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Simultaneous determination of diclofenac and oxybuprocaine in human aqueous humor with HPLC and electrochemical detection

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Abstract

A sensitive and selective bioanalytical method for simultaneous determination of diclofenac and oxybuprocaine in human aqueous humor using reversed-phase HPLC and electrochemical detection is described. Chromatographic separation was achieved by using a Regis SPS 100 RP-8 column (5 μm; 150 × 4.6 mm I.D.). This support is coated with a hydrophilic polyoxyethylenepolymer. It allows protein-containing samples to be injected directly onto the column. The electrochemical detector permit a detection limit of 500 pg diclofenac per ml (daily relative standard deviation 6.3%) and 50 ng oxybuprocaine per ml (daily R.S.D. 2.6%), respectively. Results of administered and measured drug-concentrations in time dependent decrease are presented.

Keywords: Diclofenac; Oxybuprocaine; Human aqueous humor; Electrochemical detection; Reversed-phase high-performance liquid chromatography

1. Introduction

HPLC procedures for the quantitative determination of trace concentration of drugs are important tools in clinical laboratories. In ophthalmology, the well-tolerated, nonsteroidal, anti-inflammatory drug (NSAID) diclofenac-sodium is used, for example, for perioperative applications in cataract-surgery to maintain mydriasis, for prophylaxis, and for treatment of postoperative symptoms of inflammation. Diclofenac inhibits cyclooxygenase activity in the arachidonic acid cascade and decreases prostanoid synthesis. Diclofenac sodium eye-drops (0.1%, Voltaren®) are used in ophthalmology to decrease intraoperative miosis and to reduce the breakdown of the blood-aqueous barrier. Furthermore, it shows potent analgetic and antipyretic activity [1].

Oxybuprocaine-hydrochloride eye-drops (Novesine®) are used in ophthalmology as an anaesthetic drug. It temporarily blocks sensory

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nerve impulses on sensory nerve fibres. Furthermore, it has a bacteriostatic effect.

Different procedures for quantification of diclofenac have been reported. Recently, gas chromatographic methods [2,3] have been replaced by liquid chromatographic methods [4–7]. However, the detection sensitivity was often insufficient for kinetic studies. For this reason, new methods with sufficient accuracy and sensitivity at concentrations below 15 ng ml^{-1} were needed. One possibility to achieve this goal, is the on-line post-column photoderivatization of diclofenac and the measurement of its fluorescent derivative [8,9]. Another one is the use of an electrochemical detector [10–12].

The present study shows for the first time the simultaneous detection of diclofenac and oxybuprocaine in human aqueous humor using HPLC and electrochemical detection. The great improvement in sensitivity and selectivity enables kinetic studies at very low concentrations.

2. Experimental

2.1. Chemicals

Diclofenac-sodium ((2-[2,6-dichlorophenyl)amino]benzene-acetic acid) sodium salt) was obtained from Sigma (Deisenhofen, Germany), oxybuprocaine-hydrochloride ((4-amino-3-butoxybenzoic acid-(2-(diethylamino)-ethyl)-ester hydrochloride)) were purchased from Aldrich (Steinheim, 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) and Novesine® 0.4% (eye-drops with 0.4% oxybuprocaine) were obtained from Dispersa (CIBA Vision Ophthalmics GmbH, 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 distilled again.

2.2. Instrumentation

The high-performance liquid chromatograph consisted of a constant-flow pump (E. Merck, Darmstadt, Germany), a Rheodyne injector (Cotati, USA), connected with a 20-μl external loop, an integrator (E. Merck-Hitachi D-2500, Darmstadt, Germany) and an electrochemical detector (E. Merck-Recipe, L-3500A). Only for comparison of the mobile phase and human aqueous humor as external standard solutions a different electrochemical detector was used (DIONEX, pulsed electrochemical detector, Germany). Chromatographic separation was performed with a Regis SPS 100 RP-8 column (5 μm; 150 × 4.6 mm I.D.) (Morton Grove, USA). The guard column was LiChrospher 100 RP-18 (5 μm) (E. Merck, Darmstadt, Germany). A pH meter (WTW, pH537, Weilheim, Germany) was used for pH measurements.

