



DEVELOPMENT OF VALIDATED BIO ANALYTICAL METHOD FOR THE INVITRO DETERMINATION OF DOXOFYLLINE FROM HUMAN PLASMA BY HPTLC

Nihila K, Akhil MB, Y Haribabu, Kumar Palanisamy*

Department of Pharmaceutical Analysis, Grace College of Pharmacy, Palakkad, Kerala, India.

ABSTRACT

Bio-analytical methods employed for the quantitative determination of drugs and their metabolites in biological samples plays a significant role in the evaluation and interpretation of bioavailability, bioequivalence and pharmacokinetic study data. These studies generally support regulatory findings. HPTLC is one of the most widely used methods for both qualitative and quantitative analysis. After extraction, various concentrations of drug solutions were applied on the plate and development of the plate was done by using mobile phase system consisting of Toluene: n-butanol: Triethylamine: Ammonia (3:5:2:0.1% v/v/v/v) R_f values of Doxofylline was found to be 0.47 ± 0.01 . The developed method was validated according to the US-FDA guidelines. Linear regression data revealed an excellent linear relationship between concentration and peak area ratio over a concentration range of 20-200 ng/spot. The stability of Doxofylline in prepared sample was found to be 7 hours under room temperature.

Keywords: Bioavailability, Pharmacokinetic, Interpretation, Metabolites.

Corresponding Author: - **Kumar Palanisamy** Email: nkumarpharma@gmail.com

INTRODUCTION

In order to determine the optimum dose it is necessary to know in detail the pharmacokinetics of drug and its metabolites [1]. Today such requirements are put upon all newly introduced drugs and this is the reason why so much interest is focused on the topic. The most widely employed bioanalytical techniques include but not limited to conventional chromatography methods (such as HPLC, HPTLC and GC), mass spectrometry based methods (such as LC-MS, LC-MSMS, GC-MS) and ligand bind assays. The biological samples generally used


are plasma, serum, urine and saliva. However in most of the cases plasma is the sample of choice [2]. This is due to the fact that the analyte concentration in plasma can be used reliably to interpret the analyte concentration in the site of action [3]. Sample preparation involves separation of analyte from the matrix. Sample preparation step largely depends on the nature of sample and type of method employed for estimation. Steps involved in HPTLC method development can be summarized as follows [4-7].

Selection of chromatographic layer

Pre coated plates with different support materials and different sorbents are available. Of these silica gel is far most important sorbent.

Sample preparation

The sample preparation procedure is to dissolve the dosage form with complete recovery of intact compound of interest and minimum of matrix with a

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suitable concentration of analytes for direct application on the HPTLC plate [8].

Layer pre treatments

Prior to chromatography it is a common practice to prepare the layer by any or all of the following steps; washing, activation, conditioning and equilibrium so as to avoid problems like irregular and drifting densitometric baselines, ghost peaks and reduced sample detectability.

Mobile phase optimization

A solvent of correct strength for a single development separation will migrate the sample into the R_f range of 0.2 to 0.8. Mobile phase should chose taking into consideration chemical properties of analytes and sorbent layer [9].

Chamber saturation

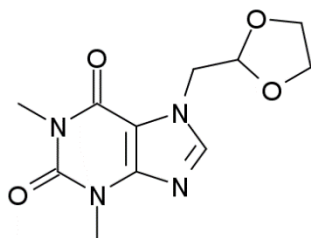
If the tank is saturated prior to development, solvent vapour soon get uniformly distributed throughout the chamber, hence less solvent shall be required to travel at a particular distance, resulting in the lower R_f values.

Development and detection

Ascending, descending, two dimensional, horizontal, multiple over run gradient, radial and anti-radial are most common methods of development. Detection may be done manually or instrumentally; instrumental detection is recommended for quantitative analysis [10].

Doxofylline

Structure



Chemical Name : 7-(1,3-dioxolan-2-ylmethyl)-1,3-dimethylpurine-2,6-dione

Molecular weight : 266.25

Molecular formula : $C_{11}H_{14}N_4O_4$

Description : White crystalline powder

Solubility : Soluble in water, acetone, benzene, chloroform

pKa value : 9.8

Therapeutic use : Antitussive and Bronchodilator

MATERIALS AND METHODS

Camag HPTLC System (with Linomat 5 as application device, TLC Scanner 3 and WinCATS software)

Determination of Doxofylline from Human Plasma by Hptlc

Stationary phase : pre-coated silica gel 60 F₂₅₄ on Aluminum sheets
 Mobile phase : Toluene: n-butanol: Ethyl acetate: Ammonia (3:5:2:0.1)
 Chamber saturation time : 20 minutes
 Pre-saturation time : 10 minutes
 Migration distance : 80mm
 Band width : 6mm
 Slit dimension : 5 × 0.45mm
 Source of radiation : Deuterium lamp
 Detection wavelength : 278nm (fig.77)
 R_f values : Doxofylline – 0.47 ± 0.01
 Spots were scanned at 278 nm in reflectance mode using Camag TLC scanner [11-15].

