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Sensitive capillary gas chromatographic–mass spectrometric–selected-ion monitoring method for the determination of diclofenac concentrations in human plasma

Michael R. Borenstein[a], Yuzhu Xue[a], Stephen Cooper[b], Tsang-Bin Tzeng[a,*]

[a]*Drug Research Unit, School of Pharmacy, Temple University, 3307 N. Broad Street, Philadelphia, PA 19140, USA*
[b]*School of Dentistry, Temple University, 3223 N. Broad Street, Philadelphia, PA 19140, USA*

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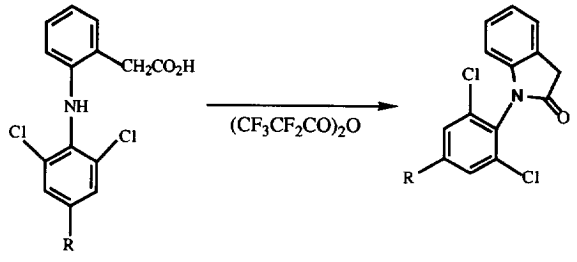
Abstract

We have modified and validated a capillary GC–MS method reported by Kadowaki et al. [J. Chromatogr., 308 (1984) 329] for the determination of diclofenac in human plasma by using heptane rather than benzene as an extraction agent. In addition, acetone was added to the samples as a deproteination agent which increased the recovery of diclofenac. These revised processes allowed clean extraction and near-quantitative recovery of analyte (>95%). Separation was achieved on an HP-1 column with helium as carrier gas. The parent ion peaks of diclofenac (*m/z* 277) and the internal standard, 4′-methoxydiclofenac (*m/z* 307), were monitored by a mass-selective detector using the selected-ion monitoring mode. The linear range for the routine assay was from 5 to 2000 ng/ml. The detection and lower quantifiable limits were 0.2 and 1 ng/ml, respectively, with no interference from plasma. The within-day and between-day coefficients of variation for high and medium concentrations were less than 5% and were less than 13% for low concentrations (10 ng/ml). This GC–MS assay method has been used for pharmacokinetic and drug interaction studies in humans.

Keywords: Diclofenac

1. Introduction

Diclofenac sodium (sodium *o*-(2,6-dichlorophenyl)aminophenylacetate) (Fig. 1) is a non-steroidal anti-inflammatory drug. It is widely used clinically for the treatment of inflammatory disorders [1,2] and acute pain [3,4]. The absorption pharmacokinetics of diclofenac in humans have not yet



R=H diclofenac
R=CH3O 4'-methoxydiclofenac

Fig. 1. Formation of the indolinone derivatives for both diclofenac and 4′-methoxydiclofenac in the presence of PFPA.

*Corresponding author.

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been well characterized, although it has been reported to exhibit highly inter- and intra-subject variability. In addition, the relationships between analgesic effect and diclofenac concentrations in plasma or in tissue have not been elucidated in the literature. To initiate these investigations, we have modified a method by Kadowaki et al. [5] and developed a gas chromatographic (GC) method with mass spectrometric (MS) detection for the determination of diclofenac concentrations in human plasma.

Many methods have been developed for the determination of diclofenac in biological specimens, using GC–MS [5–8], GC with electron-capture detection [9–11], or high-performance liquid chromatography (HPLC) with ultraviolet absorbancy detection [12–19]. HPLC with ultraviolet detection has been the most popular method for the determination of diclofenac in plasma. However, there are disadvantages. A major problem is that assay sensitivity is not sufficient to characterize the absorption kinetics of diclofenac, especially in the early absorption phase. Limit of detection for plasma diclofenac by HPLC methods has been reported in a range of about 10–100 ng/ml only [13,14,19]. Another problem is that these methods are not specific and are liable to interference from the diclofenac metabolites or plasma compounds [14]. Extensive and intensive sample preparation procedures are therefore required to ensure good reproducibility and to provide chromatograms free from interferences. GC–MS methods, on the other hand, offer better specificity and sensitivity, with a reported detection limit of between 0.2 and 2 ng/ml [5,8]. However, derivatization is required to facilitate the analysis of diclofenac in a GC system. Kadowaki et al. [5] reported a very sensitive GC–MS method for the determination of diclofenac in human plasma. Their method offered a limit of detection of 0.2 ng/ml and absolute recovery of 83.6% [5]. However, they used benzene as an extraction solvent and the limit of quantitation was not reported. In the present study, we modified their method using heptane as the primary extraction solvent. Acetone was introduced to our extraction procedures to achieve a near quantitative recovery. This method is specific, sensitive and reproducible for the determination of diclofenac in human plasma and also can be applied to other biological matrices.

