

# Determination of 4-Chloro-2-chloromethyl-5-(4-chlorophenyl)thieno[2,3-*d*]-pyrimidine in Rabbit Serum by HPLC and its Application in a Pharmacokinetic Study

Vanarote D.S<sup>1</sup>, Londhe S.V<sup>1</sup>, Arya. N<sup>2</sup>, Mulgund S.V<sup>1</sup> and Jain K.S\*<sup>2</sup>

<sup>1</sup>Department of Pharmaceutical Chemistry, Sinhgad College of Pharmacy, Vadgaon, Pune-411041, India.

<sup>2</sup>Department of Pharmaceutical Chemistry, Sinhgad Institute of Pharmaceutical Sciences(SIPS), S.No. 309/310, Kusgaon (Bk), Lonavala, Off Mumbai-Pune Expressway, Lonavala - 410401, Maharashtra, India

## ABSTRACT

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In drug development, the analytical method development including the bioanalytical method development is essential and useful for the pharmacokinetic study of the lead molecule and is an important step. A sensitive method for the quantification of potential anti lipid lowering 4-chloro-2-chloromethyl-5-(4-chlorophenyl)thieno[2,3-*d*]pyrimidine 5 (SCOP/2007/1) in rabbit serum using high-performance liquid chromatography (HPLC) was developed. Sample pretreatment involved a simple protein precipitation by the addition of 1.5 ml of acetonitrile to 0.5 ml serum sample volume. Separation was achieved on Agilent-C18 (5 $\mu$ m, 150 mm $\times$ 4.6mm) reversed-phase column at 40°C with acetonitrile: water (80:20, v/v) at a flow rate of 0.6 ml/min. The calibration curve of **5** in serum showed good linearity over the concentration range of 25–6400 ng/ml. The limit of detection and limit of quantification were 7.81 ng/ml and 23.69 ng/ml, respectively. Intra and inter-day precisions in all samples were within 15%. The validated method was successfully applied to a preclinical pharmacokinetic study of **5** in rabbits. After oral administration of 100 mg/kg **5** to rabbits, the main pharmacokinetic parameters  $T_{max}$ ,  $C_{max}$ ,  $T_{1/2}$ ,  $K_{el}$ ,  $K_a$  and  $AUC_{0-1}$  were 2 h, 1297.28 ng/ml<sup>1</sup>, 0.495 h, 1.4 h<sup>-1</sup>, 3.99 h<sup>-1</sup> and 2930.5 ng h<sup>-1</sup> L<sup>-1</sup>, respectively.

**Keywords:** Antihyperlipidemic; SCOP/2007/1; Bioanalytical; Pharmacokinetics; Rabbit Serum; Oral administration

## INTRODUCTION

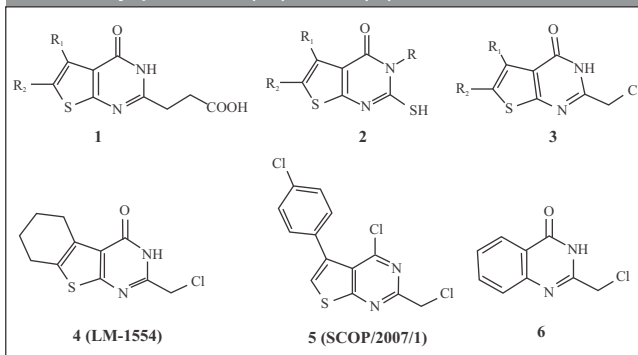
Hyperlipidemia and thereby atherosclerosis are the leading causes of cardiac illness and stroke.<sup>1,2</sup> It has been demonstrated that there exists a link between serum cholesterol levels and risk to coronary heart disease (CHD).<sup>3</sup> Despite significant medical advances, heart attacks due to coronary heart disease (due to atherosclerosis that affects the arteries supplying blood to the heart) and stroke (due to atherosclerosis that affects the arteries supplying blood to the brain) are responsible for more deaths than all other causes combined. A 1% drop in serum cholesterol reduces the risk for CHD by 2%.<sup>4</sup> In addition to this, different cholesterol lowering drugs or non pharmacological treatments can significantly reduce morbidity from CHD and stroke.

