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Short communication

Rapid determination of nimesulide in rabbit aqueous humor by liquid chromatography

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Abstract

A rapid method was developed for quantification of nimesulide (methanesulfonamide, *N*-[4-nitro-2-phenoxyphenil]) in rabbit aqueous humor. The analyses were performed by high-performance liquid chromatography using a C_{18} reversed-phase column (Ultracarb ODS) with UV detection at 300 nm. The mobile phase consisted of acetonitrile-water containing 1% triethylamine (TEA) adjusted to pH 3.2 with orthophosphoric acid. The retention time was 4.5 min. A simple pre-treatment with acetonitrile was used to deproteinize aqueous humor samples. The limit of quantitation was 50 ng/ml. The recovery was over 90%. The relationship between peak areas and concentration was linear over the range between 0.05 and 2.5 μg/ml, with r^2 values over 0.99. The assay provided good reproducibility and accuracy and proved to be suitable for pharmacokinetic studies of nimesulide.

Keywords: Nimesulide

1. Introduction

Nimesulide (Fig. 1) (methanesulfonamide, *N*-[4-nitro-2-phenoxyphenil]), a non-steroidal anti-inflammatory drug (NSAID), is a drug with potent anti-inflammatory, antipyretic and analgesic properties [1]. This is a unique NSAID, not only because of its chemical structure but also because of its specific affinity to inhibit the inducible form of cyclooxygenase (COX-2) rather than the constitutive form (COX-1). Tissue injury is associated with the release of numerous inflammatory mediators including prostaglandins that are synthesized from arachidonic acid via endoperoxide biosynthetic pathway, the initial step of which is catalyzed by the enzyme cyclooxygenase. COX-2 is the major form of the isozyme associated with inflammation and the expression of COX-2 is up-regulated in response to inflammatory stimuli in many tissues and, among the others, in ocular tissues [2,3]. Therefore, a rapid and sensitive analytic method for nimesulide could be useful for ocular pharmacokinetic studies. All published methods refer to the determination of nimesulide in plasma [4–6]. They used a quantification method based on liquid–liquid extraction [5] and back-extraction with aqueous base [4]. The simple method based only on protein precipitation developed by Ptáček et al. [6] was not applicable to aqueous humor because of interfering peaks. Moreover, the mobile phase used in published studies is elaborate and made of conspicuous quantity of salts. The objective of this study was to develop a rapid and simple high-performance liquid chromatography method for determination of nimesulide in rabbit aqueous humor, with minimal sample pre-treatment, that would be applicable to ocular pharmacokinetic studies.

2. Experimental

2.1. Chemicals

Nimesulide (purity >99%) was purchased from Sigma (Milan, Italy). Acetonitrile was obtained from Merck (Milan, Italy). Triethylamine (TEA) and 85% orthophosphoric acid were purchased from Aldrich (Milan, Italy). All solvents and chemicals were of HPLC or analytical grade.

2.2. Equipment

The HPLC apparatus (Agilent Technologies, Milan, Italy) was a Hewlett Packard HP 1100 chromatographic system

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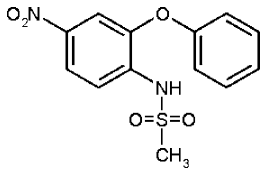

Fig. 1. Structure of nimesulide.

interfaced with the HP ChemStation software and equipped with a binary pump G1312A, a diode array detector (DAD) G1315A, a thermostated column compartment G1316A and a Rheodyne sample injector with a 20 μl loop. An Ultracarb ODS (30) reverse-phase column (150 mm × 4.6 mm i.d., 5 μm) was purchased from Phenomenex (Milan, Italy).

2.3. Chromatographic conditions

The mobile phase consisted of 60% CH_3CN and 40% water containing 1% TEA adjusted to pH = 3.2 with H_3PO_4. Prior to use, the mobile phase was filtered through a 0.2 μm nylon membrane filter. The UV detector was set at 300 nm with a flow rate of 1 ml/min. Chromatography was performed at 25 °C. The injected volume was 20 μl.

