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Short Communication

Reversed-phase high-performance liquid chromatography of ketorolac and its application to bioequivalence studies in human serum

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ABSTRACT

A reversed-phase high-performance liquid chromatographic assay was used to study the bioequivalence of the anti-inflammatory drug (±)-ketorolac in human volunteers. Following deproteinization of human serum with 5% zinc sulphate solution, ketorolac was chromatographed on a 10-μm octadecylsilica column using acetonitrile–water as mobile phase and ultraviolet detection at 313 nm. Under these conditions the method was reproducible with a coefficient of variation of less than 5%. The assay procedure was linear in the range 0.25–1.5 μg/ml, with a sensitivity of 0.01 μg/ml ketorolac. The recovery of ketorolac from serum ranged from 90 to 95%.

INTRODUCTION

Ketorolac tromethamine is chemically (±)-5-benzoyl-1,2-dihydro-3*H*-pyrrolo[1,2-*a*]pyrrole-1-carboxylic acid 2-amino-2-(hydroxymethyl)-1,3-propanediol (Fig. 1). It is a highly potent member of a new class of compounds with analgesic and anti-inflammatory activity [1]. It is efficacious in treating pain arising from a broad spectrum of causes, such as postpartum and postoperative pain, cancer pain, and pain from dental extraction [2]. This analgesic is approved by the USA Food and Drug Administration and it is non-narcotic, fast acting and non-addictive. It can be administered orally or by injection [3].

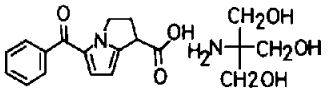

Fig. 1. Structure of ketorolac tromethamine.

Some earlier investigators used various sample clean-up procedures involving organic solvent extraction of serum or plasma samples. A sample clean-up procedure using isooctane–2-propanol (95:5, v/v) for extraction has been described: the sample is further treated with 0.2 ml of 0.6 *M* sulphuric acid and extracted in chloroform; this extract is derivatized using triethylamine [4]. The number of steps in this procedure reduces the recovery of ketorolac. Another reported method involved extraction with ethyl acetate–hexane (30:70, v/v) after acidification [5]. For the simul-

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taneous determination of ketorolac and *p*-hydroxyketorolac a three-step extraction procedure was reported [6], but the amount of sample handling was great.

Like many non-steroidal anti-inflammatory drugs (NSAIDs), ketorolac is highly protein-bound in plasma to the extent of 99%, and its binding ratio varies with age. Because of this property the recovery of the drug by extraction is low. For a better recovery of ketorolac from plasma a process is required that can free ketorolac completely from plasma proteins. With this aim, a method for sample clean-up was developed and was used for bioequivalence studies in human volunteers.

EXPERIMENTAL

Reagents

Ketorolac tromethamine standard was obtained from Chemo Iberica (Barcelona, Spain). Acetonitrile, orthophosphoric acid and methanol were purchased from E. Merck (Bombay, India). Zinc sulphate (AR grade) was purchased from Glaxo (Bombay, India). Water obtained from a Milli-Q water purification system was used for the mobile phase. The borosilicate glass culture tubes and pipettes were obtained from Borosil glasses (Bombay, India). Fresh serum was collected from healthy human volunteers for the development of method.

Equipment

The chromatographic system consisted of a Waters 510 dual-pump solvent-delivery system (Waters, Milford, MA, USA) equipped with a 100-μl loop and a Rheodyne 7125 sample injector (Rheodyne, Cotati, CA, USA). A reversed-phase μBondapak C_{18} column (300 mm × 3.9 mm I.D., 10 μm particle size) and a Waters 490 E programmable multi-wavelength UV–visible detector was used. The chromatograms were recorded on a PC/AT integrator using Oracle-2 software (Zenith computers, Pune, India). A variable pipette was used for all quantitative sampling (Scintico Instruments, Bombay, India). A Milli-Q system (Millipore, Milford, MA, USA) was used to obtain HPLC-grade water. The samples were centrifuged in a Remi RBC centrifuge (Remi Motors, Bombay, India). An analytical balance, Model AE163 (Mettler Instruments, Highstown, NJ, USA), was used to weigh the reagents. A vortex mixer (Kumar Industries, Bombay, India) was used. A Waters guard column, dry-packed with C_{18} particles (10 μm) was used throughout the experiment.

Chromatographic conditions

The mobile phase was acetonitrile–water (40:60, v/v). The pH of the mobile phase was adjusted to 2.8 ± 0.1 with 85% (w/v) orthophosphoric acid. The mobile phase was filtered through a 0.45-μm Millipore membrane filter before use and was delivered at a flow-rate of 1.4 ml/min. Ketorolac was monitored by a UV–visible absorbance detector at a wavelength of 313 nm and 0.005 a.u.f.s. The column was maintained at ambient temperature.