Antihyperlipidemic activity has been reported in some thieno[2,3-*d*]pyrimidine derivatives.<sup>1-3,5-7</sup> One of the compounds 2-chloromethyl-5,6,7,8-tetrahydrobenzo(*b*)thieno[2,3-*d*]pyrimidin-4(3*H*)-one, **4**, (LM 1554, CAS# 89567-03-38) was found to be potentially active.<sup>8</sup> Compound

**4** (Fig 1) was found to be safe during its acute and chronic toxicity studies performed on mice and rats<sup>7</sup> and was also subjected to pharmacokinetic and pharmacodynamic studies.<sup>9</sup>

In past few years we have synthesized and evaluated many analogs of **4** for antihyperlipidemic activity on Spargue Dawley rats.<sup>10,11</sup> Among these 4-chloro-2-(chloromethyl)-5-(4-chlorophenyl)thieno[2,3-*d*]pyrimidine, **5** [SCOP/2007/1] was found to be much more active than LM-1554, **4**.<sup>10</sup>

**Fig.1:** Chemical structures of thieno[2,3-*d*]pyrimidine 2-propionic acids, **1**, 2-mercapto-thieno[2,3-*d*]pyrimidin-4-ones, **2**, 2-substituted thieno[2,3-*d*]pyrimidin-4(3*H*)-ones, **3**, 2-chloromethyl-5,6,7,8-tetrahydrobenzo(*b*)thieno[2,3-*d*]pyrimidin-4(3*H*)-one, **4**, 4-chloro-2-(chloromethyl)-5-(4-chlorophenyl)thieno[2,3-*d*]pyrimidine, **5** [SCOP/2007/1] and 2-chloromethylquinazolin-4(3*H*)-one, **6**, (IS).



### \*Address for Correspondence:

Dr. K. S. Jain, Professor (P.G. Research), Dept. of Pharmaceutical Chemistry, Sinhgad Institute of Pharmaceutical Sciences(SIPS), S.No. 309/310, Kusgaon (Bk), Lonavala, Off Mumbai-Pune Expressway, Lonavala-410401, Maharashtra, India

E-mail: drkishorsjain@gmail.com

In drug development, the analytical method development including the bio analytical method development is essential and useful for the pharmacokinetic study of the lead molecule and is an important step. Hence, the present study was aimed at developing a sensitive HPLC method for determination of **5** in rabbit serum and determination of its pharmacokinetic parameters.

## MATERIALS AND METHODS

### MATERIALS

SCOP/2007/1, **5**, and internal standard (**IS**); 2-methylquinazolin-4(3H)-one, **6**, were synthesized in our laboratory. Acetonitrile was purchased from Loba Chemie (India). HPLC-grade water obtained from water purifier system of Elga, UK, (UHQ II). All other chemicals were of analytical grade. Unless otherwise specified, all solutions were filtered through a 0.2µm Ultipor® N<sub>66</sub>® Nylon 6 (Pall Life Sciences, Mumbai) prior to use. Blank Rabbit Serum (drug free) was prepared in our laboratory, the protocol for which was approved by animal ethical committee (SCOP/IAEC/2010-11/01). The samples were vortexed using centrifuge (Model R8C, Remi, Mumbai, India). A high-performance liquid chromatograph (Shimadzu, Kyoto, Japan) was composed of a LC 2010 CHT module, an autosampler injector and UV-visible detector. Separation was performed on an Agilent C18 column (5µm; 150mm×4.6mm; USA), at 40°C temperature. Chromatographic data were recorded and processed using LC-Lab solution software.

