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Bioanalysis of diclofenac as its fluorescent carbazole acetic acid derivative by a post-column photoderivatization high-performance liquid chromatographic method

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ABSTRACT

A sensitive and selective bioanalytical liquid chromatographic method for diclofenac is described. The drug was detected as a fluorescent derivative, which was demonstrated by ^{1}H NMR and mass spectrometric studies to be carbazole acetic acid. Diclofenac was derivatized by UV irradiation of the substance performed as a post-column photoreaction. The reactor was a PTFE capillary wound around a 254-nm UV lamp. Diclofenac was isolated from the plasma samples by precipitation of the proteins with acetonitrile. A 50-μl volume of the supernatant was injected onto a Nucleosil C_{18} column. The mobile phase was 32% acetonitrile in pH 6.6 buffer. Carbazole acetic acid was detected by a fluorescence detector using an excitation wavelength of 288 nm and an emission wavelength of 360 nm. The recovery was 92%, the standard curve was linear in the range 10–5500 ng diclofenac per ml plasma, and the relative standard deviation at 10 and 5000 ng of diclofenac per ml plasma was 9.0% and 3.3%, respectively. The limit of detection was 6 ng/ml at an injection volume of 50 μl. Chromatograms of human and rat plasma containing diclofenac are shown.

INTRODUCTION

Diclofenac sodium has been shown to be an effective and well tolerated pain-releasing substance. The aim of this study was to develop a bioanalytical method for the quantitation of diclofenac in rat and human plasma in order to study the pharmacokinetics of the drug after intramuscular (i.m.) and rectal administration.

In recent years, gas chromatographic methods for the bioanalysis of diclofenac [1,2] have been replaced by liquid chromatographic (LC) methods [2–9]. The LC technique requires less extensive sample clean-up and is generally sensitive and selective. It has been reported that it is possible to use UV detection at 280 nm or lower for the analysis of diclofenac in plasma [7–9]. However, the detection selec-

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tivity was too low under these conditions. At 310 nm the chromatograms were almost free from interfering peaks, but the sensitivity was then insufficient for our studies.

This paper describes a new method for the determination of diclofenac present in low concentrations in human and rat plasma. Diclofenac was transformed by a post-column photoreaction to the fluorescent carbazole acetic acid (CAA) derivative. This made it possible to obtain a dramatic improvement of both the sensitivity and the detection selectivity.

EXPERIMENTAL

Apparatus

The liquid chromatograph consisted of 2150 pump from LKB (Bromma, Sweden). The detectors were a UV detector SPD 6AV and a fluorescence detector RF-530, both manufactured by Shimadzu (Kyoto, Japan). The samples were injected by a Rheodyne 7125 (Berkeley, CA, U.S.A.) or by an autosampler 460 (Kontron Instruments, Zürich, Switzerland). The detector signal was processed by a 3000 Series chromatography data system, version 4.1, from Nelson Analytical (Cupertino, CA, U.S.A.). The column was packed by a Haskel DSTV-150 pump (Haskel, Burbank, CA, U.S.A.). Fluorescence spectra were scanned by an RF-5000 spectrofluorophotometer from Shimadzu. A Metrohm 654 pH meter (Heisan, Switzerland) was used for pH measurements. The samples were centrifuged on a Microfuge B from Beckman Instruments (Palo Alto, CA, U.S.A.). Mass spectra were recorded on a Shimadzu mass spectrometer Model QP 1000 with electron-impact (EI) ionization (70 eV, 250°C). The mass spectrometer was operated in the direct inlet mode. ^{1}H NMR spectra were recorded at 270 MHz for CD_3OD solutions at 30°C with a JEOL GSX-270 spectrometer (Tokyo, Japan). Chemical shifts are given in ppm with internal tetramethylsilane (TMS) as reference.

Chemicals

Nucleosil C_{18} (5 μm particle size) from Macherey & Nagel (Düren, Germany) was used as the chromatographic support. The diclofenac standard was used as the sodium salt of pharmaceutical grade. All other chemicals were of analytical grade from E. Merck (Darmstadt, Germany). The water was deionized, and further purified on a Milli-Q® water purification system from Millipore (Bedford, MA, U.S.A.). It consisted of one prefiltration cartridge, one super-C-carbon cartridge and two ion-X cartridges.

