# Reverse Phase High Performance Liquid Chromatographic-Diode Array Detection Method for Quantification of Rhein in Microsample of Rabbit Plasma and Application to the Pharmacokinetic Study

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**Summary:** The present work describes a reversed phase high-performance liquid chromatography (RP-HPLC) assay with diode array detection (DAD) for the determination of rhein, an active metabolite of diacerein, in spiked rabbit plasma. The plasma samples were subjected to one-step liquid-liquid extraction using a protein precipitating agent. The separation of rhein was achieved on an RP C18 hypersil-ODS column (250×4.6mm, 5µm). The mobile phase consisted of a binary mixture of methanol and water (15: 85 v/v, pH 4.7) run under the isocratic system at a flow rate of 1 $ml.min^{-1}$, the wavelength of DAD was set at 210 nm. The method was validated for linearity, accuracy, precision, and stability according to the International Council for Harmonization (ICH guidelines). The calibration curve was linear over the range of 0.015-5 µg. $ml^{-1}$ for rhein with the coefficient of regression ($r^2$) of value 0.9980. Extraction recovery for rhein from rabbit plasma was in the range of 95.0% to 98.60% to different sample concentrations. The retention time of rhein was observed at 5.34 min. Limit of detection (LOD) and limit of quantification (LOQ) were found to be 5 $ng.ml^{-1}$ and 15 $ng.ml^{-1}$ respectively. The assay method was successfully applied to the pharmacokinetic study of diacerein plain aqueous dispersion in albino rabbits.

**Keywords:** RP-HPLC, Rhein, Validation, Method development, Plasma.

## Introduction

Diacerein and its active metabolite rhein is the disease modifying agent and are effective in the symptomatic treatment of osteoarthritis with an added benefit of joint regeneration. The recommended dose of diacerein is 50 mg twice daily [1]. After an oral administration, diacerein is converted to its immediate metabolite rhein. Consequently, pharmacokinetic studies of diacerein can only be explained through plasma concentrations of rhein [2]. Therefore, the determination of rhein in biological samples is critical for pharmacokinetics, bioavailability/bioequivalence, toxicological studies, and therapeutic drug monitoring of diacerein to get better patient care and clinical evaluation.

Several liquid chromatographic methods for estimation of rhein in various pharmaceutical formulations have been discussed in the literature but limited data is available for the measurement of rhein in biological fluids with HPLC techniques. The available methods include liquid chromatography–negative electrospray ionization tandem mass/mass spectrometry [3], HPLC method using LiChrosorb RP-8, equipped with a PVDF guard column [4], HPLC with multi fluorescence detector [5], LC-MS/MS method with electrospray ionization [6], **a** method for simultaneous estimation of rhein, baicalin, and berberine with addition of column switching step [7] and an HPLC method with UV detection under gradient flow for simultaneous determination of rhein and aceclofenac [8]. These methods require acute care for maintaining optimized chromatographic conditions due to the complex intermediate steps and the challenging affordability in circumstances where large numbers of samples are to be assayed. The three criteria must be fulfilled for an HPLC assay to be functional in clinical and preclinical laboratories: greater degree of sensitivity, simple, rapid and reasonable elution time reflecting the affordability of method. Therefore, the present study has been planned and executed to develop and validate a simple, convenient and efficient HPLC method for determination of rhein in the rabbit plasma with greater sensitivity and affordability. The developed validated HPLC method was also used to determine the pharmacokinetic parameters for rhein in preclinical species (Albino rabbits). The developed method can also be applied in therapeutic drug monitoring, bioequivalence and pharmacokinetic

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evaluation of newly developed drug delivery system containing diacerein.

## Experimental

### *Materials*

Rhein was purchased from Rongsheng Biotechnology Co. Ltd, China. Analytical grade, phosphoric acid, triethylamine, trichloroacetic acid and HPLC grade methanol, and acetonitrile were purchased from Merck (Darmstadt, Germany). Fresh rabbit plasma was obtained by withdrawing a certain amount of blood from rabbits rested in Animal house facility of Faculty of Pharmacy, The Islamia University of Bahawalpur. Double distilled water was prepared in house facility (Faculty of Pharmacy, The Islamia University of Bahawalpur, Pakistan).

### *Instrumentation*

High Performance Liquid Chromatographic system (Agilent 1100 Series U.S.A), UV/Visible Spectrophotometer (IRMECO U2020 Germany), Sonicator (Elmasonic E 30 H sonicator Germany), pH meter (WTW pH 300 Germany), Centrifuge machine (SIGMA 1-14 laborzentrifugen centrifuge Germany), Analytical balance (Shimadzu AUX 220 Japan), Membrane filters 0.22µm (Sartorius Germany), Filtration assembly (Pyrex France), Vortex mixer (Seouline Bioscience Korea).

