Indian Journal of Pharmaceutical Education and Research., 2023; 57(1):278-285.
https://www.ijper.org

Original Article

Validation of Rapid and Simple HPLC-UV Method for Diflunisal Determination in Bulk Drug and Human Plasma

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ABSTRACT

Background: Transthyretin amyloidosis is a rare disease currently under wide investigation and many different therapeutic agents were developed for its control and treatment. In addition to the newly discovered drugs, it was also observed drug repurposing of some well-studied therapeutic agents. Diflunisal was developed in 1971 as an anti-inflammatory and analgesic drug but showed good results when used as a kinetic stabilizer of the transthyretin protein. **Objectives:** Present study describes a high–performance liquid chromatography method for its determination in bulk drug and human plasma by UV detection. **Materials and Methods:** Isocratic elution of the mobile phase (consisting of 0.1% trifluoroacetic acid in water and acetonitrile in the ratio 42:58 v/v) at a flow rate 1.0 ml/min was set and the developed analytical procedure became fast and simple. Chromatographic determination was performed on a Purospher® RP – 18 column at room temperature and a UV detector set at 230 nm. **Results:** The developed method was validated for linearity in the range 0.5–125 µg/ml for the bulk drug and 0.48–120 µg/ml for plasma. Calibration curves were linear over the selected ranges with correlation coefficients (R^2) greater than 0.999. The coefficients of variation for intra- and inter-assay were <2% for both methods – bulk drug and plasma determination. **Conclusion:** The developed effective and specific method can be applied in routine clinical practice for drug therapy monitoring.

Keywords: Diflunisal, Bioanalysis, HPLC-UV, Validation.

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Received: 05-05-2022;
Revised: 27-09-2022;
Accepted: 11-10-2022

INTRODUCTION

A critical step in amyloid formation is the dissociation of protein transthyretin (TTR) into monomers and the developed amyloidosis as a result. A new class of therapeutic agents, called "TTR stabilizers" was developed to affect this process of protein misfolding and stabilize the quaternary structure. Two orally administered drugs (Tafamidis (TAF) and Diflunisal (DIF)) were studied as appropriate for patients not eligible for liver transplantation with the same mechanism of action of binding to the thyroxin-binding sites and providing kinetic stabilization.[1]

DIF (2',4'-difluoro-4-hydroxybiphenyl-3-carboxylic acid) is a non-steroidal anti-inflammatory drug (NSAID) developed in 1971 and initially used in the treatment of arthritis and dysmenorrheal with its analgesic, anti-inflammatory, and antipyretic properties.[2] Another important potential of DIF was discovered a few years ago when it was applied first in animals and then in humans for the treatment of TTR amyloidosis. The results of all conducted clinical trials were incontestable in favor of DIF and its capacity to increase the survival initially in TTR polyneuropathy[3-7] and after 2019 also in TTR cardiac amyloidosis.[8-13] Efficacy and safety of the treatment were additionally confirmed for this diagnosis although it was only repurposed not newly developed. No sufficient evidence was found to demonstrate the superiority of DIF over TAF or vice versa.[14] More detailed comparisons between the pharmacokinetic and pharmacological studies for the two drugs would be needed to prove the advantage of one.

The adverse events characteristic of all NSAIDs used in practice is also common in the application of DIF. Gastrointestinal, renal, and blood-related events are only some caused by chronic DIF therapy but the careful monitoring of patients can reduce significantly the chance of their appearance.[4,15] The deficiency of DIF in its activity and affinity of binding and inhibition of the tetramer dissociation in all pathogenic genetic variants is compensated by the observed very high plasma concentration levels.[16-18] To increase the selectivity, two iodinated derivatives of DIF were studied, but more analyses were needed to confirm their application in practice.[19] An interesting relationship and cooperative stabilization have also been found between clusterin and DIF. It was found that DIF partially recovered the soluble clusterin levels and the high concentration of clusterin is a premise for inhibition of the TTR monomers aggregation.[20,21]