Preparation of stock solution

A stock solution of ketorolac tromethamine was prepared by dissolving 200 mg in 100 ml of water. It was suitably further diluted to get a stock solution containing 100 μg/ml ketorolac tromethamine. Dilutions of the stock solution were made in serum to construct the calibration curves. The retention time of ketorolac was determined by injecting an aliquot of the standard solution into the HPLC system.

Isolation of ketorolac from plasma before chromatography

The frozen serum was thawed at room temperature (25 ± 2°C) and 1.0 ml was pipetted into a clean disposable borosilicate graduated centrifuge tube. An aliquot of the standard solution of ketorolac tromethamine was added to 1.0 ml of serum and vortex-mixed for 30 s. A 100-μl aliquot of 5% (w/v) zinc sulphate solution in water was added to the serum containing ketorolac tromethamine and again vortex-mixed for 2 min. An aliquot of methanol was added to make the volume to 4 ml, vortex-mixed for 2 min and then centrifuged at 2000 *g* for 5 min at room temper-

ature. The supernatant was decanted into a fresh borosilicate culture tube. A 100-μl volume of this solution was injected into the HPLC system.

Construction of the calibration curve

A minimum of seven solutions of ketorolac tromethamine were prepared by adding enough stock solution to obtain concentrations of *ca.* 25, 50, 100, 300, 500, 1000 and 1500 ng/ml. These solutions were prepared in serum and water. Under the experimental conditions, the calibration curve was linear up to 1500 ng/ml.

Assay recovery

The recovery of ketorolac was assessed at concentrations of 50 and 1000 ng/ml. Six samples containing ketorolac were extracted by the sample preparation procedure and injected. Six injections of the same amount of ketorolac in mobile phase were injected directly. The peak areas of compound in both sets of samples were measured. The recovery of each sample was computed using the following equation: percentage recovery = [peak area of extract / mean peak area of direct injection] × 100.

RESULTS AND DISCUSSION

Fig. 2 depicts representive chromatograms of drug-free serum, serum spiked with 50 ng/ml ketorolac tromethamine, and a human serum sample taken 1 h after administration of 30 mg of ketorolac tromethamine orally in tablet form. The retention time of ketorolac was *ca.* 6 min. There were no interfering peaks from the normal components of serum. An excellent linear relationship ($r^2 > 0.996$) was found between the peak area (y) and concentrations (x). Typical regression lines through the data points in serum could be described by the equation $y = 0.5472x + 14.3$, where y indicates the peak area and x indicates concentration of ketorolac in ng/ml.

The extraction efficiencies for ketorolac were determined and the results are tabulated in Table I. This study was conducted to confirm the extractability of the drug. In the literature, the extractability has been reported to range between

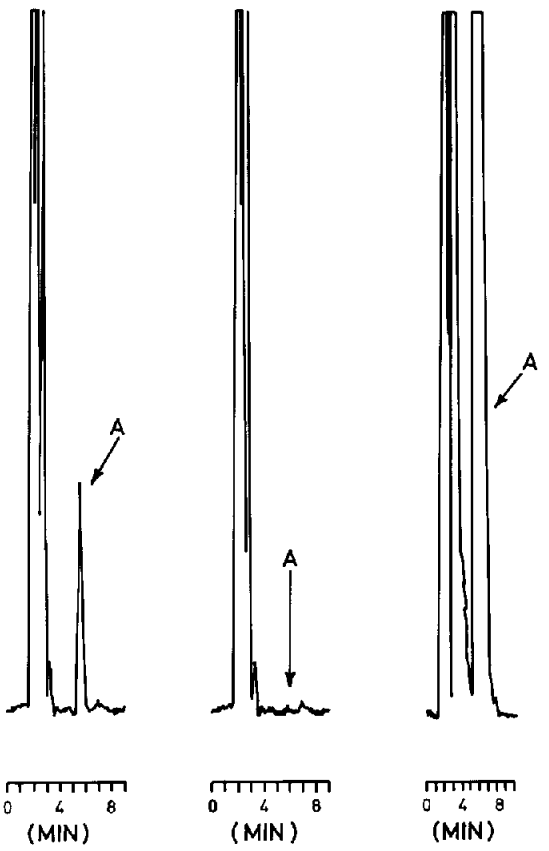

Fig. 2. Representative chromatograms of (left) serum spiked with 50 ng/ml ketorolac tromethamine, (middle) blank serum and (right) serum collected from a subject 1 h after administration of 30 mg of ketorolac tromethamine. Peak A: ketorolac. Detection at 313 nm and 0.005 a.u.f.s.

65 and 75% [4–8]. For establishing the recovery of drug, the serum was deproteinized using three systems: methanol (3 ml), 5% zinc sulphate (0.1 ml) in methanol (2.9 ml), and 5% trichloroacetic acid (3 ml). The results show that using 5% zinc sulphate methanol as solvent gives recoveries ranging from 90 to 95% ketorolac, which is better than the previously reported values.

The stock solution of ketorolac tromethamine was stored at 10°C and analysed after 0, 12, 24 and 48 h for the ketorolac tromethamine content. No marked change in the concentration was observed.