### *Optimization of the chromatographic conditions*

Experimentations were performed using high performance liquid chromatography (HPLC) with an isocratic pump and variable wavelength detector (Agilent Technologies, series 1100, USA). A reversed phase mode was adopted using ODS hypersil C18 column of dimensions 250 × 4.6 mm and 5µm particle size of internal packing Germany). Isocratic elution was achieved with an optimized composition of the mobile phase. After several trials, an optimized mobile phase of composition methanol-water (15:85 v/v, pH 4.7) was found more suitable for separation of rhein and other constitutive plasma peaks. The pH of the mobile phase was adjusted at 4.7 using orthophosphoric acid. Prior to use, the mobile phase was filtered through a membrane filter (0.22µ) and degassed by sonication for 10 minutes. The flow rate was 1 $ml.min^{-1}$ with a back pressure of 120 atmospheres and the detector was set at an optimized wavelength of 210 nm. The concentrations of rhein were determined after calculation of peak area. All the experimental work was performed at ambient temperature (25°C ± 2°C).

### *Preparation of stock solution*

1 $mg.ml^{-1}$ stock solution of rhein was prepared in dimethyl sulfoxide (DMSO). The working solutions of 5 $\mu g.ml^{-1}$, 2.5 $\mu g.ml^{-1}$, 1.25 $\mu g. ml^{-1}$, 1 $\mu g.ml^{-1}$, 0.75 $\mu g.ml^{-1}$, 0.50 $\mu g.ml^{-1}$, 0.25 $\mu g.ml^{-1}$, 0.125 $\mu g. ml^{-1}$, 0.062 $\mu g.ml^{-1}$, 0.031 $\mu g.ml^{-1}$ and 0.015 $\mu g.ml^{-1}$ were prepared in mobile phase.

### *Sample preparation*

HPLC method was developed followed by quantification of rhein in micro-sample rabbit plasma. Plasma was separated by centrifugation at 5000 rpm for 10 min and supernatant plasma samples were stored in the ultralow freezer (Sanyo, Japan) at -20°C until assayed. The blank plasma was also preserved in the ultralow freezer (-20°C) and was employed for the development and subsequent validation of the method. In the second step, a series of working solutions of rhein were prepared in the mobile phase and 500 µL of each dilution was spiked with 500 µL of blank plasma. The precipitating agent i.e. acetonitrile in a concentration of 500 µL was added to the mixture of rhein spiked plasma sample and vortexed for 3 minutes. The supernatant portion of the samples was separated by centrifugation at 14000 rpm for 10 minutes. The supernatant was transferred to clean lock-capped 1.5 ml micro-centrifuge tube after filtration from 0.45 µm samples using microfiltration assembly. This supernatant (20 µL) was injected into the HPLC system.

### *Method validation*

### *Linearity*

A calibration curve was constructed in concentrations ranging from 0.015 µg to 5 µg. The linearity of this method was established by generating a calibration curve of rhein in rabbit plasma. All concentration ranges as aforesaid were analyzed in triplicate (n=3). The peak area was plotted against drug concentration.

### *Inter-day and intra-day precision and accuracy*

The intra-day precision and accuracy were determined by analyzing six replicates of plasma samples on the same day at different time points. The inter-day precision and accuracy were assessed by analyzing each sample on different days. Precision and accuracy were established by calculating the relative standard deviation (R.S.D). The R.S.D for precision must be equal to 15% or less as it is according to ICH guidelines 2005.

*Recovery*

The efficiency of the extraction method based on protein precipitation using acetonitrile was determined. The relative recovery was calculated by comparison of peak area of rhein in the extracted plasma samples to peak area obtained with an equal concentration in the mobile phase (un-extracted samples). Each measurement was performed in triplicate to determine recovery. Following mathematical formula was used for calculating recovery;

$$\textbf{Recovery} = \frac{\textbf{Pe}}{\textbf{Pu}} \times \textbf{100} \qquad \textbf{Equation 6.1}$$ [9]

where Pe and Pu are the peak areas of the extracted and un-extracted standards, respectively.

*Limit of detection (LOD) and Limit of quantification (LOQ)*

Limit of detection (LOD) is the lowest concentration of analyte in the sample that is detectable from background noise but cannot be quantified. The limit of detection (LOD) was assessed as the amount of rhein which caused a signal that was three times the noise. The LOD was calculated using the equation given below;

$$\textbf{LOD} = \frac{\textbf{3N}}{\textbf{S}} \times \textbf{amount found} \qquad \textbf{Equation 6.2}$$ [10]

Limit of quantitation (LOQ) is the lowest concentration of analyte that is quantifiable within an acceptable range of precision and accuracy. LOD and LOQ were determined according to guidelines approved by the FDA for bioanalytical method validation [11]. LOQ was determined by spiking the plasma with a minimum concentration of calibration curve with precision (20 %) and accuracy (80-120 %) by repeated investigation for five days.

