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Statistically validated chromatographic study of salicylate and aniline derivatives of NSAID

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Abstract
A simple, precise and sensitive thin layer chromatographic experiment (TLC) had been carried out for qualitative determination of paracetamol and aspirin in pure also in pharmaceutical dosage form. The experiment was carried out using two different solvent systems used as mobile phase with the determination of R_f value. The experimented R_f value were then validated with different statistical parameters like Precision, Robustness and linearity. The proposed method was found to be precised with a single set of solvent system for pure products. Also with that solvent system it was evident that if a single fraction of that mobile phase was changed in terms of concentration still the method gave robust result. Also the method shows linearity with that solvent system after experimented with a single fraction of solvent of that solvent system. So this method can be followed with that solvent system for the drug paracetamol and aspirin which shall show satisfactory result.

Keywords: Retention factor, TLC, validation, mobile phase, aspirin

Introduction
Acetaminophen (Paracetamol) has pharmacological and pharmaceutical significance. It is a non-steroidal anti-inflammatory drug and is used for the reduction of pain and fever. Acetaminophen is commonly used for the relief of headaches and other minor aches and is a major ingredient in numerous cold and flu remedies [1].
Aspirin can also be referred as acetylsalicylic acid (ASA), is a medication used to treat pain, fever, or inflammation [2].

Introduction to TLC
Thin layer chromatography (TLC) is a chromatography technique used to separate mixtures. Chromatography was discovered by M. Tswett in 1906. Thin layer chromatography is performed on a sheet of glass, plastic, or aluminium foil, which is coated with a thin layer of adsorbent material, usually silica gel G, aluminium oxide, or cellulose (Blotter paper). This layer of adsorbent is known as the stationary phase. After the sample has been applied on the plate, a solvent or solvent mixture (Known as the mobile phase) is drawn up the plate via capillary action. Because different analytes ascend the TLC plate at different rates, separation is achieved.
The principle of TLC is the distribution of a compound between a solid fixed phases (The thin layer) applied to a glass or plastic plate and a liquid mobile phase (Eluting solvent) that is moving over the solid phase. A small amount of a compound or mixture is applied to a starting point just above the bottom of TLC plate. The plate is then developed in the developing chamber that has a shallow pool of solvent just below the level at which the sample was applied. The solvent is drawn up through the particles on the plate through the capillary action, and as the solvent moves over the mixture each compound will either remain with the solid phase or dissolve in the solvent and move up the plate. Whether the compound moves up the plate or stays behind depend on the physical properties of that individual compound and thus depend on its molecular structure, especially functional groups. The more similar the physical properties of the compound to the mobile phase, the longer it will stay in the mobile phase. The mobile phase will carry the most soluble compounds the furthest up the TLC plate. The compounds that are less soluble in the mobile phase and have a higher affinity to the particles on the TLC plate will stay behind.

R_f values: The behaviour of an individual compound in TLC is characterized by a quantity

Known as R*f* (Retention Factor) and is expressed as a decimal fraction. The R*f* is calculated by dividing the distance the compound travelled from the original position by the distance the solvent travelled from the original position (The solvent front).

$$Rf = \frac{\text{Distance of centre of spot from starting point}}{\text{Distance of solvent front from starting point}}$$

OR

R*f* = Distance travelled by the solute front / Distance travelled by the solvent front.

The R*f* value is a constant for each component only under identical experimental condition [3].

Estimation of validation parameters

Linearity

The linearity of an analytical procedure is its ability (Within a given range) to obtain test results, which are directly proportional to the concentration (Amount) of analyte in the sample. In order to determine the quantity of any analyte present in unknown sample, some kind of relationship (Mathematical/empirical) between concentration and response was essential where response should be directly proportional to the concentration.

Acceptance criteria: The correlation coefficient should be less than 1.

Range

The range of an analytical procedure is the interval between the upper and lower concentrations (amounts) of analyte in the sample (including these concentrations) for which it can be proved that the analytical procedure has a suitable level of accuracy and linearity. The range of an analytical procedure is the concentration interval within which acceptable accuracy and linearity were obtained.

