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Simultaneous HPLC–UV determination of rhein and aceclofenac in human plasma

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ABSTRACT

Diacerein and aceclofenac are prescribed for reducing the symptoms associated with osteoarthritis. We present a simple HPLC method with UV detection for simultaneous determination of rhein (the immediate metabolite of diacerein) and aceclofenac from human plasma samples. Sample preparation was accomplished through liquid–liquid extraction with ethyl acetate and chromatographic separation was performed on a reversed-phase ODS column. Mobile phase consisted of a mixture of acetate buffer and acetonitrile run under gradient at flow rate of 1.0 ml/min. Wavelength was set at 258 nm. The method was validated for linearity, accuracy, precision and stability. The calibration was linear over the range of 0.1–7.0 μg/ml for rhein and 0.5–20 μg/ml for aceclofenac using 500 μl plasma samples. Extraction recoveries were 85% for rhein and 70% for aceclofenac. The method can easily be adopted for high-throughput clinical and pharmacokinetic studies of above two-drug fixed dose combination formulations.

1. Introduction

Osteoarthritis (OA) is one of the most prevalent musculoskeletal conditions which affect the joints. The degeneration of the cartilage that protects the ends of bones causes pain and inflammation [1]. Diacerein, (diacetylrhein; [4,5-bis (acetyloxy)-9,10-dihydro-9,10-dioxo-anthracene-2-carboxylic acid]; CAS no 13739-02-1) has been found to be effective in the treatment of OA as a synthetic chemical and also in native form from many plants. It is the pro-drug which converts entirely to its main active metabolite, rhein (4,5-dihydroxy-9,10-dihydro-9,10-dioxo-anthracene-2-carboxylic acid; CAS no. 478-43-3) (Fig. 1A and B) before systemic absorption [2,3]. Rhein also occurs naturally in some plants. It belongs to the anthraquinone class of molecules. It is about 99% bound to plasma proteins. The primary route of elimination is through urine, where, 20% is removed in the native form, 60% as a glucoronide conjugate and 20% as sulfate.

Aceclofenac, ([2-(2′6′-dichlorophenyl) amino] phenyl acetoxy acetic acid; CAS no. 89796-99-6) (Fig. 1C) belongs to the class of non-steroidal anti inflammatory drugs (NSAIDs). It has pronounced anti-inflammatory, antipyretic, antirheumatoid and analgesic effect and an improved gastro-intestinal tolerance. It is well absorbed after oral administration and circulates mainly as unchanged drug. 70% of the administered dose is excreted in urine as gluconoride of aceclofenac and diclofenac [4]. This drug is also more than 99% bound to plasma proteins.

A fixed dose combination formulation containing 50 mg diacerein and 100 mg aceclofenac has been approved by Central Drug Standard Control Organization of India for the treatment of OA. Quantification of the two drugs in plasma samples is required for pharmacokinetic studies. Guidelines for bioanalytical method validation and analysis of study samples are laid out by US Department of Health and Human Services [5]. Several HPLC methods are available in literature to determine the concentration of these two drugs in plasma, individually or in combination with other drugs or metabolites. Techniques used for rhein include liquid chromatography–tandem mass spectrometry [6,7], and reversed-phase HPLC with UV [8–14] and fluorescence [15,16] detection. Methods for analysis of aceclofenac include HPLC [17–21], narrow-bore HPLC with column switching, where it has been quantified along with diclofenac [22], capillary electrophoresis [23] and LC–tandem mass spectrometry [24]. UV detector has been used with most of the HPLC methods described. The column switching method employed direct sample introduction on HPLC, sample cleanup using two columns and separation on the third column. However, there is no method available for the simultaneous determination of rhein and aceclofenac from human plasma.

Three criteria must be met for the plasma drug assay to be functional within clinical laboratories: determination of the drug at low concentrations, simple, rapid and efficient sample pre-treatment and reasonable elution time. These criteria are also valid for quantification method for bioequivalence assays, since a very large number of samples are generated in this kind of study.

