolved in 100 ml 2 M HCl in case of acid and 1M NaOH in case of alkaline stress degradation process. The solution was refluxed in a round-bottom flask for 6hrs and tested for complete degradation by TLC-densitometric method using chloroform: toluene: methanol: 10% ammonia (6:5:3:0.1 by volume) as the mobile phase.

The degraded solution was then cooled, neutralized with 2M NaOH and 1M HCl for acid and alkaline condition, respectively, till pH was approximately 7. The solution was nearly evaporated to dryness and cooled. The residue was dissolved in

methanol and quantitatively transferred into a 100-ml volumetric flask, the volume was completed to the mark with the same solvent then filtered and used to suggest the methods.

Standard solutions

PIX stock standard solution:

(1 mg/ml) for the two suggested methods. Prepared by dissolving 25 mg of PIX in a 25-ml volumetric flask, then the volume was completed to the mark with mobile phase for method (A) and with methanol for method (B).

Working standard solution for method (A)

(0.1 mg/ml) in mobile phase. Prepared by transferring 10 ml of PIX stock standard solution in a 100-ml volumetric flask, then the volume was completed to the mark with mobile phase.

Degraded standard solutions

Stock standard solutions of the acidic and alkaline degradation products derived from complete degradation of standard solution of PIX 1mg/ml in methanol.

Working degradation product solution for method (A) 0.1 mg/ml in mobile phase prepared by transferring 10 ml of stock standard solution of the degraded product in 100-ml volumetric flask, then the volume was completed to the mark with mobile phase.

Laboratory prepared mixtures containing different ratios of pipoxolan HCl and its degradation product

Solutions containing different ratios of pipoxolan HCl and its hydrolytic degradant were prepared to contain 10–90% of hydrolytic degradant of pipoxolan HCl.

Procedures

Linearity

Method A

Aliquots equivalent to (0.01-0.1) mg PIX were transferred from its working solution 0.1 mg /ml into a series of 10 ml volumetric flasks. The volume was completed to the mark with mobile phase. 20 μ l of the previously prepared solutions was injected in triplicate using the following chromatographic conditions:

- Column: Nucleosil, size 125×4mm, C18 5 μ m. The column was equilibrated with the mobile phase until steady baseline obtained and column pressure was stabilized.
- Mobile phase: The mobile phase consisted of acetonitrile: 1Mm ammonium at certain ratio (80:20 by volume). The mobile phase was filtered using 0.45 μ m membrane filters and degassed by ultrasonic vibrations for 30 min
- Temperature: The system was operated at ambient temperature.
- Flow rate: The flow rate was isocratic at 1.8 ml/ min.
- Detector wavelength: 210 nm.
- Injection volume: 20 μ l.

Then the chromatogram obtained and the average peak area ratios for each concentration of pipoxolan HCl were plotted versus concentrations, and the regression equation was computed.

Method B

Aliquots equivalent to (0.5-5) mg PIX were transferred from its stock solution 1 mg /ml into a series of 5 mL volumetric flasks. The volume was completed to the mark with methanol. Twenty μ l of the prepared solutions, using 25 μ l Hamilton syringe, was applied as separate compact bands 20 mm apart and 20 mm from the bottom of the plates. The chromatographic tank was saturated with the mobile phase for one hour in ascending manner to a distance of 7 cm from the spotting line at room temperature, air-dried, and the plates were scanned under the following conditions:

- Source of radiation: Deuterium lamp.
- Photomode: Reflection.
- Scan mode: Zigzag.
- Result output: Chromatogram and area under the peak.
- Swing width: 10 mm.
- Wavelength: 210 nm.

The scanning profile for PIX was obtained. The calibration curve relating the integrated peak area to the corresponding concentration was constructed and the regression equation was computed.

Accuracy

The accuracy of the results was checked by applying the previously mentioned procedure under linearity for different concentrations of pure PIX within the linearity range. The concentrations of the drug were calculated from the regression equation. The mean percentage recoveries and relative standard deviation were then calculated.

Precision (Repeatability and Intermediate precision)

Three concentrations of PIX (2, 4 and 6 μ g/ml) for method A and (4,6 and 10 μ g/band) for method B were analyzed three times each for inter and intra-day precision using the previously mentioned procedure under linearity. The mean percentage recoveries and relative standard deviation were then calculated.

