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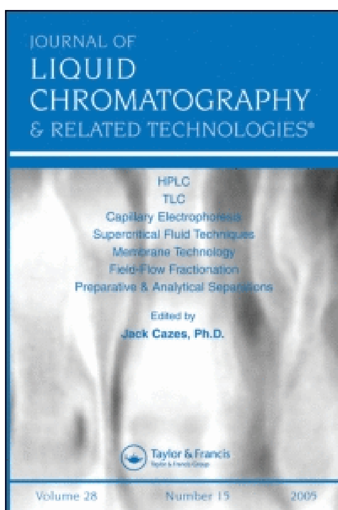

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DETERMINATION OF KETOROLAC IN HUMAN PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AFTER AUTOMATED ON-LINE SOLID PHASE EXTRACTION

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ABSTRACT

An automated solid phase extraction-based procedure for the determination of ketorolac in human plasma was developed and validated. Solid phase extraction of ketorolac tromethamine and ketoprofen (internal standard) was performed on disposable C-18 cartridges directly from plasma samples spiked with the corresponding standards after dilution with saline. All operations were performed automatically by means of a switching-valve assembly in the sample preparation module (Prospekt system), and sample analysis were carried out by on line connection with the HPLC system. Linearity of the analytical methodology was assessed at the concentration range 25-2500 ng/mL ketorolac tromethamine. However, accurate and precise determinations at 5 and 10 ng/mL were obtained by the use of weighing in calibration curve fitting. The mean absolute recoveries for ketorolac tromethamine and the internal standard were 87.7% and 72.9% respectively. Respective intra- and inter-assay precision values were below 7.2 and 18.0%, the later at the

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5 ng/mL concentration level of ketorolac tromethamine which was considered as the lower limit of quantitation (3.4 ng/mL in terms of free acid form). Intra- and inter-assay accuracy, expressed as relative error in percentage, were less than 9.5 and 8.8% respectively. The minimal sample handling and the obtained precision and accuracy at the wide range of concentration levels tested make this method suitable for routine quantitations of ketorolac in human plasma.

INTRODUCTION

Ketorolac tromethamine (KT) is an orally and parenterally active agent with potent analgesic and antiinflammatory activity associated with the inhibition of cicloo×ygenase.[1,2] KT has been shown to be effective in the management of acute pain[3,4] as well as in the improvement of occular inflammation.[5]

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Analytical methods for the determination of ketorolac in plasma and other biological fluids have been developed mainly for pharmacokinetic studies. Except for a method in which zinc sulphate-deproteinized serum is used,[6] the recently reported analytical techniques combine liquid-liquid extraction of the drug into organic solvents such as diethyl ether or ethyl acetate, with HPLC-based quantitations.[7-9] The most common disadvantages of liquid extraction are the large sample handling and the long time consuming although effective sample clean-up and relative high detection levels are often achieved using such techniques.

In this paper, a new automated solid phase extraction (SPE) based procedure for the determination of ketorolac in human plasma is described. The method uses ketoprofen (KP) as internal standard and combines minimal sample preparation with the lowest reported limit of quantitation.

MATERIALS

Chemicals and Reagents

KT was purchased from Chemo Ibérica (Barcelona, Spain), KP was obtained from Sigma (St. Louis, MO, USA) and was used as internal standard.

Sodium chloride, sodium acetate and acetic acid (reagent grade) were supplied by Merck (Darmstadt, Germany). Methanol and acetonitrile (for HPLC) were obtained from Scharlau (Barcelona, Spain). All HPLC and SPE solvents were filtered through 0.5 μm Millipore filters and thoroughly degassed in an ultrasonic bath before use. Sodium heparinate (161.4 USP heparin units/mg) was obtained from Kraeber (Hamburg, Germany). Water was purified through a Milli-Q system (18 MΩ cm resistivity).

Instrumentation

Sample injections and SPE were made with a Prospekt (Programmable On-line Solid-Phase Extraction Technique) system (Spark Holland, Emmen, Netherlands). The complete system consisted of a solvent delivery unit (SDU) with a purge pump and a six-port solvent selection valve, an autosampler (Marathon) which was cooled at 4°C, and a programmable on-line sample preparation module (the main Prospekt SPE controller unit). Chromatographic separations were performed using Waters HPLC equipment (Waters, Mildford, MA, USA) consisting of a M-600 pump, a M-486 U.V. detector, a RCM 8 x 10 radial compression module and a M-845 data and chromatography control station using Waters ExpertEase Chromatography software (V.3.0).

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METHODS

Standard Preparation

Stock solutions of KT and the internal standard KP were prepared weekly in methanol at a concentration of 1 mg/mL. Working standard solutions (0.1 to 200 μg/mL for KT and 10 μg/mL for KP) were prepared daily by dilution of stock solutions with methanol:water (1:9) mixture.

