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Short Communication

High-performance liquid chromatographic assay for antiinflammatory agents diclofenac and flurbiprofen in ocular fluids

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Abstract

An HPLC assay for diclofenac (DC) and flurbiprofen (FP) in a 100-μl sample of aqueous humour is presented. After acetonitrile extraction, the residue is analyzed using a reversed-phase octyl column and ultraviolet detection. The method is simple and reproducible. Excellent selectivity and resolution are achieved using an acetic acid–acetonitrile–triethylamine mobile phase. The lower limit of detection, defined as the amount of drug required to produce a peak twice the threshold, was *ca.* 0.3 ng DC on column and *ca.* 0.4 ng on column FP. The utility of the method is demonstrated by determining drug levels in aqueous humour of normal rabbits and of patients undergoing cataract surgery. The assay should be applicable to other antiinflammatory agents.

1. Introduction

Diclofenac sodium, 0.1%, (Voltaren) and flurbiprofen sodium, 0.03%, (Ocufen), are topical nonsteroidal antiinflammatory drugs used with increasing frequency in ocular surgery [1–5]. They act chiefly by inhibiting cyclo-oxygenase, thereby reducing prostaglandin formation and diminishing the inflammatory response in the eye [6–9]. There are numerous HPLC assays available for antiinflammatory drugs, however few are suitable for small volumes of ocular fluid. Two HPLC assays for flurbiprofen (FP) in ocular fluid have been published [10,11]. To our knowledge, a method for ocular diclofenac (DC) has not been published although DC concentrations by HPLC have been reported [12]. The methods for FP determination [10,11] were not applicable to our study of the two drugs; we used instead a wavelength of 280 nm for improved detection of DC. An acetic acid–acetonitrile mobile phase with triethylamine (TEA) enhanced sensitivity, improved peak separation and decreased k' values. The present paper describes a simple, isocratic method based on UV detection which is fast, reproducible and sensitive enough to assay the agents in a 100-μl sample of aqueous humour. The assay requires only the most basic equipment and minimum sample manipulation. Although the assay was validated only for FP and DC, ketorolac tromethamine, suprofen, indomethacin sodium and meclofenamic acid also are eluted within 12 min. The method has been developed for pharmacokinetic studies re-

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lating the time–concentration profile of DC or FP in the human eye.

2. Experimental

2.1. Chemicals

Diclofenac sodium, triethylamine, and flurbiprofen, [+]-naproxen, suprofen, ketoprofen and meclofenamic acids were obtained from Sigma (St. Louis, MO, USA). Ketorolac tromethamine 0.05% (Acular), was a gift of Allergan Pharmaceuticals (Irvine, CA, USA). Diclofenac sodium, 0.1% (Voltaren Ophthalmic) was obtained from Ciba Vision Ophthalmics (Atlanta, GA, USA). Flurbiprofen sodium, 0.03% (Ocufen) was obtained from Allergan Pharmaceuticals (Irvine, CA, USA). Other drugs used in the interference studies were commercial preparations. Acetonitrile, distilled-in-glass grade, was obtained from Burdick and Jackson Labs (Muskegon, MI, USA); other chemicals were reagent grade. Water was glass distilled and all solutions were filtered and degassed prior to use. Standard solutions were prepared in methanol every two weeks and stored at −20°C. Working standards were diluted from the methanolic solutions.

2.2. Chromatography

The Beckman (Anaheim, CA, USA) Model 330 isocratic system consisting of a Model 110 B pump, Model 210 sample injection valve with a 20-μl injection loop and a Model 166 detector for UV detection at 280 nm was used in conjunction with a Hewlett-Packard integrator (Hewlett-Packard, Avondale, PA, USA). An Altex-Beckman Ultrasphere reversed-phase octyl column, particle size 5 μm, 15 cm × 4.5 mm I.D. was used. The mobile phase was prepared by mixing 505 ml acetonitrile containing 0.65 ml TEA with 495 ml 1.65% glacial acetic acid and had an apparent pH of 4.35 after filtration. For calculation of k' values, t_0 was determined by injecting mobile phase. A Goldenfoil heating unit was employed to maintain the temperature at 30°C; mobile phase was pumped at a flow-rate of 1.0 ml/min.

2.3. Assay procedure

A 100-μl aliquot of the standard solution or sample was placed in a silanized conical tube containing 500 μl of acetonitrile. Thirty microliters of the internal standard [+]-naproxen (400 ng/ml) was added and the mixture was mixed mechanically for 90 s. The protein was removed by centrifuging for 20 min at 3000 g. The supernatant fluid was transferred to a another tube, dried under nitrogen at room temperature and the residue was dissolved in 50 μl mobile phase by swirl-mixing for 1 min. The samples were centrifuged at 3000 g for 20 s. The volume was reduced under nitrogen to 20–30 μl before injection of samples when concentrations of less than 20 ng/ml were expected.