Acceptance criteria: Linearity and Recovery are required to be shown.

Accuracy

The accuracy of an analytical procedure express closeness of agreement between the values, which is accepted either as a conventional true value or an accepted reference value and the value can be found.

Evaluation

At each concentration level % mean recovery, SD and % RSD were calculated.

Acceptance criteria

Assay recovery should be between 98%-102%. A simple logic behind this performance characteristic was whether the procedure was capable of estimating a true value or not.

Precision

Precision is the measurement of how close the data values to each other for a number of measurements under the same analytical conditions. Precision may be considered at three levels according to ICH.

Repeatability

System Precision

Precision under same operative conditions (within a laboratory over a short period of time using the same analyst with the same equipment) was determined. Mean, SD and %RSD were calculated from data. The system precision is checked by using standard chemical substance to ensure that the analytical system is working properly. In this retention time and area of six determinations is measured and % RSD should be calculated.

Acceptance criteria: % RSD should be in between 98%-102%.

Method Precision

In method precision, a homogenous sample of single batch should be analysed 6 times. This indicates whether a method is giving consistent results for a single batch. In this analysis the sample has been analysed six times with the calculation of %RSD.

Acceptance criteria: % RSD should be in between 98%-102%.

Intermediate Precision (Ruggedness)

Precision under different laboratory conditions (within-laboratory variation, as on different days, or with different analysts, or equipment within the same laboratory) has been carried out.

Acceptance criteria: % RSD should be in between 98%-102%.

Robustness

Here the closeness of the values are seen in small changes of different parameters like solvent, temperature, pH etc. Here the mean, SD, % RSD is calculated.

Acceptance criteria: % RSD should be in between 98%-102%.

Reproducibility

Precision between laboratories/intermediate precision can be considered during the standardization of a procedure before it is submitted to the pharmacopoeia. A simple logic behind this parameter was some degree of inconsistency (Occurrence of random error) was allowed for every analytical measurement. But, the extent depends on steps involved (Weighing, dilution etc.), technique used in other expected variables (Stability) and intended use of the procedure.

Limit of detection and limit of quantification

LOD: Lowest amount of analyte in a sample which can be detected but not necessarily quantitated, under the stated experimental conditions (LOD).

LOQ: Lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy (LOQ).

Procedure: SD of response (σ) and Slope (S). Five experimental values provided the standard deviation (σ) of and the slope (S) will be obtained from the standard curve of the analyte.

$$LOD = \frac{3.3\sigma}{S} \quad \text{and} \quad LOQ = \frac{10\sigma}{S}$$

Table 1: Different approaches suggested by ICH, USP & EP

Approach parameters	LOD	LOQ
Visual observation	Minimum detection level	Minimum quantifiable level
Signal to Noise Ratio	3:1	10:1
SD of response (σ) and slope (S)	{3.3x σ}/s	{10.0x σ}/s
RSD Criteria	Concentration at which RSD<33.0%	Concentration at which RSD<10.0

Specificity

Specificity generally refers to a method that produces a response for a single analyte only. Selectivity refers to a method which provides responses for a number of chemical entities that may/may not be distinguished from each other. If each response is distinguished from all other responses, then the method is said to be selective. Use of the term 'specificity' is appropriate for microbiological assay, radio-immunoassay etc. methods rather than selectivity. Use of the selectivity is appropriate for the methods based on techniques such as HPLC, GC, CE, etc., than specificity [4].

Materials and Methods

Materials

n – Butanol, acetic acid and ethanol were required and it was purchased from Merck India Pvt. Ltd. Also chloroform, methanol, ammonium hydroxide were required as it was purchased from Loba Chem Pvt. Ltd. The paracetamol tablets, that were used is of IPCA laboratories ltd. The aspirin tablets, that were used is of Reckitt and Benckiser (India) Ltd.

