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RESEARCH ARTICLE

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RP-HPLC method development, validation and pharmacokinetic applicability in preclinical evaluation of rhein treated with novel diacerein eutectics

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Abstract

The current study represents a bioanalytical method for the estimation of rhein (Rh, an active metabolite of diacerein, DIA) in rats treated with novel DIA eutectics to investigate the pharmacokinetics of DIA. A simple protein precipitation technique was used to extract Rh and the internal standard (IS), *p*-aminobenzoic acid, injected into a Phenomenex Gemini C_{18} column. The separation was achieved by a gradient elution comprising ammonium acetate (10 mM; pH 3.0) and acetonitrile in an 18 min run time at a flow rate of 0.8 ml/min with retention times of 11.8 min (Rh) and 5.9 min (IS). The results revealed that the proposed method is linear over a range of 200–20,000 ng/ml (r^2 > 0.9988) of Rh and is precise and accurate. The method was fully validated as per the US Food and Drug Administration guidelines and a pharmacokinetic study in rats was performed for Rh following oral administration of the pure DIA and newly developed eutectics. Therefore, the present method could be used to estimate DIA to illustrate a comparative pharmacokinetic analysis. This can also be applied to its related multicomponent formulations for future studies.

KEYWORDS
diacerein, eutectic, pharmacokinetics, rhein, RP-HPLC

1 | INTRODUCTION

Diacerein [$C_{19}H_{12}O_8$] (DIA; Figure 1a) is a mild analgesic and antipyretic agent approved for the clinical management of osteoarthritis (Tamura et al., 2001). The log*P* value of DIA is 2.4 (Tamura et al., 2002; Tamura & Ohmori, 2001) and it is categorized as a Biopharmaceutics Classification System Class II drug (3.197 mg/l), which hinders its oral absorption (35–56%; Elsayed et al., 2014). The bioavailable DIA converts into its active metabolic form, rhein (Rh; Figure 1b). The unabsorbed Rh irritates the intestinal mucosa and produces a weak laxative side effect that limits its usage as an effective drug. (Magnard et al., 1993; Martel-Pelletier & Pelletier, 2010; Nicolas et al., 1998) Therefore, increasing the bioavailability of DIA would allow less DIA to reach the colon, subsequently reducing its dosage and side effects. Several approaches have been reported to increase the bioavailability of DIA, including self-nanoemulsion (El-Laithy et al., 2015), complexation (Batt & Garala, 2013), solid dispersion (Aggarwal & Singh, 2011), nanoparticles (Elsayed et al., 2014; Jain et al., 2013; Patel et al., 2022) and nanofibers (Malik et al., 2016).

Recently, the crystal engineering approach has been utilized to introduce new solid forms for the diversification of multicomponent solid forms that modify the various pharmaceutical parameters without compromising the efficacy of the active pharmaceutical ingredient (API) (Desiraju, 2013; Patel et al., 2019; Patel, Raval, Pethani, & Sheth, 2020). This approach involves the development of novel eutectic, solid solution, solvate, amorphous, co-crystal,

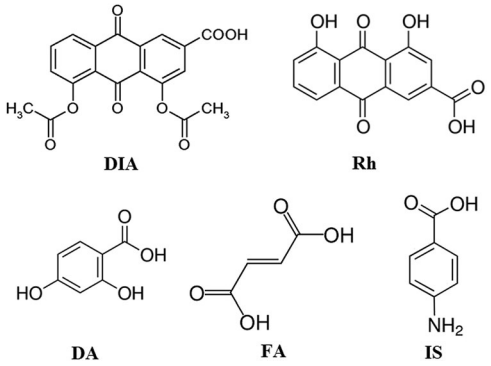

FIGURE 1 Molecular structures of diacerein (DIA), rhein (Rh), 2,4-dihydroxybenzoic acid (DA), fumaric acid (FA) and *p*-aminobenzoic acid (internal standard, IS)

polymorph and hydrate systems. Among these, a eutectic mixture and conglomerate of solid solutions is a non-covalent derivative that enhances the pharmaceutical properties of poorly ionizable APIs (Cherukuvada & Guru Row, 2014; Cherukuvada & Nangia, 2014).

Apart from the engineering of eutectic mixtures, the quantification of drugs in biological fluids is also crucial for pharmacokinetic studies (Hoelke et al., 2009; Lang & Bolton, 1991). In this regard, reversed-phase high-performance liquid chromatography (RP-HPLC) has been utilized to measure the drugs in biological matrices, which provides robust and accurate results for pharmacokinetic measurements (Prathap et al., 2013; Smolec et al., 2005). As DIA is metabolically transformed to Rh, only Rh has been quantified in biological fluids to evaluate its pharmacokinetics (Patel, Raval, & Pethani, 2020). Very few analytical methods have been reported for the analysis of Rh in biological matrices, including RP-HPLC with various detection systems (Chakrabarty et al., 2008; Dan & Xinhui, 2003; Duan et al., 2013; Ojha et al., 2009; Tang et al., 2007; Yaroshenko et al., 2014), liquid chromatography–tandem mass spectrometry (Hou et al., 2014; Jiang et al., 2012; Layek et al., 2008; Lee et al., 2003; Lv et al., 2008; Yi et al., 2006) and RP-HPLC with fluorescence detection (Mohammed et al., 2020; Wan et al., 2013; Zhang et al., 2005).