Optimization of Extraction Procedure

Selection of organic solvent for extraction

50 μ L of standard drug solution was added to the 250 μ L of human plasma and extracted with various organic solvents listed below in table 1.

Fixed extraction procedure

To the clean and dry centrifuge tube, 250 μ L plasma, 50 μ L of aliquots of standard drug solutions (2 to 20 μ g/ml) were added and vortexed for 5 minutes. About 5 mL of acetonitrile was added to the above solution and vortexed for 5 minutes. This solution was centrifuged at 3000 rpm for 10 minutes. The clear supernatant solution was transferred to eppendorf's tubes and evaporated to dryness using nitrogen gas. The residue was reconstituted with 500 μ L of methanol and 10 μ L of this solution was applied on to the plate. The plate was analysed and chromatograms were recorded. R_f values of Doxofylline found to be 0.47.

Standard Chromatograms of Doxofylline in Plasma

Validation

The validation of the developed method was carried out in terms of specificity, linearity, lower limit of quantification (LLOQ), accuracy, precision, recovery and stability studies according to US-FDA guidelines.

Specificity

Specificity of the above developed extraction method was demonstrated as follows. Blank plasma samples from six volunteers were extracted using the above procedure and chromatographed. It was found that there was no interference from blank plasma.

Linearity and Range

Linear regression data revealed an excellent linear relationship between concentration and peak area ratio over a concentration range of 20- 200ng/spot in table 2. The slope, intercept and correlation co-efficient values were found to be, 137.0773, 59.5 and 0.9989 as shown in figure 1.

Lower limit of quantification (LLOQ)

The lowest concentration at which the peak is quantified is called 'Lower Limit of Quantification' which was found to be 20ng/spot.

Accuracy

A minimum of five determinations per concentration were carried out to measure the accuracy of the method. At least three concentrations (high QC, middle QC, low QC) in the range is recommended. Accuracy can be measured by assessing the deviation of mean test result from the true value and it should be within 15%. The results are shown in table 3.

Precision

Precision of the method was determined by

Intraday precision

Intraday precision was studied by carrying out the analysis of the standard drug at three different concentrations in the linearity range of drug (high QC, middle QC, low QC) for five times on the same day and coefficient of variation (CV) was calculated in table 4.

Inter day precision

Inter day precision was studied by carrying out the analysis of the standard drug at three different concentrations in the linearity range of drug (high QC,

middle QC, low QC) for five days over a period of one week and coefficient of variation (CV) was calculated, table 5.

Recovery

The extraction efficiency was explained by recovery studies. The standard drug was extracted by the proposed extraction method. The extracts were analysed with the fixed chromatographic conditions. The peak areas of developed chromatograms were compared with that of unextracted standard peak areas. The peak area responses obtained were used for determining the extraction efficiency. Recovery should be determined by using three concentration levels (high QC, middle QC, low QC). The results are shown in table 6.

Stability

The drug may undergo degradation during the analysis. Hence it is necessary to study the stability of drug in prepared sample at two different concentrations (low QC, high QC). The stability of the drug was studied under room temperature. The samples were chromatographed at fixed time intervals and the peak area was compared with that of fresh solution. The stability of Doxofylline was found to be 7 hours under room temperature.

Table 1. Selection of organic solvent for extraction

Organic solvent	Observation
Acetonitrile	Extraction efficiency was better
Methanol	Extraction efficiency was good

Table 2. Calibration data

Concentration (ng/spot)	Peak area
20	293
40	677
60	961
80	1221
100	1505
120	1779
140	1998
160	2201
180	2520
200	2811

Table 3. Accuracy study

True concentration(ng/spot)	Mean test result (ng/spot)	% RSD*	Limit for % deviation	Number of determinations
20 (Low QC)	16.7	8.25	Within 15	5
100 (Middle QC)	92.38	7.72		
200 (High QC)	187.2	6.4		