For Paracetamol

TLC method

Method precision

a. **TLC of Paracetamol:** (n- butanol: acetic acid: water = 4: 1: 5). Then the R_f value is calculated. And this process was repeated for 6 times.
b. TLC of Paracetamol: (Chloroform: acetic acid) = 7.5:2.5. The R_f value is calculated. And this was repeated for 6 times.
c. TLC of P-650 (Marketed tablet) (n – butanol: acetic acid: water) = 4: 1: 5. Then the R_f value is calculated. And the process was repeated for 6 times.
d. TLC OF P-650 (chloroform: acetic acid) = 7.5:2.5. Then the R_f value is calculated. And the process was repeated for 6 times.

Robustness

E. Tlc Of Paracetamol: In this process pure aspirin drug is taken as analyte.
In this process the ratio of the chemical substances are changed with each mobile phase and with each mobile phase TLC of that drug was performed for single time.

E1. With the first mobile phase where n – butanol: acetic acid: water is taken with the ratio of 4: 1:5. (Here 4ml of n-butanol was taken).

E2. In the second mobile phase n–butanol: acetic acid: water is taken with the ratio of 5:1:4 (here 5ml of n-butanol was taken).

E3. In the third mobile phase n- butanol acetic acid: water is taken with the ratio of 6:1:3 (Here 6ml of n-butanol was taken).

E4. In the fourth mobile phase n-butanol: Acetic Acid: water is taken with the ratio of 7:1:2 (Here 7ml of n-butanol was taken).

Linearity

Linearity has been calculated by taking a single solvent from the mixture of the solvent involving the preparation of mobile phase and was match against the corresponding R_f value that came at that particular concentration of that single solvent which was present inside the solvent mixture involving the preparation of mobile phase for this particular analyte. Here n-butanol fraction was measured.

For Aspirin

TLC Method

Method Precision

A. TLC of Aspirin (n- butanol: acetic acid: water = 4: 1: 5). R_f value was calculated. And this process was repeated for 6 times.

B. TLC of Aspirin (chloroform: acetic acid: water: ammonium hydroxide) = 1.2:7.5:0.6:0.2. R_f value was calculated. And this was repeated for 6 times.

C. TLC of Disprin (Marketed tablet) (n – butanol: acetic acid: water) = 4: 1: 5. R_f value was calculated. And the process was repeated for 6 times.

D. TLC of Disprin (chloroform: acetic acid: water: ammonium hydroxide) = 1.2:7.5:0.6:0.2.$R_f$value was calculated. And the process was repeated for 6 times.

Robustness

E. TLC of Aspirin: In this process pure aspirin drug is taken as analyte.
In this process the ratio of the chemical substances are changed with each mobile phase and with each mobile phase TLC of that drug was performed for single time.

E1. With the first mobile phase where n – butanol: acetic acid: water is taken with the ratio of 4: 1:5.(here 4ml of n-butanol was taken)

E2. In the second mobile phase n–butanol: acetic acid: water is taken with the ratio of 3:1:2. (here 3ml of n-butanol was taken)

E3. In the third mobile phase n- butanol: acetic acid: water is taken with the ratio of 2:1:3.(here 2ml of n-butanol was taken)

E4. In the fourth mobile phase n-butanol: acetic Acid: water is taken with the ratio of 1:1:4. (here 1ml of n-butanol was taken)

Linearity

Linearity has been calculated by taking a single solvent from the mixture of the solvent involving the preparation of mobile phase and was match against the corresponding R_f value that came at that particular concentration of that single solvent which was present inside the solvent mixture involving the preparation of mobile phase for this particular chromatographic determination of analyte. Here also n-butanol fraction was measured.

Results and Discussion

Method Precision

Table 2: Method precision data of pure Paracetamol

TLC of paracetamol pure drug		**TLC of paracetamol pure drug**	
Mobile phase is		Mobile phase is	
n-butanol: acetic acid: water		chloroform : n-butanol	
04:01:05		7.5:2.5	
No. of experiment	R_f value	No. of experiment	R_f value
1	0.94	1	0.93
2	0.95	2	0.95
3	0.92	3	0.95
4	0.93	4	0.91
5	0.91	5	0.95
6	0.93	6	0.95
mean	0.93	mean	0.94
sd	0.014142	sd	0.016733
% rsd	1.52066	% rsd	1.780128
In this solvent ratio pure paracetamol is method precised		In this solvent ratio pure paracetamol is method precised	

