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Determination of Ketorolac in Human Serum by High-performance Liquid Chromatography

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A high-performance liquid chromatographic (HPLC) assay has been developed for the determination of ketorolac in human serum using a new extraction method with a good recovery. Human serum samples (1.0 ml) spiked with known concentrations of ketorolac tromethamine and 10 μg of ketoprofen as the internal standard (IS) were acidified with 200 μl of 1N HCl and extracted with 7 ml of *n*-hexane-ether (7:3 v/v). Extracts were centrifuged and organic layer was back-extracted with 400 μl of 0.1% tromethamine solution. Twenty μl of centrifuged aqueous layer was injected onto a reversed-phase octyl column and eluted with a mixture of acetonitrile, water, methanol, and triethylamine [35:55:10:0.1 (v/v), pH 3.0] at a flow rate of 1.0 ml/min. Ultraviolet detection of ketorolac and IS was carried out at 300 nm. The calibration curve obtained using peak area ratios showed a good linearity (in concentration range 10-150 ng/ml r^2=0.9944; in range 50-2000 ng/ml, r^2=0.9998). The mean intra-day accuracy and precision for this HPLC method were found to be 3.6 and 3.7%, respectively. The mean inter-day accuracy and precision were found to be 4.0 and 3.7%, respectively, in the concentration range 50-2000 ng/ml. The recovery of ketorolac from serum was 92.0 (±5.7)% at the concentration of 100 ng/ml. This method proved to be readily applicable to the assay of ketorolac in human serum.

Key words : HPLC assay, Ketorolac tromethamine, Human serum

INTRODUCTION

Ketorolac is a potent non-steroidal and non-narcotic analgesic currently used in the treatment of moderate to severe pain (Rooks *et al.*, 1985; Buckley and Brogden, 1990; Granados-Soto *et al.*, 1995). Comparative clinical studies have shown that ketorolac is remarkably more potent in pain relief than other currently used non-steroidal anti-inflammatory drugs (Forbes, *et al.*, 1990), and that it has similar potency and efficacy comparing with those of opioid drugs (Yee *et al.*, 1986; O'Hara *et al.*, 1987). One major advantage of ketorolac over other non-steroidal anti-inflammatory drugs is that the tromethamine salt (Fig. 1) of this drug can be administered either intravenously, intramuscularly, or orally (Jung *et al.*, 1988; Mroszczak *et al.*, 1990).

Various extracting solvents, such as ether (Jung *et al.*, 1989; Wu and Massey, 1990; Flores-Murrieta *et al.*, 1994), isooctane-2-propanol (95:5, v/v) (Jamali *et al.*, 1989), ethyl acetate-*n*-hexane (30:70 v/v) (Wu *et al.*, 1986; Mroszczak *et al.*, 1987; Jallad *et al.*, 1990), and dichloromethane (Sane *et al.*, 1993) have been reported for the HPLC analyses of ketorolac in plasma or serum samples. However, ether and dichloromethane extracts are not suitable for HPLC analysis owing to the presence of numerous endogenous components that interfere with the quantification of ketorolac. In addition, the extractability of ketorolac by these solvent systems has been reported to be about 70% (Jamali *et al.*, 1989; Chaudhary *et al.*, 1993). Because of high protein binding of ketorolac in plasma, its recovery by extraction is low. Chaudhary *et al.* (1993) developed a method for deproteinizing human serum with 5% zinc sulphate solution, and increased the recovery of ketorolac from serum by directly injecting methanolic supernatant of deproteinized serum. However, this method requires injection of a larger volume (100 μl), and a detection

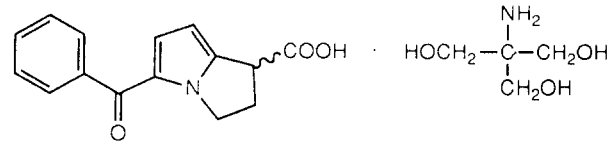

Fig. 1. Chemical structure of ketorolac tromethamine (KT)

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of higher sensitivity. On the other hand, although *p*-fluoroketorolac (Mroszczak *et al.*, 1987), ketoprofen (Jamali *et al.*, 1989) or sodium tolmetin (Flores-Murrieta *et al.*, 1994) has been employed as an internal standard (IS), their recoveries were not reported.

The objective of this study is to develop a new extraction method with a good recovery of ketorolac and ketoprofen as the IS, and to measure ketorolac in human serum by HPLC. With *n*-hexane-ether (7:3 v/v), a good linear relationship between peak area ratios of ketorolac to IS and drug concentrations was constructed, suggesting a reproducible extractability for ketorolac as well as IS.

