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Crocheted ETFE-reactor for on-line post-column photoderivatization of diclofenac in high-performance liquid chromatography [1]

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Abstract

A sensitive and selective bioanalytical method for diclofenac using reversed-phase HPLC and fluorescence detection is described. Diclofenac was detected as its fluorescent derivative after on-line post-column photoderivatization. Irradiation with UV light of diclofenac in aqueous solutions leads to the sequential loss of both chlorine substituents and ring closure. The major product, carbazole-1-acetic acid, was detected by a fluorescence detector using an excitation wavelength of 286 nm and an emission wavelength of 360 nm. The self-made reactor was a crocheted ethylene and tetrafluoroethylene (ETFE, named TEFZEL) capillary, 20 m in length, wound directly around a 253.7 nm UV lamp. The capillary was crocheted in order to overcome peak widening. Chromatographic separation was achieved by using a Regis SPS 100 RP-8 column (5 μm; 150 mm × 4.6 mm i.d.) and a LiChrospher 100 RP-18 (5 μm) guard column from E. Merck. The detection limit was 1 ng ml^{-1} at an injection volume of 20 μl. Daily relative standard deviations (RSD) were 5.5% (73 ng diclofenac/ml, $n = 9$), and 5.1% (405 ng diclofenac/ml, $n = 6$), respectively. Chromatograms of human aqueous humor and human serum containing diclofenac, and figures showing the time dependent increase/decrease of the photoderivatization product, are shown. © 1997 Elsevier Science B.V.

Keywords: Diclofenac; Carbazole-1-acetic acid; Photoderivatization; ETFE; TEFZEL; Fluorescence detection; Human aqueous humor; Reversed-phase high-performance liquid chromatography

1. Introduction

The well-tolerated nonsteroidal, anti-inflammatory drug (NSAID) diclofenac-sodium ((2-[2,6-dichlorophenyl)amino]benzene acetic acid)sodium salt) inhibits cyclooxygenase activity in the arachidonic acid cascade and decreases prostaglandin synthesis. Furthermore, it shows potent analgesic and antipyretic activity [1].

Different procedures have been reported for the quantification of diclofenac in body fluids. Lansdorp et al. (1990) [2] reported a HPLC method using UV/Vis detection, and Schneider and Degen (1981) [3] a GC method with electron-capture detection. However, both applications did not

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achieve sufficient accuracy at concentrations below 10 ng ml^{-1}. The HPLC methods, described by Zecca et al. (1991) [4] and Kuhlmann et al. (1996 and 1997) [5,6], using an electrochemical detector, are highly sensitive and have very low concentration limits (1 ng ml^{-1}, respectively 0.5 ng ml^{-1}). A very good and much cheaper alternative to the electrochemical detector was the post-column photoderivatization of diclofenac in HPLC. Preliminary photochemical studies for diclofenac [7] were followed by bioanalysis of diclofenac as its fluorescent derivative carbazole-1-acetic acid (Fig. 1) after post-column photoderivatization [8]. The use of PTFE capillaries for photoderivatization is widely used [9–12] and different techniques for geometrically deformations are known [13–15].

In this study the latest improvements are shown, resulting in a highly sensitive and selective method for measuring diclofenac in human aqueous humor and human serum.

2. Experimental

2.1. Chemicals

Diclofenac-sodium ((2-[2,6-dichlorophenyl)-amino]benzene-acetic acid) sodium salt) was obtained from Sigma (Deisenhofen, Germany), sodium acetate anhydrous from Fluka (Neu-Ulm, Germany), and acetonitrile from E. Merck KGaA, Darmstadt, Germany. Voltaren® 0.1% (eye drops with 0.1% diclofenac) came from Dispersa (CIBA Vision Ophthalmics GmbH, Wesslingen, Germany). Phosphoric acid (85%) was produced by Laborchemie, Apolda, Germany. All chemicals were used without further purification procedures. The water used in the experiments was purified by SERALPUR Pro 90 CN (SERAL, Germany) and additionally distilled.