2. Experimental

2.1. Chemicals and reagents

Unless specified, all chemicals and reagents were of analytical grade. Diclofenac sodium and the internal standard, 4′-methoxydiclofenac sodium, were supplied by Sigma (St. Louis, MO, USA) and Ciba Geigy (Basel, Switzerland), respectively. Acetone, *n*-heptane, *n*-hexane and methanol were of HPLC grade and were purchased from Fisher (Fair Lawn, NJ, USA). Pentafluoropropionic anhydride (PFPA) and sodium carbonate were obtained from Aldrich (Milwaukee, WI, USA). Phosphoric acid was purchased from J.T. Baker (Phillipsburg, NJ, USA).

2.2. Apparatus and assay conditions

Analysis was accomplished on a Hewelett-Packard Model 5890 gas chromatograph (Hewlett-Packard, Avondale, PA, USA) equipped with a 5971A mass-selective detector and a split–splitless injector. Injections were made with a Hewlett-Packard Model 7673 automatic sampler. Separation was achieved on an HP-1 (cross-linked methyl silicone gum) fused-silica capillary column (12 m×0.2 mm I.D., 0.33 μm film thickness, Hewlett-Packard). Helium (99.95%, BOC Gases, PA, USA) was used as a carrier gas at a flow-rate of 0.92 ml/min. Split injection was used at a ratio of 1:50. Carrier gas flow-rate at the split vent was 50 ml/min. The injection port temperature was 270°C, the detector temperature was 280°C. The column temperature was initially set at 215°C for 5.5 min and at 15°C/min to 240°C for 1.5 min and then at 40°C/min to 290°C for 2.8 min. The last temperature purged residual materials from the column. The mass selective detector was operated in the selected-ion monitoring mode and set at *m/z* 277 and *m/z* 307 [M^+] for the detection of diclofenac and 4′-methoxydiclofenac, respectively. Accelerating voltage was set at 3.5 kV and electron-ionization energy at 70 eV. The detector output was digitized and data processed using a Chemstation data system (Version B.00.02, Hewelett-Packard)

2.3. Sample preparation

To samples of plasma (1 ml), 1225 ng of the internal standard were added followed by acidification with 1 ml of 1 *M* phosphoric acid and deproteination with 1 ml of acetone. The mixture was extracted with 7 ml of heptane by vortex-mixing for 15 s and then by rotating the mixture for 15 min. After centrifugation at 1000 rpm/min for 2 min, the organic (upper) layer was transferred to a 15-ml tube. A 1-ml volume of 0.08 *M* sodium carbonate solution was added to the tube which was then rotated for 15 min. The organic phase was discarded after centrifugation at the same spin rate as mentioned above. The aqueous phase was acidified with 1 ml of a phosphoric acid solution and extracted again with 7 ml of heptane after rotating for 15 min. The extracts were transferred to another clean tube after centrifugation and were evaporated to dryness under nitrogen gas in a 50°C water bath. To the residues, 1 ml of hexane and 0.1 ml of PFPA were added. The samples were allowed to react for 30 min at room temperature (Fig. 1). The hexane layer was then evaporated to dryness under nitrogen gas in a 50°C water bath. The residues were reconstituted with 20 μl of chloroform and 5-μl samples were injected for analysis.