Further, pharmacokinetic assays for DIA and its eutectic formulations have not been well explored. Therefore, we attempted to develop and validate a simple and effective RP-HPLC technique for estimating DIA in rat plasma. In line with this, we synthesized and characterized new DIA eutectics with fumaric acid (FA) and 2,4-dihydroxy benzoic acid (DA) (Patel, Raval, & Pethani, 2020; Patel, Raval, & Sheth, 2020). Moreover, the proposed RP-HPLC method was validated as per the United States Food and Drugs Administration (USFDA) guidelines and applied for quantifying Rh in rat plasma.

2 | MATERIALS AND METHODS

2.1 | Reagents and chemicals

Diacerein (API) was kindly supplied by Ami Lifesciences Pvt. Ltd (Baroda, India) and Rh (active metabolite of DIA) was purchased from Yucca Enterprises (Wadala, Mumbai). The DA and FA used as coformers were received from Sisco Research Laboratories Pvt. Ltd (Mumbai, India). *p*-Aminobenzoic acid (internal standard, IS) was purchased from Qualikemes Fine Chem. Pvt. Ltd, Vadodara, India. HPLC-grade methanol, acetonitrile (ACN) and ammonium acetate were purchased from Merck Pvt. Ltd.(Mumbai, India). Analytical-grade dimethyl sulfoxide (DMSO) was purchased from Spectrochem Pvt. Ltd, Mumbai, India. Formic acid was purchased from LobaChemie Pvt. Ltd, Mumbai, India. The other solvents and chemicals utilized in the experiment were of analytical reagent grade. Adult healthy Sprague–Dawley rats were used to obtain drug-free rat plasma in the study.

2.2 | Preparation and characterization of multicomponent solid forms

The eutectics of DIA with DA and FA were formulated as described previously (Patel, Raval, & Pethani, 2020; Patel, Raval, & Sheth, 2020). The prepared eutectics were characterized and analyzed using differential scanning calorimetry (DSC), powder X-ray diffraction (PXRD), Fourier transmission infrared spectrophotometry (FT-IR), and scanning electron microscopy (SEM) as described previously (Patel & Raval, 2022; Patel, Raval, & Pethani, 2020; Patel, Raval, Pethani, & Sheth, 2020; Patel, Raval, & Sheth, 2020).

2.3 | Instrumentation and chromatographic conditions

The HPLC system (LC-20 AD, Shimadzu Corporation, Japan) comprised a high-pressure pump (15,000 psi), a SIL20AC autosampler and a thermostated column (CTO-20 AC) were employed for the mobile phase run. The chromatography separations of Rh and IS were performed on a Phenomenex Gemini C_{18} column (250 × 4.6 mm, 5 μm) maintained at 40°C. An SPD-M20A PDA detector and Lab solution software (version 5.91) were used for data acquisition and processing. The separation was achieved using a binary mobile phase consisting of ACN (A) and ammonium acetate (B; 10 mM; pH 3.0) with gradient elution as follows: 80–30% B, 0.0–8.0 min; 30–60% B, 8.0–10.0 min; 60 → 80% B, 10.0–18.0 min. The flow rate of elution was set at 0.8 ml/min with total run time 18 min. The injection volume was 20 μl and the detection wavelength was fixed at 254 nm.

2.4 | Stock and standards solution preparation

The standard stock solution of Rh (25 mg/ml) was prepared by dissolving 50 mg in 2 ml of DMSO, and further diluted in ACN to achieve a final concentration of 5 mg/ml. The stock solutions of Rh were diluted with a solvent mixture (ACN–water, 50:50, % v/v) by serial dilution to prepare working standard solutions prior to use. Similarly, the standard stock solution of IS (10 mg/ml) was prepared in methanol and diluted with ACN (1 mg/ml). The IS working solution (10 μg/ml) was prepared by further diluting the stock solution in the solvent mixture.

The working solutions (5%) of Rh were employed to spike the blank plasma to make up the calibration range 200–20,000 ng/ml. Similarly, 600, 5,000 and 18,000 ng/ml were considered as low, medium and high quality control samples (LQC, MQC and HQC) respectively.

2.5 | Extraction procedure

The protein precipitation technique was utilized to extract Rh from the rat plasma samples followed by evaporation and reconstitution. Before the analysis, the frozen plasma was thawed at room temperature. A 200 μl aliquot of plasma samples was mixed with 50 μl of the IS working solution (10 mg/ml) and vortexed for 30 s. Subsequently, 500 μl of ACN (extraction solvent) was added and vortexed for 10 min, then centrifuged at 4°C for 10 min at 10,000 rpm. A 500 μl aliquot of supernatant was transferred to a fresh centrifuge tube and dried in a nitrogen evaporator at 40°C under a moderate stream of nitrogen. The dried residue was reconstituted with 100 μl of mobile phase, vortexed for 5 min and analyzed in the HPLC system.

2.6 | Bioanalytical method validation

The chromatographic method was validated as per the recommendations of the USFDA guidelines for selectivity, linearity, sensitivity, accuracy, precision, carryover, matrix effects, extraction recovery and stability studies (US Food and Drug Administration, 2018).

2.6.1 | Selectivity

The method selectivity was evaluated by comparing the chromatograms of blank plasma (without Rh and IS), corresponding spiked plasma with Rh and IS, and actual experimental plasma samples from animals treated with Rh. The plasma samples in each group were obtained from six different Sprague–Dawley rats.