*Stability*

Freeze-thaw stability was investigated by a comparative analysis of plasma samples and freeze-thawed samples. Three dilutions at a concentration level of 5 µg. $ml^{-1}$, 1 µg. $ml^{-1}$ and 15 ng. $ml^{-1}$ were used for three cycles of freeze-thaw and analyzed in triplicate. Benchtop stability of spiked plasma was evaluated at ambient temperature for 12 hours. Long term stability of spiked plasma was investigated for one month.

*Robustness*

HPLC analytical conditions were changed to inspect the influence of different parameters on the separation of rhein. pH of mobile phase, flow rate and column age were the parameters studied for their possible effect on proposed method.

*Application to Pharmacokinetic Evaluation*

This method was applied for pharmacokinetic evaluation of diacerein in rabbits for preclinical studies. The animal study was approved by Pharmacy Research Ethics Committee, Department of Pharmacy, the Islamia University of Bahawalpur (Ref. no. 69-2013/PREC). All the *in vivo* experiments were conducted in accordance with the animal scientific procedure Act 1986 [12]. Healthy male albino rabbits (n=8) weighing 2-2.2 Kg (average weight of 2 Kg) were housed with access to water *ad libitum.* The single oral dose of Diacerein aqueous dispersion (5.4 mg/Kg) was administered to overnight fasted rabbits. 2 ml blood samples were taken from the jugular vein of rabbits using 3 ml syringe at 0, 0.5, 1, 2, 3, 4, 6, 8, 12- and 24-hours post-dosing. The 2 ml normal saline solution was administered orally to compensate probable blood loss after the withdrawal of each blood sample. The plasma was separated from blood by centrifugation and stored at -20°C prior to analysis. Pharmacokinetic parameters of rhein were determined by Kinetica software (Thermo Kinetica ver. 5.0, Thermo Fisher Scientific, Waltham, MA, USA) employing the non-compartmental model approach [13]. The area under the concentration vs. time curve from zero time to infinity ($AUC_{0-\infty}$) and area under the first-moment curve ($AUMC_{0-\infty}$) were calculated by the log-linear trapezoidal method [14]. Maximum plasma concentration ($C_{max}$) and time to reach maximum concentration ($T_{max}$) was observed by visual inspection of plasma concentration-time curves.

*Statistical analysis*

The results are expressed as mean ± S.D, percent relative standard deviation (% RSD) and coefficient of variation (CV). Analysis of variance (ANOVA) was employed in the statistical analysis of the determined parameters in this study. The results with a probability of less than 0.05 ($p < 0.05$) were considered significant.

**Results and Discussion**

*Screening of optimized conditions*

For an efficient chromatographic method, the necessities include separation of the analyte of interest with better resolution, precision, accuracy, and sensitivity.

*Selection of an optimum detection wavelength*

The UV scan of the rhein solution was recorded in the range of 200-400 nm and the spectrum was recorded (Fig. 1). The maximum absorbance of rhein was observed at 210 nm wavelength by UV/Visible spectrophotometric (IRMECO U2020 Germany) scan of 0.50 µg. $ml^{-1}$ rhein solution in the optimized mobile phase employed in this study. The optimum wavelength of 210 nm was used for further analysis of rhein in samples.

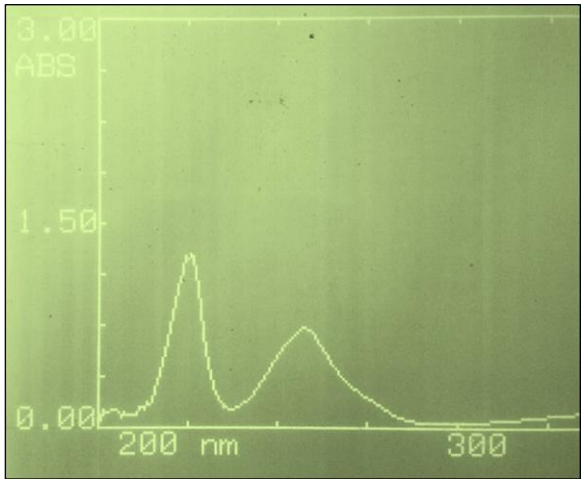


Fig. 1**:** UV Spectrum of Rhein in Mobile phase (Methanol: Water, 15:85, pH 4.7).

