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Research Article

DETERMINATION OF DRUG CODE 456/9 BY DIRECT ESTIMATION METHOD IN HUMAN PLASMA AND TABLET DOSAGE FORM BY RP-HPLC

PS Raghu[1], JSK Nagarajan[2], Amruth Kumar N[2] and Sreedevi Vanapalli[2]

[1]David Memorial College of Pharmacy, Yacharan, Tamilnadu, India.
[2]Department of Pharmaceutical Analysis, JSS University, Rock lands, Ooty, Tamilnadu, India.
*Corresponding Author: yuvas2010@gmail.com

ABSTRACT

A simple, precise, rapid and high through put, direct estimation Reverse Phase High Performance Liquid Chromatography (RP-HPLC) method has been developed and validated for quantitation of code 456/9 in human plasma and tablet dosage form. Direct estimation method involves the precipitation of plasma proteins with organic solvent. Clear supernatant was then injected in to the column. The chromatographic separation was performed using methanol: water (80:20, v/v) as isocratic mobile phase at a flow rate of 0.5ml min^{-1} and Inertsil-ODS-3 (C-18) Column (5 µm, 250mm x 4.60mm) as stationary phase. Detection was carried out using a UV PDA detector at 235 nm. The retention time for code 456/9 was 3.8 ± 0.2. The method was validated for a working range of 60-140ng/ml range and 600-1400ng/ml for plasma and tablet dosage form respectively. The average recovery of extraction procedure was found to be 76%. The limit of detection was found to be 7.7 ± 0.2 ng/ml for plasma. The R.S.D for both intra and inter day precision was found to be less than 10% for both plasma and tablet dosage form. The accuracy for tablet dosage form was found to be in the range of 99.0-100.1%.

Keywords: RP-HPLC, code 456/9, direct estimation, plasma, tablet dosage form.

1. INTRODUCTION

Code 456/9 hydrochloride (N, N-dimethylimidodicarbonimidic diamide hydrochloride) is an orally administered biguanide widely used in the treatment of type 2 (non-insulin dependent) diabetes mellitus[1,2]. It improves hepatic and peripheral tissue sensitivity to insulin without the problem of serious lactic acidosis commonly found with analogue, phenformin. Code 456/9 is a hydrophilic drug with an oral bioavailability of 50–60% and a relatively short half-life of 1.5–4.5 hrs[3].

It does not produce hypoglycemia in either patient with type 2 diabetes or normal subjects, indicated as an adjunct to diet and exercise to improve glycemic control in adult patients with type 2 diabetes.

Some HPLC methods have been reported for the measurement of code 456/9 in human plasma[4-9]. Most of these chromatographic methods either involve liquid-liquid extraction, or solid phase extractions associated with HPLC involving two or more steps and thus are time consuming. The objective of the present work was to develop a simple, sensitive, precise and accurate direct estimation RP-HPLC method for the determination of code 456/9 in biological fluids. In this method, plasma after protein precipitation by organic solvent is directly injected in to the system after filtration from membrane filter of 0.22µm. Complete validation of method was performed to access the accuracy, precision, linearity and lower limit of detection and the result presented here demonstrate that this method is feasible for analyzing code 456/9 in plasma The developed method is also applicable for analysis of tablet dosage form.

2. EXPERIMENTAL

2.1. Reagents and chemicals

Code 456/9 Hydrochloride was supplied by CRC Pvt. Ltd. (AP, India). Methanol (HPLC grade) was supplied by Merck Ltd., India. In house triple distilled water was used.

2.2 Instrumentation

A liquid chromatographic system from Shimadzu comprising of manual injector, double reciprocating plunger pump for constant flow and constant pressure delivery and Photodiode array Detector connected to software SPD-M10 ATvp for controlling the instrumentation as well as processing the data generated was used.

Cooling Ultra Centrifuge Remi Instruments Ltd.was used during extraction procedure.

2.3. Chromatographic conditions

The chromatographic separation was performed using an Inertsil-ODS-3 (C-18) Column (5 µm, 250mm x 4.60mm) as the stationary phase. Isocratic mobile phase consisted of water–methanol (20:8 0 v/v), at a flow rate of 0.5ml min^{-1} was delivered. Detection was carried out at 235 nm.

2.4 Plasma estimation

2.4.1 Precipitation procedure

To 1ml of plasma 4 ml of methanol was added, mixed thoroughly and left to stand for 5 min at room temperature. After 5 min the solution was centrifuged at 5000rpm for 15 min at 10°C. The clear supernatant liquid was separated, filtered through 0.22 µ syringe filter and injected directly into HPLC system for analysis.