2.2. Instrumentation

The high-performance liquid chromatograph from Spectra–Physics consisted of a pump (P2000), an UV/Vis detector (UV3000/FOCUS/SM5000), a fluorescence detector (FL 2000) and a Rheodyne injector Model 7125 (Cotati, U.S.A.) connected with a 20-μl external loop. Chromatographic separation was performed with a Regis SPS 100 RP-8 (5 μm; 150 mm × 4.6 mm i.d., Morton Grove, U.S.A.). The guard column was LiChrospher 100 RP-18 (5 μm) (E. Merck, Darmstadt, Germany). Between the column and the detector systems, a self-made photoderivatization reactor was inserted. This reactor consisted of a crocheted ethylene and tetrafluoroethylene (ETFE) capillary (0.25 mm i.d., 1.59 mm o.d., Plasticell Vertriebs GmbH, Radolfzell, Germany) wound directly around a low pressure 3.5-W UV lamp (length: 378 mm; 26 mm o.d.) (HNS 15 OFR, Osram, Leipzig, Germany) with spectral emission at 253.7 nm. The length of the ETFE capillary was 20 m, and after crocheting about 4.3 m. The excitation spectrum (absorption) was scanned by the UV/Vis detector, and the emission spectrum by the fluorescence spectrophotometer F-3010 from Hitachi (Hitachi, Ltd., Tokyo, Japan). A pH meter (WTW, pH537, Weilheim, Germany) was used for pH measurements. The acidified serum samples were centrifuged in a Hettich centrifuge (EBA 12; 14000 rpm/5min).

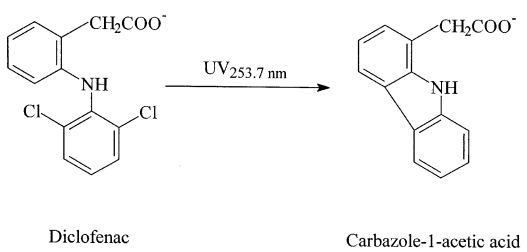

Fig. 1. Photoderivatization scheme of diclofenac.

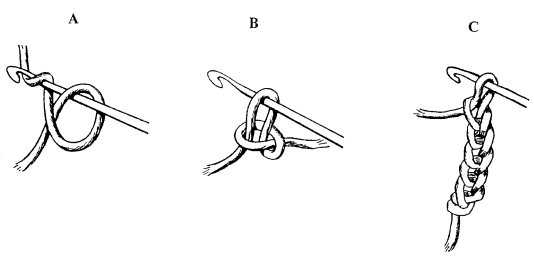

Fig. 2. Instruction scheme for the crocheting procedure.

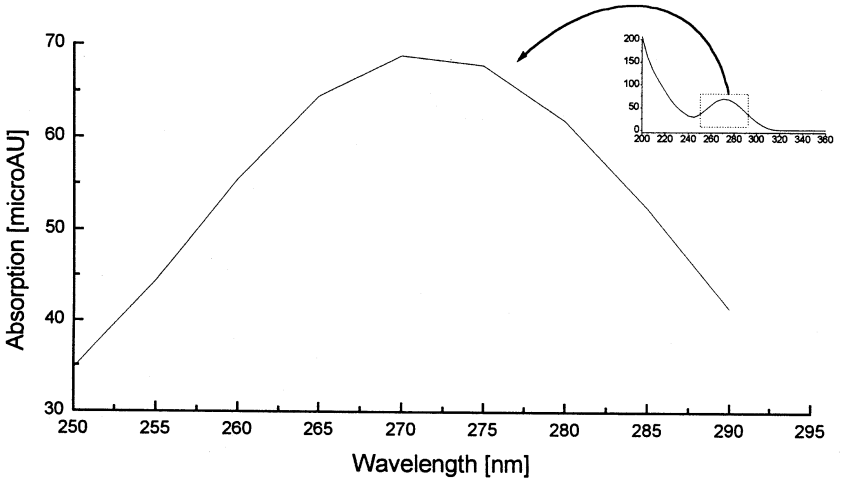

Fig. 3. Absorption spectrum for diclofenac.