*Choice of an optimized mobile phase*

The mobile phase composition was extensively studied to attain a reasonable degree of detection of rhein in the plasma samples. Various binary and ternary eluents were evaluated using different ratios of water and modifiers such as methanol, acetonitrile, and buffers at various pH values. After several attempts, an optimized mixture of methanol and water (15:85, pH: 4.7) presented better peak resolution and improved symmetry. The pH of the mobile phase was adjusted with orthophosphoric acid. The optimized mobile phase also avoided the problems of peak tailing or broadening. In the initial trials, the problems of early elution in 2-3 minutes and merging of rhein and plasma peaks were also resolved using various ratios of mobile phases at different pH conditions. The trials on different columns including hypersil BDS, Nucleosil C18, and hypersil ODS C18 were performed using different mobile phases. These experimentations fruited out a binary mobile phase of methanol and water (15:85) at pH 4.7. A reverse-phase ODS C18 column (250×4.6mm,5µm) with a flow rate of 1 $ml.min^{-1}$ and a detection wavelength $\lambda_{max}$ 210 nm was also validated for obtaining symmetric peak of rhein. The retention time of rhein was 5.34 minutes (Fig. 2). Chromatograms of blank rabbit plasma and plasma spiked with rhein are illustrated herein (Fig 3 and 4). The findings of the present study were in accordance with the previously reported methods for rhein by Emam et al., (2018) who used gradient program for simultaneous elution of rhein and aceclofenac by pumping mobile phase of MPA (ammonium acetate buffer and acetonitrile) and MPB (100% acetonitrile) at proportion of 85:15 [8]. Tang et al., (2007) also developed an HPLC method for pharmacokinetic studies of rhein after administering Chinese medicine preparation (Da-Cheng-Qi decoction) in rats employing a mobile phase consisting of methanol and 0.2% acetic acid (89:11) [15]. HPLC column switching technique for simultaneous estimation of baicalin, rhein and berberine employing a mobile phase consisting of phosphoric acid and acetonitrile at a ratio of 4:1, v/v [7]. The current method employed for the estimation of rhein in biological fluids is relatively more cost effective compared to the previously reported methods. In addition, the present method is simple and convenient as the mobile phase contain only 15% modifier to form a monolayer on the stationary phase. Thus, the method shows reproducibility, as it provides better peak using the prespecified analyte and improved sensitivity as well [16].

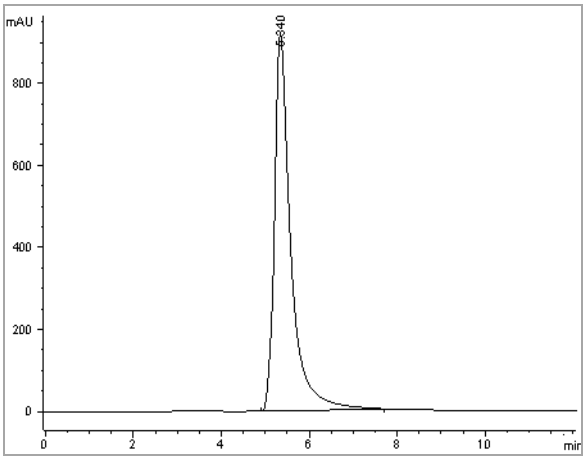


Fig. 2: Chromatogram of rhein in the mobile phase.

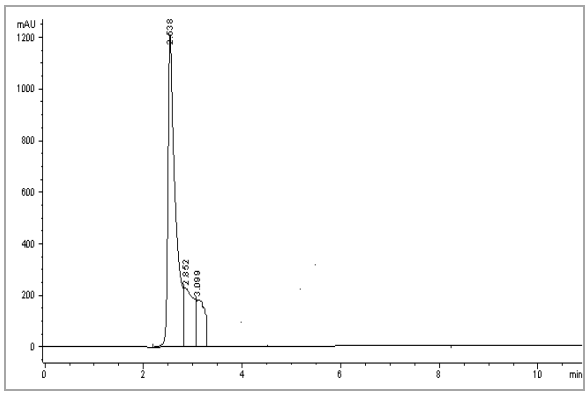


Fig. 3: Chromatogram of blank rabbit plasma.

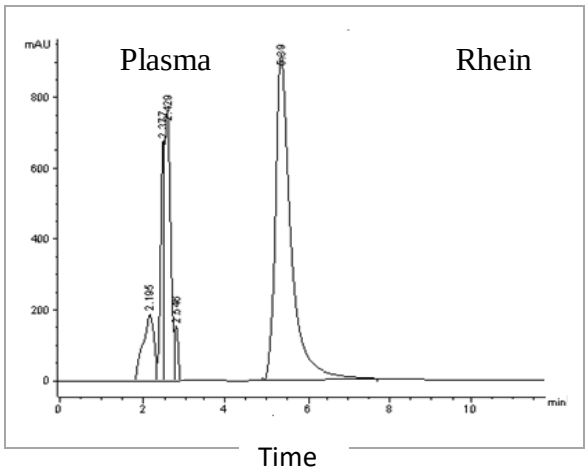


Fig. 4: Chromatogram of rabbit plasma spiked with rhein.