### METHODS

#### Chromatographic conditions

Analysis was isocratic at 0.6 ml/min flow rate with acetonitrile: water (80:20, v/v) as mobile phase. The mobile phase was prepared freshly every day. The solvents were filtered through a 0.2µm nylon filter to remove any particulate matter and degassed by sonication before use. Both, **5** and **IS** had considerable absorbance at 243 nm. Hence, the eluted peaks were detected at 243 nm. The substance was quantified using peak area ratio of **5** to **IS**. Prior to injecting solutions, the column was equilibrated for at least 30 min with the mobile phase flowing through the system.

#### Preparation of standard and quality control samples

Stock solutions of **5** (100 µg/ml) and **IS** (100 µg/ml) were prepared in acetonitrile and stored at 4 °C. The stock solution of **5** was diluted quantitatively with mobile phase to give spiking solutions at concentrations of 0.125, 0.25, 0.5, 1, 2, 4, 8, 16 and 32 µg/ml. Suitable aliquots of the spiking solutions were spiked to blank rabbit serum in order to obtain the concentrations of **5** in the analytical range of 25 to 6400 (25, 50, 100, 200, 400, 800, 1600, 3200 and 6400) ng/ml and that

of **IS** to be 2000 ng/ml for calibration curve. Quality control (QC) samples were prepared at low (35 ng/ml), medium (2000 ng/ml) and high (5500 ng/ml) concentrations in the same way as the serum samples for calibration.

#### Sample preparation

In order to investigate the practical applicability of the method in biological analysis, the present method was applied to the estimation of **5** from rabbit serum. Blank serum of a New Zealand white rabbit was spiked with known concentrations of **5** and **IS**. In one glass test tube, spiked serum along with 1.5ml of acetonitrile was taken for protein precipitation and complete extraction of drug and **IS**. The mixture was vortexed for 1 min and centrifuged (Model R8C, Remi, Mumbai, India) at 2500 rpm for 15 min. The supernatant transparent liquid was transferred into a glass test tube and evaporated to dryness at room temperature under a stream of nitrogen. The residue was reconstituted with 1 ml of mobile phase. Then, the samples were filtered through a 0.22 µm nylon filter using syringe filter holder. An aliquot of 20 µl was injected into the HPLC system for analysis.

#### Validation of the developed method

The validation process was carried out according to the "Guidance for Industry-Bioanalytical Method Validation", recommended by the US Food and Drug Administration<sup>12,13</sup> for linearity, precision, accuracy, sensitivity, and recovery. Consequently, the following parameters were evaluated.

#### Calibration curve (linearity)

Nine different concentrations of **5** with constant **IS** concentration were spiked to the blank serum as described previously and calibration curve was constructed in the specified concentration range. The calibration plot (peak area ratio of **5** to **IS** versus **5** concentration) was generated by replicate analysis ( $n = 3$ ) at all concentration levels and the linear relationship was evaluated using the least square method within Microsoft Excel® program.

#### Accuracy and precision

Both repeatability (within a day precision) and reproducibility (between days precision) were determined. Solutions containing lowest, intermediate, and highest concentrations of the calibration curve, *i.e.* 35, 2000 and 5500 ng/ml were prepared. Six injections at each of the specified concentration levels were injected within the same day for repeatability, and over a period of 3 days (6 injections/day) for reproducibility. Mean and coefficient of variance (%CV) were calculated and used to judge precision of the method. Both intra-day and inter-day samples were calibrated with standard curves concurrently prepared on the day of analysis. Accuracy was calculated as the percent of ratio of **5** amount found to that of the actual.