DOI: 10.5530/001954641926

A few chromatographic methods are published in the available literature for the determination of DIF in human plasma,[22-27] urine[25,26] or bulk drug and tablets,[28] using HPLC[22-26,28] or LC–MS/MS.[27] All DIF plasma concentrations were found in healthy volunteers.[22-27,29] Most of the analytical methods found in the literature were developed in the 1980s,[23,25,26] some in the 1990s and 2000s[22,27,29] and two in 2021;[24,28] one for separation and determination of DIF and its impurity in bulk drug[28] and one for separation of three drugs (lesinurad, febuxostat, and DIF).[24] In connection with the initiated "orphan" drug approval procedure by the EMA and the use of DIF in transthyretin amyloidosis we developed a rapid, accurate, and precise reversed-phase high-performance liquid chromatography (RP-HPLC) method for its determination in human plasma. According to International Council for Harmonization (ICH)[30] the developed analytical method has been validated, making it applicable in the routine clinical practice for drug therapy monitoring in patients with TTR amyloidosis.

MATERIALS AND METHODS

Chemicals and reagents

HPLC grade chemicals and reagents were used for method development. The analytical standard of DIF (2',4'-difluoro-4-hydroxybiphenyl-3-carboxylic acid (Figure 1) and internal standard - clofibric acid (2-(4-chlorophenoxy)-2-methylpropanoic acid (Figure 2) were provided from Sigma-Aldrich Co. Blank human plasma standard, HPLC quality acetonitrile and trifluoroacetic acid were also purchased by Sigma – Aldrich Co. All additional reagents needed for method development were proper for HPLC analysis.

Preparation of DIF stock solution I

DIF stock solution I was prepared by dissolving 50 mg in methanol to obtain a final concentration of 1000 µg/ml (50 ml volumetric flask). The calibration curve for bulk drug method validation was based on seven points; respectively seven working solutions were prepared with the following concentrations: 0.5, 12.5, 25.0, 50.0, 75.0, 100.0, and 125.0 µg/ml. For this purpose suitable aliquots

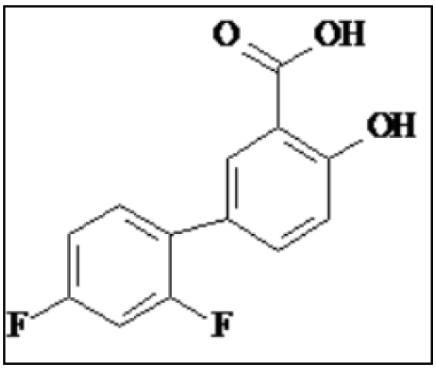

Figure 1: Structure of Diflunisal.

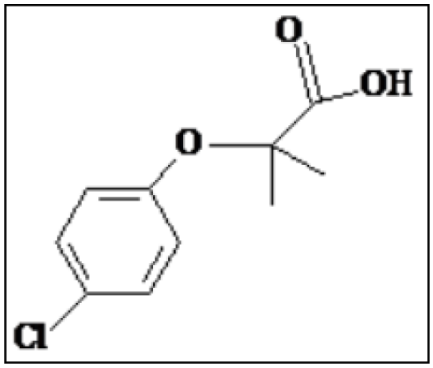

Figure 2: Structure of Clofibric acid.

of the stock solutions were futher diluted with methanol using volumetric flasks.

Preparation of DIF stock solution II and working solutions

Stock solution II (600 µg/ml) of DIF was prepared by dissolving an appropriate amount in methanol. A subsequent dilution of stock solution II was made and as a result, seven working solutions were prepared and used for blank plasma standard spiking. After proper sample preparation steps, linearity was determined and the final DIF plasma concentrations ranged from 0.48 to 120.0 µg/ml.

Preparation of stock solutions I and II of the internal standard (IS)

The stock solution I of the IS (clofibric acid) was prepared by dissolving 75 mg of the substance in methanol. The obtained concentration was 750 µg/ml and an appropriate amount of this solution was used for each of the seven calibration standards preparation. The final IS concentration was 75 µg/ml.

Working solution II was prepared by dissolving 187.5 mg clofibric acid in methanol (1875 µg/ml) and used for the construction of the plasma calibration curve. The final IS plasma concentration was 75 µg/ml. The similar chemical properties and structure of clofibric acid to the target drug - DIF were leading in our internal standard selection.