*Method validation*

*Linearity*

The current method was found linear as the values of particular parameters were within acceptable limits [17]. The proposed method was linear over the concentration range of 0.015 µg. $ml^{-1}$ to 5 µg. $ml^{-1}$ for rhein spiked in rabbit plasma. The chromatograms were constructed between the concentration of rhein and peak area as illustrated in Fig. 5. The values of calibration curve parameters were calculated using Kinetica® software and summarized in Table-1. Six replicates (n=6) were evaluated at each concentration level. The average value of $r^2$ for rhein was 0.9980 which indicated good linearity of the method over the analyzed concentration range.

Table-1: Calibration curve parameters of rhein in rabbit plasma.

| Curve Code | Slope | Intercept | r2 | Concentration Range |
|---|---|---|---|---|
| **RH -01** | **14.64** | **1555.8** | **0.9985** | |
| **RH-02** | **14.66** | **1551.3** | **0.9981** | |
| **RH-03** | **14.62** | **1553.5** | **0.9975** | **0.015 ng.mL$^{-1}$-5 µg.mL$^{-1}$** |
| **Mean** | **14.64** | **1553.8** | **0.9980** | |
| **S.D** | **0.02** | **2.250** | **0.0005** | |
| **% C.V** | **0.137** | **0.145** | **0.0504** | |

Abbreviations: S.D=standard deviation, % C.V=percent coefficient of variation, RH=rhein

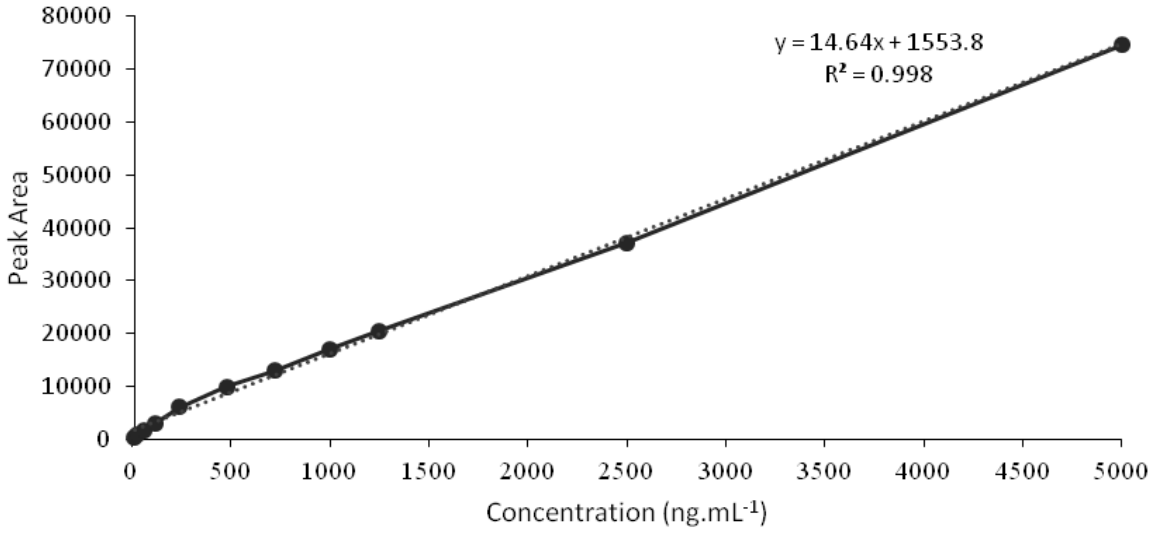


Fig. 5: Calibration curve of rhein in plasma.

*Limit of detection (LOD) and Limit of quantification (LOQ)*

LOD for rhein in rabbit plasma was established at 5ng.ml$^{-1}$ and value of LOQ was measured as 15 ng.ml$^{-1}$. The values of LOD and LOQ were found better than previous study [18] and also comparable to other reported methods [19].

*Inter-day and intra-day precision and accuracy*

Nine replicate injections of rhein standard solution in plasma at the highest, middle, and lowest concentrations were analyzed by HPLC system. The concentrations studied were 15 ng.ml$^{-1}$, 1µg.ml$^{-1}$, 5 µg.ml$^{-1}$ for both inter-day and intra-day precision and accuracy evaluation. The intra-day precision and accuracy for rhein (Table-2) were found in the range of 98.2 – 99.4%., whereas, the inter-day precision and accuracy range was found to be 95.4 – 99.7%. Intra-day and inter-day accuracy of the current method authenticated reproducibility and confirmed the applicability of the method for rhein assay of biological samples. The values of intra-day and inter-day precision and accuracy obtained in the present method were better as compared to a method developed by Emam et al (2018) calculating precision and accuracy at 92% -97.6 [8].