2.6.2 | Linearity of calibration curves and sensitivity

Eight calibration standards in the range of 200–20,000 ng/ml were freshly prepared and tested on three consecutive days to investigate the linearity. The calibration curves were created by graphing the peak-area ratios (Rh/IS) (y) against the corresponding calibration standard concentrations (x). As a mathematical equation, weighted ($1/x^2$) least-squares linear regression was used to access the linearity of Rh. The sensitivity of the method was assessed by calculating the lower limit of quantification (LLOQ), which is defined as the lowest feasible concentration in the calibration curve that yields a signal-to-noise ratio of ≥5 and can be determined with acceptable accuracy and precision not exceeding 20%.

2.6.3 | Precision and accuracy

The intra- and inter-day precision and accuracy were measured using six replicate analyses of QC samples spiked in rat plasma at four concentration levels (LLOQ, LQC, MQC and HQC) for three consecutive days. The concentrations of QC samples were selected from the calibration curve range. The precision was assessed by calculating the coefficient of variation (CV) of the measured concentrations at each QC level. The accuracy was estimated by comparing the average obtained concentration with the nominal concentration and was indicated as the percentage (%). In all cases, the criteria for acceptability of the data included the percentage difference that was limited to 15% (20% for LLOQ) of the nominal values.

2.6.4 | Carryover effects

The carryover effect of Rh was evaluated by analyzing a blank sample directly after the injection of the upper limit of quantification (ULOQ) sample. At the relevant chromatographic regions, the carryover should be <20% of LLOQ and 5% of the IS.

2.6.5 | Extraction recovery and matrix effect

The extraction recovery of Rh in the biological sample was estimated at the LQC (600 ng/ml), MQC (5,000 ng/ml) and HQC (18,000 ng/ml) ($n = 6$) by comparing the mean peak-areas obtained from the extracted plasma samples with those of the non-processed standard solutions. The matrix effect of Rh was calculated by comparing the mean peak-areas of plasma samples spiked with analyte after extraction (the presence of matrix ions) with those of the pure, authentic standard solutions (the absence of matrix ions) at three QC levels. For a method to be free from the relative matrix effect, the matrix effect could be considered acceptable when the assessed value is <15%. The recovery and matrix effect of IS were estimated in the same way using QC samples (10,000 ng/ml) as the reference.

2.6.6 | Stability and dilution integrity

The stability study of stock solutions was evaluated for Rh (5 mg/ml) and IS (1 mg/ml) at ambient temperature for 8 h and at 2–8°C for 15 days. Various stability data of Rh in rat plasma were estimated by analyzing replicates ($n = 6$) at LQC and HQC levels, which were exposed to the anticipated temperature and storage conditions as follows: bench-top stability (at ambient temperature for 6 h before processing), process stability (2 h at room temperature and 24 h at 5°C), freeze–thaw stability (three freeze–thaw cycles from −80 to 25°C), long-term stability (−80°C for 30 days) and dry extract stability (24 h at −80°C). All of the stability samples were estimated against freshly prepared QC samples. The mean concentrations of freshly prepared samples were compared with those of the stability samples and expressed as the percentage mean change. The stability was confirmed if the percentage mean change was within ±15%.

Dilution integrity was performed by analyzing the samples after 2- and 5-fold dilution. The samples were diluted with blank plasma and the results were compared with the original samples. The outcomes of the diluted samples should not exceed 15% of the outcome of the original samples.

2.7 | Pharmacokinetic applicability of the developed method

The proposed bioanalytical method was effectively employed for a comparative study of the pharmacokinetics of newly developed eutectics against pure DIA in rats. In the experiment, healthy Sprague–Dawley rats (either sex; 200–250 g) were acquired from the Central Animal House Facility of the Department of Pharmaceutical Sciences, Saurashtra University, Rajkot. Rats were kept in a room with a relative humidity of 50–60% and a temperature of 24 ± 2°C. Before the experiment, the animals were acclimatized for a week and then fasted overnight with free access to water. The experimental protocol was approved by the Institutional Animal Ethics Committee on 12 December 2016 (IAEC/DPS/SU/1609; Patel, Raval, & Pethani, 2020). Rats were randomly distributed into three groups ($n = 12$). Each group was administered an orally pure DIA suspension, DIA–DA and DIA–FA eutectic suspensions in water containing 0.2% w/v sodium carboxymethylcellulose as a suspending agent at a dose equivalent to 30 mg/kg body weight of DIA, respectively (Patel, Raval, & Pethani, 2020; Tamura et al., 2002). Following the drug treatment, blood samples were collected through the retro-orbital plexus route using heparinized tubes at predetermined time intervals, 0 (predose), 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 12 and 24 h following oral administration. The plasma samples were collected by centrifugation (Centrifuge 5418R, Eppendorf AG, Germany) at 10,000 rpm for 20 min and stored at −20°C until analysis (Patel, Raval, & Sheth, 2020).

2.8 | Calculations and statistical analysis

The pharmacokinetic evaluation was performed of the area under the plasma concentration–time curve (AUC_{0-t}) from zero to the last time point, the elimination half-life, the mean residence time, volume of distribution, the area under the moment curve (AUMC), the total clearance, the time to maximum concentration and the maximum plasma concentration of the drug were calculated using PK solver (version 2.0). Data were expressed as the mean ± standard deviation (SD).

