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Determination and pharmacokinetics of diclofenac in rabbit plasma by gas chromatography with mass spectrometry

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ABSTRACT

This paper describes a gas chromatography-mass spectrophotometry (GC-MS) method for determination of diclofenac in rabbit plasma. Diclofenac and internal standard (IS) naproxen were extracted by liquid-liquid extraction method. The samples were separated by GC on a DB-5MS analytical column and determined by a quadrupole mass spectrometer detector operated under selected ion monitoring mode (SIM). Excellent linearity was found between 5 and 600ng mL^{-1} (r=0.999) for plasma samples. Intra-day and inter-day precisions expressed as the relative standard deviation (RSD) for the method were 2.13-3.21% and 4.62-5.83%, respectively. The recoveries for all samples were >92.3%. This assay was successfully applied to a pharmacokinetic study of diclofenac in New Zealand white rabbits. As a result, the plasma half-life was 65.8 ± 16.27 min and the mean $AUC_{0\text{-}360\ min}$ was 5841.8 ± 1382.8 min ng mL^{-1}. The maximum plasma concentration (C_{max}) of 587.1 ± 74.21ng mL^{-1} reached 45.0 ± 13.41 min after administration.

KEYWORDS

Diclofenac;
GC-MS;
Liquid-liquid extraction;
Rabbit;
Pharmacokinetic study.

INTRODUCTION

Diclofenac is a nonsteroidal anti-inflammatory drug (NSAID) that is widely prescribed for the treatment of rheumatoid arthritis, osteoarthritis, musculoskeletal injuries, and post surgery analgesia in human and veterinary medicine. Patients are frequently given special formulations of diclofenac or a co-treatment agent as a therapeutic strategy to attenuate the gastrointestinal tract complications that limit the use of diclofenac and other NSAIDs[1-3]. Many patients prescribed diclofenac for arthritis also take additional drugs for other chronic health problems, such as hypertension[4,5].

Several methods have been reported for determination of diclofenac including gas chromatography-mass spectrometry (GC-MS)[6-8] high-performance liquid chromatography (HPLC)[9-23] and LC-MS-MS[24] in plasma and other biological fluids.

The present work describes the O-silylation of the hydroxyl group of diclofenac using N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) as a silylating reagent. This is followed by determination of diclofenac after a derivatization procedure in rabbit plasma using internal standard methodology by GC-MS. The developed method was validated by using linearity, stability, precision, accuracy and sensitivity

parameters according to International Conference on Harmonization (ICH) guidelines[25].

The advantages of present method include simple and single step extraction procedure using inexpensive chemicals and short run time. Also, this method was used to assay the diclofenac in plasma samples obtained from three rabbits which had been given an oral tablet of Dolorex dragee (50mg diclofenac).

EXPERIMENTAL

Chemicals and reagents

Diclofenac and naproxen were obtained from Sigma (St.Louis, MO, USA). N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA), ethylacetate, diethylether, hexane and acetonitrile were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dolorex dragee (50 mg diclofenac) was obtained by pharmacy (Erzurum, Turkey).

Apparatus and analytical conditions

Chromatographic analysis was carried out on an Agilent 6890N gas chromatography system equipped with 5973 series mass selective detector, 7673 series autosampler and chemstation (Agilent Technologies, Palo Alto, CA). HP-5 MS column with 0.25μm film thickness (30m×0.25mm I.D., USA) was used for separation. Splitless injection was used and the carrier gas was helium at a flow rate of 1mL min^{-1}. The injector and detector temperatures were 250°C. The MS detector parameters were transfer line temperature 280°C, solvent delay 3 min and electron energy 70eV.

Preparation of stock and standard solutions

The stock solution of diclofenac (1mg mL^{-1}) was prepared and diluted with acetonitrile to give standard solutions of 5-600ng mL^{-1}. Standard calibration samples were prepared daily by spiking 1.0 mL of drug-free plasma with 0.1mL of appropriate diclofenac standard solutions to achieve final concentrations of 5-600ng mL^{-1} for plasma. The working solution of IS was prepared by dissolving in acetonitrile to obtain a concentration of 100ng mL^{-1}. Also, quality control (QC) solutions were prepared from stock solution at concentrations of 10, 125 and 500ng mL^{-1} together with 100ng mL^{-1} IS.

