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SEPARATION, IDENTIFICATION AND QUANTIFICATION OF LAWSONE AND METABOLITES BY CHROMATOGRAPHIC METHODS

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ABSTRACT

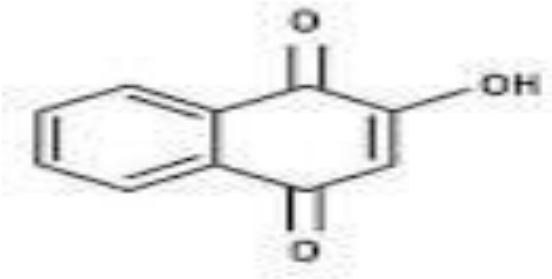
Two simple, precise and accurate chromatographic methods were developed and validated for the simultaneous estimation of Lawsone and its metabolites. In RP-HPLC the separation was carried out by C_{18} column by using mobile phase (0.06%v/v trifluoro acetic acid buffer): methanol: acetonitrile (60:30:10 v/v/v) with pH 3.7 and flow rate of 0.8ml/min in isocratic mode. The PDA detection wavelength was 235nm and 20 µl of sample was injected. For HPTLC the solvent system comprised of toluene: ethyl acetate: ethanol: formic acid in the ratio of 8:2:1:1 drop % v/v/v/v detection was done at 235nm. The R_f value of lawsone, salicylic acid and catechol were found to be 0.37, 0.33 and 0.49 respectively. Linearity was found to be 10-160 µg/ml for Lawsone, 5-80µg/ml for salicylic acid, 40-640 µg/ml for Catechol in HPLC; 20-320 µg/ml for Lawsone, 10-160µg/ml for salicylic acid, 80-1280 µg/ml for Catechol in HPTLC. The above methods were validated according to ICH guidelines and successfully employed for estimation of lawsone and its metabolites.

KEY WORDS: Lawsone, salicylic acid, catechol, RP-HPLC and HPTLC.

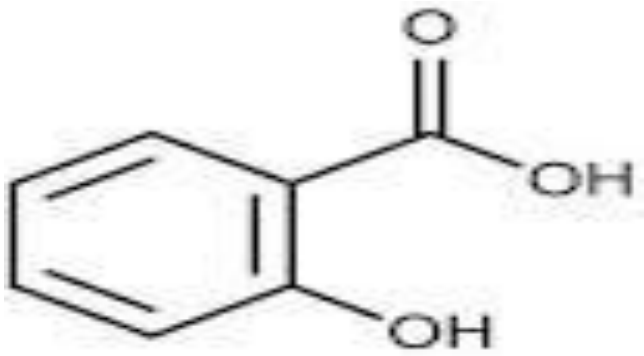
INTRODUCTION

Lawsone (2-hydroxy 1, 4-napthaquinone, figure 1) is a biomedicament from herb which is used in the treatment of Psoriasis, Acne, Eczema and blood disorders. When it is consumed internally, it is reported to be metabolized by *Pseudomonas putida* L2 [1] and forms two

metabolites *viz* Salicylic acid (2-hydroxy benzoic acid) and Catechol (1, 2-dihydroxy benzene) [2-5].



(a)



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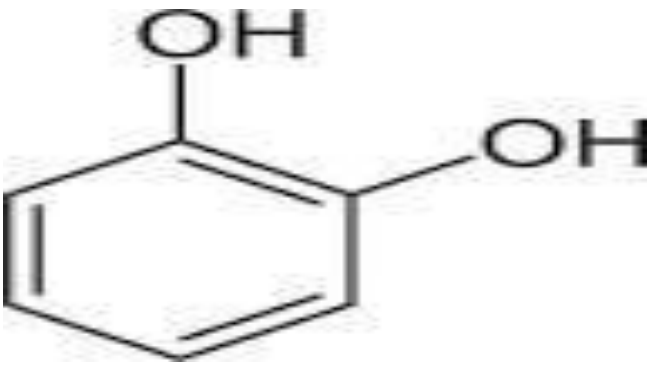

(c)

Figure 1. Chemical structure of (a) Lawsone, (b) salicylic acid and (c) catechol.

Literature survey reveals that, Lawsone is not official in any of the pharmacopeias like IP, BP, USP and European pharmacopeia. Till now no analytical method reported for lwasone. Hence an attempt has been made to develop a simple, efficient and selective method for the determination of lawsone and its metabolites. This paper describes sensitive, accurate and precise RP-HPLC & HPTLC methods which can be used successfully for routine quality control analysis for the estimation of lawsone and its metabolites.