Table-2: Intra-day precision and accuracy of rhein in rabbit plasma.

| Curve Code | LQC | MQC | HQC |
|---|---|---|---|
| Units | ng.mL$^{-1}$ | µg.mL$^{-1}$ | µg.mL$^{-1}$ |
| Theoretical Concentrations | 15 | 1 | 5 |
| Batch -01 | 14.91 | 0.992 | 4.97 |
| | 14.88 | 0.988 | 4.88 |
| | 14.94 | 0.994 | 4.91 |
| | 14.98 | 0.998 | 4.94 |
| | 14.92 | 0.989 | 4.95 |
| | 14.89 | 0.996 | 4.93 |
| | 14.95 | 0.995 | 4.96 |
| | 14.90 | 0.991 | 4.84 |
| | 14.96 | 0.994 | 4.86 |
| Mean | 14.92 | 0.993 | 4.91 |
| S.D | 0.034 | 0.0032 | 0.046 |
| % C.V | 0.227 | 0.330 | 0.94 |
| % Accuracy | 99.4 | 99.3 | 98.2 |

Abbreviation: LQC= Lowest quality control concentration, MQC= Medium quality control concentration, HQC=Highest quality control concentration, S.D = Standard deviation, C.V= Coefficient of variation

*Extraction recovery*

The extraction of rhein was carried out with acetonitrile as protein precipitant and investigated for recovery, accuracy and column pressure. The recovery was calculated at three concentration levels of standard dilutions including 15 ng.ml$^{-1}$, 1 µg.ml$^{-1}$ µg and 5 µg.ml$^{-1}$ for rhein. The peak response of rhein in spiked plasma was compared with standard dilutions in the mobile phase. The % recovery of rhein in plasma was found to be 98.5%, 94.5% and 96.2% for 15 ng.ml$^{-1}$, 2.5 µg. ml$^{-1}$ and 5 µg. ml$^{-1}$ respectively (Table-4). The recovery values obtained in this method were comparable to liquid-liquid extraction obtained elsewhere [4, 15] and relatively superior to an extraction protocol with acetonitrile [6].

Table-3: Inter-day precision and accuracy of rhein in rabbit plasma.

| Curve Code | LQC | MQC | HQC |
|---|---|---|---|
| Units | ng.mL$^{-1}$ | µg.mL$^{-1}$ | µg.mL$^{-1}$ |
| Nominal Concentrations | 15 | 1 | 5 |
| Batch-01 | 14.93 | 0.99 | 4.99 |
| | 14.98 | 0.96 | 4.95 |
| | 14.97 | 0.93 | 4.98 |
| Batch-02 | 14.99 | 0.97 | 4.92 |
| | 14.95 | 0.91 | 4.91 |
| | 14.91 | 0.91 | 4.89 |
| Batch-03 | 14.94 | 0.95 | 4.95 |
| | 14.97 | 0.92 | 4.97 |
| | 14.98 | 0.98 | 4.95 |
| Mean | 14.96 | 0.95 | 4.94 |
| S.D | 0.026 | 0.030 | 0.033 |
| % C.V | 0.179 | 3.21 | 0.671 |
| % Accuracy | 99.7 | 95.4 | 98.8 |

Abbreviations: LQC= Lowest quality control concentration, MQC= Medium quality control concentration, HQC=Highest quality control concentration, S.D = Standard deviation, C.V= Coefficient of variation

Table-4: Percent recovery of rhein in rabbit plasma.

| Spiked drug concentration | Concentration detected | % Recovery (Mean) |
|---|---|---|
| 15 ng.mL$^{-1}$ | 14.87 | 98.5 |
| 1 µg.mL$^{-1}$ | 0.945 | 94.5 |
| 5 µg.mL$^{-1}$ | 4.81 | 96.2 |

*Stability*

The highest and lowest concentrations of rhein (HQC and LQC) were selected for the determination of freeze-thaw stability (Table-5). The results of the stability studies demonstrated negligible drug degradation in plasma. Benchtop stability of spiked plasma samples stored at ambient temperature was investigated for 12 hours (Table-6). The values of % C.V for 12 hours were found as 0.235 (LQC) and 0.040 (HQC). The values of long term stability studies were at 0.221 for LQC, 1.77 for MQC and 0.447 for HQC (Table-7). These results indicated that rhein remained stable during processing, experimentation and ultralow temperature conditions. These findings were also in good agreement to that of the reported studies [19].

