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Research Article

Development and Validation of a Bioanalytical Method for Direct Extraction of Diclofenac Potassium from Spiked Plasma

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Abstract

Purpose: *To develop and validate a user-friendly spiked plasma method for the extraction of diclofenac potassium that reduces the number of treatments with plasma sample, in order to minimize human error.*
Method: *Instead of solvent evaporation technique, the spiked plasma sample was modified with H_2SO_4 and NaCl, respectively, and then the drug was extracted after vortexing the sample with acetonitrile as precipitating agent. The separation of diclofenac potassium and internal standard (ketoprofen) was achieved at preset conditions: 5 µm ODS Hypersil C-18 (4.0 mm x 250 mm) column, eluted with 50% acetonitrile in water (v/v) as mobile phase containing ammonium acetate and triethylamine (TEA), at a flow rate of 1 mL min^{-1}.*
Results: *The peaks of the drug and internal standard (I.S.) were resolved at 14 ± 1 min and 7 ± 1 min, respectively. The calibration curve and linearity were determined over the concentration range of 0.25 to 40 µg mL^{-1} and they were linear (r^2 = 0.9991 and 0.9982, respectively). The accuracy was > 81.32 %. Limit of detection and limit of quantification were 0.05 and 0.25 µg mL^{-1}, respectively, while the recovery range for diclofenac potassium and ketoprofen was more than 79 and 85 %, respectively. The absolute average difference of 0.18 between the observed concentrations for intra- and inter-day studies indicated that the sample was stable for over one month.*
Conclusion: *The proposed method may be applied to routine bioanalysis, particularly for NSAIDs, due to its high sensitivity, specificity, repeatability, reproducibility, robustness and ruggedness.*

Keywords: *Bioanalytical method, Diclofenac potassium, RP-HPLC method, NSAIDs, Plasma*

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INTRODUCTION

Diclofenac potassium is a faintly yellowish white to light beige, virtually odorless, slightly hygroscopic crystalline powder. It is freely soluble in methanol, soluble in ethanol and water, and practically insoluble in chloroform and in dilute acid. The n-octanol/water partition coefficient is 13.4 at pH 7.4 and 1545 at pH 5.2. It has a single dissociation constant (pKa) of 4.0 ± 0.2 at 25℃ in water [1]. Diclofenac potassium (Diclo-K) is a substituted phenyl-acetic acid derivative and widely used in the management of many inflammatory conditions [2-3]. It also has analgesic and antipyretic actions. In spite of its pharmacological importance, relatively few studies are known to explain analytical quantification of diclofenac potassium in human plasma.

HPLC is a powerful tool for analyzing small analytes such as drugs in biological samples. However, analysis of analytes present in matrices such as plasma requires well-designed sample preparation procedures such as protein precipitation, centrifugation, extraction and filtration that can introduce experimental errors and ultimately reduce precision and accuracy of measurement [3-4]. An attractive approach would be to reduce the number of treatments in sample preparation procedure in order to minimize human and non-human errors. Reverse phase high performance liquid chromatography (RP-HPLC), a bonded phase chromatographic technique, uses water as a base solvent. Separation based on solvent strength and selectivity also may be affected by column temperature and pH. In general, the more polar component (diclofenac potassium) elutes faster than the less polar one [4]. In the pH range 2 - 8, RP-HPLC resolves polar components efficiently [5].

The aim of this work was to develop a simple, accurate, reproducible and sensitive method for the determination of diclofenac potassium in human plasma using rapid, convenient and simple liquid-liquid extraction and reverse phase HPLC method based on UV-visible detection [6].

EXPERIMENTAL

Materials

All the reagents used were of analytical grade. Diclofenac potassium was donated by PDH Pharmaceuticals Pvt Ltd, Lahore, ketoprofen by Sanofi Aventis Limited Karachi, Pakistan. Acetonitrile, methanol, ammonium acetate, triethylamine (TEA), sodium chloride, sulphuric acid and ortho-phosphoric acid 85 %v/v were obtained from Merck, Germany. HPLC grade water was obtained using a water distillation appratus (model, IM-100, Irmeco GmbH, Germany) and filtered, prior to use, through a vaccum filter assembly (Sartorius Goettingen, Germany) containing a cellulose acetate filter of 0.45µm pore size.