Plasma Collection and Sample Preparation

Human blood was obtained from healthy donors into heparinized tubes (0.75 mg sodium heparinate/mL blood). Plasma was obtained by blood

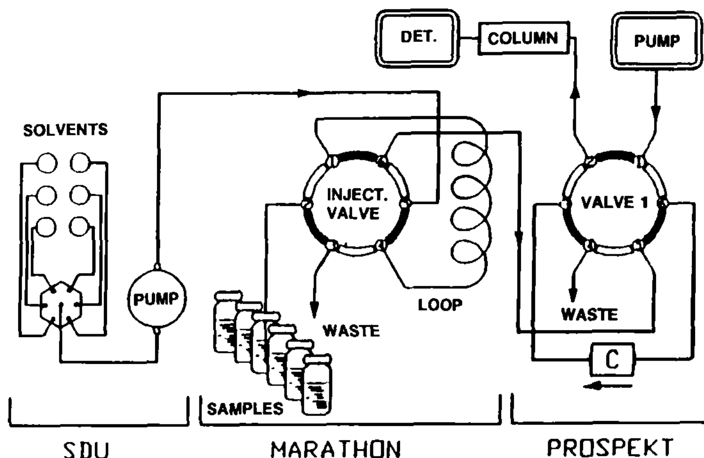

Figure 1. Schematic diagram of the SPE-HPLC system.

centrifugation at 2000 x g (4°C) for 15 minutes. After centrifugation, plasma was pooled and stored frozen at -80°C in polypropylene tubes until use.

Triplicate sets of standard calibration curves of KT in human plasma were prepared on three different days for inter-assay method validation. Intra-assay validation data was obtained using six calibration curves, all prepared on the same day. Sample preparation was performed as follows: 500 μl aliquots of pooled blank plasma were placed in 1.2 mL autosampler glass vials. 550 μl of NaCl 0.9% were further added and the mixture was vigorously vortexed. Finally, 25 μl aliquots of KT and KP working standard solutions were added to obtain the desired final plasma concentrations. Samples were analyzed on the same day of preparation.

Solid Phase Extraction and Chromatography

Automated SPE from spiked plasma samples was performed on the Prospekt system which was switched on-line to the liquid chromatograph. Samples were automatically loaded on disposable cartridges, purged with the appropriate solvents for clean-up and were subsequently eluted to the HPLC system. The configuration of the whole SPE-HPLC system is illustrated in Figure 1.

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Extraction procedure was conducted as follows: Solid phase C-18 disposable cartridges (10 x 3 mm I.D., Analytichem) were first conditioned with 2 mL of methanol (solvent 1) followed by 2 mL of Milli-Q water (solvent 2) and 2 mL of 50 mM sodium acetate pH 3.5 (solvent 3). All solvents were flushed at a flow rate of 2 mL/min. Sample loading was achieved by switching the sample loop valve and allowing the on line conection of the SDU with the C-18 cartridge through the sample loop. Sample (1 mL) was then directed to the cartridge at a flow rate of 1 mL/min solvent 3 during 2 minutes. For sample clean-up, 1.5 mL of methanol:0.1% acetic acid (20:80) mixture were flushed through the cartridge at a flow rate of 1.5 mL/min. Elution was performed by on-line connection of C-18 cartridge with the HPLC system by means of the switching valve (valve 1, Figure 1).

Chromatographic separations were carried out using a Nova-pak C-18 radial pak cartridge (10 x 0.8 cm, 4 µm, Waters) coupled to a Newguard RP-18 cartridge guard column (15 x 3.2 mm, 7 µm, Applied Biosystems, San Jose, CA, USA). The mobile phase consisted of acetonitrile (solvent A) and 0.1% acetic acid (solvent B). A linear gradient program was used from 30% to 60% solvent A in 10 min, 60% solvent A was maintained for 2 minutes and increased to 100% in 3 minutes. Flow rate was set at 2 mL/min. UV detection was performed at 313 nm from 0 to 7.2 min and at 258 nm from 7.2 min to the end of the analysis.

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Quantitation

Quantitation was performed by the internal standard method. For calibration graphs the peak area ratio of KT/KP were plotted versus known concentrations of KT. Data were fitted by weighed least-squares linear regression using the reciprocal of the square concentration values as the weighing factor. A weighed least-squares regression was used because of the clear improvement in the precision and accuracy for the back calculated values, mainly at low concentration levels. Data were automatically processed with ExpertEase Chromatography Software (V.3.0).

Linearity was assessed by comparing the obtained values after peak area ratio normalization by the corresponding KT effective concentration.