3 | RESULTS AND DISCUSSION

3.1 | Solid-state characterization of the newly developed eutectics

Differential scanning calorimetry is the most useful and comprehensive tool for interpreting the development of eutectics among other characterization techniques (Lu et al., 2008; Patel & Raval, 2022). The DSC thermographs of DIA, DA and FA showed endothermic events at 255.17, 222.05 and 289.48°C, respectively. DIA–DHA (1:3) and DIA–FMA (1:2) ground products indicated sharp melting points (201.53 and 232.97°C, respectively), which were lower than those of both of the participating components (Figure 2a). These events pointed out the generation of the new solids can be either cocrystal or eutectic. Further, stoichiometric evaluation using a binary phase diagram (data not shown) exhibited a "V" shape, indicating eutectic development. The characteristic vibration bands corresponding to DIA and coformer molecules (DA and FA) without any substantial shift were observed in FT-IR data for the grounded samples as presented by the asterisks in Figure 2b. The PXRD study revealed all of the characteristic peaks of the involved compounds that were present in the diffraction patterns of the produced samples with no changes in the 2θ values (Figure 3a). As a result, adhesive interactions between DIA and coformers (DA and FA) are unlikely to be strong enough to replace cohesive interactions, and are therefore ineffectual in changing the crystal

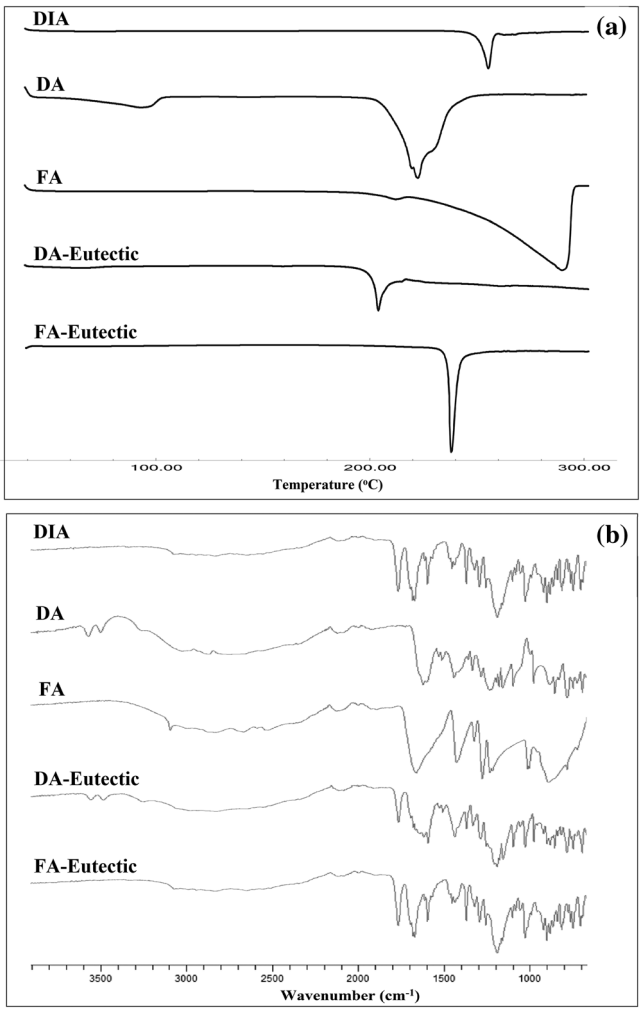

FIGURE 2 Solid-state characterization: differential scanning calorimetry (a) and Fourier transmission infrared spectrophotometry (b) of DIA, DA, FA, DA-eutectic and FA-eutectic