### Sensitivity

The accuracy and precision at the lower limit of quantitation (LLOQ) were determined by analyzing at least 5 replicates of the sample at the LLOQ concentration on one of the validation days. These samples were independent of those used for construction of the calibration curve. The accuracy as determined by the percent nominal (%Nominal) at this concentration should be within  $\pm 20\%$  and the %CV should be less than 20%.<sup>12,13</sup>

### Specificity

The specificity<sup>12,13</sup> of the method was determined by comparing the chromatograms obtained from the samples containing **5** at lower limit of quantitation (LLOQ) and IS with those obtained from blank serum. Six blank serum samples from different lots of rabbit serum were processed with and without the internal standard to evaluate presence of interfering peaks.

### Stability

Blank serum was spiked with the known amount of **5** to achieve the concentration of 35, 2000 and 5500 ng/ml ( $n = 3$ ) and stability of these samples was checked for bench top stability in replicate (eg, triplicate) QC samples in matrix were analyzed after keeping them at ambient temperature for 4 to 24 hours to cover at least the duration of time it takes to extract the samples. The observed sample concentrations were compared with their nominal values. The stability of these samples was also checked for up to 1 month by comparing the results with freshly prepared on the day of analysis. Further, the freeze-thaw ( $-20^{\circ}\text{C}$ /room temperature) stability of the **5** spiked serum samples was determined for three cycles. Samples were considered to be stable, if the assay values were within the acceptable limits of accuracy and precision. Internal standard was added just prior to the analysis.

### Extraction efficiency

Different organic solvents (dichloromethane, methanol and acetonitrile) were tried for the extraction experiments, in which, acetonitrile proved to be the most efficient in extracting **5** from rabbit serum and had very slight variation in extraction recoveries over a range of concentrations. Spiked serum samples were prepared in triplicate at three concentrations 35, 2000 and 5500 ng/ml of **5** and 2000 ng/ml of **IS**, and assayed as described above. The extraction efficiency<sup>12</sup> of **5** was determined by comparing the peak areas measured after analysis of spiked serum samples with those found after direct injection of non-biological (un extracted) samples into the chromatographic system at the same concentration levels.

### Application to pharmacokinetics study

The method described above was applied to quantify the serum concentration of **5** in a single-dose (oral) pharmacokinetic study conducted on three New Zealand White rabbits. The protocol was approved by the Institutional ethical committee at the Sinhgad College of Pharmacy, Pune, India, constituted under CPCSEA (Committee for Purpose of Control and Supervision of Experimental Animals) guidelines. The rabbits weighing 2.5–3.5 kg were housed with free access to food and water, except for the final 12 h before experimentation. After a single oral administration of 100 mg/kg of **5**, 1.5 ml of blood samples were collected from the marginal ear vein at 0, 0.5, 1, 2, 3, 4, 5, 6 and 8 h time-points into eppendorf collection tubes. The blood was allowed to stand for 1 h then centrifuged at 5000 rpm for 15 min at an ambient temperature. The supernatant serum layer was separated and stored at  $-20^{\circ}\text{C}$  until analyzed.<sup>14</sup> The serum samples were analyzed for **5** concentrations as described above. The total area under the observed serum concentration–time curve (AUC) was calculated by using the linear trapezoidal rule.<sup>14,15</sup> The first order elimination rate constant ( $k_{el}$ ) was estimated by the least square regression of the points describing the terminal log-linear decaying phase.  $T_{1/2}$  was derived from  $k_{el}$  ( $T_{1/2} = \ln 2/k_{el}$ )<sup>14, 15</sup>. The absorption rate constant ( $k_a$ ) was determined by residual method. The maximum observed **5** concentration ( $C_{max}$ ) and the time at which  $C_{max}$  was observed ( $T_{max}$ ) were reported directly from the profile.

