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ESTIMATION OF CAFFEINE CONTENT IN CHOCOLATES BY USING HPTLC

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ABSTRACT

The aim of the project is to develop a new simple, accurate, precise, rapid, selective and reproducible high performance thin layer chromatographic method for quantitative analysis of caffeine in chocolate products has been established and validated. Chocolate is a preparation of roasted andground cocoa seeds, which contains caffeine. Daily limit of caffeine 300-400mga day. More than 400mg of caffeine consumption leads to disrupted sleep, nervousness, dizziness, alsoincreases release ofacid. Inthis theextraction ofcaffeine fromchocolates had done and obtained product was quantified by using HPTLC. High performance thin layer chromatography (HPTLC) on aluminium – baked silica gel 60 F254 plates with butanol- chloroform-ammonia-acetone 4:3:2:1% (v/v/v/v) as mobile phase was followed by densitometry measurement at 254nm. This system was found to give compact bands for caffeine. Calibration plots were linear with correlation coefficient. The developed method was validated and proved to meet the requirements delineated by ICH guidelines with respect to linearity, accuracy, precision, and robustness and can be used for analysis of marketed products.

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INTRODUCTION

Caffeine (1,3,7-trimethyle xanthine) is a natural occurring as well as pharmacologically active substance. caffeine is an odorless white crystalline powder with a very bitter taste . It is a mild central nervous system (CNS) stimulant and is considered the most frequently consumed dietary stimulant of CNS. The recommended daily dose for the pharmacological effect is 200mg/day .

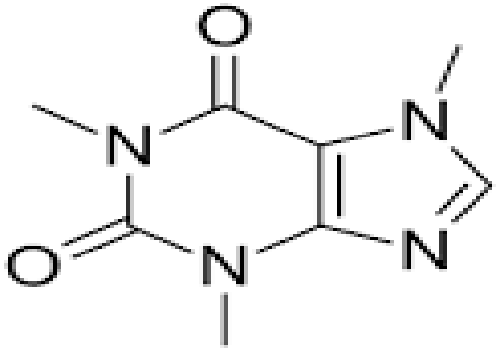

Figure .1. structure of caffeine.

The Food and Drug Administration (FDA) defines caffeine as a generally recognized as a safe (GRAS) substance but, with certain limits. When higher doses are consumed, it may leads to side effects on central nervous system of human body like Addiction and anxiety , It can affect cardio vascular system ,liver function ,its stimulates gastric secretion .Caffeine does not accumulate in the body and it is normally excreted with in several hours of consumption .A dose of 10g is lethal is equivalent to about 100cups of coffee

Purpose of study:

Now –a- days caffeine consumption by humans is increasing day by day intentionally or unintentionally examples: tea, coffee ,cool drinks and chocolates.

Regardless of age groups all these products mainly chocolates are consumed by population more and more which results in large amount of caffeine in body leads to a condition known as caffeinism.

Caffeinism habitually is a condition of caffeine dependency with a broad rangeof disagreeable mental and physical conditions as well as anxiety, headaches, insomnia, irritability, nervousness, hyperreflexia, respiratory alkalosis, heart palpitations. Caffeine activates the stomach to release excess amount of acid . Because of high usage of caffeine for long time leads to irritation in the pits of the stomach and worsen peptic ulcers in the duodenum ,erosive esophagitis and gastro esophageal reflux disease . It may also persuade non cancerous breast disease and may aggravate premenstrual symptoms in women who overuse it. To provide awareness about serious effects of caffeine which makes the people get aggravated due to its high consumption in any form.

HPTLC

High performance thin layer chromatography (HPTLC) is the most powerful advanced form of thin layer chromatography (TLC) and consists of chromatographic layers of utmost separation of efficiency .HPTLC is an entire concept that includes a standardized methodology based on facts as well as use of validated methods for qualitative and quantitative analysis. It meets all quality requirements for todays analytical labs , to increase the resolution and to allow more accurate quantitative measurements.

HPTLC methodology:

The first objective may be quantification or qualitative identification or separation of multi component mixture . HPTLC primary knowledge about the nature of sample, structure , polarity, volatility, stability and the solubility parameters. Selection of stationary phase is easy, silica gel is suitable for most of the drugs. Mobile phase optimization is carried out by using three level techniques .First level involves use of solvents in those solvents with average power of the desired drug are selected. In second level the strength of solvent was increased or decreased by use of hexane or water for respective purpose. In third mixtures will be tried instead of solvents selected from above two levels and then optimized by acid or base modifiers.

AIM AND OBJECTIVE

The main aim of the study is to develop a new analytical method for determination of caffeine content in chocolates by HPTLC .

Objectives:

The current research is done to achieve the following objectives:

a) To extract the caffeine from different chocolates.
b) To recognize the different chocolates containing caffeine.
c) To estimate the amount of caffeine present in chocolates by performingHPTLC.
d) To bring awareness in people about the caffeine content in chocolates.