Experimental

Materials and Reagents

HPLC grade Water, Methanol, Acetonitrile were procured from SD Fine Chemicals Pvt. Ltd. (Ahmedabad, India). LR grade Tri-fluoro acetic acid, Toluene, Ethanol, Ethyl acetate, and Formic acid were obtained from Qualigens fine chemicals Ltd. (Mumbai, India).

Instrumentation and chromatographic conditions

The HPLC system (Shimadzu) consisted of a pump (SPD-M10 AVP), PDA detector, a 20µl fixed volume Rheodyne injector and LC-MS solution software set at 235 nm. The Analytical column, HIBAR- 5µ [C_{18}] (250×4.6mm) was operated at room temperature. Isocratic elution with (0.06%v/v trifluoro acetic acid buffer): methanol: acetonitrile (60:30:10 v/v/v) was used at a flow rate of 0.8ml/ min. The mobile phase was prepared freshly and degassed by sonication for 5 min before use (Shimadzu).

HPTLC Camag with precoated silica gel plate 60GF_{254} (20cmX10cm), 250µm thickness was used as stationary phase. Sample application was done by using 100µl syringe and Camag LinomatV applicator. Linear ascending development was carried out in 20 x 10 cm twin trough glass chamber. The densiometric scanning was performed by using Camag TLC scanner III supported by Win cats soft ware.

Preparation of the Mobile phase

A concentration of 0.06% v/v of trifluoro acetic acid was prepared and used to adjust the pH of water to 3.7. This was used as aqueous media for mobile phase. The mobile phase was prepared by mixing 60 mL of above buffer and 30 mL of methanol and 10mL of acetonitrile. The mobile phase was degassed for 5 minutes before use.

Preparation of stock solutions

A quantity of 10mg each of lawsone, salicylic acid, and catechol were taken in a series of 10ml standard flasks and made up to volume with methanol for HPLC and ethanol for HPTLC analysis.

Preparation of working sample solution

For HPLC, from the above stock solution(1mg/ml) aliquots of solutions were prepared containing 10-160µg/ml, 5-80µg/ml, 40-640µg/ml of Lawsone, Salicylic acid and Catechol respectively and the mixture was diluted using mobile phase.

For HPTLC, the mixture of working standards were prepared in the concentration of 20µg/ml ;10µg/ml; 80µg/ml of Lawsone, salicylic acid and Catechol respectively. Ethanol was used for dilution.

Method validation

When method has been optimized it must be validated before practical use. By following the ICH guidelines for analytical method validation Q2 (R1), the validation characteristics were Addressed [6-7].

Linearity (Calibration curve)

An aliquot of 20 µL of each working solution was injected under the operating chromatographic conditions as described earlier. Chromatograms were recorded in HPLC system. Calibration curves were constructed by plotting peak areas versus concentrations, and the regression equations were calculated. Each response was average of three determinations. A good linearity was found in the range of 10-160 µg/ml for Lawsone; 5-80µg/ml for salicylic acid; 40-640 µg/ml for Catechol.

For HPTLC, from the mixture 1-16 µl was applied on a 20 ×10 pre coated TLC plate with Linomat 5 applicator. With the fixed chromatographic conditions the plate was developed and analyzed photometrically. The chromatograms were recorded. Peak areas were measured at 235nm. A good linearity was observed in the range of 20-320 µg/ml for Lawsone; 10-160µg/ml for salicylic acid; 80-1280 µg/ml for Catechol.

Precision

HPLC

The intraday (repeatability) and interday precisions of the proposed methods were determined by estimating the corresponding responses 6 times on the same day and on 6days over a period of one week in the concentration range of 80µg/ml of lawsone, 40 µg/ml of salicylic acid and 320µg/ml of catechol for intraday precision and 40µg/ml of lawsone, 20 µg/ml of salicylic acid and 160µg/ml of catechol for interday precision the response for each injection was measured. The results were reported in terms of relative standard deviation.

HPTLC

Precision is the degree of repeatability of an analytical method under normal operational condition. The precision of the assay was determined by intraday (repeatability) and interday

precision was reported as % RSD for a statistically significant number of replicate measurements. Intraday and interday precision of the method were determined by analyzing the samples in the concentration range of 80µg/ml of lawsone, 40 µg/ml of salicylic acid and 320µg/ml of catechol for interday and 20µg/ml of lawsone, 10 µg/ml of salicylic acid and 80µg/ml of catechol for intraday precision.

Limit of detection and limit of Quantification

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value. The quantification limit of an individual of an individual analytical procedure is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy. The detection limit (LOD) for the proposed was calculated using the following equation LOD=3.3S/K.