MATERIALS AND METHODS

Materials

Fresh human serum was obtained from Korea University Medical Center, Anam Hospital (Seoul, Korea) and frozen at -20°C until used. Ketorolac tromethamine (KT) was given by Whan In Pharm. Co., Ltd. (Ansung, Korea). Ketoprofen and tromethamine were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). HPLC-grade acetonitrile and methanol were products of Fisher Scientific (Pittsburgh, PA, USA). Hydrochloric acid, *n*-hexane, ether, ethyl acetate, triethylamine, and all other chemicals used in the study were of analytical reagent grade and used as such without any further purification. HPLC-grade water was obtained by passing distilled water through a Elgastat UHQ PS Water Purification System (Elga, Bucks, England).

Equipments

The chromatographic system consisted of a Perkin-Elmer Series 410 isocratic HPLC (Perkin-Elmer, Norwalk, CT, USA). The chromatograph was equipped with a variable ultraviolet detector (Perkin-Elmer, LC 90 UV), a 20 μl-loop and a Rheodyne injector (Rheodyne, Cotati, CA, USA). A Ultrasphere C8 column (5 μm, 4.6 mm×15 cm, Beckman, Fullerton, CA, USA) and Guard-Pak™ precolumn insert (μBondapak™ C18, Waters, Milford, MA, USA) were used. The chromatograms were integrated with a Varian 4290 integrator (Varian, Walnut Creek, CA, USA). Variable micropipettes (Varipette 4710, Eppendorf, Hamburg, Germany) were used for all quantitative sampling. The extracts were centrifuged in a VS-4000 centrifuge (Vision Scientific, Inchon, Korea). An analytical balance, Model B120S (Sartorius, Gottingen, Germany) was used to weigh the reagents.

Chromatographic conditions

The mobile phase was a mixture of acetonitrile, water, methanol and triethylamine (35:55:10:0.1, v/v). The pH of the mobile phase was adjusted to 3.2±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 eluted through the chromatograph at ambient temperature using a flow rate of 1.0 ml/min. The chromatographic peaks of interest were monitored by UV absorption at 300 nm (attenuation 16). Peak areas were integrated with the integrator, and concentrations of ketorolac in samples of serum were calculated by reference to the corresponding calibration curve. Since the pK_a of ketorolac acid in water was reported to be 3.5 (Gu and Strickley, 1987), and KT dissociates into the anion form of ketorolac at physiological pH after absorption, the measured concentrations are referred to ketorolac.

Preparation of stock and spiking solutions

A stock solution of KT was prepared by dissolving 100 mg in 100 ml of water. It was suitably further diluted to get a stock solution containing 100 μg/ml KT. The stock solution of IS at a concentration of 1 mg/ml was prepared in methanol, and diluted with water to make the IS solution at a concentration of 100 μg/ml. Spiking solutions of KT containing 0.1, 0.3, 0.5, 0.7, 1.0 and 1.5 μg/ml or 0.5, 1.0, 3.0, 5.0, 7.0, 10.0, 15.0 and 20.0 μg/ml in water were prepared by serial dilution of the stock solution. Dilutions of spiking solution were made in serum to construct the calibration curves.

Sample preparation

Human serum (1.0 ml) was spiked with 100 μl of the KT spiking solution to make an appropriate concentration, and 100 μl of the IS solution (100 μg/ml) was added. The mixture was acidified with 200 μl of 1N HCl, and vortex-mixed for 60 seconds to deproteinize the samples. Seven ml of a mixture of *n*-hexane-ether (7:3 v/v) were added, and ketorolac and IS were extracted from the serum samples by vortex-mixing for 10 min using a Vortex-Genie 2* mixer (Fisher Scientific, Pittsburgh, PA, USA). The tubes were centrifuged at 3000 rpm for 5 min. Five ml of upper organic layer were taken and transferred into a conical borosilicate centrifuge tube, and back-extracted with 400 μl of 0.1% tromethamine aqueous solution by vortex-mixing for 3 min. Twenty μl of aqueous layer were injected onto a HPLC system after a centrifugation at 3000 rpm for 5 min.

Construction of the calibration curve

For generation of calibration curves 1-ml aliquots of blank human serum were spiked with 100 μl of

spiking solutions of KT and 100 µl of the IS solution. These samples were processed in the manner described above under sample preparation. Calibration curves were obtained by plotting the ratio of the peak area for KT to that of IS (y) against the concentration of KT (x) in human serum and fitting the data to a straight line by linear least-squares regression analysis.