2.4.2. Standard preparation of plasma samples

1000µg/ml standard stock solution was prepared by weighing the appropriate amount of code 456/9 and dissolving it into methanol. This stock was further diluted to get a stock of 10µg/ml. 1ml from this stock was diluted with 9ml of plasma to yield spiked plasma of 1000ng/ml. Calibration standards were prepared in the range of 60-140ng/ml from 1000ng/ml spiked plasma stock in triplicates.

2.4.3 Linearity for plasma

Linearity was determined to assess the performance of the method. A linear least square regression was performed on peak area of code 456/9 verses its nominal concentration in the range of 60-140ng/ml in triplicate to generate a calibration. Samples were processed as described in (section 2.4) and injected into the HPLC system.

2.4.4 Method recovery and L.O.D

The recovery of the extraction procedure was estimated by comparing the peak heights obtained from an extracted sample with those measured amount of drug in methanol. The recovery was estimated for the concentration range of 60-140 ng/ml.

The limit of detection (L.O.D) was defined as the sample concentration of code 456/9 resulting in peak height of three times the noise level of a blank preparation.

2.4.5 Precision

The precision of the method based on within day repeatability and day to day reproducibility was determined by triplicate analysis of spiked samples with different concentrations (50, 70 and 120ng/ml).

2.4.6 Quality control

Quality control samples were prepared in the concentration of 50, 100 and 120ng/ml in each analytical batch. Using the selected linearity equation assessed revalidation.

2.5 Analysis of tablet dosage form

2.5.1 Standard stock solution

Standard stock solutions of 1000µg/ml and sub stock of 10µg/ml were prepared similarly as for plasma estimation. Calibration standards were prepared in the range of 600-1400ng/ml from sub stock in triplicate.

2.5.2. Validation

The RP-HPLC method has been intensively validated for quantitative estimation of code 456/9 using analytical validation parameters linearity, accuracy, and precision. The standard curve for code 456/9 in methanol was prepared in the range of 600-1400ng/ml. For accuracy to pre-analyzed tablet solution, a definite concentration of drug was added and then its recovery was studied. The studies were performed by varying the amount of drug solution added to the final dilution keeping the concentration of sample solution constant and then calculating the recovery. The precision of the method was determined for repeatability, day-to-day and analyst-to-analyst in the range of 600-1400ng/ml.

2.5.3 Sample preparation
For analysis, 20 tablets were taken, crushed and powder equivalent to 10 mg of code 456/9 was accurately weighed and dissolved in 9ml of solvent. The solution was sonicated for 20 min and filtered to get a solution of 1000 µg /ml. Further diluted samples (3 replicates) in the range of 800 – 1200ng/ml were prepared and analyzed.

3. RESULTS AND DISCUSSION

3.1 System suitability
System suitability parameters (RT, HETP, Tailing factors and No. of Theoretical plates) were analyzed to check the system's performance. The result of system suitability parameters is given in Table 1.

3.2 Plasma estimation

3.2.1 Optimization of precipitation procedure
Various, organic solvents like methanol, ethanol, acetonitrile and salt solution (Sodium sulphite solution of various strengths) were used to bring about precipitation. Since methanol yields better chromatographic separation, brings about complete precipitation of plasma proteins at room temperature without any astringent conditions compared to salt solution so it was selected as the precipitating agent.
The representative chromatograms of code 456/9 in methanol and plasma are given in fig 1.A and 1.B. The retention time of code 456/9 in methanol was found to be 3.6±0.3min, while in spiked plasma the drug and plasma were eluted at 3.5±0.2min, and 5.6±0.2min respectively. The chromatogram clearly shows no interference of code 456/9 with selected solvent system and other components of plasma.

3.2.2 Linearity
The standard curve for code 456/9 in plasma was found to be linear in the range of 60-140ng/ml. The standard curve was calculated by the linear regression method: $y = mx + c$, where y is the peak area of drug, x is the concentration (ng/ml), m is slope and c is the intercept.

$$AUC = 73.64X + 149.45 \ (r^2 = 0.9988)$$

3.2.3 Method recovery and L.O.D.
The average recovery was found to be 76% over the concentration range of 60-140ng/ml. The minimum detectable concentration (LOD) was determined to be 7.73±0.2 ng/ml.

3.2.4 Accuracy and Precision
Accuracy was found to be in the range of 92-97%. The R.S.D. values for both inter and intra day precisions were less than 10% for all the concentration studied. The results are shown in Table 2.

3.2.5 Quality control
For quality control samples the accuracy was with in the range of 95.8-97.3% shown in Table 3.

3.3 Quantitative estimation in tablet dosage form
All the analytical validation parameters were observed and the R.S.D. was found to be less than two, which indicates the validity of method. The results are given in Table 4.