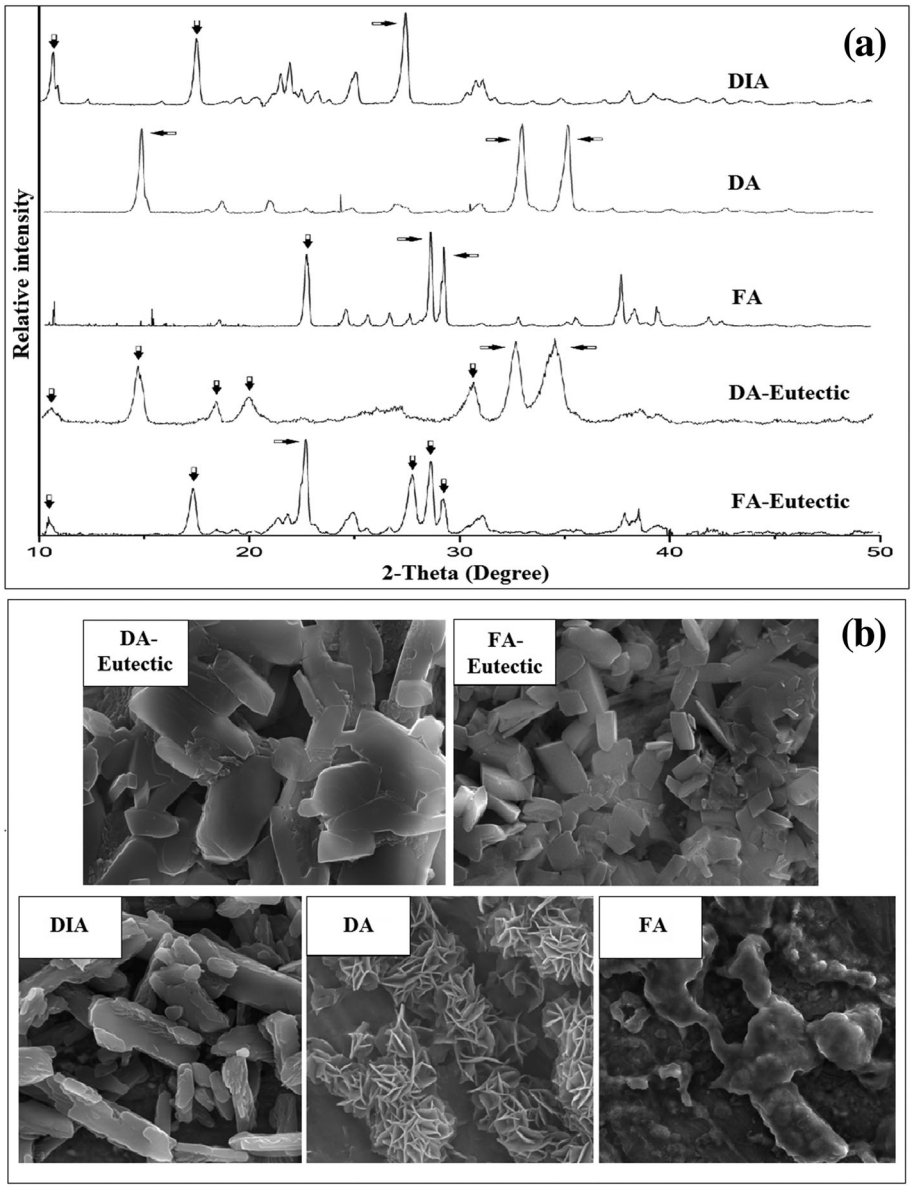

FIGURE 3 Solid-state characterization: powder X-ray diffraction (a) and scanning electron microscopy (b) of DIA, DA, FA, DA-eutectic and FA-eutectic

packing of individual components, resulting in eutectic binary mixtures (Cherukuvada, 2016; Patel et al., 2019). Moreover, SEM analysis (Figure 3b) revealed the morphological differences in newly generated solid forms, which could be advantageous for the development of oral solids (Raval et al., 2013). The eutectics data characterization has not been discussed further since our focus is to develop RP-HPLC technique for the estimation of DIA in the prepared eutectic formulation.

3.2 | Optimization of chromatographic conditions

The main goal of any bioanalytical method optimization is an efficient separation of the analytes. The mobile phase preparation can significantly influence the optimization parameters like the composition and type of organic/aqueous phase with a varity of pH values, flow rates, wavelengths, column temperatures, and many more. Therefore, these parameters were evaluated and optimized to attain better peak shape, high resolution, selectivity and sensitivity (Braggio et al., 1996; Patel & Raval, 2020). During the column selection experiments, different columns such as Gemini (250 × 4.6 mm, 5 µm), Thermo (250 × 4.6 mm, 5 µm), Symmetry (250 × 3 mm, 5 µm), Enable (250 × 3 mm, 5 µm) and Atlantis (250 × 4.6 mm, 5 µm) columns were tried with various mobile phase compositions, including organic and aqueous phases. The best separation between the two main compounds as well as their coformers within a relatively short run time was achieved via a Phenomenex Gemini C_{18} column (250 × 4.6 mm, 5 µm) column; hence, it became the column of choice for the separation of this sample mixture. Other columns revealed poor resolution between the main analytes with their coformers.