2.3. Chromatographic technique

The mobile phase was acetonitrile-sodium acetate (30 mM) (60:40, v/v). The sodium acetate was adjusted to pH 3.00 with phosphoric acid (85%). The filtered mobile phase was degassed under a constant flow of helium (10 min). A flow rate of 1.0 ml min^{-1} was established at ambient temperature. The excitation and emission wavelengths for fluorescence detection were set at 286 and 360 nm, respectively. The wavelength for UV detection was set at 272 nm.

2.4. Sample preparations

Human aqueous humor was sampled at the very beginning of cataract surgery by puncturing the anterior chamber (60–150 μl). The samples were stored until analysis at −20°C. Twenty μl were directly injected onto the column.

Blood samples were centrifuged at −4°C for 10 min (5500 rpm) and the supernatant stored at −20°C. For analysis, 250 μl human serum were deproteinized with 250 μl acetonitrile. After centrifugation (14000 rpm/5 min), 20 μl of the supernatant were injected onto the column.

2.5. Calibration curve

A 1.0 ml stock solution containing diclofenac was prepared with acetonitrile. Further dilutions were made, giving concentrations between 200–2000 ng diclofenac/ml (e.g., 200, 400, 800, 1600 and 2000 ng ml^{-1}). The external standard solutions of diclofenac were then prepared by adding 50 μl of the different stock solutions (200–2000 ng ml^{-1}) to 250 μl human serum. After shaking, 200 μl acetonitrile were added, mixed well and centrifuged for 5 min (14000 rpm). Twenty μl of the supernatant were injected onto the column. The concentrations yielded were 20, 40, 80, 160 and 200 ng diclofenac/ml.

2.6. Photochemical reactor design

Before crocheting the capillary, the following precautions should be taken in order to prevent leakage: 1. fill the capillary with water using a HPLC pump, 2. close the ends (e.g. by heating). The crocheting procedure starts with tying a loose knot about 15 cm from the end of the sleeve. Insert the crochethook through the knot and pull the tubing through to form another loop (Fig. 2).

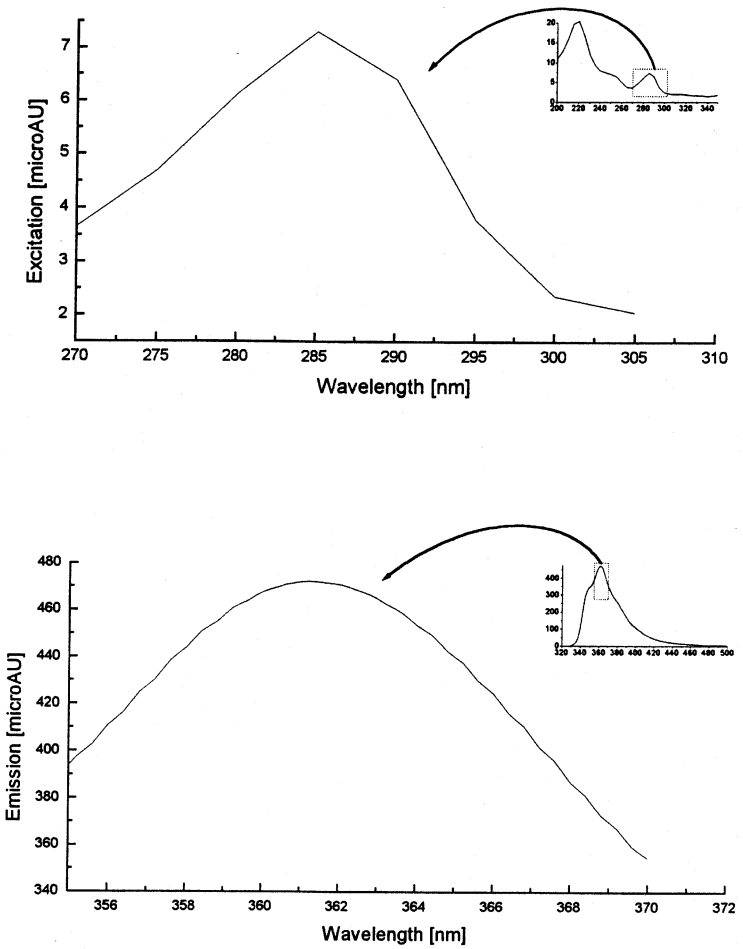

Fig. 4. Excitation- and emission-spectra for carbazole-1-acetic acid.