## RESULTS AND DISCUSSION

### Optimization of chromatographic conditions

Chromatographic separations are significantly affected by the mobile phase conditions, such as the type and composition of the organic modifiers<sup>16</sup>. Therefore, before selecting the conditions for the optimization, a number of preliminary trials were conducted with different combinations of different solvents, their compositions and different flow rates to check the chromatographic parameters suitable for **5** and **IS** peaks, individually. In order to achieve an optimum separation, following conditions were studied: (i) Mobile phase type varied from methanol: water to acetonitrile: water keeping the composition 70:30 and flow rate of 1.2 ml/min (ii) mobile phase composition varied at 60:40, 70:30 and 80:20 with acetonitrile: water and flow rate kept constant at 1.2 ml min<sup>-1</sup> (iii) flow rate was varied (1.2, 1, 0.8 and 0.6 ml min<sup>-1</sup>) with mobile phase composition fixed as acetonitrile: water 80:20. With the aim of the optimization of mobile phase one factor was changed at time, the remaining two factors were kept constant. Change in mobile phase type from methanol to acetonitrile, keeping composition (60:40, v/v) and flow rate 1.2ml/min, gives better peak symmetry. Change in mobile

phase composition (acetonitrile: water, 60:40, 70:30, 80:20, v/v) showed minimum retention times of **5** and **IS** were obtained at 80:20 v/v level, which makes the method rapid, a one of the most desirable criteria. Though retention time was shorter, and asymmetry values were achieved resolution was not good. Therefore, flow rate was changed to 0.6 ml/min to achieve better peak separation and resolution. HPLC chromatogram of rabbit serum is shown in Fig. 2, which shows (A) Blank serum (B) Serum spiked with internal standard (C) Serum spiked with **5** at LLOQ and internal standard (D) A serum sample at 2 hours after oral administration of **5** [SCOP/2007/1].

**Validation of the proposed method**

**Calibration curve (linearity)**

Calibration curve (peak area ratio of **5** to **IS** versus **5** concentration) in serum was constructed by spiking nine different concentrations of **5** and fixed concentration of **IS**. The chromatographic responses were found to be linear over an analytical range of 25–6400 ng/ml and found to be quite satisfactory and reproducible with time.

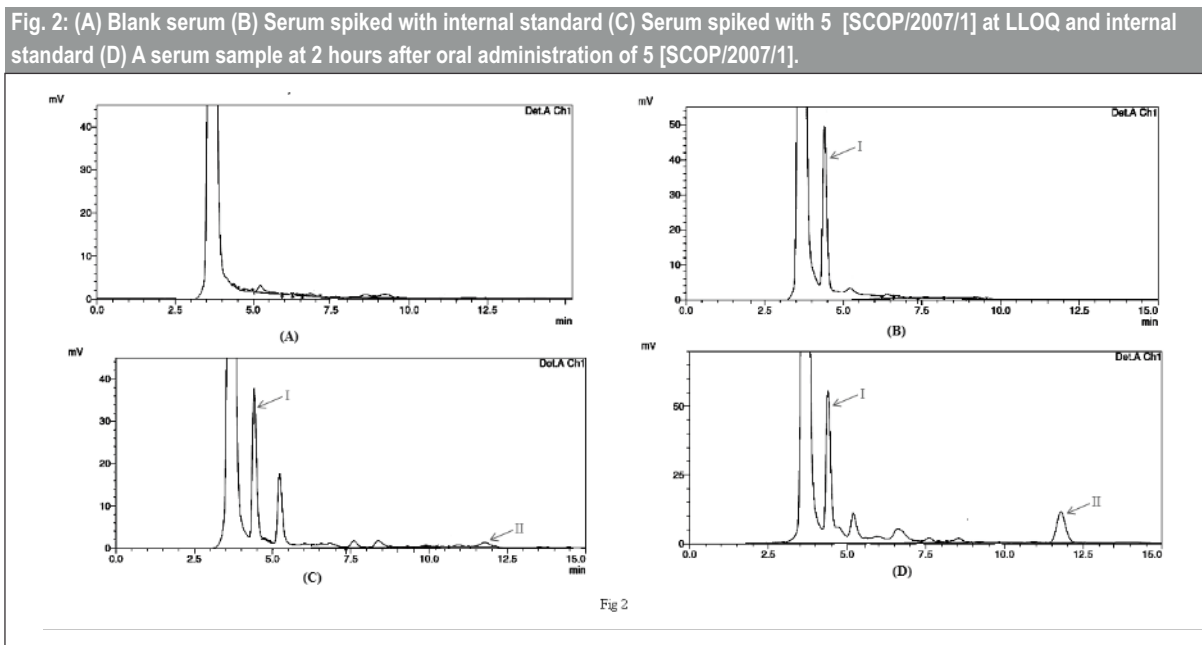
The LLOQ of **5** was 25 ng/ml and the LOD (S/N > 3) was 7.81 ng/ml, respectively. The linear regression equation was calculated by the least squares method using Microsoft Excel® program. Representative regression equation of the calibration curve was  $y = 0.222x + 0.033$  ( $r^2 = 0.997$ ), where, y is the peak area ratio of **5** to **IS** and x is the plasma concentration of **5**.