Quantitative analytical methods are essential to characterize and quantify the drug substances and drug products compositions during all phases of pharmaceutical development.Comprehensive literatures are survey reveal that few analytical methods have been reported for the estimation of caffeine which includes Thin Layer Chromatography(TLC), Reverse Phase High Performance Liquid Chromatography(RP-HPLC). But there are no reported HPTLC, other methods for estimation of caffeine.

Hence there is a scope to develop simple, accurate, sensitive, rapid, and economic method for effective quantitative determination of caffeine as an active pharmaceutical ingredient as well as food items using High Performance Thin Layer Chromatography.

Validation of the method will be done in accordance with USP and ICH guidelines to prove better performance characteristic of the method in its intended analytical applications. The method will be validated for parameters like system suitability, linearity, accuracy, precision, robustness.

EXPERIMENTAL WORK

Extraction of caffeine from dark chocolates:

Weighed 500gm of cocoa powder
↓
And dissolve in 1000ml hot water
↓ Stirre well
↓
Take 1000ml separating funnel and add 250 ml of the above solution.
↓
Then add 175 ml Dichloromethane and 175 ml of chloroform to the separating funnel.
↓
Shake well and place the separating funnel aside with out any disturbance for one and half hour.
↓
After collect the organic layer from the separating funnel into a beaker .
↓
Then again add 25 ml Dichloromethene and 25ml chloroform
↓
Keep aside for one hour and again collect the organic layer .
↓
Repeat the same procedure for above chocolate solution and collect organic layer.
↓
Add sodium sulphate to the organic solutions and stirre.
↓
Then filter the above solution.
↓
Take the filterate and kept for evaporation at room temperature.

At the room temperature the dichloromethene and chloroform is evaporatedand precipitate will be obtained.

Confirmatory tests :

Muroxide test :

To the above precipitate add
↓
Hydrochloric acid and Nitric acid
↓
Placed on the water Bath for evaporation
↓
After evaporation add Strong Ammonia
↓
It gives purple colour – presence of caffein

METHOD DEVELOPMENT

1. Layer pre-washing Sample preaparation:
2. Application of sample:.
3. Selection of mobile phase:
4. Chamber saturation:
5. Chromatographic development and drying:
6. Activation of chromatographic plates
7. Photo documentation

OPTIMISED CHROMATOGRAPHIC METHOD

After several trails with different combinations and ratios of solvents used in the extraction of caffeine from cocoa powder chromatographic parameters above trails were optimized.

Extraction of caffeine from cocoa powder

Solvent used for the solid liquid extractionButanol :Choloform : Ammonia : Acetone was prepared in the ratio of 40:30:20:10 v/v/v

Chromatographic parameters:

HPTLC plate
: 10 cm × 10 cm aluminium backed silica gel 60 F254 Mobile phase
: Butanol:Chloroform:Ammonia:Acetone(40:30:20:10) Sprayspeed
:8µl/sec
Detection of wave length
: 254nm Band volume
: 10µL
Temperature
: Ambeint

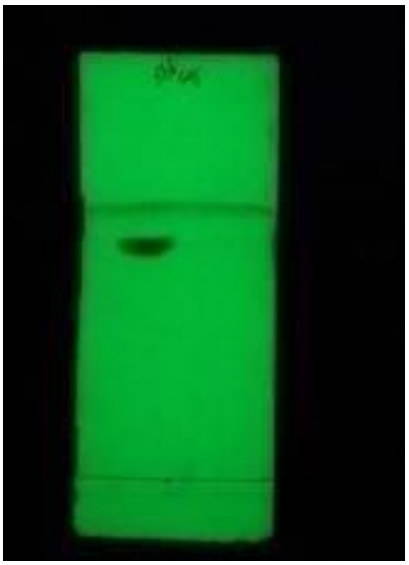

Fig :6 0ptimization of standard caffeine.

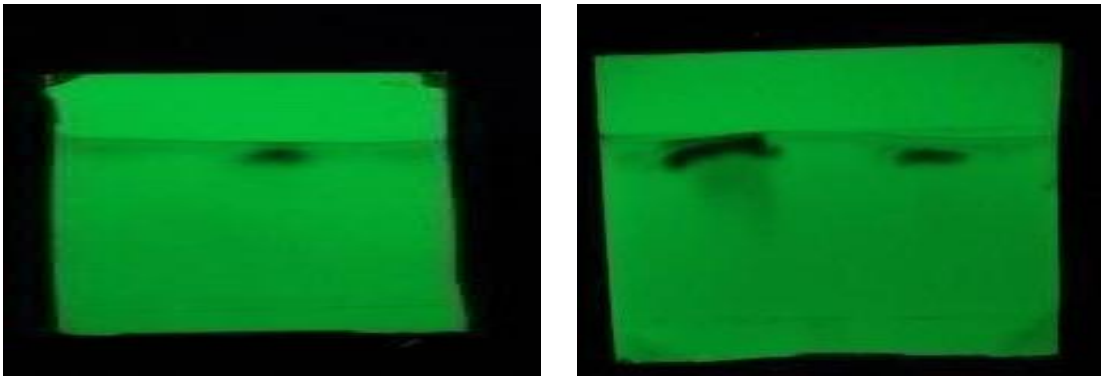

Fig:7 optimization extracted caffeine from cocoa powder.