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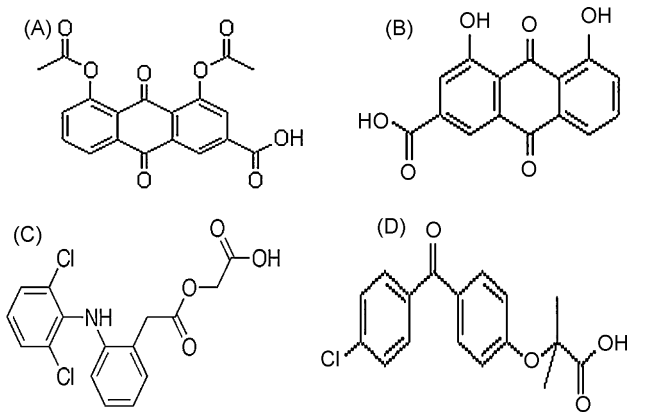

Fig. 1. Chemical structure of (A) Diacerein; (B) Rhein; (C) Aceclofenac and (D) Fenofibric acid.

In this paper, a simple and efficient HPLC method with UV detection, adequate sensitivity and short elution time is described where both rhein and aceclofenac have been determined in plasma samples using a single assay.

2. Experimental

2.1. Chemicals

Perchloric acid (analytical grade), methanol (HPLC grade), acetonitrile (HPLC grade) and ammonium acetate buffer (analytical grade) were purchased from Fisher Scientific (India). Ultra-pure water was produced by distillation. Rhein was obtained from Aldrich, aceclofenac from Lyka Labs, Mumbai, India and fenofibric acid (internal standard, I.S.) (Fig. 1D) was sourced from Council of Europe, European Pharmacopoeia, Strasbourg, Cedex 1.

2.2. Calibration standards and quality control plasma samples

Stock solutions of aceclofenac (1.0 mg/ml) in methanol, rhein (0.1 mg/ml) in tetra hydrofuran and a stock solution (0.2 mg/ml) of fenofibric acid (I.S.) in methanol were prepared and stored at 6 °C. Calibration range was selected taking into account the concentrations expected in plasma samples. Preparation of calibration standard plasma samples (0.5, 1, 2.5, 5, 10, 15 and 20 μg/ml aceclofenac and 0.1, 0.5, 1.0, 2.0, 3.0, 5.0 and 7.0 μg/ml rhein) was accomplished by introducing known amounts of stock solutions of both the drugs to pooled drug-free plasma in volumetric flasks. The highest and lowest standards were prepared in 25 ml volumetric flasks while the remaining were prepared in 10 ml flasks, aliquoted and stored at −80 °C. Quality control plasma samples (1.25, 8 and 17 μg/ml aceclofenac and 0.25, 2.5 and 6.0 μg/ml rhein) were similarly prepared in 50 and 25 ml volumetric flasks by spiking pooled drug-free plasma with known amounts of stock solutions, aliquoted and stored at −80 °C.

2.3. Sample preparation

One hundred microliters of I.S. solution in methanol (20 μg/ml) was introduced in glass tubes containing 0.5 ml plasma sample and mixed. Then, 50 μl of perchloric acid, 3.5% (prepared by 20 times dilution of 70% perchloric acid) were added and vortex-mixed for 30 s. Extraction was accomplished by adding 4 ml of ethyl acetate and again vortex-mixing for 2 min followed by centrifugation at 1900 × *g* for 10 min and organic layer transferred to conical tubes. The solvent was evaporated to dryness at 40 °C under a nitrogen stream. The dried extracts were reconstituted with 100 μl of mobile phase and 50 μl were injected into the chromatographic system.

2.4. Chromatographic conditions

The chromatographic system consisted of a Jasco series-2000 model which included binary pump (PU-2080*plus*), auto injector with variable injection valve (AS-2057*plus*), and UV–vis detector (UV-2075*plus*). The system was connected through a LC-Net interface to ChromPass 1.8.6 software in a computer system for data collection and processing. Separation was performed on a 250 mm × 4.6 mm i.d., 5 μm particle size Peerless LC-C_{18} column (Chromatopak). Mobile phase consisted of a mixture of ammonium acetate buffer (0.01 M; pH 5.65) and acetonitrile (74:26, v/v; MPA) on one pump and 100% acetonitrile (MPB) on second pump run under gradient conditions. Initial condition of 100% MPA was held for 8 min followed by linear increase of MPB to 15% in next 2 min to a final composition of 85:15 of MPA:MPB. This composition was held for 8 min. The system was brought back to the initial condition of 100% MPA by the linear gradient in the next 2 min and maintained for 3 min before the next injection.