Application of the proposed method for the analysis of laboratory prepared mixtures of pipoxolan HCl and its degradation product

Mixtures containing different concentrations of PIX and its induced hydrolytic degradant were analyzed as mentioned under each method. The concentrations were calculated from the corresponding regression equations.

Application of the proposed method for the determination of pipoxolan HCl in its pharmaceutical formulation

Ten tablets of Rowaprxin® were weighed and finely powdered. Two portions of the powder equivalent to about 25 mg of PIX for each method were accurately weighed, dissolved and

diluted with the appropriate solvent for each method, mobile phase for method A and methanol for method B. Then the solutions filtered and diluted to volume to obtain concentrations of 1mg/ml for both methods. Then the procedure was completed as described under Linearity. The concentrations of PIX were then calculated by substitution in the corresponding regression equation.

System suitability

Tailing factors, resolution factor, selectivity factor, theoretical plate count and the height of theoretical plates (HETP) were calculated.

RESULTS AND DISCUSSION

Forced degradation (FD) study is a process in which the natural degradation rate of a pharmaceutical product is increased by the application of an additional stress. FD studies (i) help to identify reactions that cause degradation of pharmaceutical product, ii) are part of the development strategy and an integral component of validating analytical methods that indicate stability and detect impurities which are formed during manufacture, storage, or use; these impurities have properties which are different from the desired product with respect to activity, efficacy and safety, and iii) are designed to generate product-related variants and develop analytical methods to determine the degradation products formed during accelerated and long term stability studies. Any significant degradation product should be evaluated for characterization and quantization for its potential hazard (ICH.,1996 ; FDA ,2000)

The regulatory authorities have also developed guidelines regarding stress testing on drug substances and drug products to predict the degradation products and degradation pathways which can help in establishing intrinsic stability of the drug substance as well as developing stability indicating assay method (SIAM). There is a significant increase in demand for the impurity reference standard by both the regulatory authorities and pharmaceutical companies (Ahuja ,1998).

Pipoxolan HCl contains a dioxolan moiety which is important for its activity (Yuh et al. ,2010) . This moiety is expected to undergo cleavage under different stress condition, so it was necessary to develop simple stability indicating method after subjecting PIX to acid or alkaline condition.

The degradation product was prepared in laboratory using either 2M HCl or 1M NaOH. The degradation processes were followed using TLC-densitometric method. The mentioned drug was found to be liable to alkaline and acidic degradation, where a single component indicated by the appearance of one spot at complete degradation.

(Hassib et al.,2011) claimed that the degradation could be attributed to the cleavage of the lactone group giving a degradation product (1) with a COOH & OH groups then decarboxylation of degradation product (1) occurs giving a degradation product containing OH, however structure elucidation using IR spectra of the obtained degradation confirms the conversion of degradation

(1) to a final degradation product namely, benzoic acid (scheme 1), Figures (1a, b). Where the IR spectrum on KBr discs of intact PIX shows peaks (7,8,9) at (2616,2463,2398) cm^{-1} , respectively, corresponding to the 3rd amine which disappeared in the IR spectrum of the degradation product (benzoic acid) (figure 1a) this is accompanied by the appearance of new peaks in the IR spectrum of the degradation product (3,4) at (3396,2911) cm^{-1} , corresponds to OH and COOH respectively (figure 1b) which gave an additional proof of the suggested structure.

Method A

HPLC has become the most versatile and wide spread technique used by the pharmaceutical industries for quality control. It has many applications in the field of pharmaceuticals including the quantitative determination of drugs present either alone or in presence of their degradation (Bajerski *et al.*, 2008; Doshi *et al.*, 2008; Rane *et al.*, 2009; Singh *et al.*, 2006).

Different mobile phases with different ratios were investigated. A satisfactory separation was obtained with a mobile phase consisting of acetonitrile: 1mM ammonium acetate (80:20, v/v) with a retention time of 4.143 ± 0.004 min for PIX and 0.927 ± 0.09 min for its degradation product. This would permit quantitative determination of PIX in presence of its degradation product (Figure 2).