2.4. Standard curve and analysis

Stock solutions of the sodium salts of diclofenac (1 mg/ml) and 4′-methoxydiclofenac (0.2 mg/ml) were prepared by dissolving amounts equivalent to 10 mg of pure diclofenac standard and 2 mg of pure 4′-methoxydiclofenac standard in 10 ml of methanol, respectively. These solutions were stored in the freezer (−20°C). The working solutions in methanol were made by serial dilution of the stock solutions to final concentrations of 0.05, 0.1, 0.2, 1, 5 and 20 μg/ml. Calibration standards were obtained by spiking 100 μl of each of these standards into 1 ml of human plasma to produce concentrations of 5, 10, 20, 100, 500 and 2000 ng/ml. The samples for the standard curve were processed as described previously. The ratio of peak area of diclofenac to that of the internal standard was plotted versus the concentration of the diclofenac in the calibration standard, and a least-squares linear regression analysis was performed. Values of unknown plasma concentrations were determined from the regression line of this calibration curve.

2.5. Variables

The absolute extraction recovery of diclofenac from human plasma was estimated using spiked plasma samples at concentrations of 10, 100, 500 and 1000 ng/ml. These samples were extracted as described earlier except that the internal standard was added to the collected extract. In addition, a set of drug-free normal human plasma samples was also analyzed. Each drug-free plasma extract was then supplemented with diclofenac (5, 10, 20, 100, 500 and 2000 ng/ml) along with the internal standard, and was analyzed to construct a calibration curve. The concentrations of the spiked plasma samples were calculated from the curve and compared to the theoretical values in order to calculate the extraction recovery. The same approach was employed for the determination of the recovery of the internal standard except that diclofenac was used as the "internal standard".

To assess the inter-assay precision [coefficients of variation (C.V.)] and accuracy, four quality control (QC) samples (concentrations at 10, 100, 500 and 1000 ng/ml) were analyzed on five separate days. To assess the intra-assay precision, these same four QC concentrations were analyzed in triplicate on one day.

3. Results and discussion

3.1. Performance of chromatographic system

Fig. 2 displays a representative chromatogram of blank plasma and plasma spiked with 100 ng/ml of diclofenac and 1225 ng/ml of the internal standard. Pooled normal human plasma yielded relatively clean chromatograms with no significant interfering peaks. Both diclofenac and the internal standard showed sharp, well-defined peaks at retention times of 4.7 and 7.8 min, respectively, with baseline separation. The complete mass spectra of diclofenac and the internal standard are shown in Fig. 3. The