The mobile phase was prepared daily and degassed before use. Flow rate was 1.0 ml/min. Wavelength was set at 258 nm.

2.5. Method validation

Assay performance was evaluated through determination of specificity, linearity, quantification limit, precision, accuracy, recovery and stability.

Specificity was investigated by analyzing six drug-free bottled plasma and volunteer samples for interference of endogenous compounds. The standard curve was obtained through analysis of calibration standard plasma samples and plot of peak area ratios of respective drugs and fenofibric acid versus the corresponding drug concentrations. Linearity of the standard curve was evaluated using least-squares linear regression analysis with weighting 1/*x*. Quantification limit was defined as the lowest drug concentration on the calibration curve. Intra-and inter-day imprecision were determined by repeated analysis of quality control plasma samples on the same day and on different days. Five replicates of each of the three quality control standards and the lowest calibrator were analysed in a batch to establish the method's precision (% relative standard deviation). Accuracy was evaluated by comparing the observed concentration with the true value of spiked controls. The recoveries of rhein, aceclofenac and IS were determined by comparing the response of quality control plasma samples with the response of identical standards prepared in the mobile phase which did not undergo sample pre-treatment. The recovery was evaluated at all the three quality control concentration levels. Stability of rhein and aceclofenac in plasma samples stored at −80 °C, in plasma samples after freeze–thaw cycles and in pre-treated plasma samples (short term) was evaluated. Stability of drugs and IS were also evaluated in respective stock solutions and in mobile phase in the processed samples. Higher and lower quality control standards were used for evaluating the stability of spiked plasma samples subjected to various conditions and the results compared with initial readings. To evaluate the freeze–thaw stability, plasma controls were subjected to three cycles of freezing and thawing. Short-term or temperature stability of spiked plasma controls and drug and IS solutions was evaluated by leaving the samples at room temperature for 6 h. The processed samples were reconstituted with mobile phase and left in the autosampler at 4 °C for 24 h to find the post-processing stability. The dried extracts of quality control samples were kept in −80 °C freezer in stoppered glass tubes for 24 h to check the stability of dry extracts.