The average peak area ratios obtained for each concentration of PIX to that of external standard 1 $\mu\text{g}/\text{ml}$ were plotted versus the corresponding concentration of the drug. The proposed method was found to be valid in the range of 1 – 10 $\mu\text{g}/\text{ml}$, and the regression equation was computed and found to be:
 $A = 38.18 C + 13.85$
 $r = 0.9999$

Where A is the peak area ratio, C is the concentration of the drug in $\mu\text{g}/\text{ml}$ and r is the correlation coefficient.

The proposed method was successfully applied for the determination of the drug in pure powdered form with mean percentage recovery of $99.38 \pm 0.672\%$, (Table 1).

System suitability test according to the (United States Pharmacopoeia, 2009) was used to verify that the resolution and reproducibility of the chromatographic system were adequate for the analysis to be done as summarized in (Table 2).

Method B

Thin layer chromatography has become a well-established technique for the assay of drugs either in binary or in multi-component mixtures (Mehta *et al.*, 2008; Sathe *et al.* 2007). The proposed method is based on the difference in the R_f between the intact drug and its degradation product. The suitable mobile phase has been selected to achieve the best separation of the drug from its degradation product, other necessary conditions have been established.

Different solvent systems with different ratios were tried until good separation of PIX and its degradation product was

obtained by using mobile phase consisting of chloroform: toluene: methanol: 10% ammonia in the ratio of (6:5:3:0.1v/v). The instrumental conditions for densitometric measurement such as scan mode and wavelength detection were optimized. The scan mode chosen was zigzag mode, and the wavelength was 210 nm. Pipoxolan HCl was completely resolved from its degradation product and its R_f value was 0.85. On the other hand the R_f value of the degradation product was 0.2, this would permit quantitative determination of PIX in presence of its degradation product, (Figure 3).

A linear relationship between the concentration of PIX and the integrated peak area was obtained (figure 4). The proposed method was found to be valid in the range of 2 – 20 $\mu\text{g}/\text{band}$ (figure 5) and the regression equation was computed and found to be:
 $A = 154.92C + 61.816$
 $r = 0.9994$

Where A is the integrated peak area, C is the concentration of the drug in $\mu\text{g}/\text{band}$ and r is the correlation coefficient.

The proposed method was successfully applied for the determination of the drug in pure powdered form with mean percentage recovery of $99.32 \pm 0.97\%$ (Table 1).

The specificity of the methods was proved by the analysis of laboratory prepared mixtures containing different percentages of the degradation product. The proposed methods were found to be specific for PIX in presence of up to 90% of its degradation product Table (3). The proposed methods have been successfully applied to assay PIX in Rowaproxin tablets. The validity of the proposed methods was further assessed by applying the standard addition technique for the analysis of Rowaproxin tablet as shown in Table (4).

Results obtained by the proposed methods for the determination of pure samples of the drug were statistically compared to those obtained by the manufacturer's method of the drug [HPLC] and no significant differences were observed, Table (5).

The accuracies were assessed by the determination of pure PIX samples within the linearity ranges, the mean accuracies are given in Table (1).

The inter and intraday precision were evaluated by assaying three freshly prepared solutions of the drug in triplicate on the same day and on three successive days respectively at concentrations within the linearity range for both methods. RSD% shows the precision of the methods Table (1).

CONCLUSION

From the data obtained it is proved that the proposed HPLC and TLC-densitometric methods are simple, accurate, precise, specific and could be applied in quality control laboratories for quantitative determination of the drug in presence of its degradation product in pure powdered form and in pharmaceutical formulation

Table 1: Results of validation parameters of the responses and the regression equations obtained by the proposed methods.

Parameters	Method (A) HPLC method	Method (B) Densitometric method
Linearity range	1-10 (µg/ml)	2-20 (µg/spot)
LOQ (µg/spot)	0.278	0.240
LOD (µg/spot)	0.092	0.73
Accuracy * Mean ± RSD%	99.38 ± 0.676	99.34 ± 0.78
Precision	99.74 ± 0.396	0.81
Repeatability ** RSD% Intermediate precision ** RSD%	100.22 ± 0.194	0.62
Regression equation		
Slope	38.18	154.9
Intercept	13.85	61.81
Correlation coefficient (r)	0.9999	0.9994

n = 5., ** n = 3×3.

Table 2: Parameters of system suitability test of HPLC Method.