**Accuracy and precision**

The accuracy and precision of the method were evaluated with QC samples at three concentrations using six replicates. The %CV for intra- and inter-day precision were not more than 10.01% and 3.038 %, respectively. The %Nominal for intra- and inter-day accuracy was less than 96.45% and 96.88%, respectively. The precision and accuracy study indicated that the developed HPLC method was reproducible and accurate (Table 1).

**Sensitivity**

Sensitivity was measured in terms of LLOQ. The lowest limit of reliable quantification for **5** was set at the concentration of



| Table 1: Precision and accuracy for the determination of <b>5</b> in rabbits serum by HPLC method. |  |                 |                     |  |                 |                     |
|--|--|-----------------|---------------------|--|-----------------|---------------------|
| Sample concentration (ng/ml)   | Intra day                              |                 |                     | Inter day                              |                 |                     |
|  | Average measured concentration (ng/ml) | Precision (%CV) | Accuracy (%Nominal) | Average measured concentration (ng/ml) | Precision (%CV) | Accuracy (%Nominal) |
| 35   | 33.82                                  | 10.01           | 96.63               | 34.21                                  | 3.03            | 97.75               |
| 2000   | 1968.03                                | 1.63            | 98.40               | 1940.01                                | 4.29            | 97.00               |
| 5500   | 5305.07                                | 1.33            | 96.45               | 5328.92                                | 2.90            | 96.89               |

the LLOQ, 25 ng/ml. The precision (% CV) and accuracy (% nominal) for **5** at this concentration was found to be 3.84% and 91.24%, respectively.

**Specificity**

Selectivity is expressed in terms of % interference. The results were within the acceptance criteria and the response of interference peak at retention time of **5** was found to be varying between 12.33% to 14.80 % and the response of interference peak at retention time of **IS** was found to be varying between 0.242% to 0.298%.

**Stability**

The stability of stock solution of **5** and the spiked serum samples was evaluated for short term stability i.e. for 6 Hrs at Room Temperature. Bench Top Stability in rabbit serum for 6 Hrs, Freeze-Thaw Stability and Long term Stability at -20°C (30 days) of spiked serum samples were evaluated. Both the stock solution of **5** and the spiked serum samples were found to be stable during above mentioned stability study (Table 2).