2.3. Chromatographic technique

The mobile phase was acetonitrile-sodium acetate (30 mM) (40:60, v/v (A), or 50:50, v/v (B)) 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 1.3 ml min^{-1} was established at constant water bath temperature (between 23–25°C). A potential of +0.95 V was applied versus the reference electrode (as described by Zecca et al., [10]).

Table 1
Calibration data for diclofenac

Diclofenac (ng ml^{-1})	Measured peak-area (microAU)	Linear regression data
1	556	561.904
5	2739	2809.523
10	5715	5619.047
25	14 547	14 047.617
50	27 606	28 095.234
100	57 245	56 190.469
500	270 083	280 952.344
1000	566 217	561 904.688

Table 2
Calibration data for oxybuprocaine

Oxybuprocaine [μg/mL]	Measured peak-area (microAU)	Linear regression data
0.4	1802	1758.265
4	17 795	17 582.650
10	42 405	43 956.626
20	86 842	87 913.252
40	197 559	175 826.503
100	447 922	439 566.258
200	876 708	879 132.517

2.4. Calibration curve

For establishing the calibration curves (Tables 1 and 2), external standard solutions of diclofenac-sodium and oxybuprocaine-hydrochloride were prepared by solving the drugs in the mobile phase. Twenty μl were injected onto the column. For diclofenac the concentrations ranged from 1–1000 ng ml^{-1}, and for oxybuprocaine from 0.4–200 μg ml^{-1}, respectively.

2.5. 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. For chromatographic analysis 20 μl were injected directly onto the column.

3. Results and discussion

The goal of this study was to establish methods for the quantification of diclofenac and oxybuprocaine (Fig. 1) in human aqueous humor. The methods had to be highly sensitive and selective. Because of the small sample volumes (average of 80 μl) and low drug-concentrations, neither preconcentration-procedures nor purification steps were possible. Therefore, simultaneous determination of the two compounds were desirable. Human aqueous humor contains 200 μg protein (or lower) per ml [13]. When a standard reversed-phase column is used, the sample-protein will be irreversibly adsorbed on the support material, resulting in an irreversible increase in column-backpressure and in decreasing selectivity. In order to prevent the fouling of the support material, an octyl-column, coated with a hydrophilic polyoxyethylenepolymer, was used. The polymer forms a semi-permeable surface (SPS), inhibiting the access of protein to the hydrophobic inner phase. This coated column is a restricted access

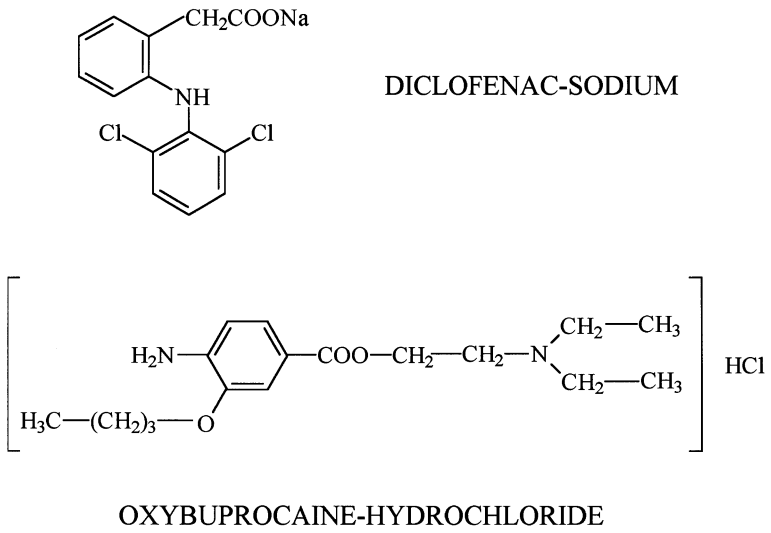

Fig. 1. Chemical structure of diclofenac-sodium salt and oxybuprocaine-hydrochloride.