Compound	PIX	Deg	Reference value
Retention time (min)	4.143	0.927	
Capacity (K')	1.22	3.18	0.5 < K' < 10
Selectivity (α)	1.83	-	> 1
Resolution (Rs)	11.61	-	> 1.5
Tailing factor (T)	0.8	0.86	0.8 ≤ T ≤ 1.5
RSD% of retention time	0.004	0.09	
Column efficiency	4681	6551	Increase with efficiency of separation

Table 3: Results of analysis of pipoxolan HCl in laboratory prepared mixtures containing different ratios of Pipoxolan HCL and its degradation product in pure powder form by the proposed methods.

Degradation %	Method (A)			Method (B)		
	Pipoxolan HCL (µg/ml)	Concentration (µg/ml) Degradation product (µg/ml)	Recovery % of method (A)	Pipoxolan HCL	Concentration (µg/band) Degradation product	Recovery % of method(B)
10	9	1	99.43	4.5	0.5	100.94
30	7	3	101.90	3.5	1.5	100.22
50	5	5	101.56	2.5	2.5	100.41
70	3	7	99.50	1.5	3.5	98.82
90	1	9	99.60	0.5	4.5	100.74
Mean			100.4			100.23
RSD			1.24			0.832

Table 4: Determination of Pipoxolan HCl in Its Pharmaceutical dosage form using the Proposed Methods and application of Standard Addition Technique.

Rowaprxin tablet, B.N 23671 claimed to contain 10mg/tablet	HPLC method				TLC-densitometric method			
	Taken µg/mL	Found µg/mL	Standard added µg/mL	Recovery% ^a of added standard	Taken µg/band	Found µg/band	Standard added µg/band	Recovery% ^a of added standard
			1	100.4			4	100.09
	4	99.423±0.451	2	100.19	8	99.75 ±0.77	8	99.8
			3	99.76			12	98.6
	Mean ± SD			100.12±0.326	Mean ± SD			99.49±0.793

^a Average of three different determinations.**Table 5:** Statistical analysis between the results obtained for the determination of Pipoxolan HCl in pure samples by the proposed methods and those obtained by the manufacturer's method.

Item	HPLC Method	TLC- densitometric Method	(Manufacturer's method)*
S.D	0.672	0.97	0.622
R.S.D%	0.676	0.97	0.625
SE	0.3	0.43	0.278
Variance	0.452	0.934	0.386
n	5	5	5
t test(2.306)	0.167	0.264	-
F test(6.400)	1.169	2.414	-

Figures in parentheses are the corresponding tabulated values at p = 0.05

*Manufacturer's method HPLC, mobile phase (0.1 M Barium Perchlorate in Methanol), UV set at 210 nm.