Assay recovery

The recovery of KT and IS from human serum was assessed at concentrations of 0.1 and 10 µg/ml, respectively. Four samples containing both compounds were extracted by the sample preparation procedure and injected. Twenty µl of the aqueous solution containing KT and IS at their final respective concentrations were injected directly three times. The peak areas of both compounds were measured. The recovery of each sample was computed using a following equation: % recovery=[peak area of extract/mean peak area of direct injection]×100.

RESULTS AND DISCUSSION

Extracting solvent

Initially, we attempted to use a mixture (7:3 v/v) of *n*-hexane and ethyl acetate (Jallad *et al.*, 1990) as an extracting solvent, but it was not acceptable for measuring ketorolac in human serum samples, because it resulted in a poor and irregular extraction. Therefore it was required to develop a new extracting solvent. When we used a mixture of *n*-hexane and ether (7:3 v/v) as an extracting solvent, and 0.1% tromethamine aqueous solution as the back-extracting solvent, it gave a good linear relationship (r=0.9999) between peak area ratios of ketorolac to IS and the concentration of KT spiked into human serum. There were no interfering peaks from the normal components of serum. This finding suggests that *n*-hexane-ether (7:3 v/v) system has a good extractability for ketorolac as well as ketoprofen.

Effect of acidifying agent on the extraction of ketorolac

Since KT is a water-soluble salt and stays in ionized form in serum, acidifying agents such as 1N HCl (Jung *et al.*, 1989; Sane *et al.*, 1993), pH 3.0 (0.5 M) acetate buffer (Mroszczak *et al.*, 1987) or pH 4.0 (0.1M) acetate buffer (Flores-Murrieta *et al.*, 1994) have been added into plasma or serum samples to extract ketorolac (free acid) into organic layer. In this study, to observe the effect of strength of acid on the extraction of ketorolac, hydrochloric acids having different normality were used. After extraction by vortex-mixing for 10 min, the tubes were centrifuged at 3,000 rpm for 5 min to remove any minute aqueous droplets from organic layer as much as possible. The organic layer was taken from the tube using a 5-ml volumetric pipet, and back-extracted with 0.1% tromethamine solution. The aqueous layer was injected onto the HPLC. The result is shown in Table I. Good extraction of ketorolac and IS was obtained by adding 200 or 400 µl of 1N HCl into 1.0 ml of the serum samples, when *n*-hexane-ether (7:3 v/v) was used as an extracting solvent.

However, in *n*-hexane-ethyl acetate system, extraction of both compounds was very poor and variable, although the normality and volume of diluted hydrochloric acid added were changed.

Effect of extracting time on the extraction of ketorolac

To observe the effect of extracting time on the extraction of ketorolac and IS from human serum, extracting time was varied from 5 to 15 min. Five or 10 min was sufficient to extract ketorolac. However, longer and/or vigorous extraction occasionally formed a gel between aqueous serum and organic layers. Therefore, 10 min was recommended as the extracting time in this study.

Effect of back-extracting solvent on the extraction of ketorolac

In order to back-extract ketorolac in organic layer into aqueous layer, the use of 0.1 N NaOH solution has been reported (Jung *et al.*, 1989; Wu and Massey, 1990). However, strong alkali is not recommended, since ketorolac is degraded by the specific base-catalyzed process in strong alkaline region (Gu *et al.*,

Table I. Effect of the volume of HCl on the extraction of KT (1000 ng/ml) from human serum

Normality of HCl (N)	Volume added (µl)	*n*-Hexane-ethyl acetate (7:3)			*n*-Hexane-ether (7:3)		
		Peak area		Ratio of ketorolac to IS	Peak area		Ratio of ketorolac to IS
		Ketorolac	IS		Ketorolac	IS	
1.0	400	ND[a]	ND	ND	161864	239612	0.6755
1.0	200	155072	139664	1.1103	173664	258149	0.6727
0.5	200	134631	206703	0.6513	114756	216099	0.5310
0.1	200	-	14551	-	-[b]	-	-

[a]Not determined.
[b]Not detected. Back-extracting solvent was 0.1% tromethamine solution of 400 µl.

1988). Therefore we tried to use tromethamine solution, as the back-extracting solvent, which is a mild organic amine base. Sufficient back-extraction of both ketorolac and IS was obtained on addition of 400 μl of 0.1-0.2% tromethamine solutions. In the case of 0.3% tromethamine solution, each chromatographic peak of ketorolac and IS was splitted into two peaks. This might be due to insufficient conversion of ionized ketorolac to acid form during the elution process. Therefore, 0.1% tromethamine solution has been selected in this study.