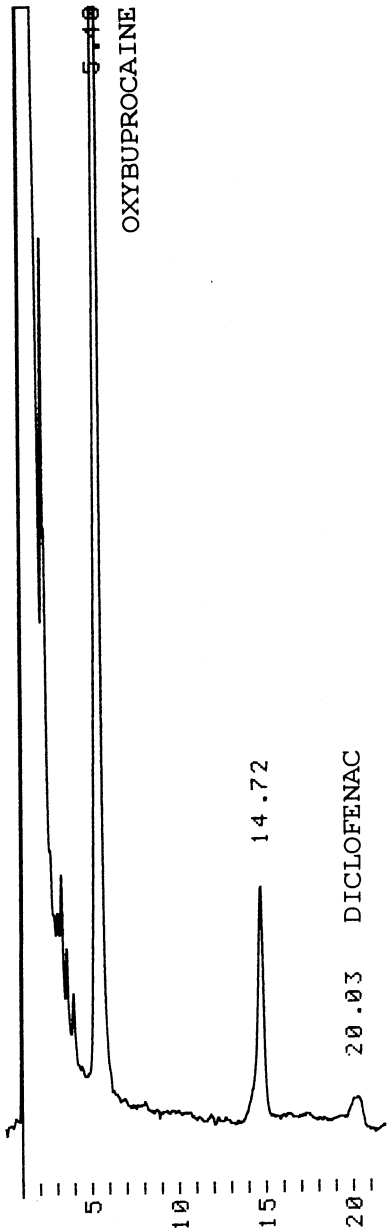

Fig. 2. Chromatogram of human aqueous humor; containing oxybuprocaine (77.069 μg ml^{-1}) and diclofenac (20 ng ml^{-1}). Chromatographic separation as described above (A).

material (RAM) and allows only the analytes to come in contact with the inner hydrophobic phase. For establishing the calibration curve, diclofenac and oxybuprocaine were solved in the mobile phase. Previous investigations indicated no statistical significant differences between solutions in mobile phase and in human aqueous humor. The chromatographic technique used shows sufficient selectivity and sensitivity for kinetic studies of human aqueous humor.

Using the described method, the effects of time dependent applications to the concentrations in the human aqueous humor of diclofenac and oxybuprocaine were examined. Fig. 2 shows a typical chromatogram of human aqueous humor, containing oxybuprocaine and diclofenac. Fig. 3 and Table 3 show the data observed for diclofenac. Oxybuprocaine was given 15 min before surgery (four drops). The concentrations at the beginning of the cataract surgery found in the aqueous humor were between 11.659 and 159.599 μg oxybuprocaine per ml (average 60.752 μg ml^{-1}; $n=25$), with one exception with 1.784 μg ml^{-1}.

When diclofenac was determined alone, the mobile phase mixture was changed from 40:60 (acetonitrile/sodium acetate (A), to 50:50 (B)), resulting in a shift of the diclofenac-peak from t_R 20 min to t_R 9 min. The following detection limits were measured: 500 pg ml^{-1} for diclofenac (daily R.S.D. 6.3% at 50 ng ml^{-1}, $n=14$) and 50 ng ml^{-1} for oxybuprocaine (daily R.S.D. 2.2% and 3.0%, both at 40 μg ml^{-1}, $n=15$), respectively. For both substances the signal-noise-ratio was 3. Diclofenac was much more sensitive detectable by electrochemical detection than oxybuprocaine. Resulting in a limit of quantification for diclofenac of 1 ng ml^{-1} (linear range: 1–1000 ng ml^{-1}) (Table 1), and for oxybuprocaine 0.4 μg ml^{-1} (linear range 0.4–200 μg ml^{-1}) (Table 2). The correlation coefficient for diclofenac was 0.99977 and for oxybuprocaine 0.99992, respectively. Constant temperature and highly purified eluents were crucial for achieving accurate data, when operating the electrochemical detector. For better sensitivity, the pump-pulsation was reduced by inserting a 60-cm long PEEK-capillary between the column and the electrochemical detector. Additionally, for low noise-values, it was essential to use freshly prepared mobile phase, cleaned capillaries and capillary connections. The total current was then about 8–10 nA. Increasing the total current during the measurement procedure can result in decreased sensitivity.