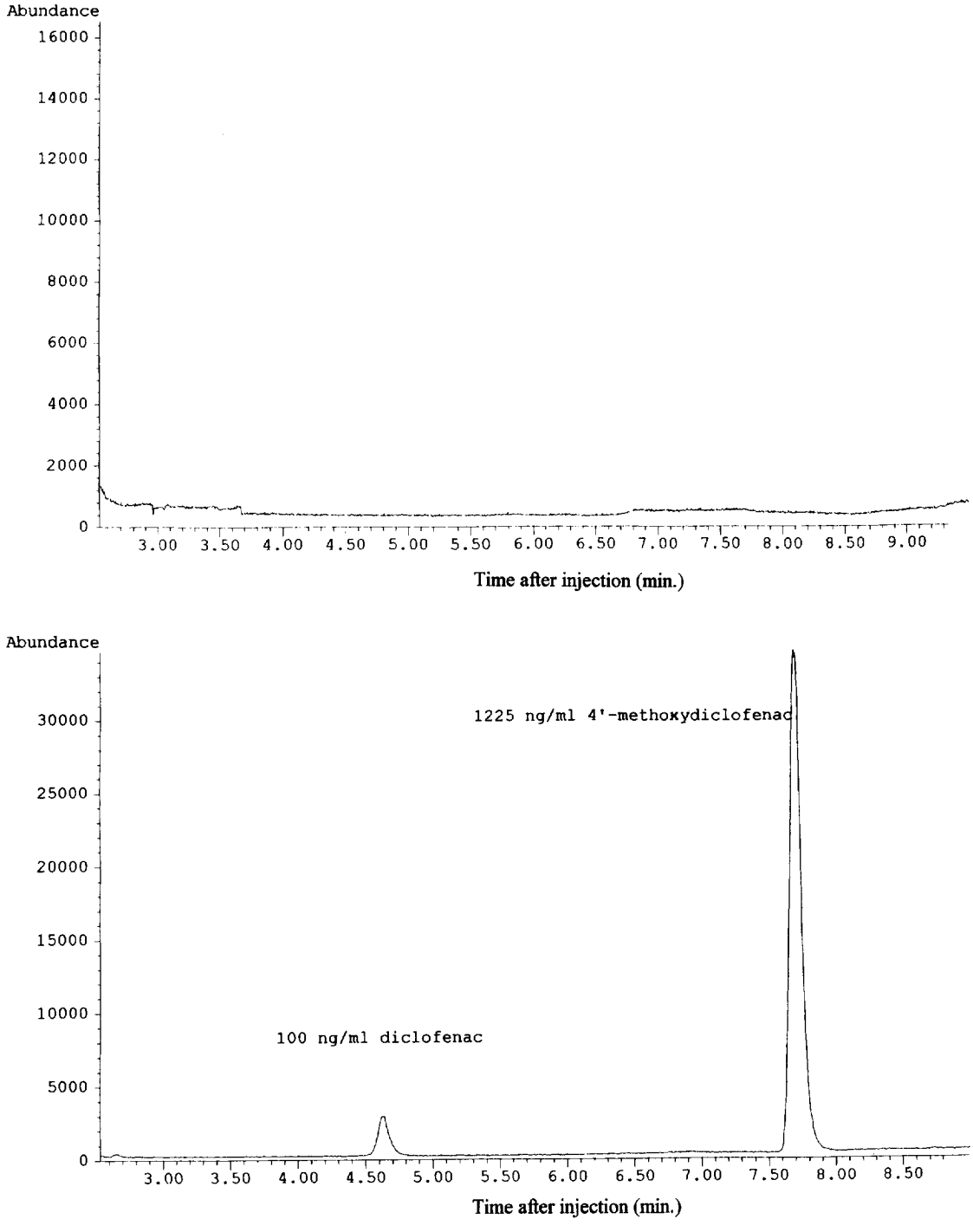

Fig. 2. Chromatograms of blank human plasma (upper) and that spiked with diclofenac (100 ng/ml) and internal standard (1225 ng/ml) (lower).