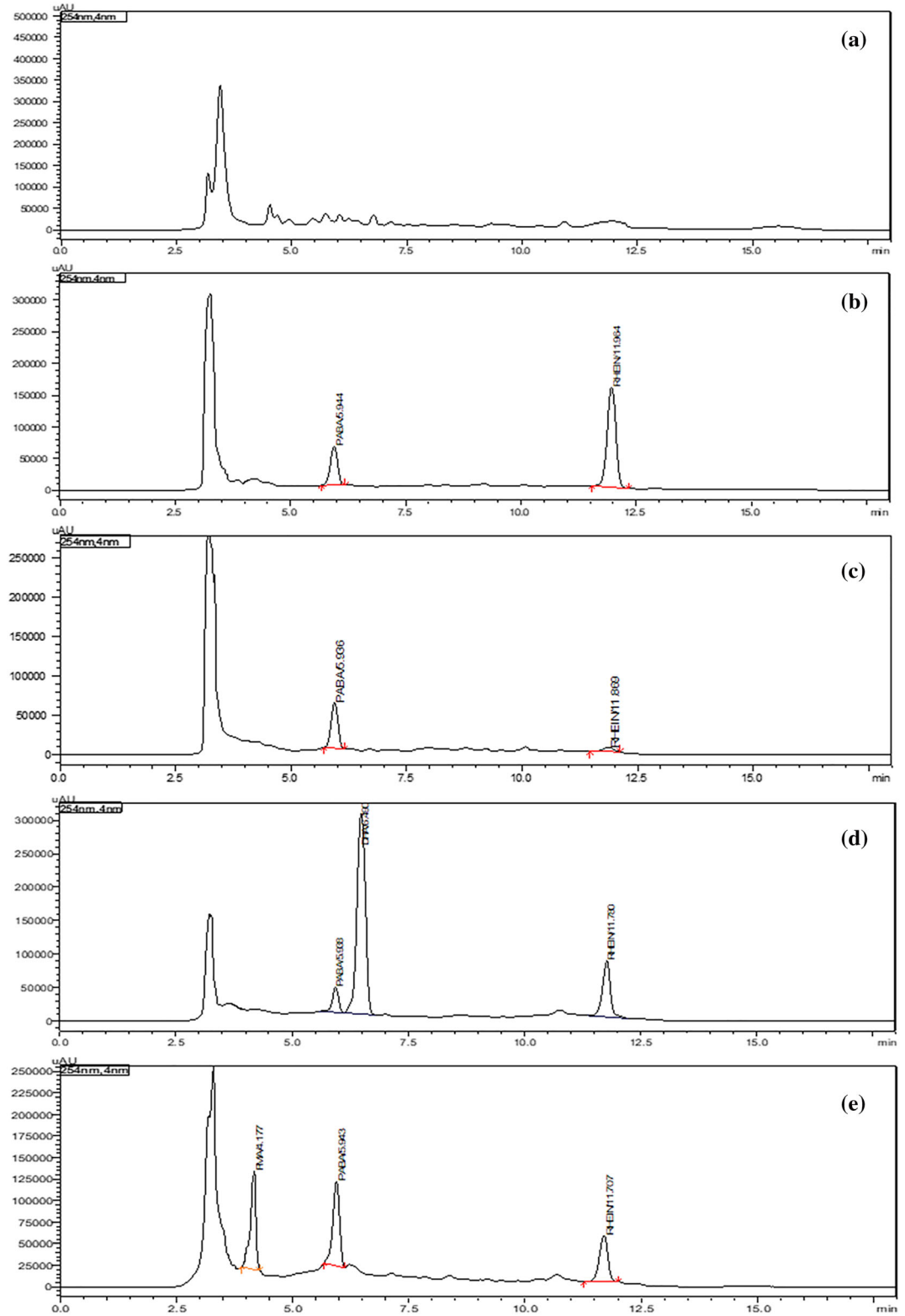

FIGURE 4 Representative HPLC chromatograms of blank rat plasma (a); a spiked plasma sample with Rh and IS (b); a spiked plasma sample with Rh and IS at LLOQ level (c); 2 h plasma samples collected after single oral administration of the DIA–DA eutectic mixture (d) and of the DIA–FA eutectic mixture (e) (spiked with Rh and IS in rat plasma)

Several mobile phase compositions were examined using various percentages of aqueous and organic modifiers. Acceptable separation of the two main analytes and coformers was achieved using ACN and ammonium acetate in terms of peak shape, retention times and resolution of the analytes and IS. The method development involved trials with different pH ranges from 2 to 6, wherein pH 3 yielded the proper peak shapes for the analyte and IS. However, poor resolutions between the analytes instigated us to choose the gradient system instead of binary elution.

The other aqueous phases such as ammonium formate, trifluoroacetic acid, potassium phosphate and 0.1% v/v triethyl amine buffers resulted in poor resolution and broad-tailed peaks. The method development experiments disclosed that using ACN as an organic modifier led to adequate separation between the DIA, Rh, IS and coformers and to symmetrical peaks.

Parameters such as sensitivity, good separation with acceptable peak shape, column efficiency, and short analysis time were evaluated and optimized. The ammonium acetate (pH 3.0; 10 mM) and ACN (mobile phase) were eluted by gradient elution in a Phenomenex Gemini C_{18} column (250 × 4.6 mm, 5 μm) at 40°C. The flow rate and injection volume were set at 0.8 ml/min and 20 μl, respectively. To obtain a high specificity for Rh and the IS, UV detection was set at 254 nm with a total run time of 18 min. Additionally, system suitability parameters such as resolution, tailing factors and a number of theoretical plates were within the acceptable limits in the finalized chromatograms (Patel & Raval, 2020).

3.3 | Optimization of sample preparation

Recent reports demonstrated that liquid–liquid and solid-phase extraction techniques can be utilized for maximum recovery of the analyte from the plasma sample (Layek et al., 2008; Mohammed et al., 2020; Yaroshenko et al., 2014). Initially, we utilized the liquid–liquid extraction technique with several organic solvents such as chloroform, *tert*-butyl methyl ether, ethyl acetate and diethyl ether–dichloromethane (70:30, v/v). However, poor analyte recovery was observed owing to its poor solubility. Moreover, the solid-phase extraction technique was not considered owning to challenges like expensive materials and time-consuming processing steps. Therefore, we employed a simple and cost-effective protein precipitation technique that gave the maximum recovery of the analyte with a short processing time. Methanol and ACN as precipitating agents were tested for the extraction of Rh from the rat plasma. Initially the samples obtained from the protein precipitation procedure were injected directly into the system, but we found a chromatography disturbance and less response. Hence, we subjected the samples to drying and reconstitution and found that the ACN gave improved extraction efficiency, better recovery of the analytes and the least amount of interference from any biological matrix and IS. *p*-Aminobenzoic acid, aspirin, naringenin, carbamazepine, salicylic acid, anthracene and mefenamic acid were evaluated for the IS. Finally, *p*-aminobenzoic acid was chosen in the present assay because it did not interact with the matrices and was well separated from Rh and formulation excipients. The chromatograms of the prepared mixtures of Rh and IS in the plasma sample are shown in Figure 4b.

3.4 | Bioanalytical method validation

3.4.1 | Selectivity

Figure 4 illustrates the representative HPLC chromatograms of blank rat plasma, a spiked plasma sample with Rh and IS, and experimental plasma samples at 3 h following oral dosing of DIA eutectic suspensions (30 mg/kg). The results showed no appearance of co-eluting interferences from the endogenous substances at the retention times of Rh (11.8 min) and IS (5.9 min). This observation confirmed that Rh and IS were well separated by the analytical conditions and resulted in satisfactory selectivity from plasma samples.