2.4. Aqueous humor collection

Male New Zealand albino rabbits (Charles River, Calco, Italy) 2.0–2.2 kg, free of any signs of ocular inflammation or gross abnormality were used. Animal procedures conformed to the Association for Research in Vision and Ophthalmology (ARVO) resolution on the use of animals in research. In order to collect the aqueous humor the rabbits were anaesthetized by an i.m. injection of 35 mg/kg ketamine HCl and 5 mg/kg xylazine HCl (RBI, Milan, Italy). Aqueous humor was collected by a 26-G needle attached to a tuberculine syringe. The needle was introduced into the anterior chamber of the eye through the cornea and 150 μl aqueous humor were withdrawn.

2.5. Sample preparation

A 100 μl aliquot of rabbit aqueous humor was pipetted into a 1 ml Eppendorf tube and a 100 μl aliquot of acetonitrile was added in order to precipitate proteins. The sample was vortex-mixed vigorously for 60 s and centrifuged at 10,000 *g* for 5 min. The supernatant was aspirated with a tuberculine syringe, filtered through a 4 mm HPLC syringe filter with 0.2 μm nylon membrane (Alltech Italia, Milan) and injected onto the HPLC system.

2.6. Standard solution and calibration curve

A standard stock solution of nimesulide (2.00 mg/ml) was prepared by direct dissolution in acetonitrile. Aliquots of standard solution containing nimesulide were transferred into a 1 ml Eppendorf and evaporated to dryness. Drug-free rabbit aqueous humor (100 μl) was added. The final concentration was in the range of 0.05–2.50 μg/ml. Spiked aqueous humor samples were taken through the assay procedure and calibration graphs were constructed by plotting nimesulide peak area versus the concentration of the analyte. Linear regression analysis was used to calculate the slope, intercept and correlation coefficient of the calibration curve.

2.7. Recovery, intra- and inter-day precision

The recovery of nimesulide from rabbit aqueous humor was determined by comparing the peak areas obtained from the direct injection of standard solutions of compound with those found by extraction ($n = 5$ for each concentration of nimesulide used) from spiked aqueous humor. The measurement of intra- and inter-day variability were utilized to determine the accuracy and precision of the developed assay. Three different concentrations of nimesulide were chosen to test both intra- and inter-day variations. Relative standard deviation (R.S.D.) was taken as a measure of precision, and the percentage difference between determined and spiked amounts was considered a measure of accuracy. Samples at each given concentration were analyzed five times for intra-day variation. While, the inter-day reproducibility was examined on five separate days (Table 1).

2.8. Stability test

The stability of nimesulide in rabbit aqueous humor was investigated. Spiked samples were prepared with drug-free aqueous humor at two concentration levels. Spiked aqueous humor samples were divided in two portions. One portion was stored at −20 °C, thawed and analyzed on weeks 0, 1, 2, and 4. The other portion was treated as described in Sample preparation, divided in two portions and stored at 4 °C and room temperature. At 6, 12, and 24 h after extraction each sample in both portions was directly analyzed by HPLC.

Table 1
Intra- and inter-day variation for the assay of nimesulide in rabbit aqueous humor

Assay	Amount added (μg/ml)	Mean calculated concentration (μg/ml) ($n = 5$)	R.S.D.[a] (%)	Accuracy[b] (%)
Intra-day	0.10	0.104 ± 0.010	9.6	104.0
	0.50	0.495 ± 0.015	7.6	99.0
	1.5	1.502 ± 0.019	1.26	100.1
Inter-day	0.10	0.105 ± 0.014	13.3	105.0
	0.50	0.506 ± 0.030	5.9	101.2
	1.5	1.491 ± 0.015	1.0	99.4

[a] R.S.D. (%) (relative standard deviation) = (S.D./mean) × 100.
[b] Accuracy (%) = [1 − (concentration spiked − mean concentration measured)/concentration spiked] × 100.