Where 'S' is the standard deviation of replicate determination values under the same conditions as for sample analysis in absence of the analyte and 'K' is the sensitivity namely the slope of the calibration graph. The limit of quantification (LOQ) was defined as LOQ=10S/K**.**

Robustness

Robustness was established by varying the chromatographic condition with respect to flow rate and organic composition of mobile phase. Standard and sample solutions were injected and the chromatograms were recorded.

RESULT AND DISCUSSION

Method development and optimization

In HPLC method, the optimization was done by changing the mobile phase, mobile phase ratio, flow rate. Different buffers like formic acid and trifluoro acetic acid were tried (at different ratios with methanol and acetonitrile). But there was no satisfiable separation with formic acid. Hence trifluoro acetic acid was chosen. Initially strength of trifluoro acetic acid was 0.06M. This strength was tried with different ratios like 60:20:20 v/v/v, 60:15:25v/v/v. But the repeatability was not occur. Keeping this strength constant, different ratios with buffer and methanol, acetonitrile were tried such as 60:30:10v/v/v and 60:40% v/v. with the selected mobile phase different pH were tried such as 4.3, 3.7, 3.5 and the pH 3.7 was chosen as it gave better peak shape. Keeping all other parameters constant different flow rates was

tried. A flow rate of 0.8ml was selected based on optimum resolution and analysis time. Hence Buffer (0.06% trifluoro acetic acid buffer – pH 3.7): methanol: acetonitrile 60:30:10% v/v/v was selected as the ratio for successive steps. The above chromatographic conditions resulted in the development of an efficient reproducible method for the determination of lawsone and its metabolites and the chromatogram is shown figure 2.

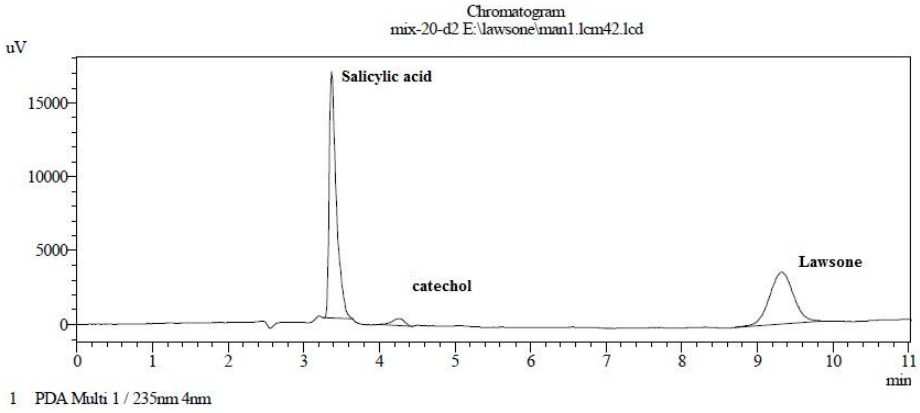

Fig.2 HPLC chromatogram of standard lawsone and its metabolites catechol and salicylic acid

For HPTLC initially plane solvents like toluene, ethyl acetate, ethanol and formic acid were tired. Different ratios were used toluene: ethyl acetate: ethanol: formic acid (9:1:1:1drop % v/v/v/v) but sample moved along with the solvent front. The Toluene: ethyl acetate: ethanol: formic acid (6:2:1:1drop % v/v/v/v) was tried. In this condition sample movement was observed but spots were not clear. Ultimately mobile phase consists of Toluene: ethyl acetate: ethanol: formic acid (8:2:1:1drop % v/v/v/v) which gave good peaks at 254nm. The R_f values for 0.37, 0.33 and 0.49 for lawsone, salicylic acid and catechol respectively (Figure 3). Well defined spots were obtained when plate was activated at 110˚C for 15min and the chamber was saturated with the mobile phase for 15min at room temperature.

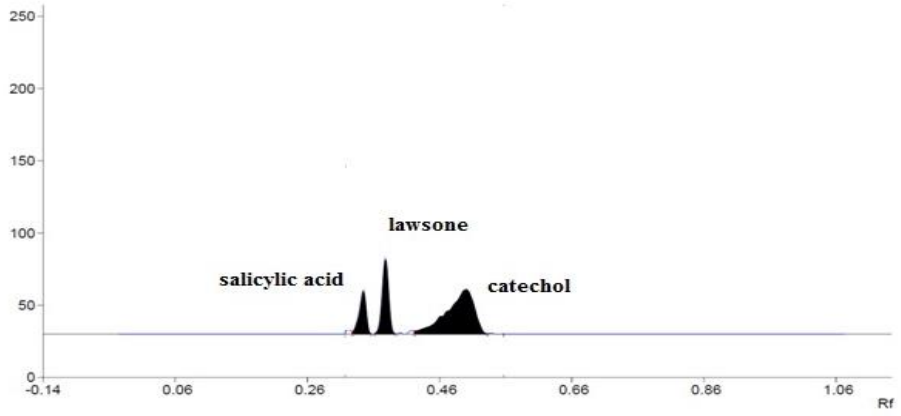

Figure.3 HPTLC chromatogram of lawsone and its metabolites

Validation of the methods

In both HPLC and HPTLC methods, linear correlation was obtained between peak area and concentration for lawsone and its two metabolites. The linearity of the calibration curves were validated by the value of correlation coefficient of the regression (*r*). The results were shown in table-1. The r values were found to be close to 1 proves the linear correlation of each component with respective concentrations. The system suitability parameters like tailing factor, number of theoretical plates, resolution were shown in Table-2.