Calibration curve and plasma sample preparation

A blank plasma standard was used to develop the bioanalytical procedure. The set of standard solutions was prepared by spiking the appropriate amount of the DIF stock solution II to blank plasma. After 10 min incubation at room temperature, samples were vortex mixed for 1 min. A plasma aliquot of 160 µl was mixed up with 40 µl of the IS working solution II and plasma protein precipitation was performed by adding 800 µl 1% (w/v) solution of trichloroacetic acid (TCA) in acetonitrile. Next 10 min vortex mixing and 15 min ultrasonic bath sonication were performed. Finally, samples were shaken at

500 rpm for 20 min at 25°C and centrifuged for 10 min at 13 000 rpm. 800 µl of the supernatant were separated, filtered through a 0.45 µm syringe filter (Whatman, PVDF syringe filter, 0.45 µm), and 20 µl were injected for analysis into the HPLC system. Blank samples were prepared from human plasma standard (Sigma Aldrich, catalog number P9523) by replacing the IS working solution II with the same amount of acetonitrile. No additional effects of plasma components on DIF and IS retention times were found.

Chromatographic conditions

A Shimadzu Prominence chromatographic system was used for method development. It consisted of a vacuum degasser, pump, auto-injector, and UV-VIS detector. Lab Solution Software was used for all results recording and processing. Stationary phase Purospher® RP-18 (150 x 4.6mm, 5µm) chromatographic column, equiped with a Guard Column ODS (TR-C-160-1) was used and the temperature was maintained at 25°C. Trifluoroacetic acid (0.1%) in water and acetonitrile in a ratio of 42:58 v/v were used as mobile phase after proper filtration and sonication. The developed analytical procedure was performed with isocratic elution of the mobile phase at a flow rate 1.0 ml / min and UV - VIS detector set at 230 nm. An injection volume of 20 µl sample was provided and typical chromatograms of bulk drug and plasma calibration standard solution were shown in Figure 3 and 4.

DISCUSSION

Method development and optimization

Based on the preliminary study regarding the physicochemical properties of the analyzed substances as well as the properties of the biological matrix, a selection of several types of columns suitable for the analysis was made. During the experimental work, changes were made only in the type of column packing and its length, and all other parameters were kept unchanged to evaluate only their influence on chromatographic separation. Different reversed-phase columns - ODS (C18) and OS (C8) were tested, with better resolution and sensitivity found for ODS compared to OS. Two columns with different lengths were

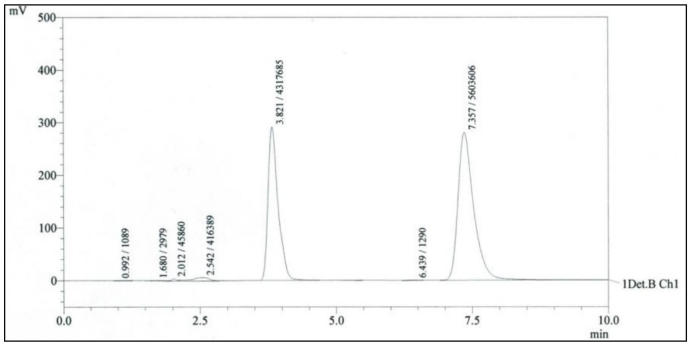

Figure 3: Typical chromatogram of DIF and IS in bulk drug. Chromatogram of DIF calibration standard solution is shown with retention time of DIF 7.36 min and retention time of IS 3.82 min.

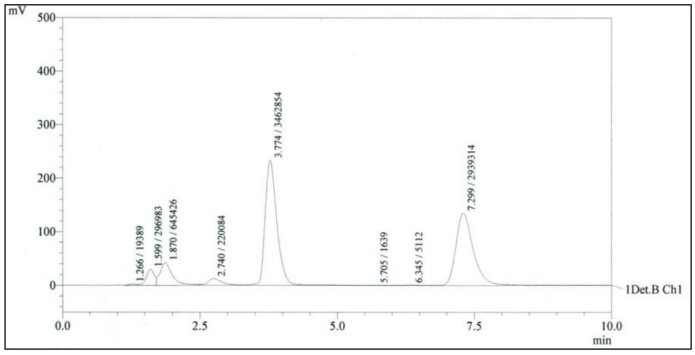

Figure 4: Typical chromatogram of DIF and IS in plasma. Chromatogram of DIF calibration standard solution is shown with retention time of DIF 7.30 min and retention time of IS 3.77 min.

