Determination of Diclofenac Potassium in Human Plasma by LC-MS

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A rapid and sensitive liquid chromatography-mass spectrometry (LC-MS) method has been developed and validated for quantification of diclofenac potassium in human plasma. The analyte was extracted from human plasma by simple precipitation technique. Bupropion was used as the internal standard. A kromasil C18 column provided chromatographic separation of the analyte which was followed by detection with mass spectrometry. The mass transition ion-pair was followed as m/z 296.10 and 214.10 for diclofenac 240.1 and 184.10 m/z for bupropion. The method involves precipitation of diclofenac from plasma, simple isocratic chromatography conditions with mobile phase acetonitrile: water and mass spectrometric detection that enables detection at nanogram levels. The retention times were 1.58 and 1.21 min for diclofenac and bupropion, respectively. The proposed method has been validated with linear range of 150-3181 ng mL for diclofenac. The precision and accuracy values are within ± 10 %. The overall recovery of diclofenac was 41.9 %.

Key Words: Diclofenac, LC-MS, Human plasma.

INTRODUCTION

Diclofenac potassium, a substituted phenyl-acetic acid derivative, designated chemically as 2-[(2,6-dichlorophenyl)amino]benzene acetic acid mono potassium salt. It is a non-steroidal antiinflammatory drug (NSAID) widely used in the management of many inflammatory conditions[1]. In addition to its antiinflammatory effect, diclofenac has analgesic and antipyretic action.

The analytical methods available for the estimation of diclofenac are official in IP[2], BP[3] and USP[4], where as the reported methods for the estimation of diclofenac sodium in the literature are by UV spectrophotometry[5], high performance liquid chromatography (HPLC)[6-8], and gas chromatography(GC)[9] methods.

Different methods have been reported for monitoring of plasma levels of diclofenac. The analytical method reported requires laborious extraction procedure like liquid-liquid extraction or solid-phase extraction (SPE) involving drying and reconstitution, long run time and high quantification limit. It is necessary, therefore, to develop a

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simple, specific, rapid and sensitive analytical method for the quantification of the diclofenac. This paper describes development and validation of a simple, specific, rapid and sensitive LC-MS method for the determination of diclofenac in human plasma with a limit of quantification (LOQ) of 150 ng mL for diclofenac, with a run time of 2.2 min and bupropion used as internal standard.

EXPERIMENTAL

The reference standards of diclofenac potassium and bupropion were obtained from, Aurobindo Pharma (Hyderabad, India), respectively. High purity water was prepared in-house using a Milli-Q water purification system obtained from Millipore (India) Pvt. Ltd. (Bangalore, India). HPLC grade acetonitrile and formic acid were purchased from E. Merck Ltd. (Mumbai, India). Drug free (blank) heparinized human plasma was stored at -70 °C prior to use.

Calibration curves: The stock solutions of diclofenac and bupropion were prepared in methanol at free base concentration of 1000 µg mL. Different mobile phases of methanol:water (30:70 %, v/v), acetonitrile:water (70:30, 80:20 %, v/v), were used in different compositions of mobile phases at different flow rates (1.0, 1.2, 1.5, 1.8 mL/min). The composition of the mobile phase acetonitrile:water in the ratio of 80:20 % v/v at flow rate of 1.0 mL/min gave sharp peaks with minimum tailing and good resolution for diclofenac and internal standard whereas broad peaks and pronounced tailing was observed with other compositions of mobile phases and other flow rates. Blank human plasma was screened prior to spiking to ensure it was free of endogenous interference at retention times of diclofenac and the internal standard bupropion. A eight point standard curve of diclofenac was prepared by spiking the blank plasma with appropriate amount of diclofenac. The calibration curve ranged from 150 to 3181 ng/mL. The samples were vortexed and stored at -70 ± 10 ºC until processing.

A 0.5 mL aliquot of human plasma sample was mixed with 0.1 mL of internal standard working solution (1.0 µg mL of bupropion and 1 mL of acetonitrile as precipitating agent. The resulting solution was vortexed for 5 min and centrifuged at 4000 rpm for 10 min. Supernatants from the above solutions were separated and used for the analysis. 20 µL of the supernant was injected into the LC-MS system.