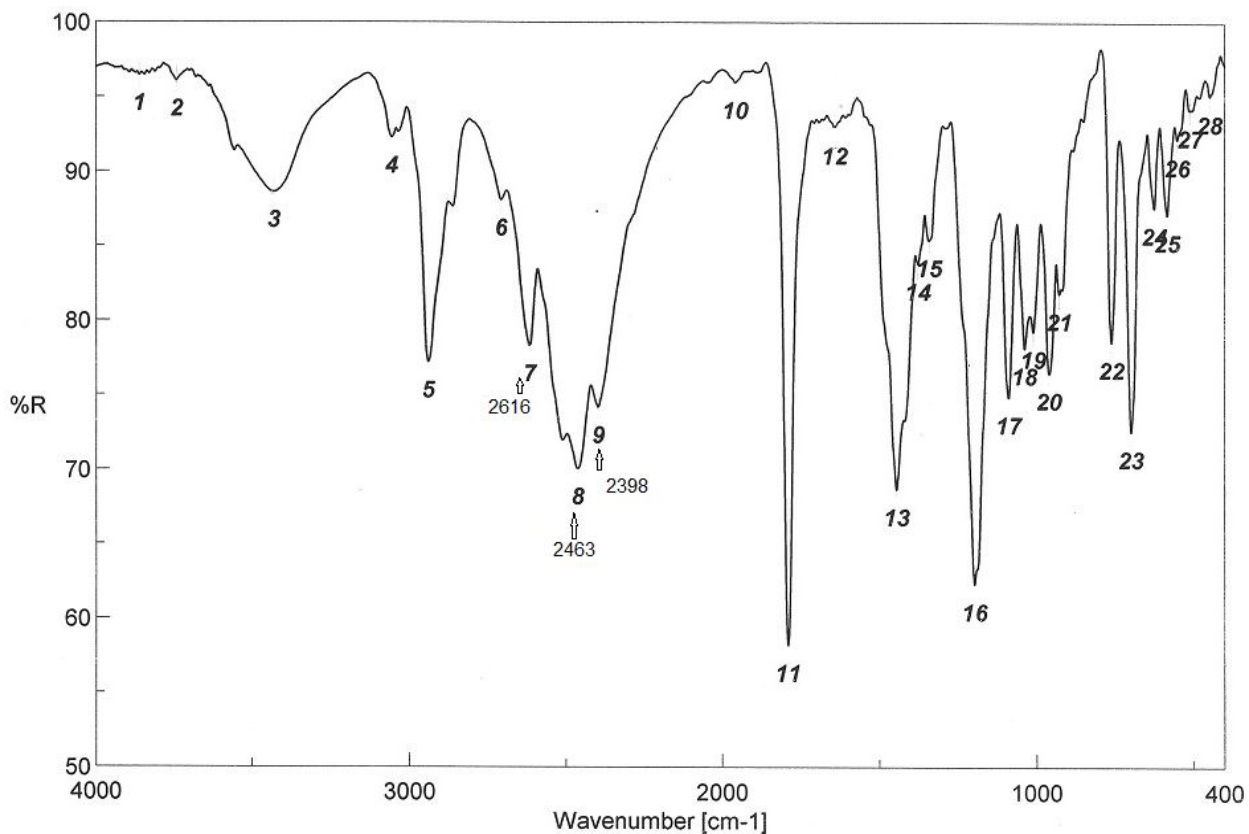


Fig. 1a: IR spectrum of Pipoxolan HCl.