used in the development of the analysis (150 × 4.6 mm and 250 × 4.6 mm), but the column with a length of 150 mm was chosen in connection with providing a shorter total time for analysis of each sample. The results obtained using columns with a particle size of 5 µm were satisfactory, providing stable and reliable conditions for analysis without the risk of clogging the chromatographic column during the elution of biological samples. According to previously collected literature data, different compositions and ratios of solvents as mobile phase components were tested. Although in much of the literature, methanol is preferred as the organic solvent, the results obtained by replacing it with ACN are equally good in terms of preserving the shape and areas of the chromatographic peaks. Three different mobile phase compositions were tested: 0.01 mol/L ammonium formate: ACN, phosphate buffer pH = 3: ACN, and 0.1% TFA in an H_2O: ACN mixture. The mobile phases containing ammonium formate and phosphate buffer pH = 3 did not show satisfactory results, and the chromatographic peaks obtained were broad, asymmetric, and with too short retention times, which makes the method inapplicable in a biological matrix. The best results were obtained using the third mobile phase 0.1% TFA in H_2O: ACN mixture. The selectivity of the method was optimized by varying the organic solvent percentage (from 60:40 v/v to 40:60 v/v). The ratio of 42:58 v/v proved to be suitable, as it showed sufficiently good separation of the internal standard from the matrix components and provided the shortest analytical time. The assay procedure was developed using isocratic elution and a flow rate 1.0 ml/min. Using lower flow rates resulted in a delay in analysis time and peak symmetry breaking. The influence of temperature on the analytical procedure was also evaluated in connection with the published literature data, but no significant improvement in the chromatographic conditions was found when the temperature was increased to 40°C. Room temperature (25°C) was preferred for conducting the quantitative analysis. It was the most favorable and does not pose a risk to the stability of the analyzed substances and the matrix. The selection of the optimal wavelength of the UV detector was based on the obtained spectra of the analytes, taken from solutions of the substances prepared in methanol

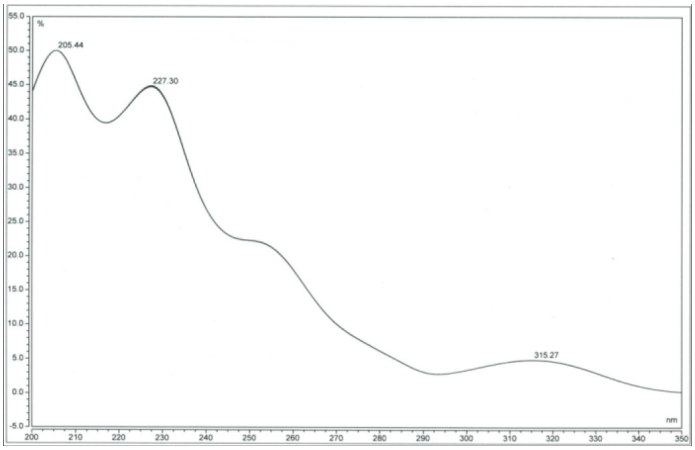

Figure 5: UV spectra for DIF.

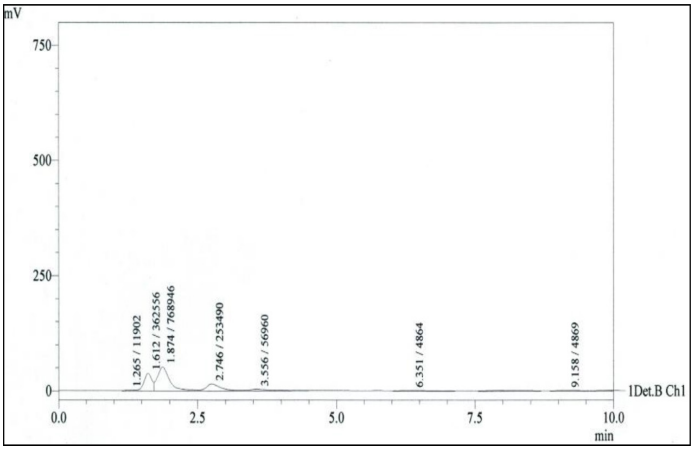

Figure 7: Typical chromatogram of blank plasma sample.