2.4. Standard curve, precision and recovery

Standard curves were constructed by analyzing samples of aqueous humour from New Zealand White rabbits [13] containing known amounts of the free acids in concentrations of 3–500 ng/ml DC and 6.25–600 ng/ml FP. The correlation coefficients and the regression equations were calculated using least-squares linear regression analysis by correlating the peak height against the corresponding spiked concentrations. Within-day variability was determined by analyzing nine replicate samples containing DC or FP at three different concentrations. Samples were analyzed on nine different days to determine between-day variability. Recovery of the drug was calculated by comparing aqueous standard solutions (no extraction) with extracted spiked aqueous humour samples.

2.5. Selectivity and interference

To identify potential interference by endogenous components, aqueous humour obtained from patients or animals not receiving FP or DC was analyzed without addition of internal standard. Stability was assessed by assaying human aque-

ous humour obtained during cataract surgery at weekly intervals for two months after freezing at −20°C. Interference by other therapeutic agents was evaluated (see Table 2). All drugs were tested for interference at 0.5 μg/ml except cyclopentolate-HCl which was tested at 10 μg/ml.

2.6. Human and animal studies

New Zealand White rabbits (Star Pines Rabbitry, Denver, CO, USA) weighing approximately 2.5–3 kg were given a 50-μl topical dose of the test drug in one eye. The contralateral eye served as a control. The animals were sedated and anesthetized and the aqueous humour was withdrawn and assayed as described. Procedures were performed in accordance with the ARVO Resolution in the Care and Use of Animals in Research.

Informed consent of the patient was obtained following an explanation of the procedure. One drop of topical antiinflammatory agent, FP or DC, was instilled onto the cornea before surgery and the time of instillation noted. Approximately 0.2 ml of aqueous humour was aspirated at the beginning of surgery and samples were frozen and assayed within 2 weeks.

3. Results and discussion

Under the chromatographic conditions described, the retention times were: DC 7.14 min, FP 6.04 min, and the internal standard, naproxen, 3.89 min; t_0 was 0.845 min. The k' values were calculated: 7.45, DC; 6.15, FP; and 3.60, internal standard. DC and FP were stable in rabbit aqueous humour for two weeks when frozen at −20°C. Human aqueous humour samples appeared stable for at least one month. Analysis of a series of spiked rabbit aqueous humour samples containing known amounts of FP or DC yielded standard curves in which the concentration of the drug was linearly related to the drug/internal standard peak-height ratios. The data for DC fit the equation of a straight line: peak-height ratio = $0.00819x + 0.08738$. Least-squares analysis yielded a coefficient of correlation (r) of 0.9999. FP data also produced a straight line: peak-height ratio = $0.00524x + 0.01164$. The coefficient of correlation (r) from least-squares analysis was 0.9991. The lower limit of detection is *ca.* 0.3 ng on column for DC and *ca.* 0.4 ng on column for FP, ($n = 7$). Recovery of drug (mean ± S.D.) from aqueous humour was 101 ± 4% (DC), $n = 8$; 99 ± 2% (FP), $n = 8$. Curves constructed from the drugs extracted from water were essentially identical to those extracted from aqueous humour. Within-day and between-day variability are summarized in Table 1. No interference from endogenous compounds was found when aqueous humour was analyzed from untreated subjects, human (Fig. 1A) or rabbit (Fig. 2A). Drugs tested for interference are listed in Table 2. Of 42 agents tested, only ketoprofen interfered with the assay. Ketoprofen eluted near the internal standard and did not exhibit a separate peak when injected with this agent. Naproxen was chosen as internal standard; it is extracted sufficiently and is not an ocular medication. It has not been reported in aqueous humour following systemic administration, although it is a common agent in the treatment of arthritis [14].

A single topical dose of 0.03% FP instilled into the human eye produced an aqueous humour concentration of 34.9 ng/ml at 50 min, 68 ng/ml at 88 min and 43 ng/ml at 130 min. A single topical dose of 0.1% DC produced aqueous humour concentrations of 22 ng/ml at 50 min, 20 ng/ml at 85 min and 52.6 ng/ml at 125 min. Fig. 1B was obtained by analyzing an aqueous humour sample taken at surgery following topical treatment of the patient with FP and DC. Fig. 2B is a chromatogram of the two drugs assayed in rabbit aqueous humour. As previously reported [11], there is no crossover of FP into the primary aqueous humour of the contralateral eye (Fig. 2A). Neither is DC observed in the aqueous humour of the contralateral eye (Fig. 2A). Fig. 3 is a chromatogram obtained by assaying aqueous humour containing six antiinflammatory agents and the internal standard. The determination of ketorolac or suprofen would require adjustment of the mobile phase for adequate separation of the peaks from the