Chromatographic technique

The analytical column (150 mm × 4 mm I.D.) was packed by a modified balanced-density technique [10]. It was made of stainless steel and equipped with modified Swagelok™ (Solon, OH, U.S.A.) connections and filters of 2-μm poros-

ity. A NewGuard™ column (15 mm × 3.2 mm I.D.) packed with 5-μm ODS (Brownlee Labs., Santa Clara, CA, U.S.A.), was used as guard column.

The mobile phase in the bioanalytical method was sodium phosphate buffer - acetonitrile (68:32, v/v). It was made by mixing 40 ml of 1 *M* sodium dihydrogenphosphate with 40 ml of 0.5 *M* disodium hydrogenphosphate; 320 ml of acetonitrile were added, and the mixture was diluted with water up to 1000 ml. This gave a phosphate concentration of 0.06 *M*. The pH of the buffer before addition of an organic modifier was 6.6. The flow-rate was 0.7 ml/min. In some experiments the concentration of the phosphate buffer salts was varied and, in the chromatography at low pH, an acetate buffer was used. The mobile phases were degassed in an ultrasonic bath a few minutes before being used.

Photoreaction studies

Batch experiments were performed in a quartz cell, 1 × 1 × 4 cm. Diclofenac was dissolved in the mobile phase used in the bioanalysis, and the quartz cell was placed *ca.* 10 mm from a UV lamp taken from a Waters 440 UV detector. Samples were taken after different times and analysed by LC. The chromatographic conditions were as in the bioanalytical method, except that the detection was made by UV monitoring at 280 nm. In the purification of CAA, a solution of *ca.* 5 mg of diclofenac was irradiated in the cell overnight. Aliquots of 100 μl were then injected into the LC system, and the peak of interest was monitored by UV detection; this fraction was collected. The pooled fractions were acidified to pH 3 and extracted into chloroform. After evaporation, a pale white-to-yellow substance was deposited. About 1 mg of the substance was used for structure determination by mass spectrometry (MS) and NMR.

Post-column photoderivatization

A UV viewing lamp was used as light source. The lamp was a TUV 6W, TYP 103314, made by Philips (Eindhoven, The Netherlands). This lamp gives its maximum energy at 254 nm, corresponding to 0.09 W, and the lifetime is > 1000 h. A PTFE tube (1.0 mm I.D., 1.8 mm O.D.) was wound directly against the glass of the lamp. The length of the PTFE capillary was 1.3 m, which give an inner volume of 1.0 ml. The diameter at the ends was reduced by stretching the tubing so ferrules could be put on. The reactor was then connected to standard low-dead-volume unions between the outlet of the column and the fluorescence detector. The detector was operated at the highest sensitivity (sensitivity = high and range = 1). The excitation wavelength was 288 nm, and the emission was measured at 360 nm.

Isolation of diclofenac from plasma samples

To 50 μl of plasma, 50 μl of acetonitrile were added for protein precipitation in a 1.5-ml conical polypropylene tube equipped with a cap. After efficient mixing the tube was centrifuged for 2 min at 12 000 *g*, and the supernatant was then

ready for injection into the LC system. The injection volume in the bioanalytical method was 50 μl.

Standard samples

From stock solutions of diclofenac sodium in methanol, appropriate amounts (5–25 μl) were added to 1-ml samples of rat or human plasma. The stock solutions were stored at 4°C, and the plasma samples at −20°C. No degradation of the drug was observed during four months.

Clinical samples

Blood plasma samples were taken from healthy volunteers who had received a single 100-mg i.m. injection of diclofenac sodium. The samples were then analysed by the bioanalytical method.

RESULT AND DISCUSSION

Photochemical reaction of diclofenac

When a diclofenac solution was exposed to UV radiation (254 nm) as described under Experimental, a number of products were found when the solution was separated by LC combined with UV detection. A chromatogram of such a solution is demonstrated in Fig. 1. Some of the peaks that appeared after the photoreaction were short-lived and were further degraded to new products,

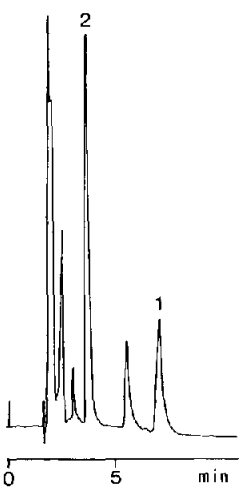


Fig. 1. Chromatogram of a diclofenac solution after 80 min UV irradiation according to the batch experiment described under Experimental. Column, Nucleosil C_{18}, 150 mm × 4.0 mm I.D.; mobile phase, phosphate buffer (pH 6.6)–acetonitrile, (68:32, v/v); flow-rate, 0.9 ml/min; UV detection at 280 nm. Peaks: 1 = diclofenac; 2 = CAA.