Table-5: Freeze-thaw stability of rhein in rabbit plasma.

| Concentration Codes | Cycles | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Cycle 0 | | Cycle 1 | | Cycle 2 | | Cycle 3 | |
| | LQC (15 ng.mL$^{-1}$ ) | HQC (5µg.mL$^{-1}$) | LQC (15 ng.mL$^{-1}$) | HQC (5 µg.mL$^{-1}$) | LQC (15 µg.mL$^{-1}$) | HQC (5 µg.mL$^{-1}$) | LQC (15 µg.mL$^{-1}$) | HQC (5 µg.mL$^{-1}$) |
| | 14.81 | 4.89 | 14.67 | 4.84 | 14.53 | 4.74 | 14.47 | 4.92 |
| Rhein | 14.66 | 4.53 | 14.74 | 4.76 | 14.44 | 4.69 | 14.87 | 4.65 |
| | 14.88 | 4.94 | 14.45 | 4.68 | 14.86 | 4.95 | 14.66 | 4.73 |
| Mean | 14.78 | 4.79 | 14.62 | 4.76 | 14.61 | 4.79 | 14.67 | 4.77 |
| S.D | 0.112 | 0.224 | 0.151 | 0.08 | 0.221 | 0.138 | 0.200 | 0.139 |
| % C.V | 0.760 | 4.67 | 1.03 | 1.68 | 1.51 | 2.87 | 1.36 | 2.90 |

LQC= Lowest quality control concentration, MQC= Medium quality control concentration, HQC=Highest quality control concentration, S.D = Standard deviation, C.V= Coefficient of variation

Table-6: Bench top stability of rhein in rabbit plasma.

| | 0 hour | | 3 hour | | 6 hour | | 12 hour | |
|---|---|---|---|---|---|---|---|---|
| LQC (15 ng.mL$^{-1}$) | HQC (5µg.mL$^{-1}$) | LQC (15ng.mL$^{-1}$) | HQC (5µg.mL$^{-1}$) | LQC (15ng.mL$^{-1}$) | HQC (5µg.mL$^{-1}$) | LQC (15ng.mL$^{-1}$) | HQC (5µg.mL$^{-1}$) | LQC (15ng.mL$^{-1}$) |
| | 15.014 | 4.990 | 14.997 | 4.962 | 14.98 | 4.962 | 15.014 | 4.960 |
| Rhein | 15.000 | 4.970 | 14.969 | 4.921 | 14.969 | 4.984 | 15.01 | 4.975 |
| | 15.043 | 4.983 | 14.97 | 4.954 | 14.97 | 4.934 | 15.022 | 4.983 |
| Mean | 15.02 | 4.981 | 14.97 | 4.94 | 14.97 | 4.96 | 15.01 | 4.97 |
| S.D | 0.022 | 0.010 | 0.016 | 0.021 | 0.0061 | 0.025 | 0.0061 | 0.012 |
| % C.V | 0.15 | 0.203 | 0.11 | 0.439 | 0.041 | 0.0051 | 0.040 | 0.235 |

Abbreviations: LQC= Lowest quality control concentration, HQC=Highest quality control concentration, S.D = Standard deviation, C.V= Coefficient of variance

Table-7: Long term stability of rhein in rabbit plasma.

| Concentration | Units | Week 1 | Week 2 | Week 3 | Week 4 | Mean | S.D | %C.V |
|---|---|---|---|---|---|---|---|---|
| LQC | ng.mL$^{-1}$ | 14.94 | 14.93 | 14.87 | 14.89 | 14.90 | 0.033 | 0.221 |
| MQC | µg.mL$^{-1}$ | 0.98 | 0.96 | 0.97 | 0.94 | 0.96 | 0.017 | 1.77 |
| HQC | µg.mL$^{-1}$ | 4.98 | 4.95 | 4.97 | 4.93 | 4.95 | 0.022 | 0.447 |

LQC= Lowest quality control concentration, HQC=Highest quality control concentration, S.D = Standard deviation, C.V= Coefficient of variation