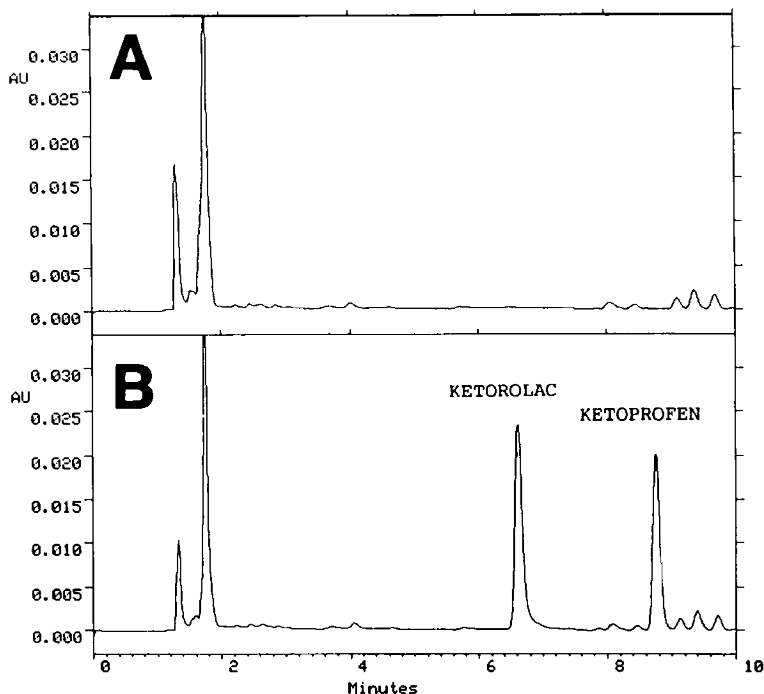

Figure 2. Representative chromatographic profiles of: (A), human blank plasma sample and (B), plasma sample spiked with 500 ng/mL KT and 250 ng/mL KP (internal standard).

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Recovery, Accuracy and Precision

To estimate the recovery of the analytical procedure, stock standard solutions of KT and KP were diluted with methanol:water (1:9) mixture and were directly injected into the HPLC system (reference solutions, injection volume of 100 µl). Dilutions were properly corrected to ensure that the same total standard amounts were injected into the HPLC system when compared with those introduced to the C-18 cartridges from spiked plasma samples. The absolute recoveries were established by comparing the absolute peak areas for spiked plasma samples after the extraction procedure with those of the reference solutions. Mean recovery values were obtained from duplicate sets of calibration standard samples prepared each day of the validation process.

Intra- and inter-assay precision and accuracy were determined at seven concentration levels of KT (5, 10, 25, 50, 250, 500 and 2500 ng/mL plasma). For this purpose, the first analyzed set of standard spiked plasma samples was used to construct the calibration graph. The remaining samples were analyzed as unknowns and KT concentrations determined from these samples against the initially obtained calibration curve. Precision was expressed as coefficient of variation (C.V.) and accuracy as relative error (R.E.), both in percentage.

Statistics

One way analysis of variance was used to perform comparisons among the different calculated parameters at the concentration levels tested.

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RESULTS AND DISCUSSION

HPLC chromatographic profiles for human blank plasma and plasma spiked with KT and KP (internal standard) are depicted in Figures 2-A and B respectively. As it is shown in Figure 2-B, both peaks were well resolved. No endogenous interfering peaks were found in blank plasma samples at their retention times (Figure 2-A) indicating a good selectivity for the chromatographic method. UV detection for KT was performed at 313 nm whereas wavelength was changed to 258 nm for KP detection due to the low molar absorptivity of this compound at 313 nm. This mechanism prevented the use of higher concentrations of internal standard in plasma samples which could presumibly influence the solid phase extraction process.

The linearity of the analytical procedure was assessed in the concentration range 5-2500 ng/mL KT. For this purpose KT/KP peak area ratios were normalized by KT effective concentration (Table 1). Comparison by means of analysis of variance showed significant differences ($p < 0.05$) among the normalized ratios. These differences were further attributed (Newman-Keul's multiple comparison test) to the higher responses obtained at the lower KT concentrations (5 and 10 ng/mL). However, the use of weighed linear regression in calibration curve fitting allowed accurate and precise measurements at these concentration levels (see Tables 2 and 3). For twelve

calibration graphs the obtained mean regression coefficient and slope values were r = 0.9986 (0.08% C.V.) and b = 0.003470 (2.2% C.V.) respectively.