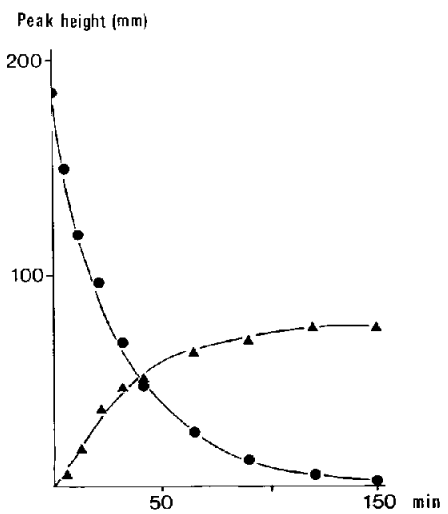
whereas especially the peak of CAA increased steadily during the time of UV irradiation which is demonstrated in Fig. 2. The effluent containing CAA was collected, and its fluorescence properties were investigated. Fig. 3 shows the emission spectrum of CAA.

MS and NMR characterization of CAA

The CAA fraction was purified as described under Experimental. The purity of the substance was determined by LC with UV detection at 280 nm. The chromatogram showed one single peak on the expected retention time. The isolated substance was used for MS and NMR studies.

The mass spectrum of the compound shows a molecular ion, m/z 225, and a base peak ion, m/z 179. The base peak ion arises from the loss of formic acid from the molecular ion.

A ^{1}H NMR spectrum of the compound showed a singlet at δ 3.94 and two spin systems in the aromatic region with signals from four and three protons, respectively. The chemical shift of the singlet corresponds to two benzylic protons in α-position to the carbonyl group. The four-proton spin system consists of signals at δ 7.48 (o, m, p), δ 7.35 (o, o, m), δ 7.14 (o, o, m) and δ 8.03 (o, m, p), and the three-proton spin system signals at δ 7.26 (o, m), δ 7.12 (o, o) and δ 7.97 (o, m). The chemical shifts and coupling patterns (in parenthesis) of the signals clearly show the structure to be carbazole acetic acid (CAA) presented in Fig. 4. This is also supported by the MS studies.



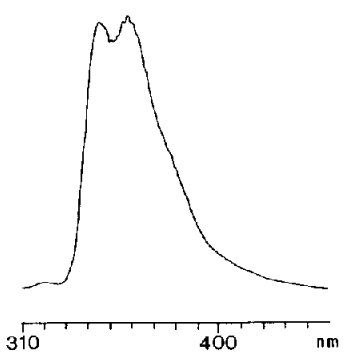
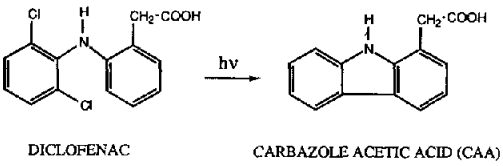


Fig. 2. Formation rate of CAA at UV irradiation of diclofenac performed as batch experiment according to Experimental. Chromatographic conditions as described in Fig. 1: (●) diclofenac; (▲) CAA.

Fig. 3. Emission spectrum of the collected CAA peak in Fig. 1: excitation wavelength, 288 nm.



DICLOFENAC CARBAZOLE ACETIC ACID (CAA)

Fig. 4. Photoreaction of diclofenac to produce CAA.

Optimization of the post-column photoderivatization

A post-column derivatization method was chosen for the bioanalysis of diclofenac. PTFE capillaries have been described as an excellent material for UV photochemical reactors [11], even in comparison with those of quartz. The mercury lamp used as light source was cool enough for the PTFE tubing to be wound directly onto the glass of the lamp. This made the photochemical reactor simple to construct, and it was easy to regulate the length and diameter of the capillary. The reactor has been stable without any change in the detected peak for *ca.* 200 h of use. A reaction performed post-column in some type of a reactor involves a balance between a good reaction yield and a small band broadening of the sample zone. Different ways of reducing the band broadening in the reactor have been suggested [12], *e.g.* packed-bed reactors, small-diameter coiled tubing, or segmented flow.