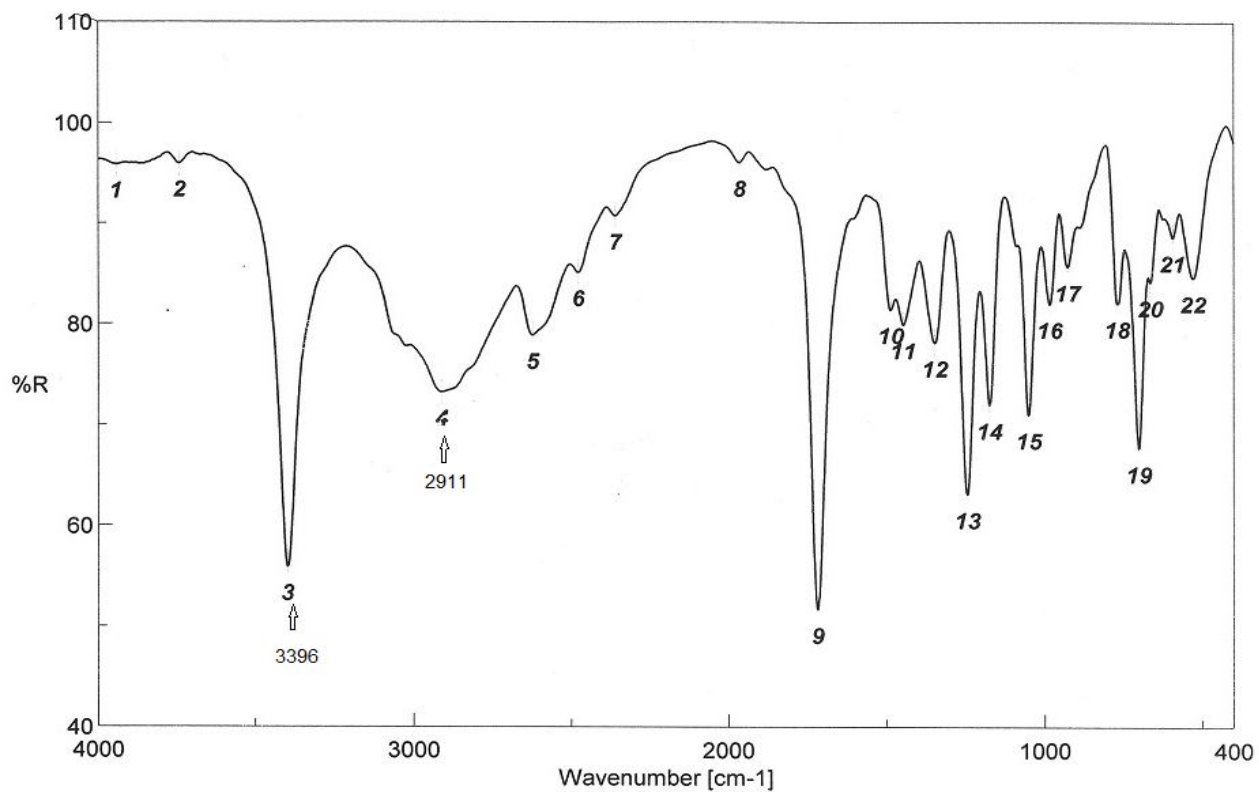
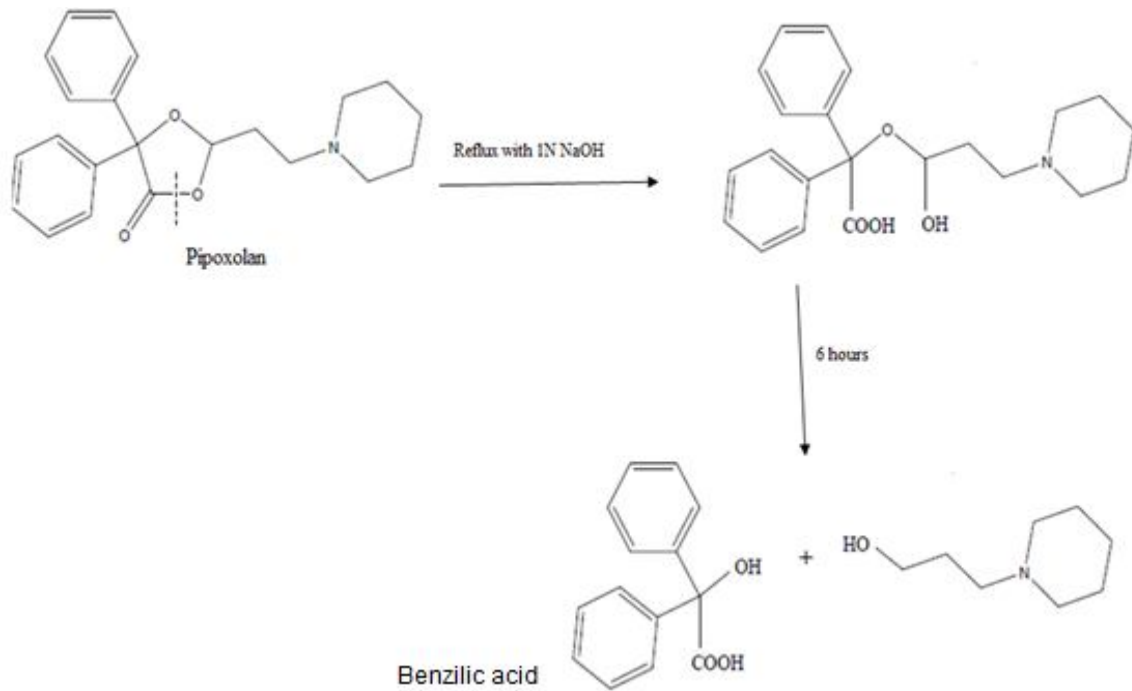


Fig. 1b: IR spectra of Benzoic acid.



Scheme. 1: The degradation pathway of PipoxolanHCl.