3.4.2 | Linearity and sensitivity

The calibration curve of Rh exhibited excellent linearity over the range of 200–20,000 ng/ml. The atypical equation was obtained as $y = 0.144x + 0.032$ ($r^2 = 0.9988$) where x is the plasma concentration of Rh and y represents the peak-area ratio of Rh to that of IS. The proposed bioanalytical method can quantify LLOQ (200 ng/ml) with a simple PDA detector, which was sufficient for the pharmacokinetic assay of Rh in rats. This LLOQ was lower than that for the reported method (Chakrabarty et al., 2008) and was slightly higher than that for the previously reported method using a fluorescence detector (145 ng/ml) (Mohammed et al., 2020; Wan et al., 2013; Zhang et al., 2005).

TABLE 1 Summary of intra- and inter-day precision and accuracy data for rhein (Rh) in plasma samples

	Intra-day ($n = 12$)		Inter-day ($n = 18$)	
Nominal concentration (ng/ml)	Precision (CV, %)	Accuracy (%)	Precision (CV, %)	Accuracy (%)
200	3.49	97.58	7.33	99.04
600	3.37	98.29	4.37	97.94
5,000	1.52	99.13	1.42	99.37
18,000	2.63	101.31	2.81	100.76

TABLE 2 Assessment of extraction recovery and matrix effect of Rh (at three quality control, QC, levels) and internal standard (IS) in rat plasma samples

		Extraction recovery		Matrix effect	
Analytes	Concentration (ng/ml)	Mean extraction recovery (%)[a]	CV (%)	Mean matrix effect[b]	CV (%)
Rh	600	90.78	2.96	0.92	2.18
	5,000	91.16	4.82	0.95	3.69
	18,000	94.30	3.56	0.91	4.84
IS	10,000	84.64	2.52	0.93	2.39

[a]Extraction recovery is expressed as the percentage of the mean peak area of the analytes prepared in the mobile phase relative to that of analytes extracted from rat plasma.
[b]Matrix effect is expressed as the percentage of the mean peak area of the analytes spiked in blank extracted plasma samples relative to that of analytes prepared in the mobile phase.

TABLE 3 Stability data of Rh at low and high QC levels

Stability study	Nominal concentration (ng/ml)	Mean stability (%)[c]
Bench-top	600	−1.29
	18,000	−2.13
Process stability[a]	600	−4.09
	18,000	−2.39
Process stability[b]	600	−2.56
	18,000	−3.23
Freeze–thaw stability (third cycle)	600	1.42
	18,000	−4.75
Dry extract stability	600	−3.54
	18,000	−6.13
Long-term stability (30 days)	600	−6.04
	18,000	−4.73
Dilution integrity	15,000 (2 times)	3.74
	6,000 (5 times)	4.83

[a]Process stability evaluated after 24 h storage at 5°C.
[b]Process stability estimated after 2 h at room temperature.
[c]Mean stability = percentage mean change in the concentration of the stability samples when compared with the freshly spiked samples.

3.4.3 | Precision and accuracy

The obtained results of accuracy and precision for Rh are listed in Table 1. The results show that the intra- and inter-day precisions were <3.49 and 7.33%, respectively. Likewise, the intra- and inter-day accuracies were between 97.58 and 101.31%. The results of inter- and intra-day precision (CV, %) and accuracy (%) suggested that the method met the accepted concentration limits for assaying Rh in rat plasma. Additionally, these data were well comparable with the previously published methods, where the mean accuracy was ranged between 96.55 and 110.5% and the precision was <4.0% in plasma samples (Mohammed et al., 2020; Yaroshenko et al., 2014). Therefore, the proposed method to extract Rh from plasma is highly robust and reproducible.

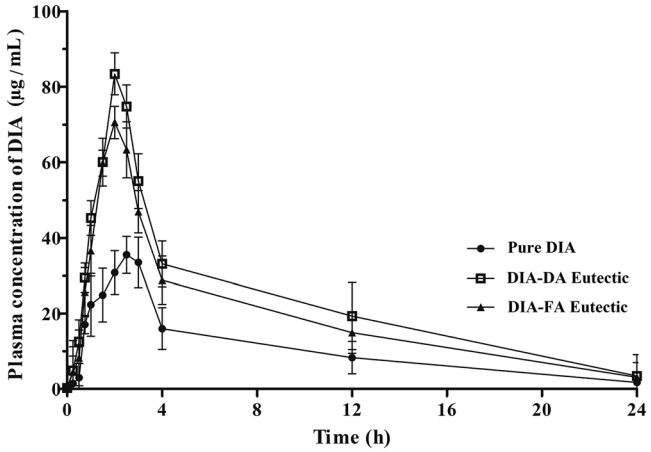

FIGURE 5 The plasma level–time profiles of pure DIA, DIA–DA and DIA–FA eutectic mixtures after an oral dose of 30 mg/kg in Sprague–Dawley rats

3.4.4 | Carryover effects

The carryover effect was assessed and no significant carryover (compared with LLOQ) in the chromatographic regions of Rh and IS was observed, which was analyzed just after the ULOQ. Hence, the developed method is considered to be free from any carryover effects during analysis.

3.4.5 | Extraction recovery and matrix effect

Three spiked QC samples were utilized for the extraction recovery study (Table 2). The mean recovery for the IS (10,000 ng/ml) was 84.64%. The mean extraction recoveries (CV %) for Rh and IS were <4.82%. There was no significant change in Rh extraction recoveries using the protein precipitation method, indicating that the proposed method to analyze Rh is consistent and reliable.