Fig. 5 shows the results of a study of the photoreaction time of diclofenac in a post-column reactor. The reactor design is described under Experimental. In this experiment the reaction time was varied by variation of the flow-rate of the

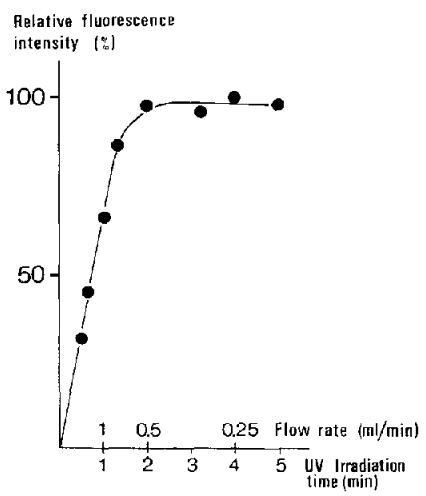


Fig. 5. Relative fluorimetric responses at different UV irradiation times of diclofenac in the post-column reactor. The photoreaction time was varied by different flow-rates through the reactor. Sample: 50 ng of diclofenac in 10 μl of mobile phase. Mobile phase as described in Fig. 1.

mobile phase. As can be seen from Fig. 5, the maximum response measured by the fluorescence detector was obtained after UV irradiation for 2 min. With the reactor design described under Experimental this corresponds to a flow-rate of 0.5 ml/min.

The band-broadening in this reactor was calculated by comparing the bandwidth of the peak of diclofenac with or without the reactor mounted before the detector. The peak was monitored by UV detection after separation on the analytical column. The UV lamp of the reactor was turned off. The bandwidth was found to increase by *ca.* 30% after passage through the reactor.

A flow-rate of 0.7 ml/min was chosen because this gave almost complete photoderivatization of diclofenac within a reasonable time of analysis. The response was linear in the concentration range 10–5500 ng diclofenac per ml plasma.

Chromatography of diclofenac

The carboxylic acid functional group of diclofenac made it easy to regulate the retention by changing the pH of the mobile phase. An increase in the retention of the substance was achieved at pH less than 5. Experiments showed, however, that the fluorimetric response after photoreaction of diclofenac to CAA was 30% lower at pH 2.3 of the mobile phase than at pH 6.6. Hence a neutral pH of the mobile phase was chosen. By varying the sodium phosphate buffer concentration at pH 6.6, it was possible to influence the retention of diclofenac. The k' was increased from 1.5 at 0.006 *M* buffer in the mobile phase to 2.5 at 0.06 *M* buffer, whereas the k' of the large front peak was not unaffected. A concentration of 0.06 *M* buffer in the mobile phase was chosen to achieve a good resolution.

Isolation of diclofenac from plasma

In pharmacokinetic studies in small animals it is not possible to take a large sample volume at each sampling. As the described bioanalytical method is very sensitive, only 50 μl of plasma are required at each sampling. The high detection selectivity and sensitivity also made it possible to use a minimum of isolation and concentration steps prior to the analysis. The plasma proteins were precipitated by mixing equal parts of acetonitrile and plasma, followed by centrifugation and injection of the supernatant. This precipitation method reduces the content of proteins in the supernatant to 2–3% [13]. The degree of protein binding of diclofenac in plasma at the therapeutic drug level is reported to be *ca.* 99% [14]. The recovery of diclofenac in human plasma after protein precipitation was found to be 91 and 93%, at concentrations of 10 and 5000 ng/ml diclofenac, respectively, compared with injection of diclofenac in water–acetonitrile (50:50 v/v). It seems likely that some of the drug is included in the precipitated protein fraction. Deterioration of the analytical column caused by endogenous lipophilic substances, unprecipitated proteins and particles in the sample was eliminated by the use of a guard column. The guard column was changed after *ca.* 100 injections.