Sample preparation and derivatization procedure

MSTFA is an effective trimethylsilyl (TMS) donor. MSTFA reacts to replace labile hydrogens on a wide range of polar compounds with a TMS group and is used to prepare volatile and thermally stable derivatives for GC-MS[26] To increase the performance of the gas chromatographic separation, diclofenac and IS were derivatized using MSTFA (Figure 1). The hydroxy (-OH) groups were converted to the corresponding silyl (-O-TMS) groups.

Blood samples were collected into the tubes containing disodium EDTA and centrifuged at 4500×g for 10 min. A 1.0mL of the resultant plasma samples were spiked with 0.1mL of diclofenac, 0.1 mL of internal standard and 0.5 mL H_3PO_4 solutions were added. After vortex mixing for 5s, 4mL of hexane and ethylacetate was added (4:1, *v/v*), the mixture was vortexed for 30s and then centrifuged at 3000×g for 3 min. The organic layer was transferred into another tube and evaporated to dryness at room temperature under nitrogen gas. The dry residue was dissolved in 100μL of a mixture of acetonitrile and MSTFA (50:50, *v/v*). The mixture was vigorously shaken and then delayed at room temperature for 10 min. 1μL sample was injected into the GC-MS system.

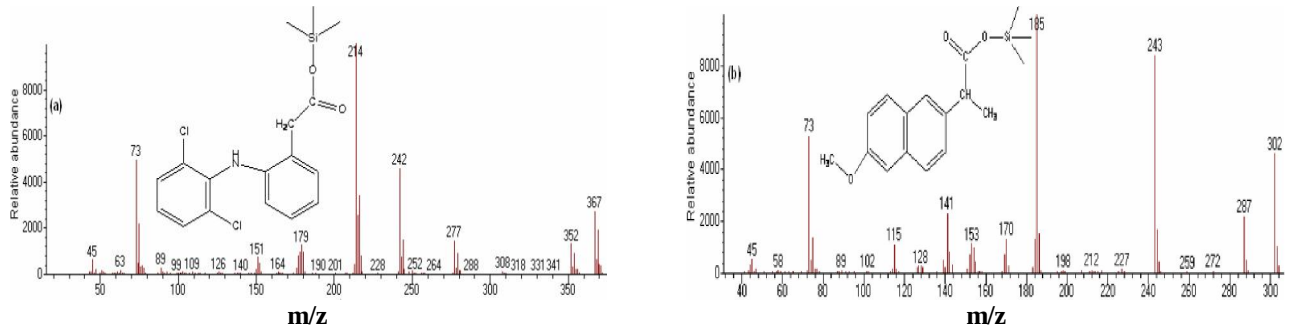

Figure 1 : MS spectra after derivatization of diclofenac (a) and naproxen (IS) (b) with MSTFA

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Rabbits

The study was conducted in accordance with the Animal Ethical Guidelines for Investigations in Laboratory Animals and was approved by the Ethical Committee for Medical Experimental Research and Application Centre of Ataturk University. The rabbits are male which is 4.8-5.2kg weight. The rabbits were housed with free access to food and water, except for the final 2 h before experiment. After a single oral administration of 50mg of diclofenac (Dolorex dragee), 2.5mL of blood samples were collected from the marginal ear vein at 0, 15, 30, 45, 60, 75, 90, 120, 180, 240, 300 and 360 min time-points into EDTA collection tubes. The blood samples were centrifuged at 4000rpm for 10 min and the plasma was taken and stored at -20°C until analysis.

RESULTS AND DISCUSSION

Method development and optimization

Today, GC-MS is a powerful technique for highly specific and quantitative measurements of low levels of analytes in biological samples[26]. During method development, it became evident that diclofenac and IS were very sensitive to matrix effects during the derivatization process in rabbit plasma. Sample preparation techniques, such as liquid-liquid extraction was used in order to minimise matrix suppression effects.

A capillary column coated with 5% phenyl and 95% dimethylpolysiloxane was used for separation. The injection port and detector temperature was set to 250°C. Different temperature programs were investigated to give an optimum temperature program as follows: initial temperature was 150°C, held for 1 min, increased to 220°C at 20°C min^{-1} held for 1 min, and finally to 300°C at 10°C min^{-1} with a final hold of 1.0 min. The injector volume was 1µL in splitless mode.