The absolute matrix effects for Rh at three concentrations of 600, 5,000 and 18,000 ng/ml were 92.24, 95.22 and 90.78%,

TABLE 4 Pharmacokinetic parameters of diacerein (DIA) and prepared eutectics after single oral dose administration to Sprague–Dawley rats

Parameters[b]	DIA	DIA–DA eutectic	DIA–FA eutectic
C_{max} (μg/ml)[a]	35.54 ± 5.72	83.43 ± 4.57	70.58 ± 4.46
T_{max} (h)	2.5 ± 0.92	2 ± 0.66	2 ± 0.82
$AUC_{0-24\ h}$ (μg h/ml)[a]	249.91 ± 15.24	542.02 ± 20.30	453.11 ± 28.97
AUC_{total} (μg h/ml)[a]	298.60 ± 14.12	621.27 ± 38.94	528.05 ± 26.26
$T_{1/2}$ (h)	9.8 ± 2.39	8.02 ± 1.87	8.44 ± 2.51
V_D (L)[a]	21.22 ± 4.01	15.10 ± 2.94	17.96 ± 3.04
CL (L/h)	0.74 ± 0.26	0.65 ± 0.19	0.72 ± 0.14
AUMC (μg h^2/ml)[a]	1,174.28 ± 308.50	2,348.56 ± 653.13	2,113.71 ± 428.03
MRT (h)[a]	3.93 ± 1.53	4.68 ± 2.04	4.37 ± 1.04
F_{rel}	1	2.08	1.77

Note: $F_{rel} = (AUC_{total-eutectic}/AUC_{total-drug})$

[a]Pharmacokinetic parameters obtained with the newly developed eutectics were significantly different from the parent DIA at $p < 0.05$.

[b]Indicates data shown as means ± SD ($n = 6$).

DA, 2,4-Dihydroxy benzoic acid; FA, fumaric acid; C_{max}, maximum plasma concentration; T_{max}, time to maximum concentration; AUC, area under the plasma concentration–time curve; $T_{1/2}$, the elimination half-life; V_D, volume of distribution; *CL*, total clearance; AUMC, area under the moment curve; MRT, mean residence time.

respectively (Table 2). The matrix effect for IS at 10,000 ng/ml in plasma was 93.44% (Table 2). The matrix effect study showed that the CV values for all samples were <15%. These results of matrix performance demonstrated that negligible interference of endogenous substances was observed for the quantification of Rh in rat plasma samples.

3.4.6 | Stability and dilution integrity

The working standard solutions of Rh and IS (stock solutions) were stable at room temperature for 8 h (short-term stock solution stability) and at 2–8°C for 15 days (long-term stock solution stability). Table 3 summarizes the results of the stability experiments. We did not find any degradation in the spiked plasma samples under the described conditions, suggesting that the Rh was stable under all of the diverse conditions.

The percentage mean changes in the diluted samples at 2 and 5 times dilution were observed to be 3.74 and 4.83%, respectively. This outcome indicated that the dilution steps have an acceptable dilution effect on the accuracy and precision.

3.5 | Pharmacokinetic applicability of the developed method

The proposed method was used to estimate the amount of Rh in rat plasma obtained after the application of a novel DIA eutectics formulation. Figure 5 depicts the average plasma level–time profiles of the pure drug and the prepared eutectics after an oral dose of 30 mg/kg in Sprague–Dawley rats and the pharmacokinetic evaluation parameters are tabulated in Table 4. Oral administration of DIA and eutectics to rats revealed a higher maximum concentration of DIA–DA and DIA–FA eutectics (83.43 ± 4.57 and 70.58 ± 4.46 μg/ml, respectively) as compared with the parent DIA (35.54 ± 5.72 μg/ml). Moreover, the time required to attain the maximum plasma concentration of DIA eutectics was reduced from 2.5 to 2 h as compared with the pure drug. The obtained AUC_{total} and AUMC in the case of eutectics were significantly superior ($p < 0.05$) compared with DIA alone (Table 4). Furthermore, the relative bioavailability of DIA–DA and DIA–FA samples were 2.08- and 1.77-fold higher compared with DIA alone. The suitability of the developed bioanalytical method was demonstrated by the bioavailability improvement of the newly developed eutectic formulations against the pure DIA in the rat plasma. In addition, this assay allows the quantification of plasma concentrations of DIA and its eutectics at a lower level (200 ng/ml of LLOQ). This LLOQ was lower than that in the previously reported method (Chakrabarty et al., 2008) and was slightly higher than that in the previously reported method (145 ng/ml) that was achieved by applying a fluorescence detector (Mohammed et al., 2020; Wan et al., 2013; Zhang et al., 2005). According to the above established assay, the validated bioanalytical method in rat plasma was effectively utilized for the quantitative measurement of DIA in the current investigation. This can be also applied to its related formulations in future studies.

4 | CONCLUSIONS

In summary, we have developed a simple method for the quantification of Rh as the active metabolite of DIA in its pure form and newly developed eutectic mixtures in rat plasma. The proposed method exhibits better separation efficiency and sensitivity for hydrophobic (Rh) and hydrophilic (coformers) analytes. The LLOQ was achieved with a simple processing method, which shows minimum matrix effect

with good recoveries of Rh and IS. Further, the pharmacokinetics of DIA and its eutectic formulations (after an oral dose of 30 mg/kg) revealed a significant improvement in the bioavailability and could be a possible alternative to commercial DIA. Altogether, the developed method offers a novel approach to estimating DIA in the form of active metabolite Rh in the biological samples following the dosing of multicomponent adducts in routine pharmacokinetic and drug monitoring studies.

CONFLICTS OF INTEREST

All authors declare that they have no actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations that could inappropriately have influenced, or be perceived to have influenced this work.

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DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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