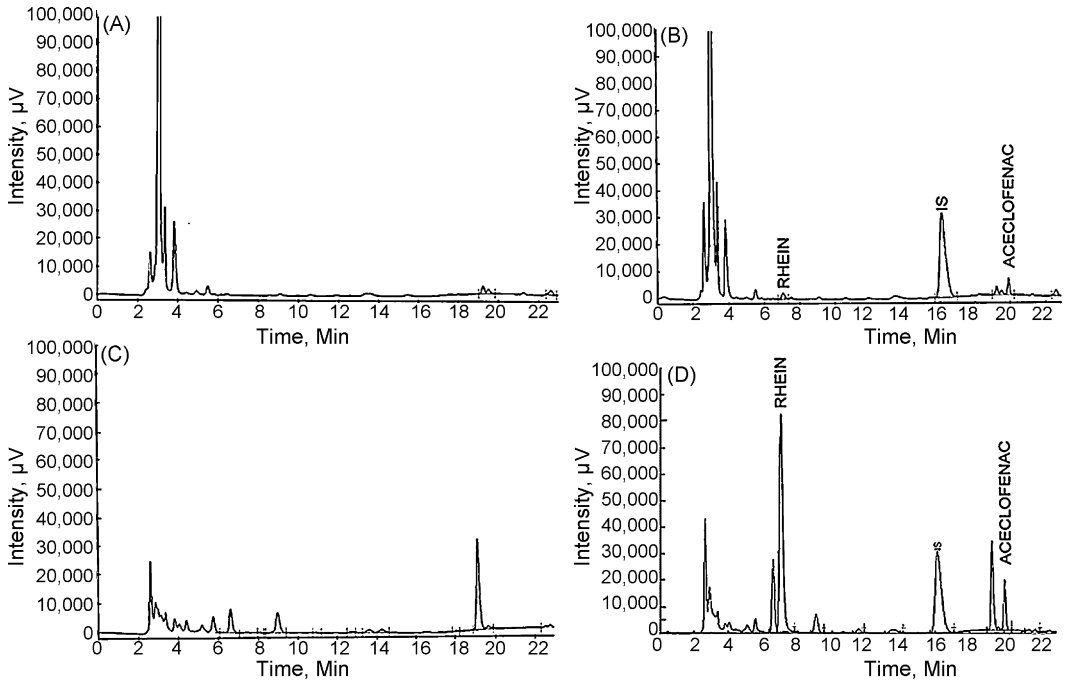

Fig. 2. (A) HPLC–UV chromatogram obtained for blank plasma used for preparing calibration standards and quality control samples; (B) Blank plasma spiked with 0.1 μg/ml rhein and 0.5 μg/ml aceclofenac; (C) A representative zero-hour volunteer plasma sample and (D) 0.5 h volunteer plasma sample.

2.6. Application

The method was used to determine rhein and aceclofenac concentration in plasma samples from 13 healthy volunteers, after oral administration of a tablet containing 50 mg of diacerein and 100 mg of aceclofenac. The dose was administered under overnight fasting conditions. Blood samples were withdrawn at given time intervals and heparin used as the anticoagulant. Plasma was separated by centrifugation at 4000 rpm for 10 min and samples stored at −80 °C.

3. Results and discussion

3.1. Validation

Short elution time, good separation between rhein, aceclofenac and fenofibric acid and endogenous substances and baselines with low background were accomplished by using an octadecyl column with low carbon load. C_{18} analytical columns with different carbon loads showed different selectivity. Synergi Fusion-RP, 250 mm × 4.6 mm, 4 μm gave almost similar selectivity. Increasing the pH of acetate buffer to 7.8 reduced the retention times of aceclofenac and IS but there was interference from plasma components. Finally, a lower pH buffer with gradient elution was found to give the best results. Of all the solvents checked for recovery (ethyl acetate, dichloromethane and ether) ethyl acetate gave good recovery of both the compounds under acidic conditions (pH 4–5). Reducing the pH further gave dirty extracts. Though both the drugs have λ_{max} at different wavelengths (Rhein 230 nm and aceclofenac 275 nm) but 258 nm was selected as both the molecules had good absorption at this wavelength and interference from endogenous substance was also relatively less.

The proposed method is suitable for quantification of rhein and aceclofenac in plasma samples. It showed specificity, since I.S. and drug were well resolved and no interfering peaks from endogenous components of normal plasma were observed, as can be seen from Fig. 2A and B. Average recovery was 85% for rhein, 70% for aceclofenac and 87% for fenofibric acid. The method was linear over the range 0.100–7.0 μg/ml for rhein and 0.500–20.0 μg/ml for aceclofenac. The calibration ranges were selected based on the expected concentration in study samples to which the method was to be applied. The calibration curve could be described by the equation $y = 2.34(\pm0.139)x + 0.036(\pm0.043)$ ($r^2 = 0.999$) for rhein while that for aceclofenac was $y = 8.188(\pm0.761)x + 0.033(\pm0.131)$ ($r^2 = 0.999$). The retention times were 6.7 min (rhein), 15.0 min (fenofibric acid, I.S.) and 19.7 min (aceclofenac). Retention time of diacerein was 5.5 min on the system. The lower quantification limit (LLOQ) was 0.1 μg/ml for rhein and 0.5 μg/ml for aceclofenac. The method precision (%RSD) at LLOQ were 5.71% (intra-day) and 5.39% (inter-day) and accuracy of 96% for rhein (Table 1). The same for

Table 1
Intra- and inter-day precision and accuracy determination of rhein concentration in plasma samples ($n = 5$ at each concentration for intra-day and $n = 3$ days for inter-day precision).

Concentration (μg/ml)	Standard deviation		Precision (%RSD)		Accuracy (%)
	Intra-day	Inter-day	Intra-day	Inter-day	
0.10	0.005	0.002	5.71	5.39	96.0
0.25	0	0.0233	0	10.39	92.0
2.5	0.071	0.061	2.95	3.12	96.6
6.0	0.184	0.079	3.14	2.56	97.6

%RSD, relative standard deviation; %Accuracy = (concentration found/concentration expected) × 100, mean of five replicates.