To confirm the complete derivatization of diclofenac and IS each compound was derivatized and analyzed separately. After establishing the optimum reaction conditions, the compounds were mixed together and then derivatized in order to perform a simultaneous analysis. The fragment ions (m/z 214 and 185) were used for quantitation of diclofenac and IS in SIM mode. The retention times of diclofenac-TMS and IS-TMS derivatives were 9.2 and 7.7 min, respectively and the total run time of analysis was 10 min.

The effects of time and temperature on the reaction were investigated. Therefore, diclofenac and IS were dissolved in acetonitrile. To 100µL of 500µg mL^{-1} diclofenac solution and 100µL of MSTFA solution were added and reacted at room temperature, 50 and 75°C for 5, 10 and 20 min. The resulting samples were quantitated by GC-MS system. After standing for 10 min at

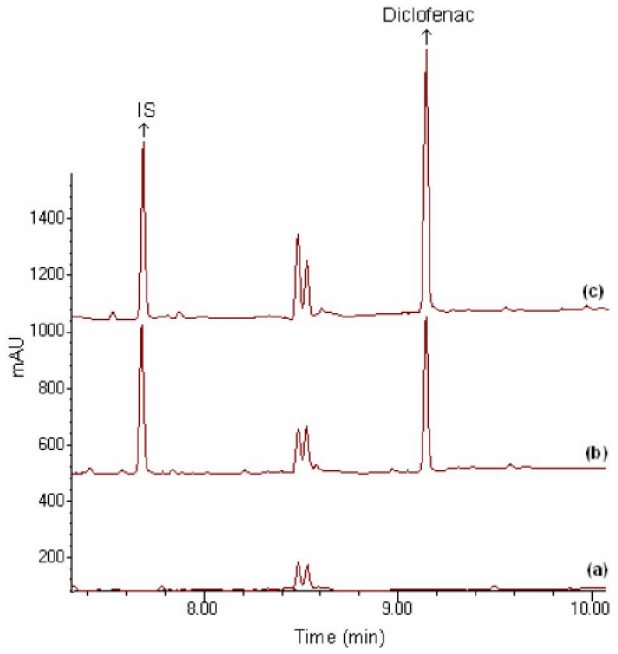

Figure 2 : Representative chromatograms of (a) drug-free rabbit plasma, (b) the rabbit plasma spiked with diclofenac (250µg mL^{-1}) and IS (100µg mL^{-1}), (c) the rabbit plasma obtained at 45 min after a single oral dose of 50mg diclofenac

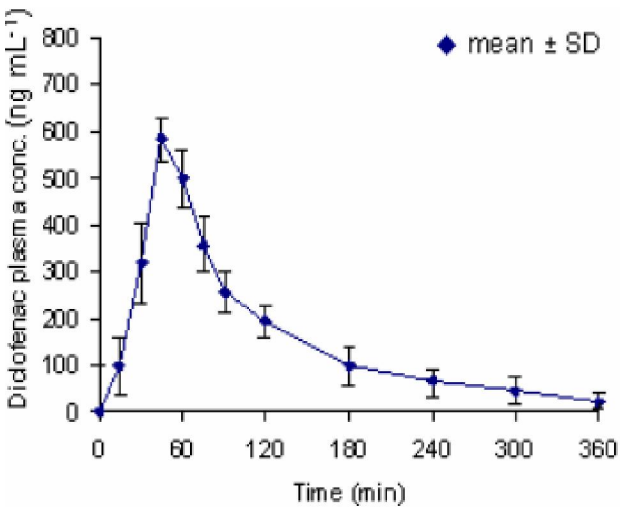

Figure 3 : Mean plasma diclofenac concentration-time profile for three rabbits after 50mg diclofenac

room temperature, maximum peak areas were quantitated.

Validation of the method

The validation was carried out by establishing specifity, linearity, intra- and inter-day precision, accuracy, recovery and sensitivity parameters according to ICH[25].

Specificity

The specificity of the method was verified by investigating the peak interference from the endogenous plasma substances. The chromatogram of the rabbit plasma spiked with diclofenac and IS was compared to that of the blank plasma sample.