HPLC chromatograms

Fig. 2 shows the typical HPLC chromatograms obtained after the injection of serum extracts, which were prepared according to Sample preparation described above. Retention times were ca. 5.9 and 9.8 min for the peaks of ketorolac and IS, respectively; no interfering peaks occurred at these times. Chromatographic peaks of ketorolac and IS obtained from the serum of a healthy volunteer, who received a tablet containing 10 mg of KT, were also sharp, and separated well each other.

Recovery

Recoveries of both ketorolac and IS from human serum were found to be 92.0 (±5.7) and 98.3 (±1.2)% at the respective concentration of 0.1 and 10 μg/

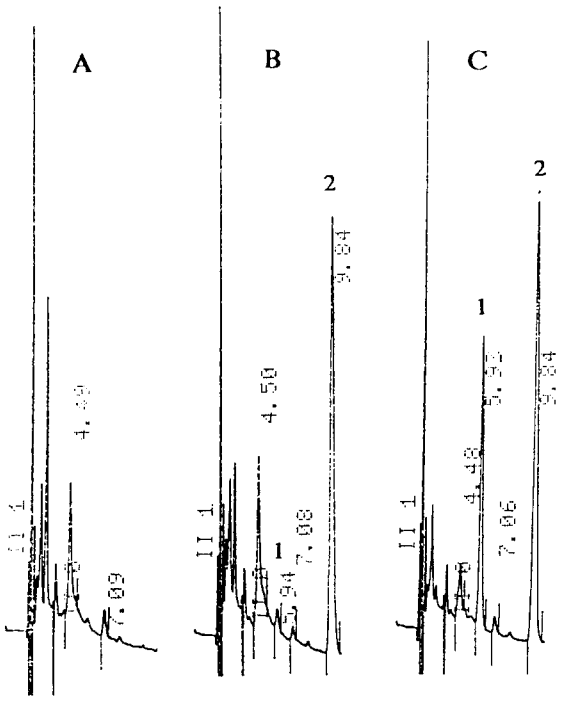

Fig. 2. Typical chromatograms resulting from the injection of serum extracts into the chromatographic system for drug-free serum (A), serum spiked with 50 ng of KT (1) and 10 μg of the IS (2) (B), and serum obtained from a healthy volunteer 2 hr after administration of an oral dose of 10 mg KT, spiked with 10 μg of the IS (2) (C)

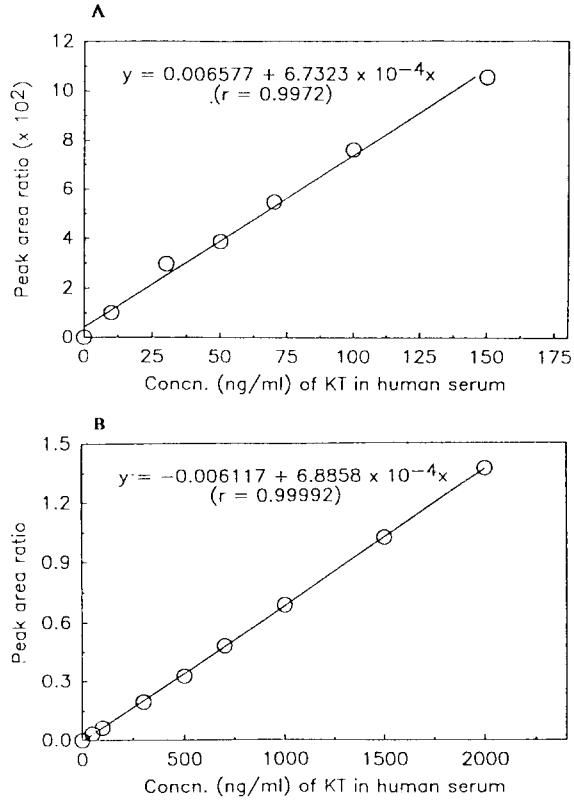

Fig. 3. Calibration curves for ketorolac in human serum over concentration ranges of KT at 10-150 ng/ml (A) and 50-2,000 ng/ml (B)

Table II. Serum linearity of known KT concentrations at nanogram level

Serial No.	Concn. of KT spiked (ng/ml)	Peak area ratio (×10²)			mean (±SD)	C.V. (%)
		1	2	3		
1	10	1.0582	0.9527	1.0326	1.0145 (±0.0550)	5.42
2	30	2.8938	2.9354	2.8149	2.8814 (±0.0612)	2.12
3	50	3.8758	3.7659	3.5058	3.7158 (±0.1900)	5.11
4	70	5.4626	5.7881	5.2465	5.4491 (±0.2726)	4.96
5	100	7.4702	7.6510	7.7467	7.6226 (±0.1404)	1.84
6	150	10.3609	10.6812	10.5482	10.5482 (±0.1669)	1.58
Mean (Intra-day precision)						3.51