Limit of detection and limit of Quantification

HPLC

LOD and LOQ were determined by analyzing progressively lower concentrations of the mixture by both methods. In HPLC, LOD and LOQ of the mixture were found to be 0.1µg/ml & 0.3 µg/ml for lawsone; 0.05µg/ml& 0.15µg/ml for salicylic acid; 0.4µg/ml& 1.2µg/ml for catechol respectively. In HPTLC, LOD and LOQ of the mixture were found to be 1µg/ml & 5µg/ml for lawsone; 0.5µg/ml& 2.5µg/ml for salicylic acid; 4µg/ml& 20µg/ml for catechol respectively.

Precision

Precision is the degree of repeatability of an analytical method under normal operational conditions. The precision of the assay was determined by repeatability (intraday) and intermediate precision (interday) and reported as %RSD for a statistically significant number of replicate measurements. The %RSD was <2 in every study proves the precision of method. The reports of analysis were shown in table 3 & 4.

Table.1. Regression Analysis of the Calibration Curve for Lawsone and its metabolites for the Proposed RP-HPLC and HPTLC

Parameters	HPLC			HPTLC		
	LAWSONE	SALICYLIC ACID	CATECHOL	LAWSONE	SALICYLIC ACID	CATECHOL
Concentration(µg/ml)	10-160	5-80	40-640	20-320	10-160	80-1280
Slope	14873.6	4253.28	206.021	5.80868	7.36103	3.23967
intercept	60210	3771.62	2505.54	73.2417	165.392	534.946
Regression equation	0.999267	0.999579	0.998999	0.998445	0.996426	0.988492
LOD	0.1µg/ml	0.05µg/ml	0.4µg/ml	1µg/ml	0.5µg/ml	4µg/ml&
LOQ	0.3µg/ml	0.15µg/ml	1.2µg/ml	5µg/ml	2.5µg/ml	20µg/ml

Table-2 System Suitability Parameters

Drug	Tailing factor	Retention time	Plate count	Resolution
Lawsone	1.016	9.323	4133.629	11.372
Salicylic Acid	1.201	3.366	5388.728	-
Catechol	0.933	4.260	3056.283	3.667

Table-3 Precision Studies for HPLC method

Precision	Concentration (µg/ml)			Peak area			%RSD*		
				L	S	C	L	S	C
	L	S	C	1386848	186896	78632			
	80	40	320	1386720	187040	78736			
Intraday				1386888	187128	79200	0.01	0.06	0.38
				1388646	187218	78362			
				1386724	187074	78376			
				1387186	186809	79020			
				690017	90708	39600			
				689108	91004	39368			
Interday	40	20	160	689254	90185	39316	0.07	0.40	0.39
				689001	90917	39426			
				688497	90814	39510			
				687416	90913	39374			

Table-4 Precision Studies for HPTLC method

Precision	Concentration (µg/ml)			Peak area			%RSD*		
				L	S	C	L	S	C
	L	S	C	228	161.1	570.8			
	20	10	80	228.9	159.4	569.8			
Intraday				227.6	159.9	570.2	0.06	0.01	0.38
				228.1	158.9	570.1			
				227.9	160.1	569.9			
				227.6	159.7	571.1			
				810.8	283.9	1733.8			
				809.9	282.7	1732.9			
Interday	80	40	320	810.1	283.8	1733.6	0.04	0.50	0.19
				810.9	282.9	1732.7			
				809.8	283.4	1733.2			
				808.9	282.9	1733.5			

Robustness

The robustness of the method was evaluated by analyzing the system suitability parameters of the standards after the performance by two different analyst and LC system. The methods were found to be robust under mentioned chromatographic conditions.

CONCLUSION

Two new, accurate and precise chromatographic methods like RP-HPLC and HPTLC were proposed for the determination of lawsone and its metabolites validated as per the ICH guidelines. The methods were found to be simple, cost effective and robust. Hence, it can be used successfully for the quality control tests and pharmacokinetic studies of lawsone and its metabolites.

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