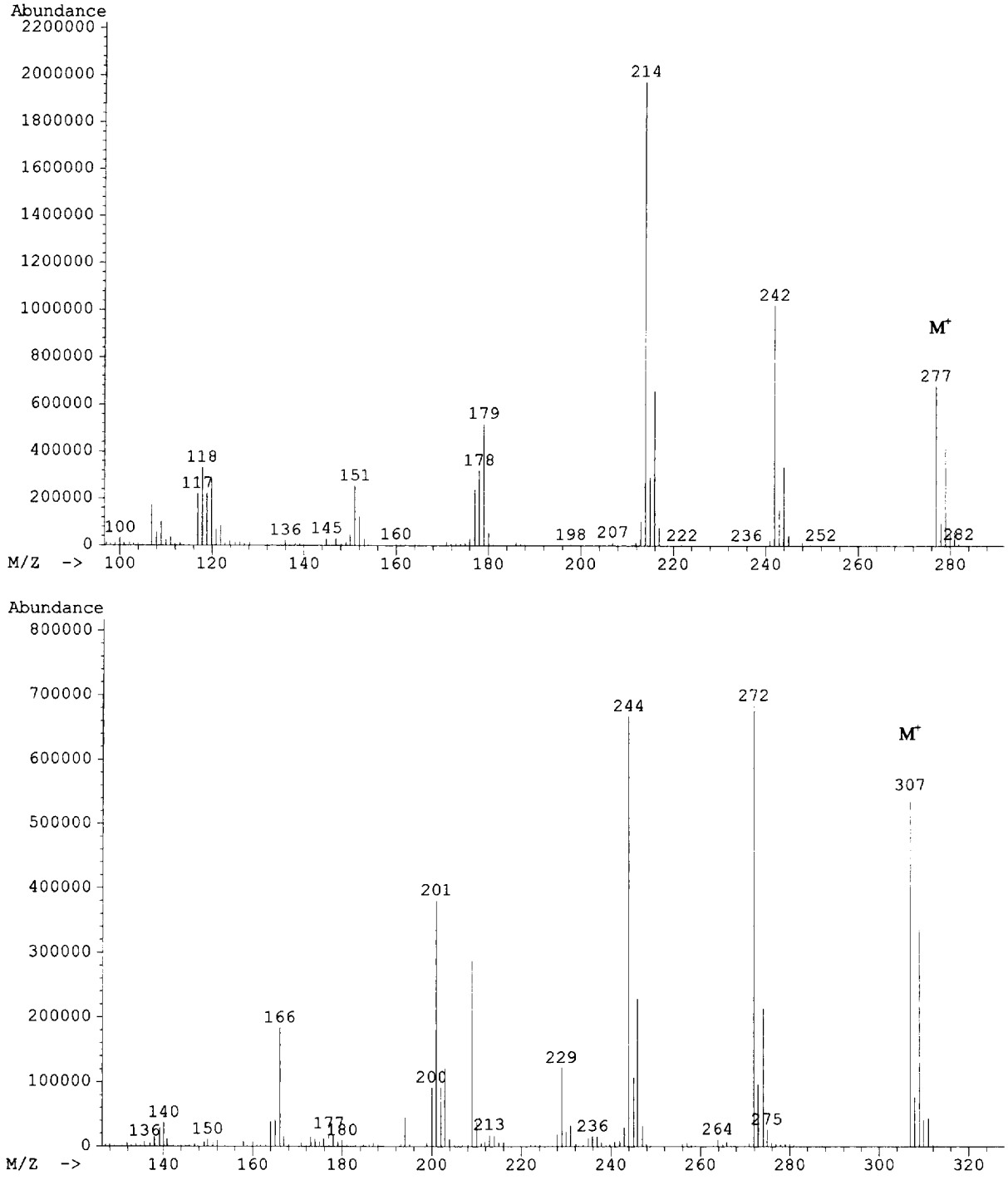

Fig. 3. Mass spectra of diclofenac (upper) and 4′-methoxydiclofenac (lower). Molecular ions for diclofenac and 4′-methoxydiclofenac were monitored in the SIM mode.

method was relatively selective since only the molecular ions, i.e. m/z 277 for diclofenac and m/z 307 for the internal standard, were monitored. To our knowledge, no potential metabolites of diclofenac in human plasma have the same molecular ions as those of diclofenac and the internal standard [19]. Because of the relatively short retention times, each chromatographic run required about 15 min to complete, including conditioning of the column after analysis. This made it possible to analyze about 80 clinical samples per day, including those used for standard curves and quality controls. Sensitivity and reproducibility of this assay were critically dependent on the clean up of the MS detector ion source after approximately 1000 injections.