Discussion:
As the elution was done properly and spots were clearly visible, this method was continued for validation.

METHOD VALIDATION
Method validation was performed as per the ICH guidelines. The development method was validated for the following parameters.

- System suitability
- Linearity
- Accuracy
- Precision
- Robustness

System suitability testing:
System suitability is a test should be carried out to verify the analytical system is working properly and can give accurate and precise results. Standard solutions are prepared as per the test method and injected into the chromatographic system.

Linearity:
The linearity of the analytical method was carried out to check its ability to elicit test results that are directly, or by a well-defined mathematical trasformation, proportional to the concentration of analyte in sample within a given range. Different levels of standard solutions were prepared and injected into the HPTLC and the chromatograms were recorded.

Procedure :
Preparartion of stock solution: 1mg of standard caffeine in 10ml of methanol(1000µg/ml)

Preparation of serial dilutions :
From the stock solution 0.1, 0.2 ,0.3, 0.4, 0.5 ml of solution was pipette out to 10ml with methanol in 10ml volumetric flask.

Acceptance criteria :
The accuracy of an analytical method is the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.
The accuracyof an analytical method is the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.
Resulting mixture Accuracy was perfomed by fallowing direct comparision method. The study was perfomed by giving same standard concentrations three times of known amounts of studied drugs. The accuracy of an analytical method should be established across its range. Finally, the final volume made up with diluents and mixed well. The were analysed by the proposed HPTLC method at 254nm. The excellent mean recoveries and standard deviation suggested good accuracy results of the propose method.

Procedure :
Sample solutions prepared were injected three times into the chromatographic system and recorded the chromatograms.

Acceptance criteria:
The % RSD for the volume of three standard injections results should be more than 2%

PRECISION:

The precision of an analytical method is a measure of the random error and is defined as the agreement between replicate measurements of the same sample .it is expressed as the percentage of relative standard deviation (% RSD) of the replicate measurments.

METHOD PRECISION:

Intraday precision

preparation of sample solution:

Accurately weighed about quantities of 3 mg of caffeine were transferred into 10 ml clean volumetric flask and is make up with 10 ml methanol.

procedure:

For precision studies 3 replicate injections of caffeine standard were performed
%RSDwas determined for volumes

Acceptance criteria :

The %RSD for the area of three standard injections results should not be more than 2%

Interday precision

Preparation of sample solution:

Accurately weighed about quantities of 3 mg of caffeine were transferred into 10ml clean volumetric flask and is make up with 10ml methanol.

Procedure:

For inter day method precision studies 3 replicate injections of caffeine samples were performed. %RSD was determined for volumes of caffeine.

Acceptance criteria:

The %RSD for the area of three standard injection results should not be more than 2%

Robustness:

The robustness of the analytical method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

The robustness of the proposed method was determined by analysis of aliquots from homogenous lots by differing physical parameters like volume, mobile phase volumes. The standard solution and sample solutions were injected into the chromatograph at varied conditions of flow ±5ml/min , mobile phase volumes ±5ml.

CHROMATOGRAPHIC MODE	OPTIMIZED CONDITION
HPTLC system	HPTLC-Aetron
Stationary phase	Precoated silica plates (10×10cm)
Mobile phase	Butanol:Chloroform:Ammonia:Acetone (40:30:20:10)
Detection wave length	257nm
Injection volume	20µl

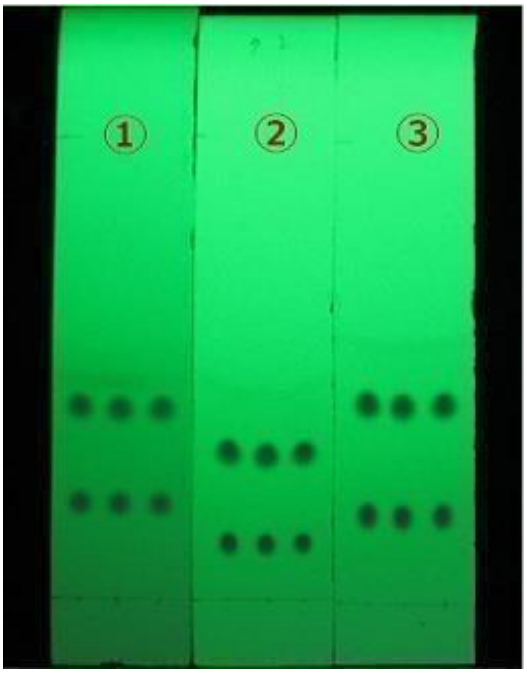

Fig : 1 Linearity of caffeine.