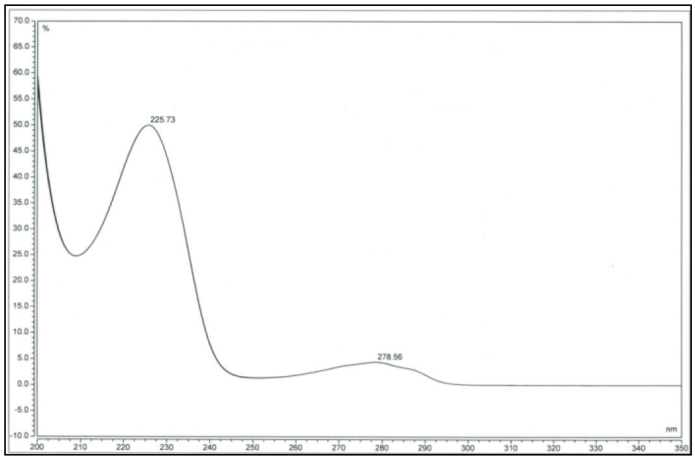

Figure 6: UV spectra for IS (clofibric acid).

(Figure 5 and 6). The wavelength of 230 nm was determined as the most suitable for the analysis, with the maximum absorption observed for both substances and, at the same time, minimum absorption by the organic solvents. The apparent similarities in the physical and chemical properties of TAF and DIF enable the use of the same chromatographic conditions for their quantitative analysis (previously developed by our research team)[31] and the possibility of their simultaneous determination if necessary.

RESULTS

Method validation

The proposed methods for DIF bulk drug and plasma quantification were validated according to ICH bioanalysis guidelines[30] by parameters selectivity, linearity, accuracy, precision, limit of detection, and limit of quantification.

Selectivity

Selectivity of an analytical method represents the ability to determine and measure a target analyte despite the possible presence of interfering substances in the biological matrix. Pure biological plasma (blank sample) obtained from different sources was analyzed in detail and no significant responses of interfering side components at the retention times of the analyte and the IS were established. Blank plasma samples without addition of analyte solution or IS solution were also prepared and analyzed. The mandatory requirement for representative matrix and test samples matrix equivalence (including anticoagulants and additional reagents) was met. Scientifically significant results required maximum acceptable response of the matrix component not more than 20% of the analyte response in LLOQ and not more than 5% of the IS response.

Under the optimized chromatographic conditions no interferences from the matrix components on DIF and IS retention times were observed. Drug substances were well separated with more than 3 min delay in elution of DIF. The representative chromatogram of the blank plasma sample, shown in Figure 7, indicated a high degree of specificity and selectivity of the developed method.

System suitability tests

System suitability tests ensured the system's performance in the course of the analysis of unknowns and verify its resolution and reproducibility. Six consecutive analyses of each test sample a determined concentration level were made, and the following parameters were determined - retention time, peak area, tailing factor, resolution between peaks, and column efficiency (theoretical plates of the column).The optimal chromatographic conditions provided fast and effective drug and IS separation with results meeting the requirements of the European Pharmacopoeia. The number of theoretical plates for both was > 2000, peak asymmetry was < 1.5, and resolution factors were > 2.1. The detailed results of the system suitability parameters are given in Table 1.

Linearity and range

The linearity of the analytical method is the proportional relationship between the concentration and the test results

Table 1: System suitability parameters.

Parameter	Acceptance criteria	Bulk drug		Plasma	
		DIF	IS	DIF	IS
Retention time (min)	-	7.39	3.83	7.30	3.78
Ph. Eur. Theoretical plates	NLT 2000	3373	2169	2735	2097
Ph. Eur. Asymmetry	NMT 2.0	1.43	1.40	1.39	1.38
Ph. Eur. Resolution	NLT 2.0	2.18	2.10	2.12	2.42

Ph. Eur.: European Pharmacopoeia; NLT: not less than; NMT: not more than.