Instrumentation: Chromatographic separation was carried out in Shimadzu HPLC with Kromasil C18 (100×4.6 mm id, 5µ), column, purchased from Shimadzu Corporation, Japan. Quantitative LC-MS was performed on a binary gradient HPLC with Azilent 1100, API 2000 mass detector, peak scientific-nitrogen generator, Galilio-Roughing vacuum pump, MDS Sciex-electron source ionization. The output signal was monitored and integrated using Analust 1.4 vesion software.

A mobile phase consisting of a mixture of acetonitrile and water (80:20 %, v/v) was used with a flow rate of 1.0 mL min. The total run time for each sample analysis was 2.0 min. Sample introduction and ionization was done in the positive ion mode. The spray voltage and capillary temperature were 1.3 KV and 400 ºC, respectively.

The mass transition ion-pair was selected as m/z 296.0 and 214.10 for diclofenac m/z 240.1 and 184.10 for bupropion. The data acquisition was ascertained by LC-MS solution data station. For quantification, the peak area ratios of the target ions of the drugs to those of the internal standard were compared with weighted (1/amount) calibration curves in which the peak area ratios of the calibration standards were plotted *versus* their concentrations.

Validation: The method was validated for selectivity, sensitivity, linearity, precision, accuracy and stability. The selectivity of the method was evaluated by comparing the chromatograms obtained from the samples containing diclofenac and the internal standard with those obtained from blank samples. Sensitivity was determined in terms of LLOQ (lower limit of quantification) where the response of LLOQ was at least five times greater than the response of interference in blank matrix at the retention time or mass transitions of the analyte. For linearity, different concentrations of standard solutions were prepared to contain 150 ng/mL to 3181 ng/mL of diclofenac containing 1.0 μg/mL of bupropion. These solutions were analyzed and the peak areas and response factors were calculated. The calibration curve was plotted using response factor *vs.* concentration of the standard solutions. Standard curve fitting was determined by applying the simplest model that adequately describes the concentration-response relationship using appropriate weighing and statistical tests for goodness of fit. The precision of the method was determined by intraday precision and interday precision. The intra-assay precision and accuracy was calculated for 6 replicates at each lower limit of quantification (LLOQ), low quality control (LQC), middle quality control (MQC) and high quality control (HQC) levels, each on the same analytical run and inter-assay precision and accuracy was calculated after repeated analysis in three different analytical runs. Accuracy of the developed method was determined by relative and absolute recovery experiments. The relative recovery of the drug was calculated by comparing the amount of the drug obtained from the drug supplemented plasma with the actually added amount. Recovery studies were carried out for three levels at 6 times and the percentage recovery, mean, standard deviation and coefficients of variation were calculated.

RESULTS AND DISCUSSION

Method development: The objective of this work is to develop and validate a simple, rapid and sensitive assay method for the quantification of diclofenac which is suitable to determine the pharmacokinetics in clinical studies. To achieve the objective, different options was evaluated to optimize sample extraction, detection parameters and chromatography during method development. The standard solutions of diclofenac were analyzed by LC-MS system using direct injection probe with ESI and APCI interfaces. From the mass spectrum recorded, the detection molecular ion selected was for diclofenac 214.10.0 m/z and for bupropion 184.10 m/z.