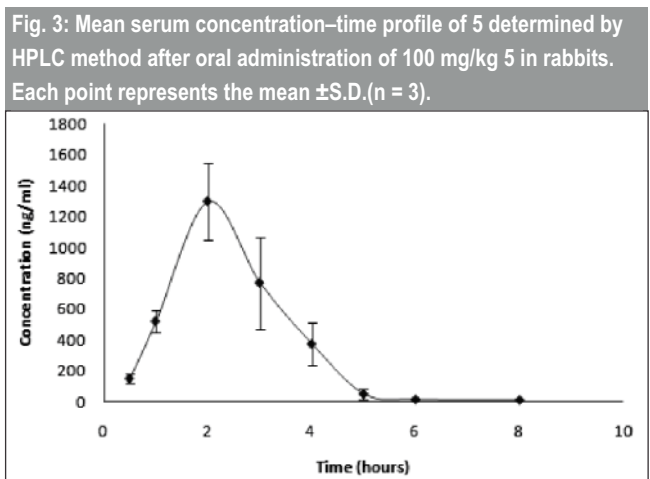
**Extraction efficiency**

Extraction efficiency was performed to verify the effectiveness of the extraction step and the accuracy of the proposed method. The extraction efficiency of **5** from rabbit serum samples was found to be 91.84% with precision 1.53. The mean overall recovery of internal standard was found to be 91.55%.

**Pharmacokinetics from rabbits**

The validated method was applied to a pharmacokinetic study in rabbits. Representative mean serum concentrations versus time profiles following a single oral administration of **5** (100

mg kg<sup>-1</sup>) to three rabbits are presented in Fig. 3. The C<sub>max</sub> for **5**, 1297.28 ng/ml was reached after 2 hrs (T<sub>max</sub>), after oral administration. The rate of absorption was found good (3.99 hr<sup>-1</sup>). The rate of elimination was higher (1.4hr<sup>-1</sup>). The half-life was found to be short (0.495 hr). Area under serum concentration (AUC) was found to be 2930.5 ng/hr/ml. various other pharmacokinetic parameters have been summarized in Table 3.



**Table 3: The pharmacokinetic parameters of 5 in rabbits serum following an oral administration of 100 mg/kg 5.**

| Parameters                                   | Mean    |
|--|---------|
| Absorption rate constant (h <sup>-1</sup> )  | 3.99    |
| Elimination rate constant (h <sup>-1</sup> ) | 1.4     |
| Area Under Curve (ng/h/ml)                   | 2930.50 |
| Half-life t <sub>1/2</sub> (h)               | 0.495   |
| C <sub>max</sub> (ng/ml)                     | 1297.28 |
| T <sub>max</sub> (h)                         | 2.0     |

**Table 2: Stability of 5 in rabbits serum different conditions determined by HPLC method (n = 3).**

| Sample concentration (ng/ml)                  | Average measured concentration (ng/ml) | Accuracy RE (%Nominal) | Precision R.S.D (%CV) |
|---|--|------------------------|-----------------------|
| <b>Short-term stability (about 25°C, 6 h)</b> |  |                        |                       |
| 35  | 33.43                                  | 95.51                  | 4.65                  |
| 2000  | 1867.10                                | 93.35                  | 1.31                  |
| 5500  | 5084.44                                | 92.44                  | 1.85                  |
| <b>Long-term stability (-20°C, 1 month)</b>   |  |                        |                       |
| 35  | 31.635                                 | 3.35                   | 90.38                 |
| 2000  | 1837.900                               | 2.97                   | 91.89                 |
| 5500  | 5017.16                                | 2.06                   | 91.22                 |
| <b>Freeze-thaw stability.</b>                 |  |                        |                       |
| 35  | 31.73                                  | 4.32                   | 90.66                 |
| 2000  | 1852.32                                | 1.40                   | 92.61                 |
| 5500  | 5034.86                                | 1.35                   | 91.54                 |

**CONCLUSION**

A simple, sensitive and reliable method for the determination of **5** over the concentration range of 25–6400 ng/ml in rabbit serum by HPLC was developed and validated. The method consisted of sample preparation by protein precipitation and extraction into mobile phase, followed by chromatographic separation and UV detection. No interfering peaks were observed at the elution times of **5** and **IS**. The method was accurate, reproducible, specific and applicable to the evaluation of pharmacokinetic profiles of **5** in rabbits. The developed HPLC method was found to be suitable for the analysis of **5** in rabbit serum and used for the estimation of pharmacokinetics parameters after administration of **5** by oral route, where it showed good rate of absorption, shorter half-life and higher rate of elimination.

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