3.3.1 Linearity
The standard curve for code 456/9 in methanol was found to be linear in the range of 600-1400ng/ml. The standard curve was calculated by the linear regression method.

$$AUC = 75.6C - 2349.1 \quad r^2 = 0.9983$$

3.3.2 Accuracy
Results of accuracy were found with in the acceptable range shown in Table 5.

3.3.3 Precision
The precision of the method was determined for repeatability, day to day and analyst to analyst in the range of 600-1400ng/ml.

3.4 Tablet analysis
The content of code 456/9 found in the tablet by the proposed method is shown in Table 6. The low values of R.S.D. indicate that the method is precise and accurate.

4 CONCLUSION

Direct estimation RP-HPLC method for determination of code 456/9 in plasma meets the criteria for its application to routine clinical drug level analysis. The advantages of this method over that previously reported are basically its simplicity, sensitivity and specificity. This method offers a rapid sample preparation time and achieves excellent

sensitivity without resorting to extraction and evaporation techniques for plasma. The validation results have demonstrated satisfactory precision and accuracy of the method across the calibration range. The method has been successfully employed for estimation of code 456/9 in tablet dosage form.

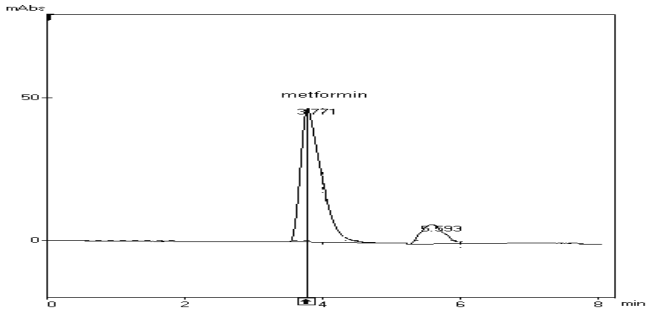

Fig.1: Representative Chromatogram of code 456/9 in methanol

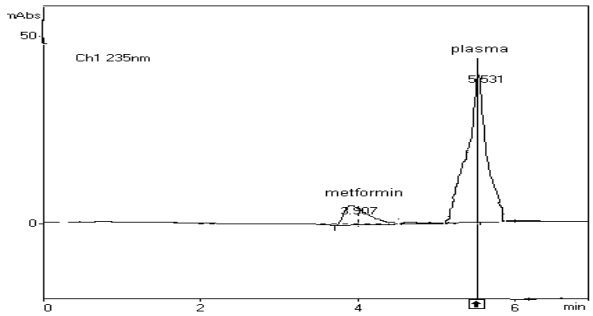

Fig. 2: Representative Chromatogram of code 456/9 in plasma

Table 1: Results of system suitability parameters

System suitability parameters	Retention time	AUC	No. of theoretical plate	Tailing factor	HETP
Mean*	3.71	73545	1037	1.28	0.241
S.D	0.065	99.23	9.33	0.020	0.002
R.S.D	0.017	0.001	0.009	0.016	0.009

*Mean of six readings

Table 2: Precision, accuracy of the assay (n = 3) in plasma

Nominal conc. (ng/ml)	Conc. found * (ng/ml)± S.D	R.S.D (%)	Accuracy (%)
Intra assay			
50	46.1±0.8	6.0	92.00
70	66.5±1.1	1.8	95.00
120	118.1±0.5	4.0	98.30
Inter assay			
50	47.3±0.4	9.7	96.61
70	66.1±0.3	4.0	94.20
120	117.1±0.8	7.0	97.50

* Mean of three readings

Table 3: Results of quality control samples in plasma

Nominal concentration (ng/ml)	Concentration found* (ng/ml) ± S.D	R.S.D (%)	Accuracy (%)
50	47.9±0.7	1.5	95.80
100	96.2±0.7	7.0	96.25
120	116.8±0.6	6.8	97.30

* Mean of 3 readings

Table 4: Validation Parameters for tablet

Validation Parameters	% Mean*	S.D	R.S.D
Linearity	98.39	0.55	0.005
Accuracy	100.04	0.138	0.001
Precision			
Repeatability	99.89	0.032	0.0003
Day to day	100.00	0.022	0.0003
Analyst to analyst	99.99	0.216	0.0002

* Mean of 3 concentrations

Table 5: Results of accuracy for tablet

Nominal conc. (ng/ml)	Amount of drug added (ng/ml)	Concentration found ng/ml ± S.D.	% R.S.D.	% Accuracy
600	500	498±0.5	2.0	99.60
800	500	499.5±0.4	8.0	99.80
1000	500	500.2±0.1	3.0	100.04
1200	500	500.7±0.7	1.5	100.10

* Mean of 9 determinations at 5 concentration level

Table 6: Result of tablet analysis

Parameters	Code 456/9
% Mean*	99.57
S.D	0.699
R.S.D	0.007

* Mean of 9 determinations at 3 concentration level.

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