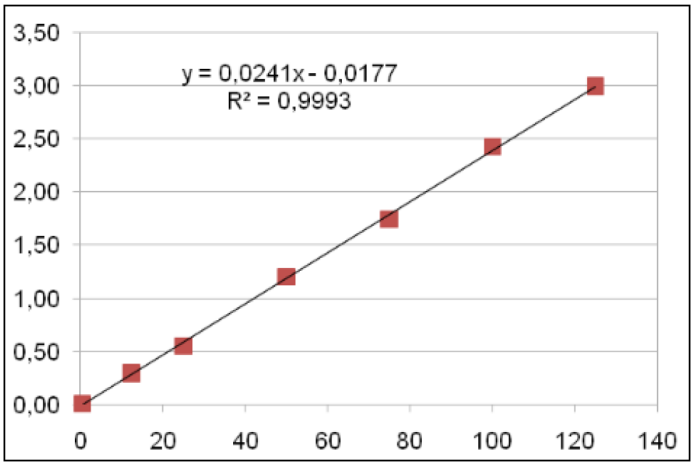

Figure 8: Calibration curve of DIF and IS bulk drug obtained between area ratio of the chromatographic peaks S_{DIF}/S_{IS} and plotted against concentration.

obtained as area or height of the chromatographic peaks. It was verified by the construction of the calibration curve and the obtained R^2 coefficient. In the present analytical procedure all standard solutions were prepared by spiking a certain amount of analyte into an empty biological matrix. Analyses were carried out with freshly prepared calibration standards or frozen samples within their specified stability period. LLOQ (lowest calibration standard) and ULOQ (highest calibration standard) determined the calibration range of at least 6 standard solutions with increasing concentrations of the required analyte. Accuracy on each calibration level must be ±15% of the theoretical concentration (ICH) and ±20% at the LLOQ. Statistically significant results were obtained after measurements were repeated three times in the same sample and follow-up analysis of the area ratios (analyte/IS) for curve shaping.

Seven calibration standards were used for linearity estimation of the developed methods and the results proved that they were sensitive to the determination of DIF in bulk drug and plasma. Linearity was shown in the range 0.5 - 125 µg/ml (Figure 8) and 0.48 – 120.0 µg/ml (Figure 9) in bulk drug and plasma, respectively. Correlation coefficients (R^2) were 0.9993 and 0.9996. According to the requirements of the internal standard method validation, the ratio of DIF/IS areas versus solution concentrations was used for linearity graph construction.

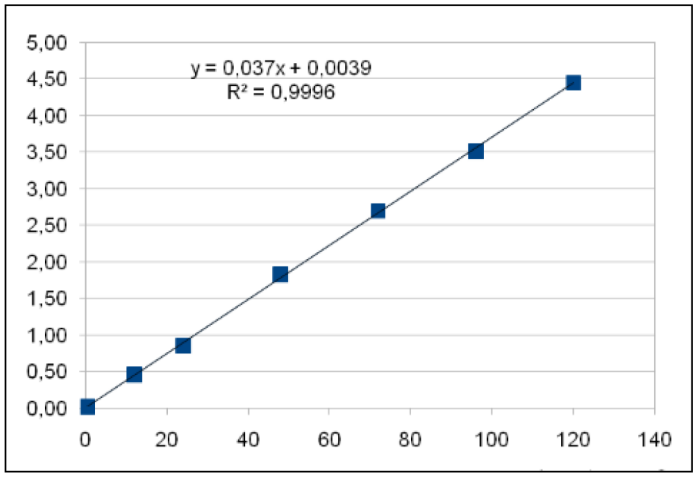

Figure 9: Calibration curve of DIF and IS in plasma obtained between area ratio of the chromatographic peaks S_{DIF}/S_{IS} and plotted against concentration.

Table 2: Results for linearity, accuracy, and precision of the calibration curves in bulk drug and plasma samples (*n*=3).