Optimization of the chromatographic conditions: Optimization of the chromatographic conditions are intended to take into account the various goals of method development and to weigh each goal (resolution, run time, sensitivity, peak symmetry, *etc.*) accurately, according to the requirement of LC-MS and HPLC methods being used for the estimation of drugs in biological fluids. Different mobile phases, namely, acetonitrile, methanol and tetrahydrofuran in aqueous phase were used at a flow rate of 1.0 and 0.5 mL min. For the initial separation conditions, acetonitrile was used because of its favourable UV transmittance, low viscosity and better solubility. When acetonitrile was substituted by other solvents, the solvents to buffer ratios were calculated using solvent strength. The resulting ratios of the mobile phase were prepared and the drugs chromatographed. These mobile phases gave well retained and symmetrical peaks. The standard solution was chromatographed with mobile phases of different ratios of organic and aqueous phases at a flow rate of 1 mL/min and 0.5 mL/min. Water:acetonitrile in the ratio of 20:80 was selected as the mobile phase. Reversed phase C18 stationary phases were used and the chromatograms were recorded. Based on the retention and peak shape, Kromasil C18 column was selected for diclofenac. Typical chromatogram of diclofenac and bupropion are given in Fig. 1.
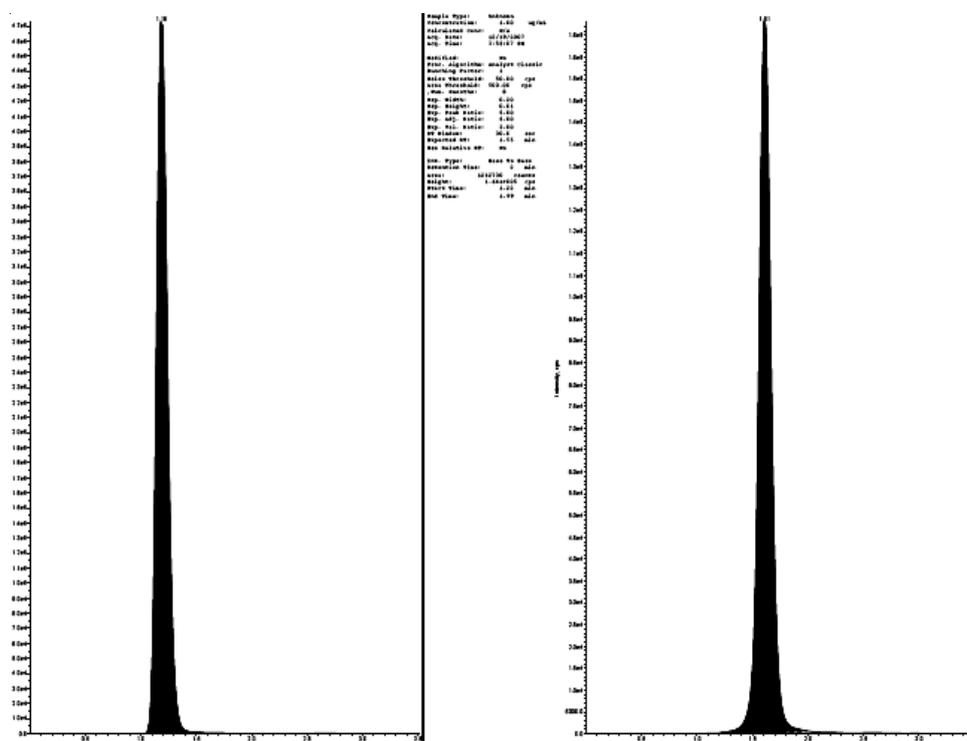

Fig. 1. Diclofenac (drug) and bupropion (internal standard) chromatograms

Validation: Estimation of diclofenac in plasma samples from the volunteers was carried out using optimized chromatographic conditions. The validation parameters such as accuracy, precision (repeatability and reproducibility), linearity and range, sensitivity (limit of detection and limit of quantitation) robustness/ruggedness, selectivity/specificity and system suitability were evaluated. The validation results are given in Tables 1 and 2.

TABLE-1
OPTIMIZED CHROMATOGRAPHIC CONDITIONS

Parameters	Method
Stationary phase (column)	Kromosil ODS C-18, 100 x 4.6 mm, 5 µm packed with 5 µ)
Mobile phase	Acetonitrile:water (80:20) with formic acid 460l
Flow rate (mL/min)	1.0 µmL
Column temperature (ºC)	Ambient
Volume of injection loop (µL)	20
Detection limits	
Drug	Q1 mass 296.10;Q3 mass 214.1 (m/z)
ISTD	Q1 mass 240.10;Q3 mass 184.10(m/z)
Polarity	Positive
Internal standard	Bupropion
Drug RT (min)	1.50 ± 0.30 min
Internal standard RT (min)	1.20 ± 0.30 min
Run time	2.2 min

TABLE-2
RECOVERY OF DICLOFENAC

	Drug					
	LQC		MQC		HQC	
	Aqueous area	Extracted area	Aqueous area	Extracted area	Aqueous area	Extracted area
Mean	34093.3	15899.5	252810.3	108460.7	491614.0	178815.3
SD	1023.7	2015.6	24641.1	5737.4	6078.3	11214.9
% CV	3.0	12.7	9.7	5.3	1.2	6.3
% Recovery	46.6		42.9		36.4	

Accuracy: The accuracy of the optimized methods was determined by relative and absolute recovery experiments. The percentage recovery values for diclofenac ranged from 36.4 to 46.6. The coefficient of variation (%) of these values was less than 10.00 %. It is indicative that the developed methods are accurate and reliable.