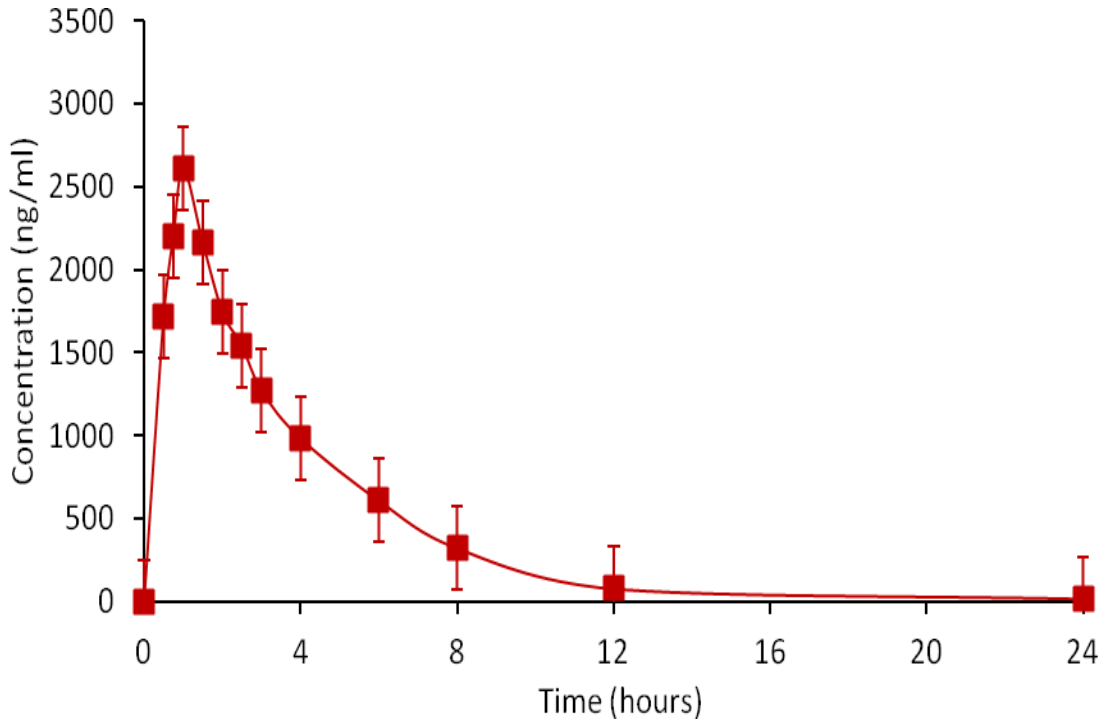


Fig. 6: Plasma level time profile of Diacerein aqueous dispersion administered in oral dose (5.4 mg. Kg$^{-1}$) to albino rabbits (n=8).

*Application of the validated method to pharmacokinetic studies*

Although diacerein was administered in albino rabbits for pharmacokinetic study, due to the rapid conversion of diacerein to rhein (through the first pass effects in gut and liver), intact diacerein levels were not practically measurable. Therefore, we did not attempt to measure intact diacerein but were interested to quantify the levels of rhein. In all pharmacokinetic studies of diacerein, rhein is the component that is being monitored to provide the pharmacokinetic assessment of diacerein. Validated methods are essential for the determination of rhein concentrations in plasma for preclinical pharmacokinetics, toxicokinetic studies and clinical studies. The current validated HPLC method developed for rhein is very specific and sensitive and it utilizes a short run time of 5 minutes for each sample analysis. Owing to very low sensitivity (LOQ 15 $ng.ml^{-1}$) of the assay, it offers **a** suitable platform for the determination of rhein in preclinical studies. Sample preparation is very simple, and it involves protein precipitation of plasma with acetonitrile. The applicability of the method in preclinical pharmacokinetic studies has been demonstrated in albino rabbits. The plasma concentration versus time curve after 5.4 mg/Kg oral administration of diacerein to albino rabbits is shown in Fig. 6.

Non-compartmental pharmacokinetic parameters were calculated by Kinetica software (Thermo Kinetica ver. 5.0, Thermo Fisher Scientific, Waltham, MA, USA) employing a non-compartmental model. The mean $C_{max}$ and $T_{max}$ of rhein were 2608.62 ng/ml and 1 hr respectively. Another study also revealed the same trend that the $C_{max}$ appeared after 1 hour of administered drug [20]. The value of $AUC_{0-\infty}$ and $AUMC_{0-\infty}$ were calculated 9880 ± 45.41 $ng.h.ml^{-1}$ and 42404.21± 28.88 $ng.h^2.ml^{-1}$. $T_{1/2}$ and MRT were found to be 3.12± 0.11 and 4.21 ± 0.19 which were substantially similar to a previous study conducted by Liang *et al* (1995) [21].

## Conclusion

A simple, fast, accurate and reliable HPLC method was developed for measuring rhein in rabbit plasma. An optimum selectivity, sensitivity, precision, accuracy and appropriate retention time nominates the technique fit for high-throughput pharmacokinetic study. Simple HPLC conditions and straightforward sample pre-treatment procedure**s** make the method quick, easy, and convenient to perform. The above-mentioned method has been successfully applied to the pharmacokinetic studies of diacerein aqueous dispersion in albino rabbits. This study describes the rapid and specific determination of rhein in rabbit plasma samples. Also, this method has the potential for the determination of rhein levels in patient blood samples for therapeutic drug monitoring.

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## Conflict of interest

The authors declare no conflict of interest.

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