*mean of five observations

Table 4. Intraday precision

Concentration (ng/spot)	Coefficient of variation (CV)*	Limit	Number of determinations
20 (Low QC)	7.25 %	Within 15 %	5
100 (Middle QC)	6.56 %		
200 (High QC)	5.65 %		

*mean of five observations

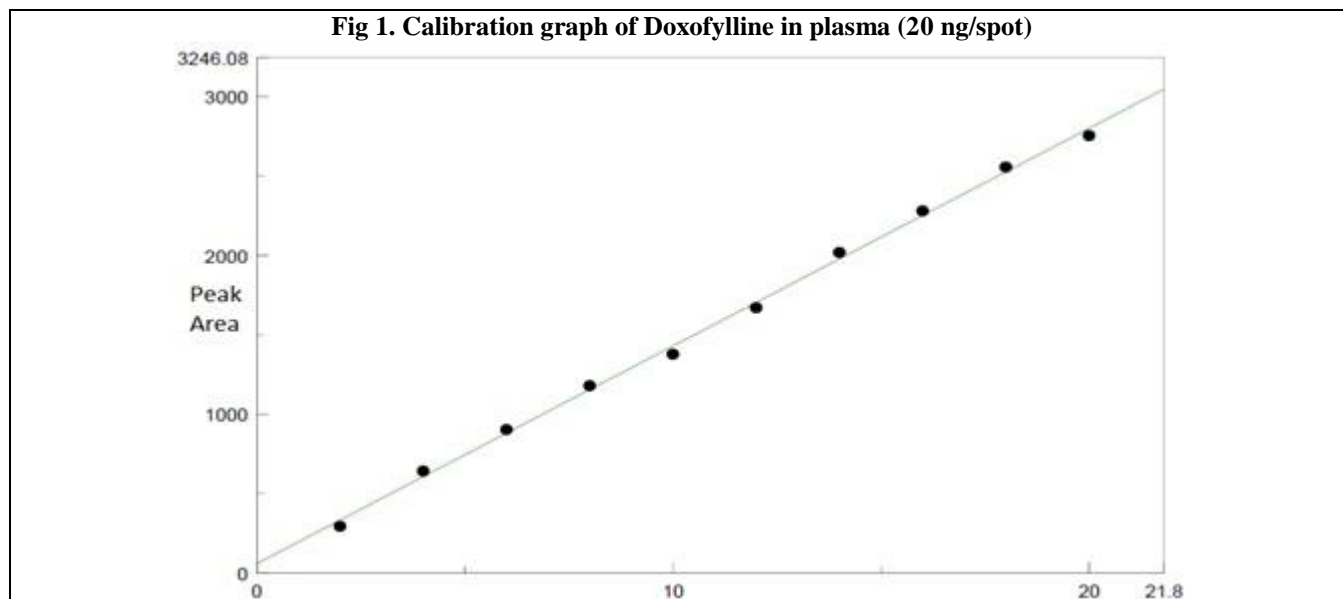
Table 5. Inter day precision

Concentration (ng/spot)	Coefficient of variation (CV)*	Limit	Number of determinations
20 (Low QC)	8.64 %	Within 15 %	5
100 (Middle QC)	6.78 %		
200 (High QC)	5.25 %		

*mean of five observations

Table 6. Recovery study

Concentration (ng/spot)	Mean % Recovery	Number of determinations
20 (Low QC)	89.36	5
100 (Middle QC)	90.35	
200 (High QC)	91.62	

Fig 1. Calibration graph of Doxofylline in plasma (20 ng/spot)**SUMMARY AND CONCLUSION**

In order to extract the Doxofylline from human plasma, Protein precipitation method was selected. After extraction, various concentrations of drug solutions were applied on the plate and development of the plate was done by using mobile phase system consisting of Toluene: n-butanol: Triethylamine: Ammonia (3:5:2:0.1% v/v/v/v) R_f values of Doxofylline was found to be 0.47 ± 0.01 . The developed method was validated according to the US-FDA guidelines. Linear regression data revealed an excellent linear relationship between concentration and peak area ratio over a concentration range of 20-200 ng/spot. The slope, intercept and correlation co-efficient

values were found to be 137.0773, 59.5 and 0.9989, respectively.

The concentration of 20 ng/spot was found as a Lower Limit of Quantification. Good recovery values demonstrated the extraction efficiency. Accuracy and precision of the developed method was found to be within the permitted limit. The stability of Doxofylline in prepared sample was found to be 7 hours under room temperature. Hence the developed method can be used for the analysis of Doxofylline from human plasma.

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Nil

CONFLICT OF INTEREST

Nil

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