TABLE I

EXTRACTION RECOVERY OF KETOROLAC

Deproteinizing agent	Recovery (mean ± S.D., n = 6) (%)		Mean recovery (%)
	50 ng/ml	1000 ng/ml	
Methanol	55.0 ± 3.2	56.0 ± 3.9	55.5
5% Zinc sulphate–methanol	95.0 ± 4.1	94.0 ± 2.3	94.5
5% Trichloroacetic acid	60.0 ± 3.2	63.0 + 2.5	61.5

TABLE II

REPRODUCIBILITY AND ACCURACY FOR KETOROLAC DETERMINATION

Amount spiked (ng/ml)	Amount assayed (mean ± S.D., n = 6) (ng/ml)	Coefficient of variation (%)	Accuracy of assay (%)
Within-day			
48.8	48.7 ± 0.5	5.2	0.2
98.2	96.8 ± 1.3	2.8	−2.8
297.5	298.8 ± 1.0	1.0	1.4
998.4	1003.5 ± 4.4	2.2	2.2
Day-to-day			
48.8	48.6 ± 1.3	3.2	−0.3
999.54	997.9 ± 2.3	2.9	2.3

Reproducibility and accuracy

The reproducibility and accuracy were determined for four spiked serum samples with respect to a calibration graph (Table II). The within-day coefficients of variation (C.V.) were 1.0 5.2%. The day-to-day C.V. for analysis of the same serum samples on three different days over a period of one week were 3.2% at 48.8 ng/ml (n = 6) and 2.9% at 999.4 ng/ml (n = 6). The accuracy of the method, expressed as the mean deviation of all concentrations from the theoretical value, was in the range 2.0–2.3%.

Serum concentration *versus* time profiles of ketorolac in human volunteers are depicted in Fig. 3, and Table III containes average pharmacokinetic data of all twelve volunteers. The peak serum concentration (C_{max}) of ketorolac and the time to reach peak levels (T_{max}) were determined from the individual serum concentrations–time curves. The serum half-life ($t_{1/2}$) was computed using linear regression on the log of the serum concentrations in the terminal phase of serum

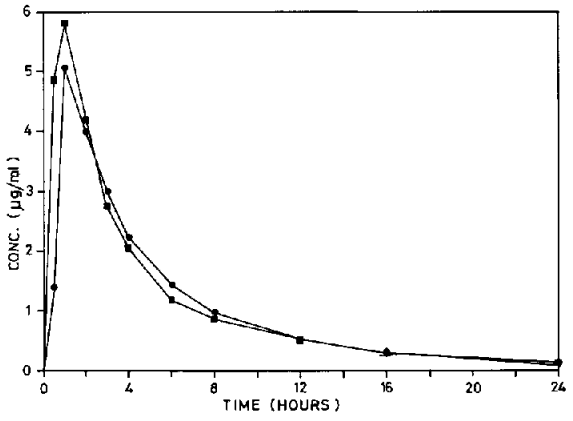

Fig. 3. Serum concentration–time curve following oral administration of 30 mg of two formulations of ketorolac tromethamine: (●) formulation A; (■) formulation B.

TABLE III

PHARMACOKINETIC PARAMETERS OBSERVED FOR PRODUCTS A AND B

Parameter	Product A (mean ± S.E., n = 12)	Product B (mean ± S.E., n = 12)	P Value
C_{max} (μg/ml)	5.240 ± 0.299	6.651 ± 0.393	0.676
T_{max} (h)	1.250 ± 0.131	0.667 ± 0.071	1.265
AUC (μg h/ml)	25.488 ± 1.413	26.405 ± 1.053	3.811

concentration–time curve. The total area under the serum concentration–time curve (AUC) was calculated using the linear trapezoidal rule up to the last measurable time point, and addition of the term CP-Last (concentration of drug for the last determined point)/k_e (elimination rate constant for the drug). Analysis of variance (ANOVA) associated with a replicated Latin-square design was performed. The P value of less than 0.05 was considered to be statistically significant. The results are in agreement with an earlier study, which demonstrated the single-dose pharmacokinetics of ketorolac tromethamine [9,10].

After oral administration or absorption, ketorolac tromethamine dissociates into the anionic form of ketorolac at physiological pH, which is measured in serum. Although previous studies in humans have used higher dosages (1.3–1.7 mg/kg) of ketorolac tromethamine, the reported average parameters are in very close agreement with the current study [1].

CONCLUSION

The method described permits the detection of as little as 10 ng/ml ketorolac in human serum with a 1.0-ml sample. The use of 5% zinc sulphate as deproteinizing agent breaks the binding of ketorolac with plasma proteins efficiently, and it is then easily extractable in methanol, with good recovery. No interference from serum components is observed in the chromatograms. No significant alterations were observed with a column used continuously for the analysis of 300 serum samples. The proposed method is sensitive and rapid enough for pharmacokinetic studies.

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