Table 2
Intra- and inter-day precision and accuracy determination of aceclofenac concentration in plasma samples ($n = 5$ at each concentration for intra-day and $n = 3$ days for inter-day imprecision).

Concentration (μg/ml)	Standard deviation		Precision (%RSD)		Accuracy (%)
	Intra-day	Inter-day	Intra-day	Inter-day	
0.5	0	0.049	0	9.99	108.0
1.25	0.028	0.072	2.34	8.84	95.5
8.0	0.125	0.278	1.54	3.52	101.8
17.0	0.294	1.176	1.76	6.21	98.3

%RSD, relative standard deviation; %Accuracy = (concentration found/concentration expected) × 100, mean of five replicates.

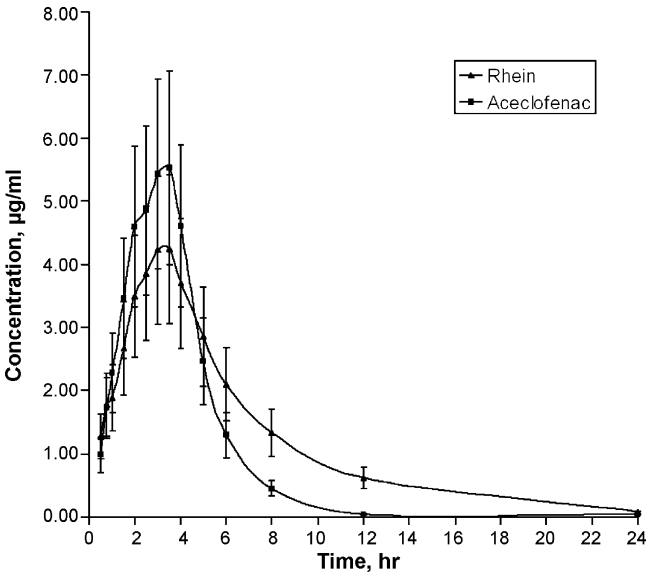

Fig. 3. Mean plasma concentration–time curve of rhein and aceclofenac after oral administration of 50 mg diacerein and 100 mg aceclofenac to 13 healthy volunteers. Vertical bars indicate standard error of mean.

aceclofenac were, precision of 0% (intra-day) and 9.9% (inter-day) and accuracy of 108% (Table 2). Precision and accuracy for quality control plasma samples ranged from 0% to 10.39% and from 92% to 100.3% for rhein and 1.54% to 9.99% and 95.5% to 108%, respectively for aceclofenac. Stability of the two drugs in plasma samples was observed for three freeze–thaw cycles and for 45 days at −80 °C. Reconstituted organic extracts were stable for 24 h in the auto sampler at 4 °C. The spiked plasma samples as well as the solutions were stable for a minimum of 6 h at room temperature. The dried extracts of samples showed stability when left overnight at −80 °C.

The proposed method allows determination of rhein and aceclofenac at concentrations that are good enough for the given dose, with a simple, rapid and efficient sample pre-treatment and has short elution time, thus fulfilling all required criteria for the plasma assay to be considered functional in pharmacokinetic and bioequivalence studies in healthy volunteers.

3.2. Application

Fig. 2C and D are the representative chromatograms of pre-dose subject sample and 0.5 h post-dose sample, respectively. Mean plasma concentration–time curve of rhein and aceclofenac, after oral administration of 50 mg diacerein and 100 mg aceclofenac to 13 healthy volunteers is shown in Fig. 3. The mean C_{max} and t_{max} observed for rhein were 5.05 ± 1.25 μg/ml and 2.88 ± 0.68 h, respectively. The same for aceclofenac were found to be 7.46 ± 1.35 μg/ml and 2.81 ± 0.92 h, respectively. The observed C_{max} and t_{max} are in agreement with those reported in literature [2,21,25].

4. Conclusion

The method has proved to be simple, specific, precise and accurate and is suitable for simultaneous quantification of rhein and aceclofenac in plasma samples from bioequivalence, bioavailability and pharmacokinetic studies in healthy volunteers.

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