3.2. Calibration curve and sensitivity

During the validation study, calibration curves were generated over a diclofenac concentration range of 5 to 2000 ng/ml. The curves were all linear with a mean coefficient of determination of 0.9988 (S.D.= 0.000712, n=4), indicating good linearity. To evaluate the curve, the observed responses for the individual standards were substituted back into the equation to calculate the predicted concentrations based on the calibration curve. The results of back calculations for the plasma standard curve generated for diclofenac are shown in Table 1. The difference between back-calculated concentrations and theoretical values ranged from 0.2 to 13% with coefficient variations from 0.4% to 7.1%, indicating good precision for the assay. The limit of quantitation was 1.0 ng/ml with a mean of 0.98 ng/ml and an associated coefficient of variation (C.V.) of 20% (n= 10, detailed data not shown here). Using a signal-to-noise ratio of 2.5 as a measure, the estimated limit of detection was 0.2 ng/ml.

3.3. Recovery of diclofenac and the internal standard

As shown in Table 2, the absolute recovery of diclofenac was almost quantitative, being more than 95% over a 100-fold concentration range. The recoveries were consistent from sample to sample as evidenced by CVs of less than 5.3% for the 1000 and 500 ng/ml levels and of less than 11% for the 10 and 100 ng/ml levels. Recovery was apparently not concentration-dependent; this was also reflected in good linearity of the calibration curves. The recovery of internal standard at the concentration used for the assay was 90.1% with a coefficient of variation of 0.5%, indicating excellent consistency from sample to sample.

The addition of acetone afforded both deproteination and better recovery. Indeed, acetone proved to be essential for the complete recovery of diclofenac from human plasma in this study. Preliminary data indicated that, without acetone, the average recovery for diclofenac from plasma was only approximately 55% after one heptane extraction and approximately 80% after two consecutive heptane extractions.

3.4. Accuracy and precision

Intra- and inter-assay precision and accuracy are illustrated in Table 3. Intra-assay C.V. values were relatively low, with values ≤4.0% at concentrations of 100, 500 and 1000 and ≤11.8% at a concentration

Table 1
Average back-calculated calibration standards for diclofenac (n= 4)

Theoretical value (ng/ml)	Estimated value (ng/ml)	E.T.V.[a] (%)	S.D. (ng/ml)	C.V. (%)
5	5.25	5.55	0.25	4.76
10	9.48	−5.50	0.44	4.68
20	18.02	−9.90	0.48	2.64
100	87.02	12.98	3.48	4.00
500	514.6	2.92	36.43	7.08
2000	1995.62	−0.22	7.86	0.39

[a] E.T.V.=errors from theoretical values.

Table 2
Absolute recovery of diclofenac and 4′-methoxydiclofenac in normal human plasma (n=3)

Added (ng/ml)	Found (ng/ml)	Recovery (%)	S.D. (%)	C.V. (%)
Diclofenac				
10	9.89	98.93	0.79	7.99
100	95.23	95.23	10.36	10.88
500	490.24	98.05	2.82	0.57
1000	963.63	96.36	51.09	5.30
4′-Methoxydiclofenac				
1225	1111.95	90.77	5.62	0.51

Table 3
Accuracy and precision of the diclofenac assay

Added (ng/ml)	Found (ng/ml)	E.T.V.[a] (%)	S.D. (ng/ml)	C.V. (%)
Within-day (n=3)				
10	9.07	−9.3	1.07	11.8
100	92.4	−7.6	2.00	2.2
500	481	−3.9	8.18	1.7
1000	974	−2.6	38.9	4.0
Between-day (n=5)				
10	9.56	−4.4	0.73	7.6
100	93.5	−6.6	4.55	4.9
500	475	5.0	18.7	3.9
1000	990	−1.0	79.9	8.1

[a] E.T.V.=errors from theoretical values.

of 10 ng/ml. The inter-assay C.V. values at concentrations of 10, 100, 500 and 1000 ng/ml were 7.6, 4.9, 3.9 and 8.1%, respectively. The percentage deviation of the mean assayed concentrations from the target values were all within 9.3% for intra-assay and within 6.6% for inter-assay.

