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Simultaneous determination of cloricromene and its active metabolite in rabbit aqueous humor by high-performance liquid chromatography

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Abstract

A rapid and simple method was developed for the simultaneous separation and quantification of cloricromene, a coumarine derivative, and its active metabolite, cloricromene acid, in rabbit aqueous humor. The analyses were performed by high-performance liquid chromatography using a C_{18} reversed-phase column (Hypersil ODS) with UV detection at 318 nm. The mobile phase consisted of acetonitrile–water containing 1% triethylamine pH 3.5, adjusted with orthophosphoric acid. An acetonitrile gradient was necessary to achieve good separation within 13 min. Timolol was found to be a suitable internal standard. The retention times ranged from 5.72 to 11.25 min. A simple pre-treatment with acetonitrile containing 0.6% $HClO_4$ was used to deproteinize aqueous humor samples. The limit of quantitation ranged between 10 and 20 ng/ml. The recovery was >90%. The relationship between peak areas and concentration was linear over the range between 0.01 and 3.8 μg/ml, with $r^2 > 0.99$. The assay provided good reproducibility and accuracy for both analytes and proved to be suitable for pharmacokinetic studies of cloricromene. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: UV detection; Cloricromene

1. Introduction

Cloricromene (Fig. 1) is a coumarine derivative (8-mono-chloro-3-beta-diethylaminoethyl-4-methyl-7-ethoxycarbonyl methoxy coumarine) with beneficial effects on splanchnic, hemorrhagic and endotoxic shock [1–3]. The drug inhibits TNFα [4] and PAF [5], and prevents the expression of iNOS and COX-2 protein [6]. Recently, Bucolo et al. showed that cloricromene attenuates the degree of inflammation and tissue damage associated with endotoxin-induced uveitis in the rabbit eye [7] and protects against experimental rat uveitis, reducing the expression of adhesion molecules such as P-selectin and ICAM-1 [8]. It was also found that cloricromene is rapidly metabolized in the blood, both in vitro and in vivo [9], into a stable catabolite through the hydrolysis of an ester bond within the molecule (Fig. 1). To our knowledge, the only documented method for the determination of the metabolite in rabbit plasma and platelets is an HPLC assay with fluorescence detection [10]. However, these authors described an

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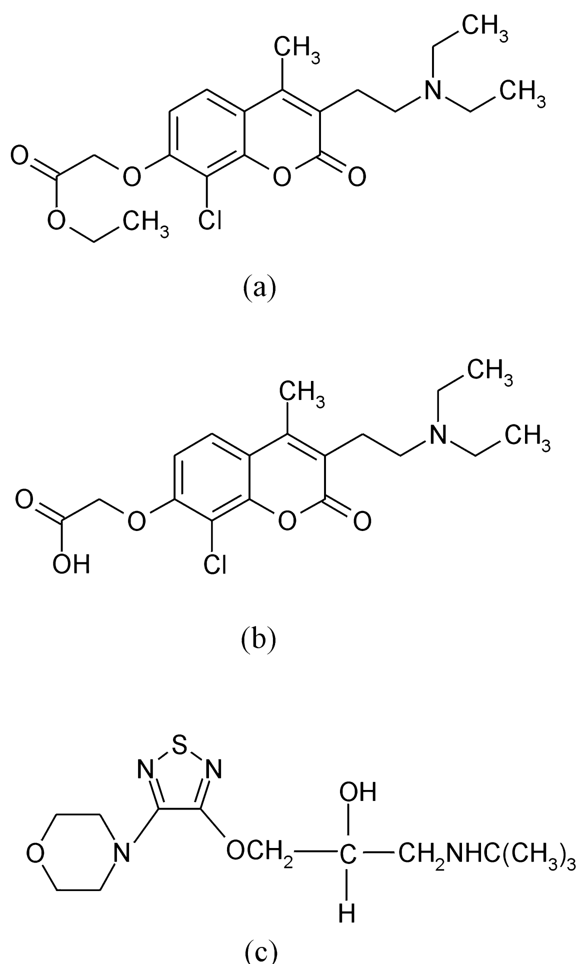


Fig. 1. Structure of (a) cloricromene, (b) its metabolite and (c) timolol.

HPLC method that is not able to simultaneously quantify cloricromene and its metabolite. Therefore, the present study, for the first time, reports the development of a specific HPLC method with UV detection for the simultaneous qualitative and quantitative analysis of cloricromene and its metabolite in rabbit aqueous humor.