Instrumentation

HPLC (Perkin Elmer, Series 200, USA) with TCNav software, consisted of a binary pump solvent delivery system, an ultraviolet-visible (UV-Vis) variable wavelength detector, integrator NCI 900 was used. Samples were introduced into a Rheodyne 20 µL fixed-loop injector with a 50 µL glass syringe. Chromatographic separation was performed on a 5 µm particle size, 4.0 mm x 250 mm ODS Hypersil C18 stainless steel analytical column (Thermo Electron Corporation, UK). All solvents were degassed with a sonicator (Elma D78224, Germany) and pH were checked with a pH meter (Inolab, Series WTW, Germany) prior to use.

Preparation of mobile phase

To prepare 1 L of mobile phase, 500 mL of HPLC grade water was added to 500 mL of acetonitrile. Freshly prepared 0.1 M ammonium acetate (2 mL) and 100 µL TEA were added, consecutively, to the mixture. This order of mixing was strictly maintained throughout the study. The pH of the mobile

phase was adjusted to 3.0 using orthophosphoric acid. The mobile phase was prepared daily and was not recycled during use.

Preparation of stock solution

Stock solution of diclofenac potassium was freshly prepared in 50% (v/v) aqueous acetonitrile to give a final concentration of 1 mg mL^{-1} (1000 µg 1000 $µL^{-1}$). Working solutions of diclofenac potassium were prepared in 50% (v/v) acetonitrile by appropriate dilutions to give appropriate concentrations of 0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10, 20 and 40 µg mL^{-1}. All the solutions were stored at -20 ^{0}C and protected from light.

Stock solution of the internal standard (ketoprofen) was prepared with 50% (v/v) acetonitrile to give a concentration of 1 mg mL^{-1} (1000 µg 1000 $µL^{-1}$) and then further diluted to 250 µg mL^{-1}.

Spiked plasma method

The modified method [7,8] involved acidifying 500 µL of plasma sample, containing 20 µL of Diclo-K and 50 µL of 250 µg mL^{-1} ketoprofen (I.S), with 500 µL of 1.74 M sulphuric acid. The plasma sample was modified with 20 µL of 3M NaCl and vortexed for 1 min [9,10]. Acetonitrile (500 µL) was added and vortexed for 30 s. The order of mixing was maintained throughout the study. The resultant mixture was centrifuged at 3000 rpm for 10 min. The supernatant was separated in Eppendrof tubes. An aliquot (20 µL) of this supernatant was chromatographed.

Preparation of standard curve

A standard curve was prepared by spiking 500 µL plasma samples with 20 µL of one of the working solutions prepared above to produce calibration curve points equivalent to 0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10, 20 and 40 µg $mL^{-1}$of diclofenac potassium.

Each suppernatant (20 µL) was injected with a 50 µL HPLC syringe and absorbance was taken at 276 nm. All the tests were performed in triplicate.

Extraction recovery

Recovery was determined by comparing the values of the peak area of spiked plasma and its relevant solution using Eq 1.

$$\% \text{ Recovery} = (\text{Diclo-K}_{ext}/\text{I.S})/ (\text{Diclo-K}_{non\text{-}ext}/\text{I.S}) \times 100 \quad \ldots\ldots (1)$$

where Diclo-K_{ext}/I.S = area ratio of extracted diclofenac potassium to ketoprofen, and
Diclo-$K_{non\text{-}ext}$/I.S = area ratio of solution of diclofenac potassium to ketoprofen

Method validation

The method was validated in accordance with European Medicines Evaluation Agency (EMEA) guidelines [11] on validation of bioanalytical methods. General tests performed were: recovery, accuracy, precision, reproducibility, linearity, specificity, limit of detection and quantification and ruggedness [12-14].

Specificity (Selectivity)

To evaluate the specificity of the developed method, placebo and appropriate test sample (spiked plasma) were run in triplicate on HPLC the preset conditions and the results compared, statistically.

Linearity of the calibration line/range

The calibration plots for the analyte in plasma were prepared by spiking drug-free plasma with standard stock solutions to yield concentrations of 0.25 - 40.0 µg mL^{-1} for diclofenac potassium. The linearity among nominal concentrations and observed peak area ratio was calculated by least squares method using regression line.