3.5. Assay application

This analytical method has been successfully applied to clinical samples obtained after administration of 50 mg of oral diclofenac sodium in dental patients participating in pharmacokinetic studies. No interference from endogenous compounds or metabolites of diclofenac have been found. A typical chromatogram from a study is shown in Fig. 4. A representative diclofenac plasma concentration–time profile is shown in Fig. 5. The observed maximal

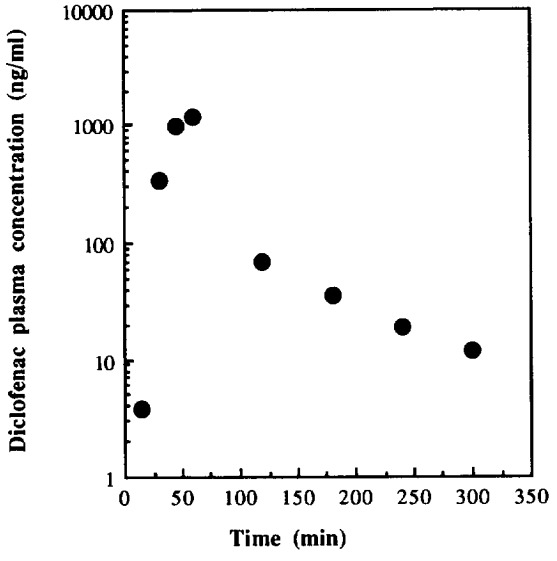

Fig. 5. Representative concentration versus time profile of plasma diclofenac in a dental patient.

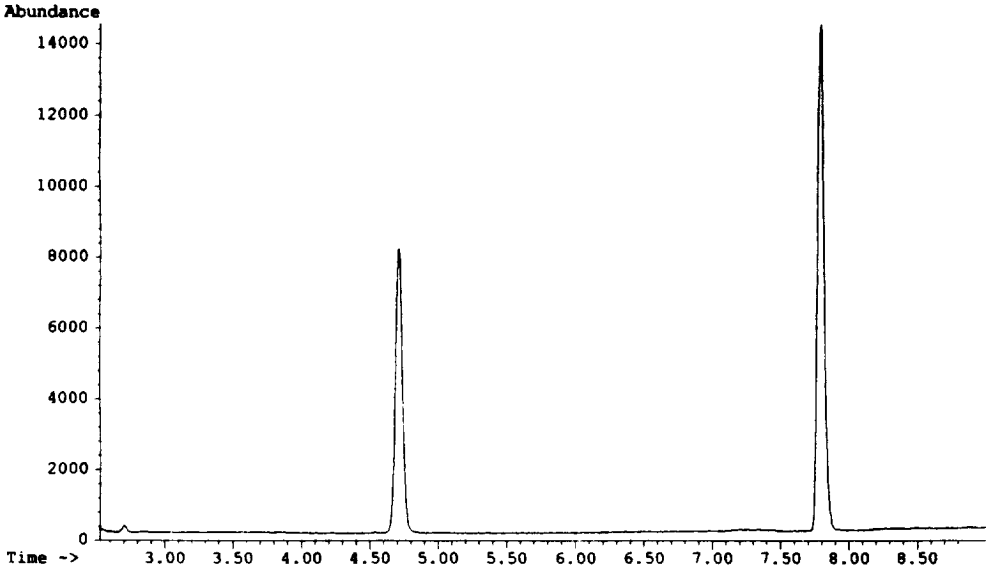

Fig. 4. Typical chromatogram of a plasma diclofenac sample in a dental patient.

plasma concentration, time to maximal plasma concentration and elimination half-life in this patient were 1.2 μg/ml, 1 h and 1.2 h, respectively. A detailed analysis of the oral pharmacokinetics of diclofenac will be presented elsewhere.

4. Conclusion

This paper describes a GC–MS method modified from that reported by Kadowaki et al. [5] for the determination of diclofenac in human plasma. This method offered better recovery and utilized heptane instead of benzene, a relative toxic solvent, as an extraction solvent. In addition, this method is robust, sensitive, specific, reproducible and has proven suitable for use in pharmacokinetic studies of diclofenac.

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