2. Experimental

2.1. Chemicals

Cloricromene hydrochloride and cloricromene acid (purity >99%) were obtained from Fidia (Abano Terme, Italy). The internal standard (Fig. 1), timolol maleate (purity >99%), was purchased from Sigma (Milan, Italy). All solvents and chemicals were of HPLC or analytical grade. Acetonitrile was obtained from Merck (Milan, Italy). Triethylamine, 85% orthophosphoric acid and perchloric acid were purchased from Aldrich (Milan, Italy).

2.2. Instrumentation

The pH was determined using a Metrohm 691 pH meter (Metrohm, Herisau, Switzerland). The sample preparation procedure utilized a Heidolph REAX 2000 vortex mixer and an ALC microcentrifugette 4214 centrifuge (ALC International, Milan, Italy). The HPLC apparatus (Agilent Technologies, Milan, Italy) was a Hewlett-Packard HP 1100 chromatographic system interfaced to HP ChemStation software and equipped with a binary pump G1312A, a diode-array detector (DAD) G1315A and a thermostated column compartment G1316A. A Hypersil ODS C_{18} reversed-phase column (150 mm×4.6 mm I.D., 5 μm) and a Hypersil ODS C_{18} column (7.5 mm×4.6 mm I.D., 5 μm), utilized as a guard column, were purchased from Alltech (Milan, Italy).

2.3. Standards

A stock solution containing both cloricromene hydrochloride (210 μg/ml free base) and the metabolite (380 μg/ml) was prepared by direct dissolution in acetonitrile. Aliquots of the stock solution containing the two analytes were added to blank aqueous humor to give an appropriate ranges of concentrations, from 0.01 to 2.10 μg/ml cloricromene and from 0.02 to 3.80 μg/ml metabolite, for the calibrations. The internal standard stock solution (timolol maleate, 320 μg/ml) was prepared in acetonitrile containing 0.6% $HClO_4$ and stored at 4 °C. This solution was diluted to a concentration of 4.8 μg/ml and used as working standard.

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 ARVO (Association for Research in Vision and Ophthalmology)

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 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 aqueous humor was pipetted into a 1-ml Eppendorf tube and a 100 μl aliquot of the internal standard solution 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 0.2 μm nylon membrane filter and injected onto the HPLC system.

2.6. Chromatographic conditions

The mobile phase consisted of 10% CH_3CN and 90% of an aqueous solution containing 1% TEA adjusted to pH 3.5 with H_3PO_4; the percentage of CH_3CN was increased to 53% in 13 min at a flow-rate of 1 ml/min. The system returned to 10% CH_3CN in 5 min and was kept under this condition for 3 min to re-equilibrate. Prior to use, the mobile phase was filtered through a 0.2 μm nylon membrane filter. The UV detector was set at 318 nm. Chromatography was performed at 25 °C. The injected volume was 20 μl.

2.7. Calibration curves

Drug-free rabbit aqueous humor (100 μl) was spiked with standard solutions containing cloricromene and the metabolite in the range 0.01–2.10 and 0.02–3.8 μg/ml, respectively. Spiked aqueous humor samples were taken through the assay procedure and calibration graphs were constructed by plotting cloricromene and metabolite to internal standard peak-area ratios versus the concentration of the analytes. Linear regression analysis was used to calculate the slope, intercept and correlation coefficient of each calibration curve.

2.8. Recovery, intra- and inter-day precision

The recoveries of cloricromene, metabolite and internal standard, timolol, from the aqueous humor were determined by comparing the peak areas obtained from the direct injection of standard solutions of compounds with those found by extraction ($n=5$ for each concentration of cloricromene and metabolite used) from spiked aqueous humor. The measurements of intra- and inter-day variability were utilized to determine the accuracy and precision of the developed assay. Three concentrations of each analyte were chosen to test both intra-day and inter-day variations. The relative standard deviation (RSD) 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. The inter-day reproducibility was examined on five separate days (Tables 1 and 2).

2.9. Stability test

The stability of cloricromene and its metabolite in rabbit aqueous humor was investigated. Spiked samples were prepared with drug-free aqueous humor at two concentrations for each analyte. Spiked aqueous humor samples were divided into two portions. One portion was stored at −20 °C, thawed and analyzed in weeks 0, 1, 2, and 4. The other portion was treated as described in Sample preparation, divided into two portions and stored at 4 °C and room temperature,

Table 1
Intra-day variation for the assay of cloricromene and metabolite

Analyte spiked (μg/ml)	Mean calc. conc. (μg/ml) ($n=5$)	RSD[a] (%)	Accuracy[b] (%)
Cloricromene			
0.52	0.50±0.02	3.5	96.2
1.05	1.02±0.06	5.7	97.14
2.10	2.03±0.17	8.5	96.67
Metabolite			
0.95	0.92±0.05	5.5	96.84
1.90	1.92±0.11	5.8	101
3.80	3.82±0.13	3.4	100.5

[a] RSD (%) (relative standard deviation) = (SD/mean)·100.