Table 1

Normalized Response and Recovery for Ketorolac and Ketoprofen from Spiked Plasma (n=12)

Effective Ketorolac Tromethamine (ng/mL)	Normalized Response Ratio ($x10^3$)	Recovery of Ketorolac (%)	Recovery of Ketoprofen (%)
	Mean ± S.D.	Mean ± S.D.	Mean ± S.D.
5	4.16 ± 0.52	92.2 ± 26.46	75.0 ± 4.14
10	4.03 ± 0.24	90.9 ± 7.52	72.3 ± 2.45
25	3.53 ± 0.22	85.3 ± 4.60	73.1 ± 3.95
50	3.50 ± 0.09	84.4 ± 3.18	72.6 ± 3.75
250	3.52 ± 0.06	87.1 ± 6.49	71.2 ± 2.19
500	3.47 ± 0.09	85.5 ± 5.31	72.4 ± 3.64
2500	3.49 ± 0.10	88.4 ± 5.33	73.8 ± 2.45
Overall		87.7 ± 8.41	72.9 ± 3.22

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The recovery of the analytical procedure for KT was calculated at the entire concentration range tested, whereas recovery for KP was estimated at the working concentration of 250 ng/mL plasma. Results are summarized in Table 1. No statistically significant differences ($p < 0.05$) were found among recovery values obtained at the different KT concentration levels, which ranged from 84.4% at 50 ng/mL to 92.2% at 5 ng/mL, the mean overall value being 87.7 ± 8.41. Likewise, no statistically significant differences ($p > 0.05$) concerning the recovery of the internal standard were found among the concentration levels of KT indicating that KP recovery was unaffected by the presence of increasing amounts of KT in the plasma samples. Recovery for KP ranged from 71.2% to 75.0%. The mean overall recovery for KP was 72.9 ± 3.22.

Precision and accuracy for the determination of KT were calculated from the measured concentration data obtained in replicate calibration curves as described in material and methods. Results are shown in Table 2. Intra-assay precision values ranged from 1.0% at 250 ng/mL to 7.2% at 5 ng/mL

Table 2

Intra- and Inter-Assay Precision and Accuracy for Ketorolac

Effective KT Concentration (ng/mL)	Concentration Found (ng/mL)	C.V. (%)	R.E. (%)
Intra-assay (n= 5)			
5	5.5	7.2	9.5
10	10.8	2.1	7.7
25	23.8	3.6	-4.8
50	49.6	2.8	-0.7
250	254.6	1.0	1.8
500	512.5	1.2	2.5
2500	2582.9	2.7	3.3
Inter-assay (n = 6)			
5	5.3	18.0	5.8
10	10.9	10.6	8.8
25	25.2	7.7	0.7
50	49.8	3.6	-0.4
250	253.7	2.6	1.5
500	503.1	2.6	0.6
2500	2529.4	5.1	1.2

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whereas intra-assay accuracy ranged from -4.8% at 25 ng/mL to 9.5% at 5 ng/mL. Overall precision and accuracy were also estimated for the three days of analysis (inter-assay), in this case, the coefficients of variation ranged from 2.6% at 250 and 500 ng/mL to 18.0% at 5 ng/mL, and the relative errors ranged from -0.4% at 50 ng/mL to 8.8% at 10 ng/mL (Table 2).

To evaluate the goodness of the calibration curve fitting, back-calculated concentration values for KT were obtained from each calibration graph used during method validation. The obtained mean back-calculated values, as well as the precision and accuracy estimations for each KT concentration level, are summarized in Table 3. Precision and accuracy were always below 5.3% at the entire range of concentrations tested.

Table 3

Precision and Accuracy of Back-Calculated Values from Calibration Curves for Ketorolac (n = 12)

Effective KT Concentration (ng/mL)	Concentration Found (ng/mL)	C.V. (%)	R.E. (%)
5	4.9	2.9	1.9
10	10.5	4.1	5.2
25	24.2	5.3	2.9
50	49.1	2.2	1.6
250	252.1	1.1	1.0
500	499.1	2.9	0.4
2500	2515.6	3.5	0.6

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In conclusion, the method described here allows rapid and fully automated extraction and chromatography of ketorolac from human plasma samples, with minimal sample preparation (virtually only plasma dilution and internal standard addition is required). Minimal sample handling as well as the fully automation of the extraction process made this technique specially useful for routine analysis and, probably, would justify the good accuracy and reproducibility obtained. The experimental limit of quantitation for KT was set at 5 ng/mL (3.4 ng/mL in terms of ketorolac free acid form) taking into account the acceptable precision obtained at this concentration (7.2% and 18.0% for intra- and inter-assay C.V. respectively). This limit of quantitation provides enough sensitivity to follow thoroughly the plasma levels of ketorolac for pharmacokinetic and biopharmaceutical studies. The high sensitivity of the proposed methodology could be useful to measure the low plasma levels obtained after the screening of some transdermal formulations where plasma levels below 92 ng/mL (C_{max} value) have been recently reported.[10]

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