Repeat this procedure until only 15 cm are left and tie a second loose knot. The crocheted capillary thus formed was wound directly around the 253.7 nm UV lamp. For increased photon flux through the reactor, and to protect the staff from UV-radiation, aluminium foil was wrapped around the UV lamp and capillary. After a PEEK-sleeve was fitted to the crocheted ETFE capillary, it was connected to the HPLC system (between column and detector).

3. Results

3.1. Off-line photoreaction studies

A quartz cell (Hellma, 100-QS, K 82) $1 \times 10 \times 40$ mm, filled with 400 μl diclofenac-solution (4050 ng ml^{-1}), was placed 3 mm in front of the UV lamp. After different UV-irradiation times (between 15 sec and 20 min), 20 μl of these solutions were injected onto the column. The ab-

sorption wavelength for the measurement of diclofenac was set at 272 nm. The excitation wavelength for the measurement of the carbazole-1-acetic acid was set at 286 nm, and the emission wavelength at 360 nm. The wavelength spectra are shown in Figs. 3 and 4, respectively the time dependent decrease of a diclofenac peak and increase of the carbazole-1-acetic acid peak (Figs. 5 and 6).

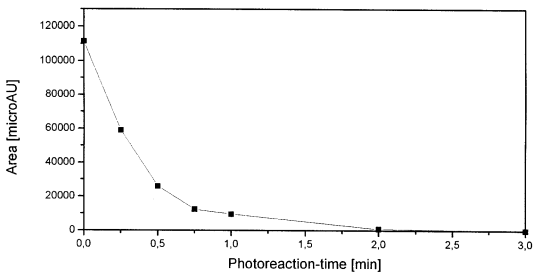

Fig. 5. Time dependent decrease of diclofenac under off-line-photoderivatization.

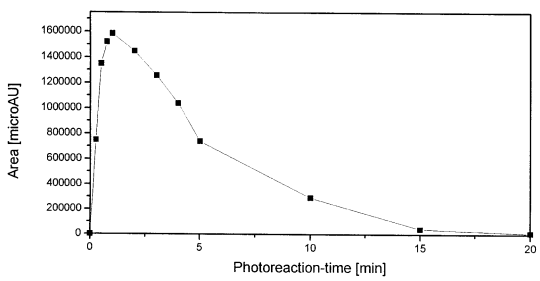

Fig. 6. Time dependent increase/decrease of carbazole-1-acetic acid under off-line-photoderivatization.

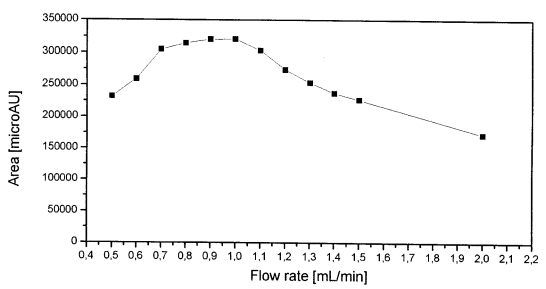

Fig. 7. Flow rate dependent increase/decrease of carbazole-1-acetic acid under on-line-photoderivatization.

3.2. On-line photoreaction studies

The 20 m long ETFE capillary (respectively 4.3 m, crocheted) gives an inner volume of 1 ml (0.25 mm i.d.). Different flow rates were tested to achieve the highest possible signal for the fluorescent derivative carbazole-1-acetic acid. Signals resulting from different flow rates are shown in Fig. 7.