[b] Accuracy (%) = [1 − (concentration spiked − mean concentration measured)/concentration spiked]·100.

Table 2
Inter-day variation for the assay of cloricromene and metabolite

Analyte spiked (μg/ml)	Mean calc. conc. (μg/ml) ($n = 5$)	RSD[a] (%)	Accuracy[b] (%)
Cloricromene			
0.52	0.50± 0.04	8.2	96.15
1.05	1.15± 0.19	16.5	109.5
2.10	2.23± 0.19	8.5	106.2
Metabolite			
0.95	0.94± 0.04	4.5	98.9
1.90	1.94± 0.07	3.6	102.1
3.80	3.86± 0.09	2.4	101.6

[a] RSD (%) (relative standard deviation) = (SD/mean)·100.
[b] Accuracy (%) = [1 − (concentration spiked − mean concentration measured)/concentration spiked]·100.

respectively. At 6, 12 and 24 h after extraction, each sample in both portions was directly analyzed by HPLC.

3. In vivo rabbit study

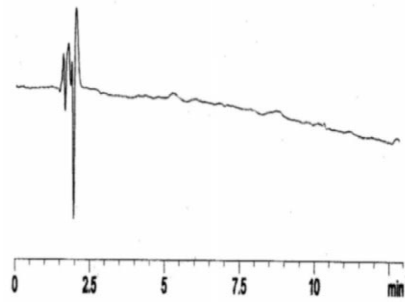
In order to demonstrate the suitability of our newly developed cloricromene HPLC assay method for pharmacokinetic studies, we performed a pilot study in the rabbit. A cloricromene eye drop (0.1% w/v) in an isotonic and phosphate buffered (pH 7.4) solution was prepared. Fifty microliters of the formulation were instilled in the conjunctival sac of rabbits. Aqueous humor samples were collected 15, 30, 60, 120, and 240 min after a single instillation of drug formulation. The rabbits were anaesthetized and aqueous humor (about 150 μl) was withdrawn through the limbus with a syringe with a 26G needle, and stored at −20 °C until analysis by HPLC.

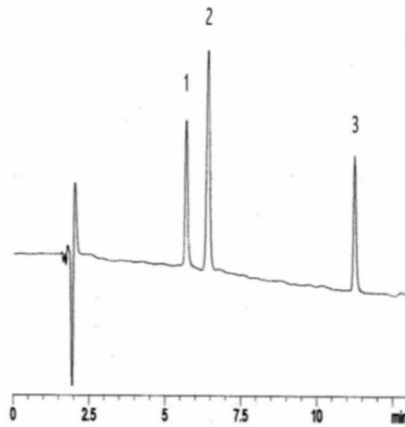
4. Results and discussion

This study was performed to determine the optimal conditions for the simultaneous extraction of cloricromene and its metabolite from rabbit aqueous humor and for their chromatographic separation.

Under the chromatographic conditions described in this study, retention times of 5.72, 6.48 and 11.25 min were observed for metabolite, timolol and cloricromene, respectively. No interfering peaks were observed in the blank aqueous humor chromatogram, indicating the efficient clean-up method used. Although the sample preparation is very straightforward, we used internal standard calibration to compensate for losses of the analytes during sample workup. Representative chromatograms for cloricromene, metabolite and timolol in rabbit aqueous humor are shown in Fig. 2. High recovery levels were achieved at all the concentrations studied with a range of 98.5–102%. The detector response was linear over the concentration range from 0.01 to 2.10 μg/ml for cloricromene and from 0.02 to 3.80 μg/ml for the metabolite. Linear regression analysis performed for calibration curves yielded correlation coefficients of 0.9955 for cloricromene and 0.9996 for the metabolite. Good linearity with a negligible intercept was found for cloricromene ($y = 2.5038x + 0.0308$) and for the metabolite ($y = 2.5752x - 0.0165$). The limit of quantitation in aqueous humor was determined to be 10 ng/ml for cloricromene with an RDS of 3.8% and accuracy of 97% ($n = 4$) and 20 ng/ml for the metabolite with an RDS of 1.98% and accuracy of 98% ($n = 4$). Validation of our assay method consisted of intra- and inter-day reproducibility studies at three concentrations: 0.52, 1.05 and 2.10 μg/ml for cloricromene and 0.95, 1.90 and 3.80 μg/ml for the metabolite ($n = 5$). Tables 1 and 2 show the intra-day precision, with RSD range from 3.4 to 8.5%, and the inter-day precision, with RSD range from 2.4 to 16.5%, 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 cloricromene was stable for more than 12 h at 4 °C and for at least 6 h at room temperature, while the metabolite was stable for more than 24 h at room temperature. Furthermore, both analytes were stable for up to 4 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. To demonstrate the applicability of the method to pharmacokinetic studies, an in vivo pilot pharmacokinetic study was carried out in the rabbit. The pharmacokinetic profile of cloricromene and its metabolite in the rabbit aqueous humor is shown in Fig. 3. Cloricromene was detectable for up to 60 min