Standard curve, accuracy and limit of detection

A reference factor was calculated from the average of ten 500 ng/ml diclofenac plasma samples, analysed according to the bioanalytical method. This reference factor was used by the chromatographic data processor to calculate the concentrations of the samples. Two standard curves for diclofenac in plasma, in the intervals 10–100 and 100–5500 ng/ml, were made by plotting the found concentrations on the y-axis *versus* the spiked concentrations on the x-axis. The linear regression equations with standard deviation for sloop and intercept were found to be: $y = 0.997x\,(\pm 0.023) - 0.099\,(\pm 1{,}076)$; and $y = 1.089x\,(\pm 0.025) - 6.532$ (± 11.34), respectively. The correlation coefficients (r) were in all cases greater than 0.999. Samples with concentrations greater than 5.5 μg diclofenac per ml plasma had to be diluted or the injection volume should be reduced. The within-day reproducibility, given as the relative standard deviation, was performed as a single analysis of spiked human plasma according to the bioanalytical method. It was found to be 9.0% ($n = 9$), at a concentration of 10 ng/ml diclofenac and 3.3% ($n = 8$) at 5 μg/ml. The day-to-day reproducibility of spiked human plasma, which was stored frozen before the analysis, was 3.9 and 3.6% ($n = 6$), at concentrations of 100 and 2000 ng/ml diclofenac, respectively.

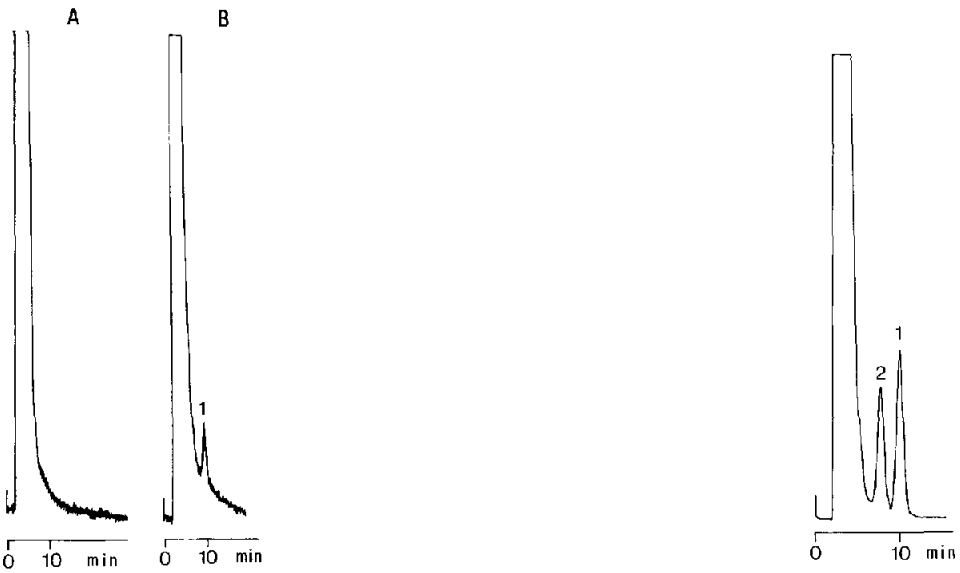


Fig. 6. Chromatograms of rat and human plasma obtained by the bioanalytical method. (A) Blank plasma from rat; (B) human plasma spiked with 10 ng diclofenac per ml plasma. Column and mobile phase as in Fig. 1; flow-rate, 0.7 ml/min; excitation wavelength, 288 nm; emission wavelength, 360 nm. Highest detector sensitivity (sensitivity = high; range = 1); recorder, 10 mV full scale. Peak 1 = diclofenac.

Fig. 7. Chromatogram of patient's plasma sample obtained 30 min after a single 100-mg i.m. injection of diclofenac sodium. Chromatographic conditions as in Fig. 6; recorder, 100 mV full scale. Peaks: 1 = diclofenac; 2 = unknown.

The limit of detection was calculated as three times the baseline noise and found to be 6 ng diclofenac per ml plasma. This corresponds to a minimum detectability of 150 pg diclofenac per injection with this arrangement of the equipment. As no interfering substances could be detected it would be possible to inject larger volumes of the supernatant in order to decrease the limit of detection. Fig. 6 shows chromatograms of blank plasma from rat and spiked human plasma, and Fig. 7 a chromatogram of diclofenac in human plasma after i.m. administration.

CONCLUSION

A sensitive and selective LC method for the bioanalysis of diclofenac in plasma is described. The method requires small sample volumes with a minimum of pretreatment, and diclofenac is detected as its fluorescent derivative, carbazole acetic acid. The derivatization is performed as a UV photochemical post-column reaction.

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