When a flow rate of 1.0 ml min^{-1} was used, the diclofenac peak appeared after about 6.9 min (RT). The capacity factor was 1.5. The linearity was measured over the range of 2.2–1740 ng diclofenac/ml. The correlation coefficients were greater than 0.9995. The within-day relative standard deviations (RSD) were 5.5% (73 ng diclofenac/ml, $n = 9$), and 5.1% (405 ng diclofenac/ml, $n = 6$), respectively.

Fig. 8 shows fluorescence emission-chromatograms (286/360 nm) from human aqueous humor with and without UV-irradiation for the observation of carbazole-1-acetic acid, and chromatograms from human plasma (Fig. 9), respectively. The increase in sensitivity on comparing UV-detection (272 nm) of diclofenac with the fluorescence emission-detection (286/360 nm) of its derivative is shown in Fig. 10.

When using the 20 m long capillary, an increase in back-pressure from 63 to 76 bars, together with a prolonged retention time from 5.5 to 6.9 min, were observed. The peak-width, measured at the base of the peak, rose from 0.227 min (t_R, 5.5 min) to 0.277 min (t_R, 6.9 min).

4. Discussion

For the determination of kinetics in biological samples, applications with sufficient accuracy at concentrations below 10 ng diclofenac/ml are necessary. The previously reported HPLC-method using UV/Vis detection [2], or the GC-method with electron-capture detection [3], did not achieve this goal. HPLC with an electrochemical detector [4–6] is sufficiently sensitive, but not as simple to handle as the described photoderivatization. Furthermore, the self-made photoreactor is much cheaper.

Human aqueous humor contains 200 μg protein (or lower) per ml [16]. Because of the small sample volumes (averaging 80 μl) and low drug concentra-

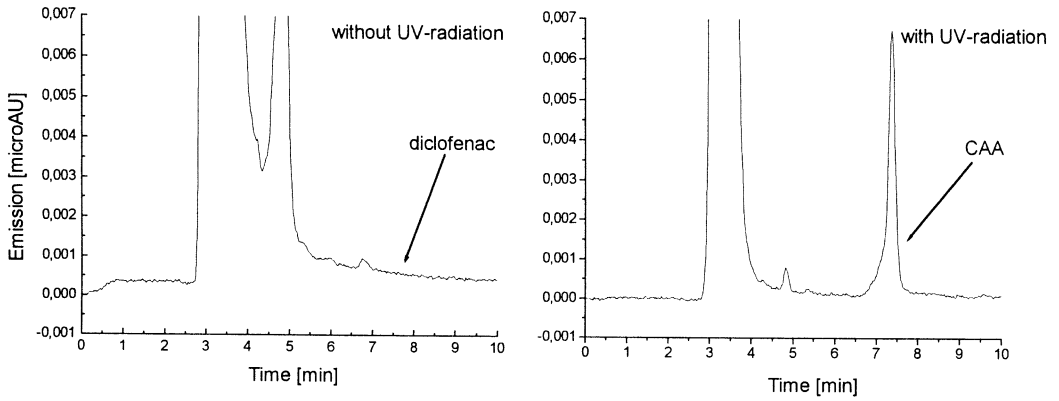

Fig. 8. Fluorescence emission-chromatograms (286/360 nm) for human aqueous humor, containing 73 ng diclofenac/ml, with and without UV-radiation (photoderivatization).