Accuracy and bias

Accuracy and bias were determined for the developed method by performing measurements in consecutive and non-consecutive sequences. For single analyte detection, the linearity was determined within the range of 50 – 150 % or 60 – 140 % or 80 –120 % of the target concentration. Accuracy was calculated as in Eq 2.

$$Accuracy\ (\%) = (C_{Obs.\ Mean} / C_{Nom}) \times 100 \quad \ldots\ldots\ldots (2)$$

Experimental errors in the test were determined using the values of standard deviation (SD) and standard error mean (SEM) while the accuracy of the results in consecutive and non-consecutive fashion was defined by values of the 95 % confidence of interval (95% CI) at lower (L) and upper limits (U).

Precision

It was desirable to have an injection precision in terms of relative standard deviation (RSD). The precision of the method was defined with the existence of the results in range, i.e., 0 - 10 [15-18]. The results of the developed method were validated by inter-day precision after one month.

Limits of detection (LOD) and Quantification (LOQ)

The LOD for extracted diclofenac potassium from spiked plasma sample was measured at signal to noise (S/N) ratio of 2:1 with precision and accuracy. To determine LOD and LOQ, the plasma samples were chromatographed with descending order of the spiked concentrations of analyte. Based on the relative standard deviation (RSD) obtained, the LOQ was subsequently validated by replicate analysis (≥ 6) of samples prepared around this concentration. The LOD was taken as 0.2 x LOQ [19] and validated by comparing the known low concentrations with blank samples (n ≥ 6) [20].

RESULTS

First, this method was implemented for the extraction of diclofenac potassium. Thereafter, it was applied to extract ketoprofen as well. The peak areas were noted for each concentration under preset conditions shown in Table 1.

Table 1: Optimal chromatographic conditions

Parameter	Specification
Column	5 µm ODS Hypersil C-18 (4.0 mm x 250 mm)
Guard column	Uniguard TEC
Mobile phase	50 % acetonitrile (100 mL) + 0.1 M ammonium acetate (2 mL) + TEA (10 µL)
Flow rate	1.0 mL min^{-1}
Detection wavelength	276 nm
Sensitivity	0.2 AUFS
Injection volume	20 µL
Retention time of IS	7 ± 1 min
Retention Time of drug	14 ± 1 min
Temperature	Ambient

The recovery values (mean ± SEM) of diclofenac potassium and ketoprofen were 98.09 ± 5.42 and 104.5 ± 6.76, respectively. Bias of the results are shown in Table 2.

Table 2: Recovery data for diclofenac potassium and ketoprofen

Nominal conc. (µg/mL)	Ketoprofen		Diclofenac potassium	
	Recovery (%)	Bias	Recovery (%)	Bias
0.25	85.54	14.46	116.91	-16.91
0.50	96.10	3.90	104.06	-4.06
1.00	126.51	-26.51	79.04	20.96
2.50	141.77	-41.77	70.54	29.46
5.00	99.54	0.46	100.46	-0.46
10.00	94.58	5.42	105.73	-5.73
20.00	95.60	4.40	104.61	-4.61
40.00	96.77	3.23	103.34	-3.34

The selectivity of the developed method was good based on the fact that there was no interfering peak in the chromatograms (not shown) at the retention time of diclofenac potassium. Both drugs (diclofenac potassium and ketoprofen, i.e., IS) were separated sufficiently at 14 ± 1 min and 7 ± 1 min, respectively. The calibration equation ($y = 20132x - 1740$, with $R^2 = 0.9991$) indicated a high degree of linearity as well as specificity of the method. At lower concentrations of diclofenac potassium, i.e., 0.25 and 0.5 µg mL^{-1}, % recovery of the sample was in a wider range thus indicating lower accuracy of the results due to sensitivity of the HPLC method at lower concentrations as shown in the Table 3.

It was observed that the accuracy of the results for developed method was better for higher concentrations than for lower concentrations of the drug as shown in Table 3. Precision was considered with intra- and inter-day performance of the developed method, as shown in Tables 4 and 5.

All the results were within the defined range except for the lower concentration, i.e., 0.25 µg mL^{-1} in intra-day study. Inter-day statistics also showed the stability of the extracted sample at -20 ºC. The LOD was 0.05 µg mL^{-1}. The quantifiable concentration for diclofenac potassium in biological sample was 0.25 µg mL^{-1} under the stated HPLC conditions.