Table III. Evaluation of the intra-day and inter-day accuracy and precision for the assay of ketorolac in human serum

Amount spiked (ng/ml)	Amount recovered (mean±S.D., n=3) (ng/ml)	Absolute deviation (mean±S.D., n=3) (%)	CV (%)
Intra-day variance			
50	51.7 (±3.1)	5.2 (±3.6)	5.90
100	98.5 (±6.1)	4.8 (±2.3)	6.15
300	298.7 (±9.2)	2.5 (±1.2)	3.09
500	487.2 (±15.1)	2.4 (±0.7)	3.03
700	676.5 (±31.3)	4.6 (±2.7)	4.63
1000	961.3 (±33.8)	3.9 (±3.4)	3.51
1500	1436.8 (±32.5)	4.2 (±2.2)	2.26
2000	1976.9 (±15.2)	1.2 (±0.8)	0.77
Mean		3.6	3.67
Inter-day variance			
50	53.2 (±1.9)	6.4 (±3.9)	3.57
100	97.6 (±2.0)	3.6 (±1.0)	2.05
300	308.2 (±17.2)	5.2 (±2.0)	5.58
500	511.6 (±21.9)	4.0 (±1.8)	4.27
700	708.5 (±36.5)	4.5 (±0.5)	5.15
1000	1019.5 (±36.0)	3.0 (±2.2)	3.56
1500	1496.6 (±69.9)	3.4 (±2.0)	4.61
2000	2025.2 (±36.1)	1.6 (±1.3)	1.08
Mean		4.0	3.73

ml. Coefficients of variation for peak area ratio were 5.12 and 0.50% at the concentration of KT at 100 and 500 ng/ml, respectively. This recovery of ketorolac was better than those obtained by other extraction methods (Jamali *et al.*, 1989; Jallad *et al.*, 1990), and comparable with the result obtained by directly injecting methanolic solution of deproteinized serum (Chaudhary *et al.*, 1993).

Reproducibility, calibration curve, accuracy, precision and application

Serum linearity of known KT concentrations is shown in Table II. Over a range of 10-150 ng/ml of KT, reproducibility (C.V. %) was 3.5±1.8%. Various concentrations of KT spiked into human serum were used to construct a calibration curve. As shown in Fig. 3, calibration curves were obtained over both ranges of 10-150 ng/ml and 50-2,000 ng/ml. Good linear relationships for both concentration ranges were obtained when the ratios of the peak areas of ketorolac to IS were plotted against KT concentrations. The sensitivity of the assay was 10 ng/ml for KT.

Tables III shows the intra-day and inter-day variances. In a concentration range of KT at 50-2,000 ng/ml, mean intra-day accuracy and precision for this HPLC method were found to be 3.6 and 3.7 %, respectively. Also, mean inter-day accuracy and precision were found to be 4.0 and 3.7 %, respectively. These results indicate that the sensitivity, precision, and accuracy of this method is better than those of other methods (Mroszczac *et al.*, 1987; Flores-Murrieta *et al.*, 1994).

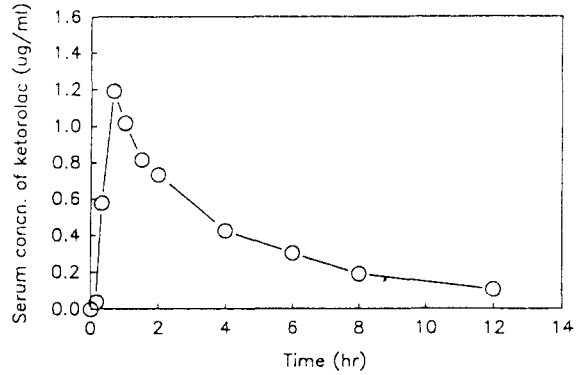

Fig. 4. Serum concentration-time curve of ketorolac after oral administration of a commercial tablet containing 10 mg of KT to a healthy volunteer

Serum samples obtained after oral adminstration of a commercial tablet containing 10 mg of KT to a healthy volunteer were analyzed by this HPLC method. Fig. 4 shows the serum concentration-time profile of ketorolac. Oral absorption of KT was rapid. Peak serum ketorolac level of 1.19 μg/ml was achieved at 40 min after oral administration. This result is similar to that of pharmacokinetic study by Flores-Murrieta *et al.* (1994).

These facts suggest that this HPLC method is successfully applicable to the bioequivalence and pharmacokinetic studies of KT preparations.

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