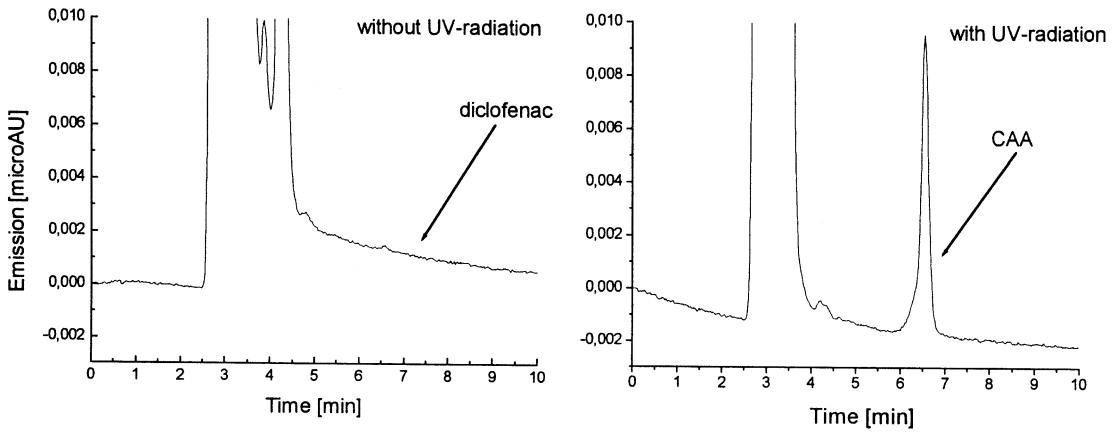

Fig. 9. Fluorescence emission-chromatograms (286/360 nm) for human serum, containing 146 ng diclofenac/ml, with and without UV-radiation (photoderivatization).

tions, neither preconcentration nor purification steps were possible. In order to prevent the fouling of the support material, a C8 5-μm column coated with a hydrophilic polyoxyethylenepolymer was used. This coated column is described as a restricted access material (RAM) and allows only the analytes to come into contact with the inner hydrophobic phase. This special phase allows the injection of 20 μl of human aqueous humor directly onto the column.

Human serum was first deproteinized, then centrifuged and only the supernatant was injected onto the column. This procedure could be chosen because of large sample volumes (5 ml) and to guard the column against unnecessary effects of protein loading.

The capillary material used for photoderivatization was ETFE (called TEFZEL). This copolymer of ethylene and tetrafluorethylene is strong, stable to pressure, and its translucence for UV light is better than that for the widely used PTFE (TEFLON) [17]. Furthermore, it is more stable to UV light [17]. Batley [18] observed the release of F^- and H^+ from PTFE capillaries irradiated at short UV wavelength, which reduced the life-span of the capillary to about 200 hrs. No leakages for the ETFE capillary used in this study were observed until now (after about 370 hrs).

Since for minimum peak–band broadening a small inner diameter is required, a 0.25 mm i.d. capillary was used. The increase of retention time from 5.5 min to 6.9 min, using the 20 m capillary, was equivalent to 25%. While the change in peak-width, determined at the base of the peak, from 0.227 to 0.277 min, was equivalent to an increase of about 22%.

These differences in expected retention time (theoretically plus 1 min; 20 m capillary, 0.25 mm i.d. = total inner volume of 1 ml) and that observed (plus 1.4 min), may be caused by variation of the tubing diameter or by stretching during the crocheting procedure and use [15]. The tolerance limits for ETFE capillaries (0.25 mm i.d.) given by the company (Plastizell Vertriebs GmbH), were between 0.20 and 0.30 mm. Taking this information into account, the difference in retention time is reasonable.

5. Conclusion

A highly sensitive and selective reversed-phase HPLC method for the bioanalysis of diclofenac in human aqueous humor and human serum is described. Diclofenac was detected as its fluorescent derivative, carbazole-1-acetic acid, after on-line post-column UV-photoderivatization. This reliable and precise method is simple to handle, has a detection limit of 1 ng ml^{-1}, and is very good for the determination of kinetics in biological samples.

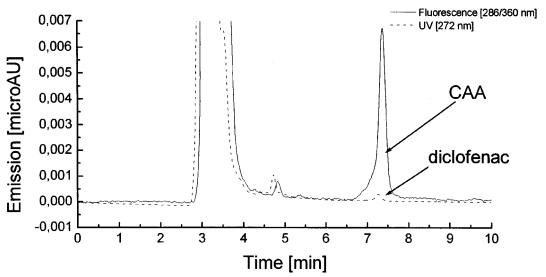

Fig. 10. Comparison of an UV-chromatogram (272 nm) and a fluorescence emission-chromatogram (286/360 nm) for human aqueous humor, containing 73 ng diclofenac/ml, after on-line photoderivatization.

Acknowledgements

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