DIF – bulk drug			
Concentration, µg/ml	C_{DIF} ± SD	Precision %	Accuracy %
0.5	0.45±0.01	2.90	90.63
12.5	12.22±0.13	1.09	97.78
25.0	22.56±1.03	4.55	90.25
50.0	49.26±1.27	2.59	98.52
75.0	71.31±0.08	0.11	95.08
100.0	99.38±0.36	0.36	99.38
125.0	122.56±0.49	0.40	98.04
DIF - plasma			
Concentration, µg/ml	C_{DIF} ± SD	Precision %	Accuracy %
0.48	0.50±0.00	0.63	100.13
12.0	12.57±0.03	0.26	100.58
24.0	23.51±0.06	0.27	94.02
48.0	50.33±0.62	1.24	100.65
72.0	74.42±0.74	0.99	99.23
96.0	96.91±1.83	1.89	96.91
120.0	122.88±1.76	1.43	98.30

SD: standard deviation

The high values for R^2 and obtained regression equations (y = 0.0241x – 0.0177 and y = 0.0370x + 0.0039) were indicators of good method linearity. The calibration points employed for the creation of the calibration curves had a low standard deviation (SD) value (Table 2) and slope, which demonstrated their significant validity.

Accuracy and precision

The degree of agreement of the obtained test results and the true values marked as method accuracy was analyzed at three concentration levels - low quality control sample (LQC), medium quality control sample (MQC), and high-quality control sample

(HQC) with the following values 12.5, 50.0 and 100.0 µg/ml for bulk drug and 12.0, 48.0 and 96.0 µg/ml for plasma samples. Three injections of each standard solution were made before calculating the analyte's percent recovery. The developed sample preparation and analytical procedures were suitable to assure accurate quantification of the target analyte in the sample matrix.

As critical parameters, accuracy and precision were analyzed carefully and thoroughly. The inter- and intra-day variability were evaluated and recovery and % RSD was calculated. Values fall within the defined limits and showed very good accuracy of the analytical method (Table 3). Separately prepared plasma samples with a final concentration of DIF in plasma 12.0, 48.0, and 96.0 µg/ml were analyzed on three consecutive days and the results obtained for % RSD was < 1.9% with a recovery range of 96.2 – 102.1%.

The closeness between a set of measurements of the same homogeneous sample taken under the established conditions following multiple sampling is expressed by an analytical procedure's precision (repeatability). It was determined using a six-fold analysis at the average concentration level, and the percentages of standard deviation (RSD) were calculated using the findings of repeated measurements. By analyzing three samples at six different times during the same day and three separate days, respectively, intra- and inter-day precision were also determined. According to the descriptions in the ICH recommendations, the determined concentrations had to be within 15% of the nominal value for at least two out of the three concentration levels. Precision validation tests of the proposed methods were developed by conducting six consecutive assays of the test samples with concentrations 50 µg/ml and 48.0 µg/ml for bulk drug and plasma, respectively. All results were united in Table 4 and clearly show recovery rates from 96.2 to 100.9% which falls into the acceptance criteria for an analytical method.

Table 3: Inter- and intra – day accuracy for determination of DIF in bulk drug and human plasma (*n*=6).

			Nominal concentration, µg/ml	Calculated concentration, µg/ml and SD	Precision (%RSD)	Recovery (%)
DIF – bulk drug	Inter day	LQC	12.5	12.22±0.02	0.19	97.72
		MQC	50.0	50.87±0.91	1.79	101.74
		HQC	100.0	99.58±0.79	0.80	99.58
	Intra day	LQC	12.5	12.16±0.04	0.32	97.28
		MQC	50.0	48.10±0.07	0.15	96.20
		HQC	100.0	99.21±0.16	0.16	99.21
DIF – plasma	Inter day	LQC	12.0	12.18±0.23	1.85	101.46
		MQC	48.0	47.97±0.16	0.34	99.94
		HQC	96.0	94.49±1.82	1.93	98.43
	Intra day	LQC	12.0	12.24±0.08	0.68	102.01
		MQC	48.0	48.48±0.26	0.54	101.00
		HQC	96.0	95.77±1.53	1.60	99.77

SD: standard deviation; RSD: relative standard deviation

Table 4: Results of the recovery test for the precision parameter (*n*=6).