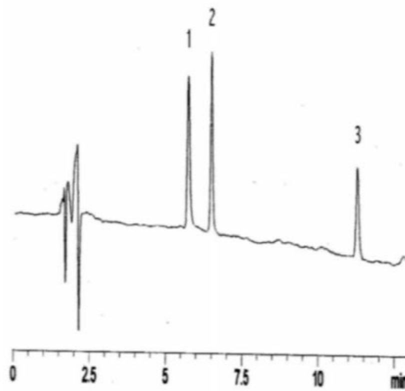


Fig. 2. Representative chromatograms of (a) blank aqueous humor, (b) standard of the metabolite (1), timolol (2) and cloricromene (3), and (c) aqueous humor sample spiked with the metabolite (1), timolol (2) and cloricromene (3).

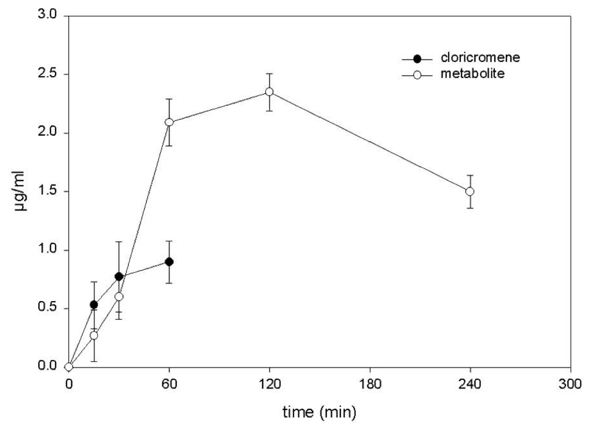


Fig. 3. Pharmacokinetic profile of cloricromene and metabolite in rabbit aqueous humor. Values represent the mean±SD of eight samples.

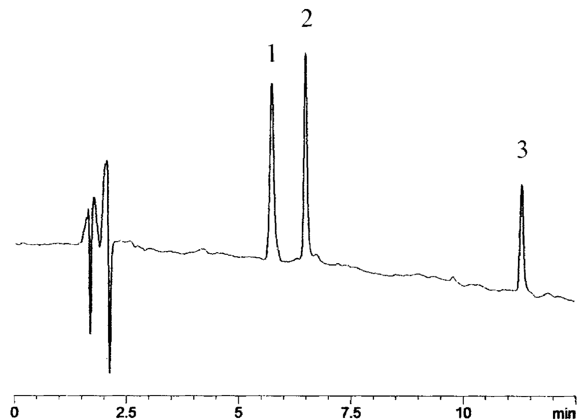


Fig. 4. Representative chromatogram of rabbit aqueous humor withdrawn 60 min after a single instillation of 50 μl of cloricromene eye drop.

because, in vivo, it is rapidly hydrolyzed. A representative chromatogram of rabbit aqueous humor, withdrawn 60 min after a single instillation of 50 μl of cloricromene eye drop, is shown in Fig. 4.

5. Conclusions

A specific HPLC method using UV detection was developed and fully validated for the simultaneous determination of cloricromene and its active metabolite in rabbit aqueous humor. The assay is simple, selective and accurate, and is the first reported

method for the simultaneous analysis of cloricromene and its active metabolite. Furthermore, the extraction procedure is rapid and simple. We believe that the described method satisfies the need for an accurate, sensitive, specific and convenient assay method in rabbit aqueous humor for ocular pharmacokinetic studies of cloricromene and its metabolite.

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