As mentioned above, under the described analysis procedure, the peaks of diclofenac and IS were well resolved with good symmetry and desirable retention time from endogenous compounds in the blank rabbit plasma. Representative chromatograms of blank plasma and plasma samples spiked with diclofenac and IS were shown in figure 2. There were no interference peaks near the retention times of diclofenac and IS.

Linearity

The linearity of the method was evaluated by a calibration curve in the range of 5-600ng mL^{-1} of the drug (n = 3). Drug-free plasma was spiked with diclofenac standard solutions to achieve final concentrations of 5, 15, 50, 100, 250, 400 and 600ng mL^{-1}. Calibration curve was obtained by plotting peak area ratios of diclofenac to IS versus the diclofenac concentrations with least-squares linear regression analysis. The calibration equation from three replicate experiments, $y = 0.0247x + 0.0543$ ($r = 0.999$), demonstrated the linearity of the method. This method is as good as to that reported in the other papers[11,13,17,20,21,23,24].

TABLE 1 : Intra-day and inter-day precision and accuracy of diclofenac in plasma (n=6)

Sample	Concentration (ng mL^{-1})		Precision % RSD	Accuracy %
	Added	Found (Mean ± SD)		
Plasma	10	10.12 ± 0.325	3.21	101.2
Intra-day	125	119.5 ± 2.545	2.13	95.6
	500	461.5 ± 13.706	2.97	92.3
Inter-day	10	10.26 ± 0.474	4.62	102.6
	125	116.8 ± 5.804	4.97	93.4
	500	482.5 ± 28.129	5.83	96.5

Precision and accuracy

Intra-day and inter-day precision and accuracy were determined by replicate analysis of six sets of samples spiked with three different concentrations of diclofenac (10, 125 and 500ng mL^{-1}) within a day or during three consecutive days. The precision was calculated from the ratio of the standard deviation to the mean (relative standard deviation, RSD). The accuracy of the method was examined by comparing the concentrations of spiked samples to the theoretical concentrations. Both values were expressed as percentage. The results of precision and accuracy were presented in TABLE 1.

The intra-day precision and accuracy were varied between 2.13 and 3.21%, and 92.3 and 101.2%, respectively. The inter-day precision and accuracy ranged from 4.62 to 5.83% and 93.4 to 102.6%, respectively. All the values of precision and accuracy including LOQ were within the specified ranges and therefore acceptable. The acceptable range of intra-day and inter-day accuracy and precision are below 15% bias or RSD. In statistical comparison ($p > 0.05$) with other methods in the literature[10,16,17,19,20,23,24] the proposed method has indicated high accuracy and precision.

Sensitivity [limits of detection (LOD) and quantification (LOQ)]

The sensitivity was evaluated by the limit of quantification (LOQ), the lowest concentration of the plasma spiked with diclofenac in the calibration curve. The LOQ was defined as the concentration producing a precision less than 20% and accuracy between 80% and 120% of the theoretical concentrations. The LOQ was determined to be 5ng mL^{-1}. The intra-day precision and accuracy were 6.32% and 103.6%, respectively. The inter-day precision and accuracy were 8.71% and 102.8%, respectively. This method is as good or superior to that reported in the other papers[7,13,20,21,23].

TABLE 2 : Recovery of diclofenac in plasma (n=6)

Sample	Concentration (ng mL^{-1})		Recovery %	RSD %
	Added	Found(Mean±SD)		
Plasma	15	15.390 ± 0.828	102.6	5.38
	250	234.3 ± 8.622	93.7	3.68
	500	461.5 ± 13.706	92.3	2.97

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TABLE 3 : Stability of diclofenac in plasma (n=3)

Treatment	Recovery (Mean ± SD) Plasma concentration (ng mL^{-1})	
	10	500
Stock solution stability	98.2±3.462	97.4±5.764
Three freeze-thaw cycles	93.7±4.782	96.6±5.748
Short-term stability for 6 h	98.3±3.245	98.4±4.364
Short-term stability for 12 h	97.6±2.473	99.5±5.732
Long-term stability for 2 weeks	89.1±6.412	94.2±5.428

Recovery

The recovery was determined by comparing peak area of diclofenac after extraction to that before extraction at concentrations of 15, 250 and 500ng mL^{-1}. The mean extraction recovery of diclofenac from rabbit plasma was 96.2%. The mean relative recovery for IS at 100ng mL^{-1} was 91.8 (n = 6). Recovery data are shown in TABLE 2. Diclofenac was extracted from plasma with a solid phase extraction procedure by Hirai et al.[13] and Arcelloni et al.[22]. These methods are also the most comprehensive method which can extract diclofenac in a single extraction procedure. The mean recovery is better for plasma than those of the studies reported by Borenstein et al.[6], Kadowaki et al.[7], Roskar et al.[20] and Kaphalia et al.[21].