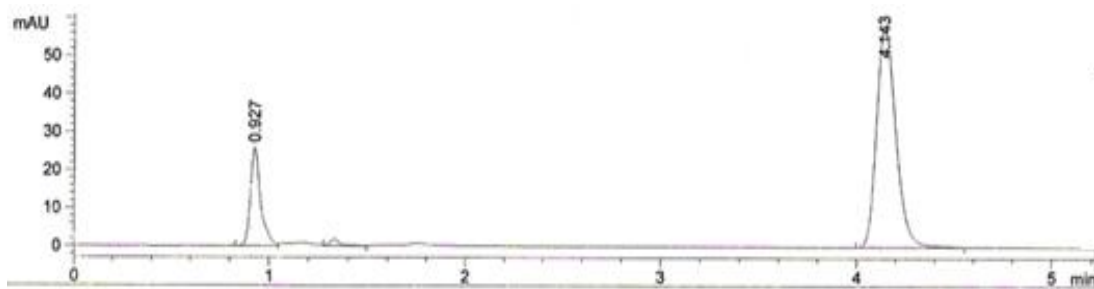


Fig. 2: The chromatogram of pipoxolanHCl (9 μ g/ml) and its acid or alkaline-induced degradation product.

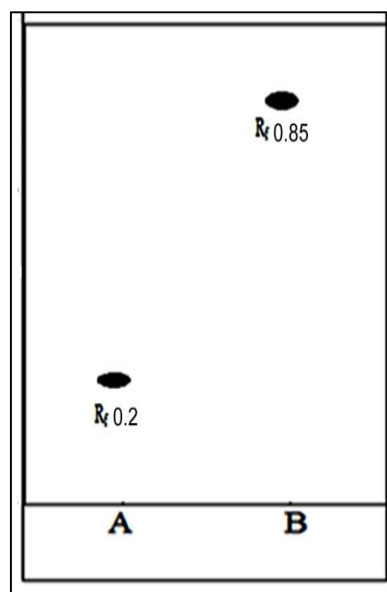


Fig. 3: Thin layer chromatogram of Pipoxolan HCl (B) and its degradation product (A) Developing system: chloroform-toluene-methanol-10% aqueous ammonia (6:5:3:0.1, by volume) at ambient room temperature.

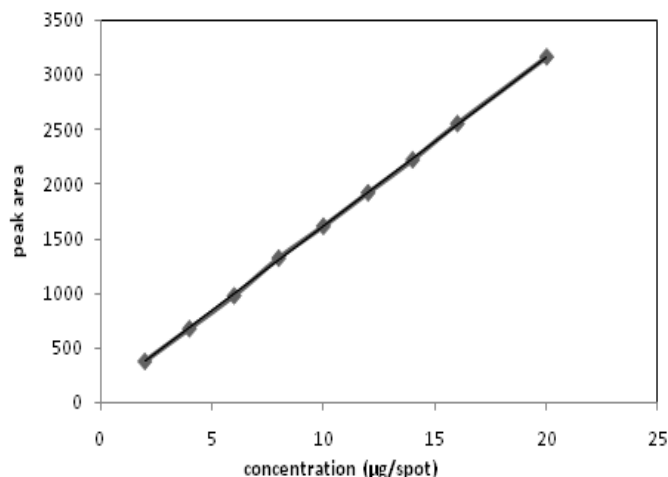


Fig. 4: Linearity of the peak areas at 210 nm to the corresponding concentrations of Pipoxolan HCl (2 – 20 µg/spot).

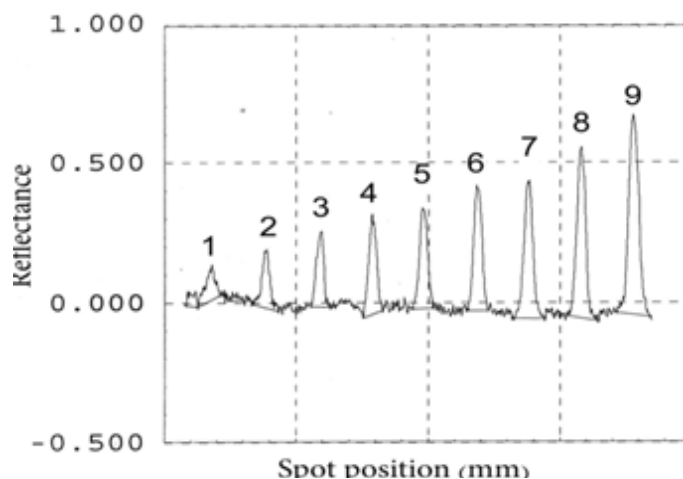


Fig. 5: Densitogram of Pipoxolan HCl in the concentration rang of 2.0-20.0 µg/ band from track 1 to track 9 at 210nm.

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