Table 3: Accuracy and bias data for diclofenac potassium

Nominal conc. (µg mL^{-1})	**0.25**	**0.50**	**1.00**	**2.50**	**5.00**	**10.00**	**20.00**	**40.00**
Mean observed conc. (µg mL^{-1})	0.43	0.67	0.93	2.03	4.60	10.37	20.00	39.90
SD	0.12	0.15	0.15	0.21	0.20	0.21	0.00	0.17
SEM	0.07	0.09	0.09	0.12	0.12	0.12	0.00	0.10
95% CI (L)	0.15	0.29	0.55	1.52	4.10	9.85	20.00	39.47
95% CI (U)	0.72	1.05	1.31	2.55	5.10	10.88	20.00	40.33
Bias	0.18	0.17	-0.07	-0.47	-0.40	0.37	0.00	-0.10
Accuracy (%)	173.32	133.34	93.33	81.32	92.00	103.70	100.00	99.75

Table 4: Intra-day statistics for diclofenac potassium

Nominal conc. (µg mL^{-1})	**0.25**	**0.50**	**1.00**	**2.50**	**5.00**	**10.00**	**20.00**	**40.00**
Mean observed conc. (µg mL^{-1})	0.33	0.57	0.93	2.13	4.70	10.67	19.80	39.93
SD	0.12	0.06	0.06	0.06	0.17	0.50	0.20	0.12
SEM	0.07	0.03	0.03	0.03	0.10	0.29	0.12	0.07
95% CI (L)	0.05	0.42	0.79	1.99	4.27	9.42	19.30	39.65
95% CI (U)	0.62	0.71	1.08	2.28	5.13	11.92	20.30	40.22
RSD	34.65	10.19	6.19	2.71	3.69	4.72	1.01	0.29

Table 5: Inter-day statistics for diclofenac potassium

Nominal conc. (µg mL^{-1}) ➡	0.25	0.50	1.00	2.50	5.00	10.00	20.00	40.00
Mean observed conc. (µg mL^{-1})	0.26	0.54	1.06	2.50	5.20	10.65	20.00	40.30
SD	0.02	0.04	0.03	0.04	0.10	0.64	0.00	0.21
SEM	0.01	0.02	0.02	0.02	0.06	0.37	0.00	0.12
95% CI (L)	0.22	0.45	0.99	2.40	4.94	9.05	20.00	39.78
95% CI (U)	0.29	0.63	1.13	2.61	5.45	12.24	20.00	40.83

DISCUSSION

From the recovery data, the developed method is suitable for diclofenac potassium and ketoprofen. There were neither overlapping nor interfering peaks at the retention times of the drugs so it was presumed that all the plasma proteins precipitated efficiently during the plasma treatment process. Thus this reduced sample processing time. Although there was variation in bias this may be reduced by treating the samples more carefully during the extraction process. The mobile phase used in this work was so simple and economical; so also was the extraction procedure which was short in duration. Thus, the chances of column damage and degree of human errors were minimal. Recovery of both drugs (diclofenac potassium and ketoprofen) in the different assays under the same pre-set conditions may indicate the suitability of the developed method for NSAIDs and its analogs. Regarding the stability of the drug in human plasma, the intra- and inter day statistics were favourable. The use of sulphuric acid and sodium chloride was critical to the effectiveness of the extraction procedure.

Precautionary measures

During sample pretreatment, the following precautionary measures must be followed: (a) Molar solutions of NaCl and H_2SO_4 that 24-h old must not be used. It was found that old solutions did not precipitate plasma proteins efficiently. (b) The solutions must be accurately prepared at the exact molar concentrations; otherwise, there will be improper extraction of the drugs from the plasma. (c) Do not inject spiked plasma if it is turbid. (d) Be careful regarding the order of mixing. Any change in mixing order may affect the results.

CONCLUSION

The proposed method may be applied to routine analysis of diclofenac potassium due to its high sensitivity, specificity, repeatability, reproducibility, robustness and ruggedness. The method is also applicable to ketoprofen which was used as the internal standard (IS). The extraction procedure is especially time-saving for *in vivo* analysis of NSAIDs.

CONFLICT OF INTEREST

The authors declare no conflict of interest. No financial support was received for this work by any of the authors

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