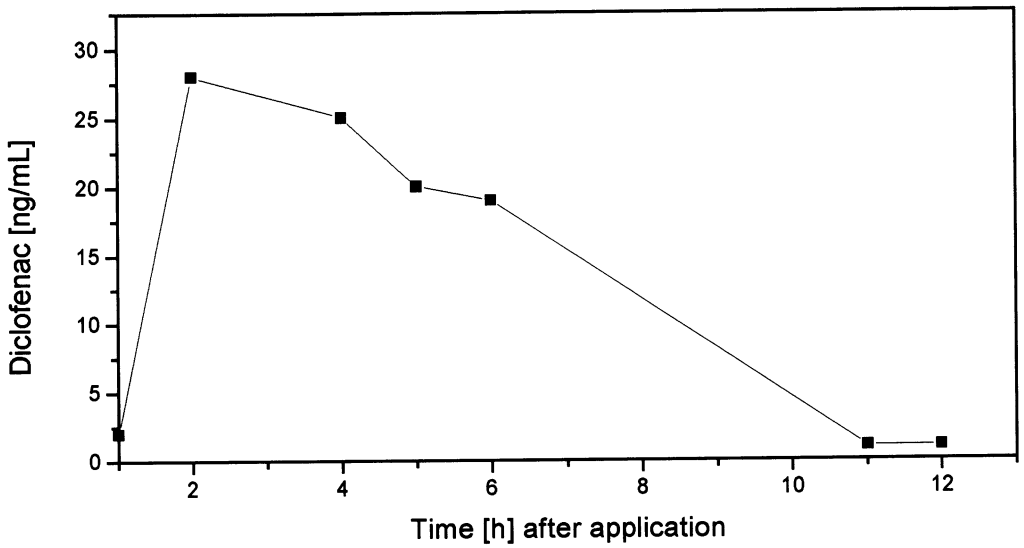

Fig. 3. Time dependent decrease of diclofenac in human aqueous humor after application of 2 drops of 0.1% Voltaren®.

4. Conclusion

A highly sensitive and selective reversed-phase HPLC method for the simultaneous bioanalysis of diclofenac and oxybuprocaine in human aqueous humor is described. By using an octyl-column, coated with a hydrophilic polyoxyethylenepolymer, it was possible to inject human aqueous humor directly onto the column without further purification steps. This reliable and precise method is simple in use and has detection limits of 0.5 ng ml^{-1} for diclofenac and 50 ng ml^{-1} for oxybuprocaine, respectively. The method is sufficient sensitive for kinetic studies.

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Table 3
Time dependent increase/decrease of diclofenac concentrations in human aqueous humor after application of two drops of 0.1% Voltaren®-eye-drops (50 clients: 23 male and 27 female; between 43 and 90 years of age, average: 74.2 years)

Sampling period (min)	Average (min)	Average diclofenae (ng ml^{-1})	Standard deviation (ng ml^{-1})	Clients
0–36	18	0	0	2
68–95	82.2	16.2	6.3	6
103–124	112.8	30.0	27.9	4
103–196	156.3	49.9	36.9	8
207–226	221.3	58.5	32.3	3
240–290	273.6	44.5	14.2	4
297–361	340.5	46.9	38.2	6
367–430	389.2	36.0	24.0	5
435–515	461.7	12.5	6.3	3
521–855	657.8	11.7	2.6	5
875–995	940.8	38.8	98.2	4

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