Table 3: Method precision data of marketed Paracetamol

TLC of paracetamol tablet (Marketed)		**TLC of paracetamol tablet (Marketed)**	
mobile phase :		mobile phase	
n-butanol: acetic acid: water		chloroform: n-butanol	
04:01:05		7.5:2.5	
No. of experiment	Rf value	No. of experiment	Rf value
1	0.92	1	0.95
2	0.91	2	0.96
3	0.91	3	0.95
4	0.87	4	0.95
5	0.93	5	0.93
6	0.91	6	0.93
mean	0.908333	mean	0.945
sd	0.020412	sd	0.012247
% rsd	2.247238	% rsd	1.296026
In this solvent ratio pure paracetamol is not method precised		In this solvent ratio pure paracetamol is method precised	

Robustness

Table 4: Robustness data of Paracetamol

TLC of paracetamol pure drug	**TLC of paracetamol pure drug**	**TLC of paracetamol pure drug**	**TLC of Paracetamol pure drug**
mobile phase:	mobile phase:	mobile phase:	mobile phase:
n-butanol: acetic acid: water	n-butanol: acetic acid : water	n-butanol: acetic acid : water	n-butanol: acetic acid: water
04:01:05	05:01:04	06:01:03	**07**:01:02
Rf value= 0.93	Rf value =0.94	Rf value= 0.94	Rf value= 0.95
n-butanol (ml)	Rf values		
4	0.93		
5	0.94		
6	0.94		
7	0.95		
Mean	0.94		
Sd	0.008164966		
% rsd	0.868613384		
In this different n-butanol concentration pure paracetamol is robust		In this experiment different n-butanol concentration in TLC of pure paracetamol is robust.	

Linearity

Table 5: Linearity table for Paracetamol

n-butanol	**Rf values**
4	0.93
5	0.94
6	0.95
7	0.96
Linearity rage was found to be 4-6 ml	

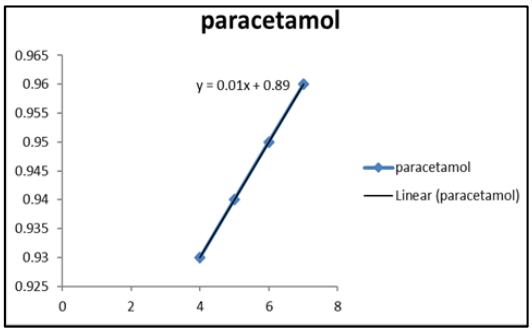

Fig 1: Linearity graph of Paracetamol

Method precision

Table 6: Method precision data of pure Aspirin

TLC of aspirin pure drug		TLC of aspirin pure drug	
mobile phase:		mobile phase:	
n-butanol : acetic acid: water		chloroform: methanol: water: ammonium hydroxide	
04:01:05		1.2: 7.5: 0.6: 0.2	
No. of experiment	Rf value	No. of experiment	Rf value
1	0.92	1	0.95
2	0.92	2	0.95
3	0.95	3	0.93
4	0.9	4	0.95
5	0.94	5	0.93
6	0.92	6	0.94
mean	0.923333	mean	0.941667
sd	0.023381	sd	0.009832
% rsd	1.032228	% rsd	1.044098
In this solvent ratio pure aspirin is method precised		In this solvent ratio pure aspirin is method precised	

Table 7: Method precision data of marketed Aspirin

TLC of dispirin (Marketed tablet)		TLC of dispirin (Marketed tablet)	
mobile phase:		mobile phase:	
n-butanol : acetic acid: water		chloroform: methanol: water: ammonium hydroxide	
04:01:05		1.2: 7.5: 0.6: 0.2	
No. of experiment	Rf value	No. of experiment	Rf value
1	0.94	1	0.95
2	0.94	2	0.95
3	0.89	3	0.92
4	0.9	4	0.93
5	0.93	5	0.93
6	0.92	6	0.92
mean	0.92	mean	0.933333
sd	0.020976	sd	0.013663
% rsd	2.280019	% rsd	1.46385
In this solvent ratio pure aspirin is not method precised		In this solvent ratio pure aspirin is method precised	