Table 1
Precision and accuracy of the assay

Theoretical concentration (ng/ml)	Within-day variability ($n = 9$)		Between-day variability ($n = 9$)	
	Concentration found (ng/ml)	C.V. (%)	Concentration found (ng/ml)	C.V. (%)
Diclofenac				
11.6	11.5	6.6	11.8	3.9
139.0	133.8	2.73	137.5	4.8
240.0	235.7	2.4	233.3	4.0
Flurbiprofen				
25.0	24.1	6.2	25.3	3.9
120.0	123.0	3.3	119.8	5.3
175.0	172.2	5.0	176.2	4.2

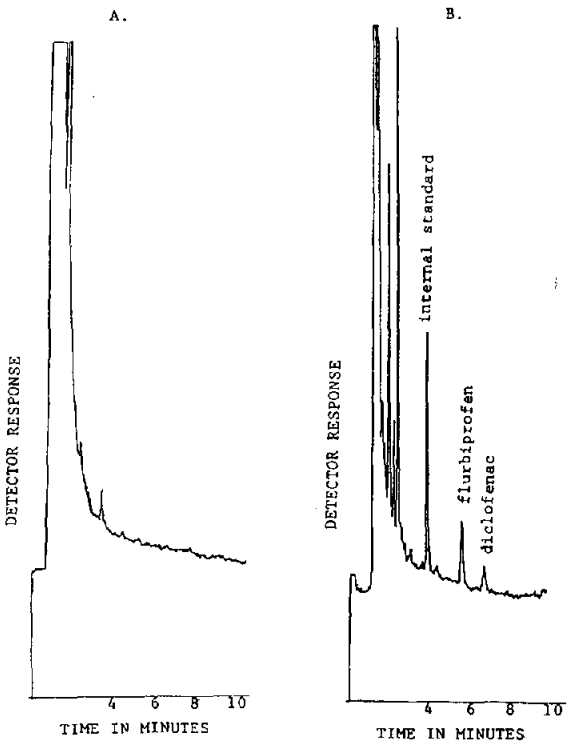

Fig. 1. (A) Chromatogram of aqueous humour from a surgery patient not receiving FP or DC and without internal standard. (B) Chromatogram of FP and DC assayed in aqueous humour of a patient undergoing cataract surgery 50 min after administration of a topical dose of 0.03% FP, and 20 min after 0.1% DC administration. FP concentration 34.9 ng/ml; DC concentration 6 ng/ml.

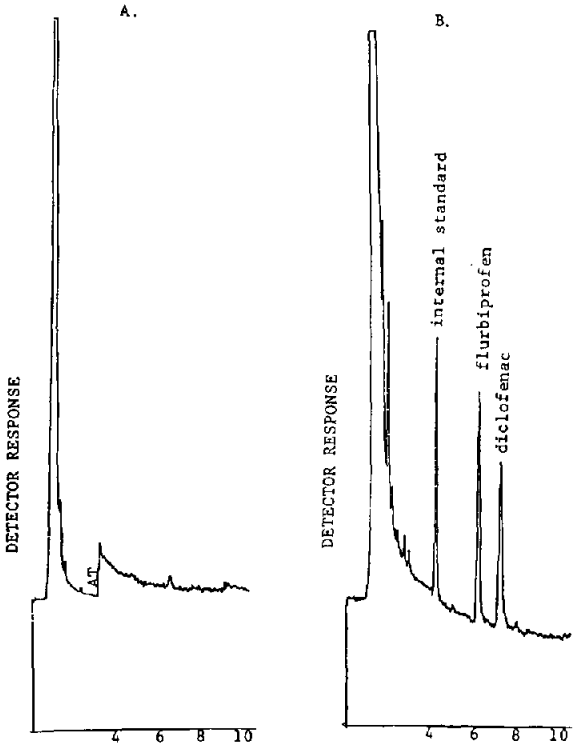

Fig. 2. (A) Chromatogram of aqueous humour from untreated rabbit eye without internal standard. (B) Chromatogram of rabbit aqueous humour following topical administration of a 50-μl dose of each antiinflammatory medications. FP concentration: 146.3 ng/ml at 15 min. DC concentration: 40.3 ng/ml at 30 min.