		DIF – bulk drug			DIF - plasma		
	Sample No.	Drug taken (µg/ml)	Total amount recovered (µg/ml)	Recovery (%)	Drug taken (µg/ml)	Total amount recovered (µg/ml)	Recovery (%)
	1	50.0	50.29	100.57	48.0	47.42	98.78
	2	50.0	48.12	96.24	48.0	47.98	99.96
	3	50.0	50.17	100.34	48.0	48.14	100.29
	4	50.0	50.40	100.79	48.0	46.92	97.76
	5	50.0	48.76	97.51	48.0	47.95	99.90
	6	50.0	49.02	98.04	48.0	48.43	100.89
Average		50.0	49.46	98.92	48.0	47.81	99.60
SD		-	0.95	1.91	-	0.54	1.13
% RSD		-	1.93	1.93	-	1.14	1.14

SD: standard deviation; RSD: relative standard deviation

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

The limit of quantification (LOQ) refers to the lowest amount of analyte that can be accurately and precisely quantified by the analytical technique, while the limit of detection (LOD) refers to the lowest amount of analyte that can be detected but not necessarily quantified as an exact number. Two samples were needed for the LOD determination process: a blank and a sample with a known low analyte concentration. A comparison of the signals acquired for both following the regular measurements were made. The signal-to-noise ratio might also be used to calculate LOD and compare it to an acceptable value (3:1). The same is true for LOQ determination when signal-to-noise ratios of 10:1 are used to compare measurement sample signals to blank samples.

In the developed methods LOD and LOQ were expressed as LOD = 3.3 σ/S and LOQ = 10 σ/S, where σ is the standard deviation of the response and S is the slope of the calibration curve. The smallest measurable DIF sample concentration was 0.05 µg/ml and LOQ was determined as 0.10 µg/ml. Therefore, the developed method was estimated exact and appropriate for the determination of even minimum plasma concentrations in the therapeutic range.

CONCLUSION

The presented RP-HPLC isocratic method was validated for rapid, accurate, and sensitive plasma DIF quantitation within 9 min. UV detection at 230 nm was used and the obtained retention times for the main analyte and the IS were 7.30 min and 3.77 min, respectively. Good resolution, precision, linearity, and isocratic elution were only some of the advantages of the present analytical procedure that made it suitable for application in routine quality control or clinical practice with many samples analysis for a short period in respect of pharmacokinetic studies and therapeutic drug monitoring.

ACKNOWLEDGEMENT

Declared none.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest

ABBREVIATIONS

%; percentage; **°C:** degree Celsius; **µg:** microgram; **µl:** microliter; **µm:** micrometer; **DIF:** diflunisal; **HPLC:** high-performance liquid chromatography; **HQC:** high quality control sample; **ICH:** International Council for Harmonization; **IS:** internal standard; **LC-MS/MS:** liquid chromatography-tandem mass spectrometry; **LLOQ:** lower limit of quantification; **LOD:** limit of detection; **LOQ:** limit of quantification; **LQC:** low quality control sample; **mg:** milligram; **min:** minute; **ml:** milliliter; **mm:** millimeter; **MQC:** medium quality control sample; **NSAID:** non-steroidal anti-inflammatory drug; **RP-HPLC:** reversed-phase high-performance liquid chromatography; **rpm:** rotations per minute; **RSD:** relative standard deviation; **SD:** standard deviation; **TAF:** Tafamidis; **TCA:** trichloroacetic acid; **TTR:** transthyretin; **ULOQ:** upper limit of quantification.

SUMMARY

In the present study, RP-HPLC method was developed for the quantification of Diflunisal in bulk drug and human plasma. The analytical method was validated according to the ICH guidelines for various parameters. Before analysis, all biological samples were prepared using a protein precipitation technique. This analytical procedure can be useful in routine clinical practice for therapeutic drug monitoring.

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Cite this article: Smerikarova M, Bozhanov S, Maslarska V. Validation of Rapid and Simple HPLC-UV Method for Diflunisal Determination in Bulk Drug and Human Plasma. Pharmacog Res. 2023;57(1):278-85.