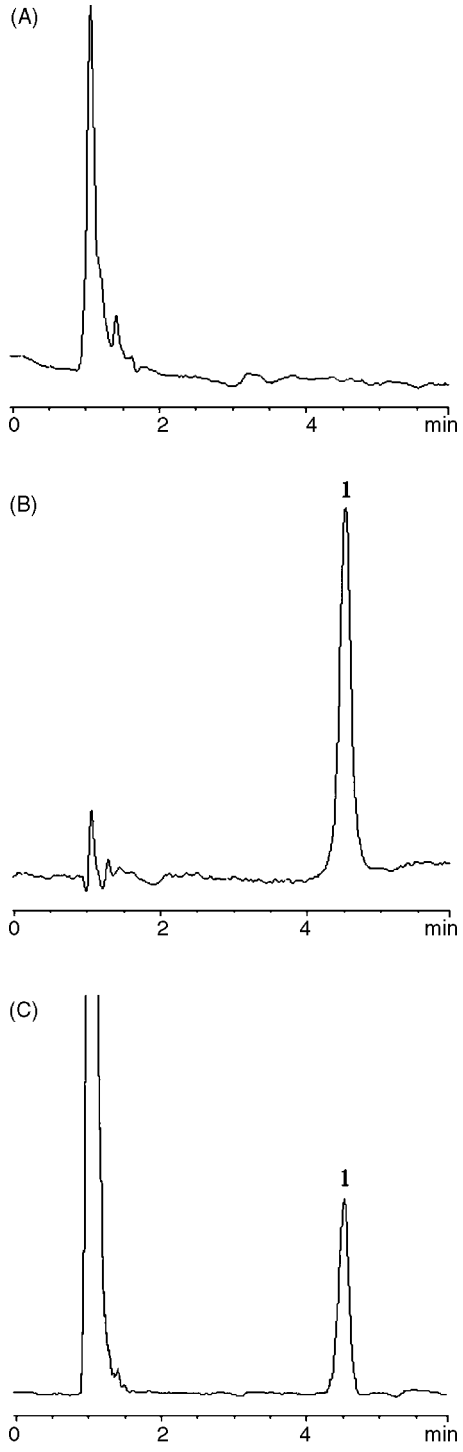

Fig. 2. Representative chromatograms of (A) blank aqueous humor, (B) standard of nimesulide (1), and (C) aqueous humor sample spiked with nimesulide (1).

3. Results and discussions

Under the chromatographic conditions described in our study, a retention time of 4.5 min for nimesulide was observed. No interfering peaks were observed in the blank aqueous humor chromatogram, indicating the efficient clean up method used. A sharp peak corresponding to nimesulide was clear on the chromatogram with stable retention time, therefore we did not use an internal standard. Fig. 2 shows representative chromatograms for nimesulide in rabbit aqueous humor. High recovery levels were achieved at all the concentrations studied with a range of 99–102%. Good linearity was found for nimesulide ($y = 18.01x + 6.5$) in the range from 0.05 to 2.5 μg/ml. Linear regression analysis performed for calibration curve yielded a correlation coefficient of 0.9968. Under the described experimental conditions, the limit of quantitation was 50 ng/ml. Validation of our assay method consisted of intra- and inter-day reproducibility studies at three concentration levels of nimesulide: 0.1, 0.5, and 1.5 μg/ml ($n = 5$). Table 1 shows the intra-day precision, with R.S.D. range from 1.26 to 9.6%, and the inter-day precision, with R.S.D. range from 1.0 to 13.3%, indicating the very good reproducibility of this method.

A stability study was conducted to determine the best storage temperature for aqueous humor samples. The results demonstrated that nimesulide in extracted samples was stable for 24 h at 4 °C and at room temperature. Furthermore nimesulide in rabbit aqueous humor were stable up to four weeks when stored at −20 °C. Therefore, all extracted samples were stored refrigerated at 4 °C for the same day analysis; whereas aqueous humor samples were frozen at −20 °C until analysis by HPLC.

4. Conclusions

In conclusion, this HPLC method is rapid, sensitive, reproducible and well suited to routine measurements. Both assay and extraction procedure are simple and rapid, so it is possible to analyze large number of samples within a short period of time. We believe that this method is suitable for ocular pharmacokinetic studies.

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