Stability

The stabilities of drug and IS in a biological fluid are affected by the chemical properties of drug and IS, the storage conditions, the matrix and the container systems.

Standard stock solution stabilities at the concentration of 10 and 500ng mL^{-1} of diclofenac and of 100ng mL^{-1} of IS were obtained by analyzing samples left at 4°C for 6 h. Freeze-thaw stability of the plasma samples was determined by the following three freeze-thaw cycles. The spiked plasma samples at concentrations of 10 and 500ng mL^{-1} were frozen at -20°C for 24 h and thawed at room temperature. After completely thawed, the samples were refrozen and this cycle was repeated three times. For the short-term stability, plasma samples were kept at room temperature for 6 and 12 h before the sample preparation. The long-term stability was evaluated after freezing the plasma samples at -20°C for 2 weeks. The stabilities were determined by the difference of measured sample concentration from the concentration of sample at 0 h. The stability of standard stock solution of IS was 96.1%. Also, the stability of the prepared plasma samples was 98.3% at 6 h and 97.6% at 12 h. Other results of stability experiments were shown in TABLE 3.

TABLE 4 : Mean pharmacokinetic parameters of diclofenac for three rabbits after dolorex dragee (50mg)

Parameters	(Mean ± SD)	% RSD
Maximum plasma concentration C_{max}(ng mL^{-1})	587.1 ± 74.21	12.64
Time required for maximum plasma concentration (T_{max}) (min)	45.0 ± 13.41	29.78
Area under curve $AUC_{(0\text{-}360\ min)}$ (min ng mL^{-1})	5841.8 ± 1382.8	23.67
Area under curve at infinite time $AUC_{(0\text{-}\infty)}$ (min ng mL^{-1})	6452.4 ± 1256.3	19.47
Elimination rate constant (K_{el}) (min^{-1})	0.012± 0.002	16.67
Plasma half life ($T_{1/2}$) (min)	65.8 ± 16.27	24.73

Pharmacokinetic analysis

The maximum plasma concentration (C_{max}) and the time to reach maximum concentration (T_{max}) were directly determined from the plasma concentration versus time curves. The area under the curve from 0 to t ($AUC_{0\text{-}t}$) was calculated by the linear trapezoidal rule. The area under the curve from 0 h to infinity ($AUC_{0\text{-}\infty}$) was estimated by summing the area from 0 to t ($AUC_{0\text{-}t}$) and t to infinity ($AUC_{t\text{-}\infty}$), where $AUC_{t\text{-}\infty} = C_t/K_{el}$, with C_t defined as the last measured plasma concentration at time t, and k_{el} the slope of the terminal portion of the ln(plasma concentration) versus time curve. The elimination half-life ($t_{1/2}$) was calculated using the pharmacokinetic relationship $t_{1/2} = \ln(2)/k_{el}$.

The plasma samples obtained three rabbits were assayed with the validated method described above. The peaks of diclofenac and IS were completely separated from endogenous peaks with similar retention times to those of the samples used for the validation studies (Figure 2). The mean plasma concentration-time curve was shown in figure 3.

The mean values of pharmacokinetic parameters estimated by the computer program WinNonlin with non-compartmental method were shown in TABLE 4.

CONCLUSION

In the present work, a simple and sensitive GC-MS method has been developed for the determination of diclofenac in rabbit plasma. Also, the method was

completely validated by using sensitivity, stability, specificity, linearity, accuracy and precision parameters for determination of diclofenac in rabbit plasma. Additional advantages of this method include small sample volume (1.0mL), good extraction recovery from plasma and a readily available internal standard. To our knowledge, this is the first description of diclofenac pharmacokinetics in rabbit plasma by GC-MS method in the literature. It can be very useful and an alternate to performing pharmacokinetic studies in determination of diclofenac for clinical use.

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