Robustness

Table 8: Robustness data of Aspirin

TLC of aspirin pure drug	TLC of aspirin pure drug	TLC of aspirin pure drug	TLC of aspirin pure drug
mobile phase:	mobile phase:	mobile phase:	mobile phase:
n-butanol acetic acid :water	n-butanol :acetic acid: water	n-butanol: acetic acid :water	n-butanol: acetic acid: water
04:01:05	03:01:02	02:01:03	01:01:04
Rf value= 0.95	Rf value= 0.92	Rf value= 0.92	Rf value= 0.93
n-butanol (ml)	Rf values		
4	0.93		
3	0.92		
2	0.92		
1	0.95		
mean	0.93		
sd	0.014142136		
% rsd	1.520659744		
In this different n-butanol concentration pure aspirin is robust.			

Linearity

Table 9: Linearity table of Aspirin

n-butanol fraction	Rf values
1	0.92
2	0.93
3	0.94
4	0.96
Linearity range was found to be 1-4 ml	

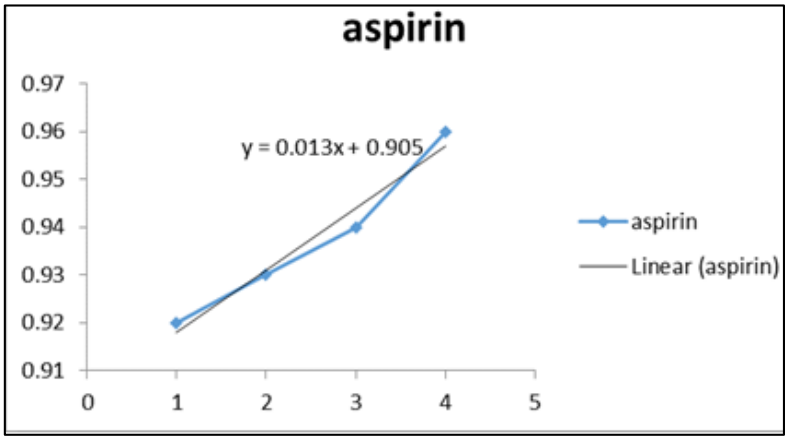

Fig 2: Linearity graph of Aspirin

Table 10: Summary of the results

Validation parameter	Pure drug (Paracetamol)	Marketed Tablet (Paracetamol)	Pure drug (Aspirin)	Marketed drug (Aspirin)
Method Precision (Solvent system: N-butanol: Acetic acid: water= 4:1:5)	Precised	Not Precised	Precised	Not precised
Method Precision (Solvent system: chloroform : n-butanol= 7.5:2.5)	Precised	Precised	N.A	N.A
Method Precision (Solvent system: chloroform: methanol: water: ammonium hydroxide=1.2: 7.5: 0.6: 0.2)	N.A	N.A	Precised	Precised

Conclusion

It appeared from above experiment that the solvent system having fraction of n-butanol: acetic acid: water was promised to give robust results for the both pure aspirin and paracetamol where as it only showed satisfactory precised result for both the pure aspirin and paracetamol entity. It indicated that for the marketed product of aspirin and paracetamol the above solvent system is not sufficient. But separately if we compare in case of paracetamol the precised solvent system appeared to be chloroform: n-butanol where as in the case of aspirin, chloroform: methanol: water: ammonium hydroxide seemed to be precised result for both the pure and marketed formulation. Also the linearity calculation also was carried out by taking the fraction of n-butanol from the solvent system of n-butanol: acetic acid: water for both the case of aspirin and paracetamol.

So it can be concluded that there was a marked characteristics differences between two solvent systems while analysing same drug entity in respect to obtaining precised result in pure and marketed form as excipient's role may be under consideration. By in large separate solvent system for separate drug may be the way to go although the drug characteristics may appear to be same in marketed formulation.

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