Table 2
Agents tested for interference in the chromatography of diclofenac (t_R 7.14 min), flurbiprofen (t_R 6.04 min) and naproxen (t_R 3.89 min)

Compound	t_R	k'
Acebutolol		no peak
Acetaminophen		no peak
Acetazolamide		no peak
Alprenolol		no peak
Apraclonidine-HCl		no peak
Atenolol		no peak
Atropine sulfate		no peak
Bacitracin	t_R 5.22[a]	5.18
Betamethasone		no peak
Betaxolol-HCl		no peak
Bupivacaine-HCl		no peak
Caffeine		no peak
Cortisone acetate	t_R 3.53 min	3.18
Cyclopentolate-HCl		no peak
Dexamethasone		no peak
Diazepam	t_R 4.97 min	4.88
Diphenhydramine-HCl		no peak
Erythromycin		no peak
Fluorometholone	t_R 3.19 min	2.78
Haloperidol		no peak
Hydrocortisone acetate	t_R 2.11 min	1.50
Imipramine-HCl	t_R 2.57 min	2.04
Indomethacin	t_R 6.64 min	6.86
Ketoprofen	t_R 3.81 min	3.51
Ketorolac tromethamine	t_R 2.65 min	2.14
Levobunolol-HCl	t_R 1.64 min	0.94
Lidocaine-HCl		no peak
Meclofenamic acid	t_R 11.2 min	12.25
Metipranolol	t_R 1.95 min	1.31
Neomycin sulfate	t_R 1.99 min	1.36
Phenylephrine-HCl		no peak
Polymyxin B sulfate		no peak
Prednisolone acetate	t_R 3.00 min	2.55
Procaine-HCl		no peak
Proparacaine-HCl	t_R 2.33 min	1.76
Propranolol	t_R 2.10 min	1.49
Salicylic acid	t_R 1.72 min	1.04
Scopolamine-HCl		no peak
Sulfacetamide-Na	t_R 1.69 min	1.00
Suprofen	t_R 3.22 min	2.81
Timolol maleate		no peak
Tropicamide		no peak

[a]Increased solvent front.

solvent front, however the present assay should suffice for indomethacin and meclofenamic acid as well as FP and DC.

The concentration of 34.9 ng/ml FP determined in human aqueous humour at 50 min (Fig. 1B) is in excellent agreement with the work of Strobel who found a mean of 37 ng/ml at 1 h by high-performance thin-layer chromatography [15]. Anderson and Chen reported 200 ng/g in the aqueous humour 30 min after a 15-μg FP dose [16]; we observed a concentration of 146.3 ng/ml FP at 15 min (Fig. 2B). Using this assay, we found lower levels of DC (135 ng/ml after 1 h) than the radioactivity levels reported by Agata *et al.* (280 ng/ml, 1 h) [17].

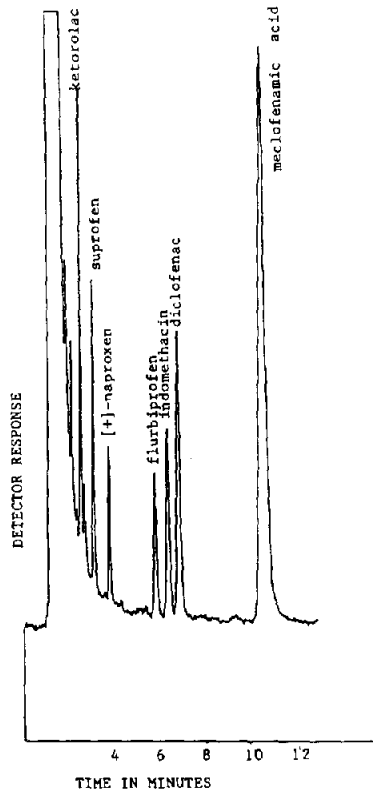

Fig. 3. Chromatogram of rabbit aqueous humour containing diclofenac, 217.7 ng/ml; flurbiprofen, 178 ng/ml; ketorolac, 368 ng/ml; suprofen, 79 ng/ml; indomethacin, 145 ng/ml; meclofenamic acid, 1600 ng/ml; and the internal standard [+]-naproxen.

The assay proved satisfactory for the analysis of either drug in human or rabbit aqueous humour. A considerable number of assays exist for DC and FP, however few HPLC analyses have dealt with the low ocular volumes and nanogram concentrations of these agents [10–12]. Although the methodology is not novel,

modification of the mobile phase with TEA and acetic acid rather than using an inorganic buffer or acetic acid alone provided better sensitivity and separation of the compounds as well as lower k' values and reduced solvent consumption. As sample residues were difficult to solubilize in small volumes of mobile phase, swirl-mixing for at least 1 min greatly improved reproducibility. The method described here is simple, reproducible and selective and should be of value in ocular pharmacokinetic studies.

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