Table -2 Accuracy and precision of caffeine.

Theoritical concentrations (µg/ml)			Determined (µg/ml)	Precision	%RSD	Accuracy (˚)
Original	**Added**	**Total**		**Intra-day**	**Inter-day**	**Intra-day**
11.1	5.0	16.1	16.1	0.42	0.90	99.7
11.1	10.0	21.4	21.4	0.85	1.44	100.8
11.1	20.0	31.1	31.1	0.51	1.67	98.9

Inference:

The accuracy of the method was determined by measuring caffeine content. The mean recovery was in acceptable range. The excellent mean recoveries and standard deviation suggested that the good accuracy of the proposal method.

Data interpretation :

The volume for caffeine obtained from six replicate injections are consistent as evidence by the values of relative standard deviation. Hence it can be concluded that the system precision parameter meets the requirement of method validation.

Robustness

Chromatogram of change in spray volume of sample 25ml

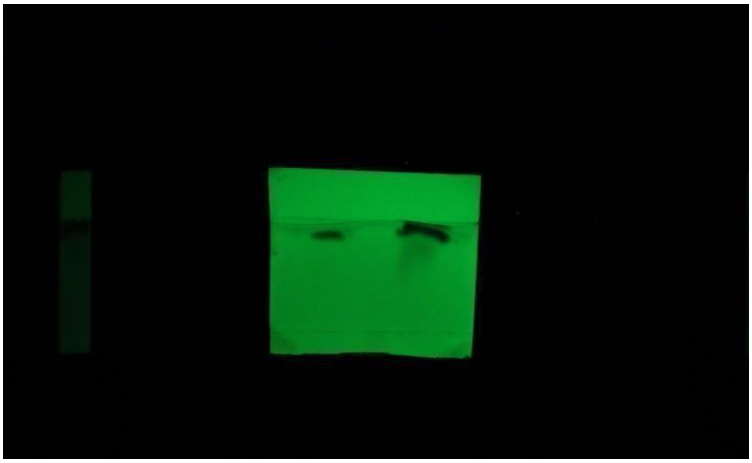

Table -4 Robustness of caffeine volume changed.

Concentration	Area	RF value	Volume	Average	SD	° RDS
2500	1139	0.439	36.35			
2500	1116	0.439	36.22	36.285	0.091924	0.28333

Table -5 Robustness of caffeine volume changed Butanol :chloroform.

Concentration	Area	RF value	Volume	Average	SD	° RDS
3500	960	0.526	40.72			
3500	965	0.531	40.68	40.65	0.098995	0.24353

Ammonia:Acetone

Table -6 Robustness of caffeine mobile phase ratios changes.

Concentration	Area	RF values	volume	Average	SD	° RSD
3000	1144	0.75	48.59			
3000	1056	0.75	48.06	48.356	0.374767	0.775513

RESULTS AND DISSCUSSION

A simple high performance thin layer chromatographic method was developed for the qualification of caffeine using silica as stationary phase equilibrated with mobile phase containing combination of Butanol: Chloroform: Ammonia :Acetone.

- The RF values was found to be 0.495,0.563,0.733,0.662,0.705 respectively the platewas scanned quantified at 254nm.
- Calibration curve for each drug was plotted using to parameters concentrationv/speak height. The linearity range of caffeine were 1000,2000,3000,4000,5000 ng/spot. The validation of the developed method was performed in accordance with ICH guidelines(Q,B validation of analytical procedure methodology)

CONCLUSION

Considering the broad range of biological activities determined through pharmacological investigations and widespread occurrence of caffeine in chocolates, a HPTLC method was developed for the quantification of caffeine in various chocolate products and cocoa powder. HPTLC technique was successfully used for estimation of caffeine in chocolates. Caffeine was extracted from chocolates and then is dissolved in methanol.This HPTLC method for quantitative analysis of caffeine in chocolates is simple, fast, accurate, precise, specific, rugged, reproducible. The method was validated in accordance with ICH guidelines. The method reduces analysis time and found to be cost effective and seems to be suitable for routine analysis of compound present in chocolates in quality control laboratories, where economy and speed are essential to the best of our knowledge.The validation data showed that this HPTLC method is a reliable high-throughput alternative for measurement of caffeine. To the best of our knowledge, the online identification by HPTLC–ESI-MS of caffeine compound was successfully achieved.

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