Precision: The optimized method for the estimation of diclofenac was found to be precise. This was evident from the coefficient of variation values, which were less than 10.00 % at all concentrations.

Selectivity: The selectivity of the method was evaluated by comparing the chromatograms obtained from the samples containing diclofenac and the internal

standard with those obtained from blank samples. These chromatograms were compared with the chromatograms obtained from standard solutions. Each chromatogram was tested for interference. The combination of the sample preparation procedure and chromatography provided an assay which is free from significant interfering endogenous plasma components at the retention times of the selected drugs and the internal standard. These observations show that the developed assay method is specific and selective.

Linearity: It was observed that the optimized methods were linear within a specific concentration range for diclofenac. The calibration curves were plotted between response factor and concentration of the standard solutions. The linearity ranges were found to be 150 to 3181 ng mL^{-1}. The results indicated no significant interday variability of slopes and intercepts over the optimized concentration range.

Limit of detection: The limit of detection (LOD) value was found to be 125 ng/mL for diclofenac and their limit of quantification (LOQ) value was 150 ng/mL. These observations indicate that the developed methods have adequate sensitivity. This value, however, may be affected by the separation conditions (*e.g.*, column, reagents and instrumentation and data systems), instrumental changes (*e.g.*, pumping systems and detectors) and use of non HPLC grade solvents and may result in changes in signal to noise ratios.

Ruggedness: The ruggedness of the methods were studied by changing the experimental conditions. No significant changes in the chromatographic parameters were observed when changing the experimental conditions (operators, source of reagents and column of similar type) and optimized conditions (pH, mobile phase ratio and flow rate).

From the linearity it was found that the drug obeys linearity within the concentration range of 150-3181 ng/mL for diclofenac. From the results shown in recovery (Table-2), it was found that the recovery for drug and internal standard was reproducible. The percentage recovery value of drug and internal standard from human plasma is 36.4 and 78.8 %, respectively.

Conclusion

The proposed method was found to be simple, precise, accurate and rapid for determination of diclofenac in human plasma. The mobile phase is simple to prepare and economical. The sample recoveries in all formulations were in good agreement with their respective label claims and they suggested non-interference of other endogenous materials in the estimation. Hence, this method can be easily and conveniently adopted for routine analysis of diclofenac in human plasma using LC-MS/MS.

REFERENCES

1. B.G. Katzung, Basic and Clinical Pharmacology, The Lange Medical Publications, International Edition, edn. 9, Ch. 36, p. 383 (2004).
2. Indian Pharmacopoeia, Government of India, Ministry of Health and Family Welfare, The Controller of Publications, Delhi, Vol. 1, p. 242 (1996).
3. British Pharmacopoeia, Published on the Recommendations of the Medicines Commission, Department of Health, United Kingdom, Vol. 1, edn. 1, p. 559 (2001).
4. USP 24, The United States Pharmacopoeia, NF 19, Asian Edition, United States Pharmacopoeial Convention, Inc., Twinbrook Park Way, Rockville, MD, p. 546 (2000).
5. A.K. Shakya, G.K. Joshi and P. Mishra, *Indian J. Pharm. Sci.*, **54**, 44 (1992).
6. Y.K. Agrawal and K. Shivramchandra, *J. Pharm. Biomed. Anal.*, **9**, 97 (1991).
7. J. Moncrieff, *J. Chromatogr. Biomed. Appl.*, **115**, 185 (1992).
8. D. Grandjean, J.C. Beolor, M.T. Quincon and E. Savel, *J. Pharm. Sci.*, **78**, 247 (1989).
9. H. Kadowaki, M. Shiino, I. Uemura and K. Kobayashi, *J. Chromatogr.*, **308**, 329 (1984).

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