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SHORT COMMUNICATION

Development of an HPLC method for determining the alpha$_2$-adrenergic receptor agonist brimonidine in blood serum and aqueous humor of the eye

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ABSTRACT: A procedure for determining brimonidine [5-bromo-6-(2-imidazolidinylideneamino) quinoxaline] in biological samples using a reversed-phase isocratic HPLC method is described. The application in blood serum and eye aqueous humor of patients treated with the Alphagan^{TM} ophthalmic solution was carried out by enrichment of samples in brimonidine with solid-phase liquid extraction. Brimonidine reached maximum levels in aqueous humor and serum within 2–2.5 h, whereafter a declining pattern was obtained. An approximate 50% level of brimonidine was identified in serum at 12 h after ocular administration, whereas in aqueous humor this percentage was determined after a period of 4–5 h. Copyright © 1999 John Wiley & Sons, Ltd.

INTRODUCTION

Glaucoma is estimated as the third leading cause of blindness. Identification and treatment of patients who will develop glaucoma and progressive visual loss by the disease, are very important and crucial parameters to prevent the structural change in the optic nerve head (Sommer *et al.*, 1991). The topical ocular medication is of great value in the management of glaucoma patients. Water soluble brimonidine L-tartrate has been shown to act as a highly selective alpha$_2$-adrenergic receptor (Munk *et al.*, 1996). It causes a hypotensive effect by lowering the intraocular pressure (IOP) in patients with open-angle glaucoma or ocular hypertension (Burke *et al.*, 1995). This effect is associated with a decrease of aqueous humor production and an increase of overscleral outflow (Toris *et al.*, 1995; Munk *et al.*, 1996). Its use, therefore, is of importance in patients with elevated IOP, which is a major risk factor in glaucomatous optic nerve damage and visual field deterioration.

Quantitation of brimonidine levels in serum and aqueous humor by a highly selective analytical method, such as HPLC, so that brimonidine could be accurately determined and identified, has not been previously described. In this paper, we report on a biochemical procedure for sample clean-up, and HPLC determination of brimonidine in serum and the eye aqueous humor.

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Abbreviations used: IOP, intraocular pressure; R.S.D., relative standard deviation.

EXPERIMENTAL

Biological material. The collection of biological material and the procedure followed were in accordance with the ethical standards of the Helsinki Declaration of 1975, and its latest version. Aqueous humor (100 μL) was aspirated by inserting a 25-gauge needle into the anterior chamber, and collected in test tubes without preservatives. All aqueous humor and serum (100–300 μL) samples used were from three non-glaucomatous patients admitted for cataract extraction (males, 65–70 years old). Samples were immediately transported to the laboratory packed in dry ice. In all cases, individuals were treated by ocular administration of one drop into one eye, from the commercially available 0.2% (w/v) brimonidine L-tartrate sterile ophthalmic solution (Alphagan^{TM}).

Sample preparation. Samples were first treated by the addition of two volumes of 10 mM triethylamine buffer, pH 3.2, adjusted by the addition of phosphoric acid. The mixtures were centrifuged at 11,000 *g* for 10 min and the supernatant was enriched in brimonidine by chromatography on Sep-Pak C-18 cartridges (Waters). Following washing with 5 mL of triethylamine buffer, brimonidine was recovered by eluting with 5 mL of 50% (v/v) acetonitrile in triethylamine buffer. Following evaporation under N_2, the dry residue was dissolved in 100 μL of 10% (v/v) acetonitrile in triethylamine buffer. Aliquots of 10–20 μL were taken for HPLC analysis.

Chromatographic conditions. The analytical column was a Supelcosil LC-18, 5 μm, 250 × 4.6 mm i.d., stainless steel (Supelco, Bellfonte, PA, USA) equipped with a RP-18 precolumn, 20 × 4.6 mm i.d. (Supelco). The mobile phase was 10% (v/v) acetonitrile in 10 mM triethylamine buffer, pH 3.2. The separation was performed at room temperature, at a flow-rate of 1.0 mL/min, and the detection of brimonidine at 248 nm.

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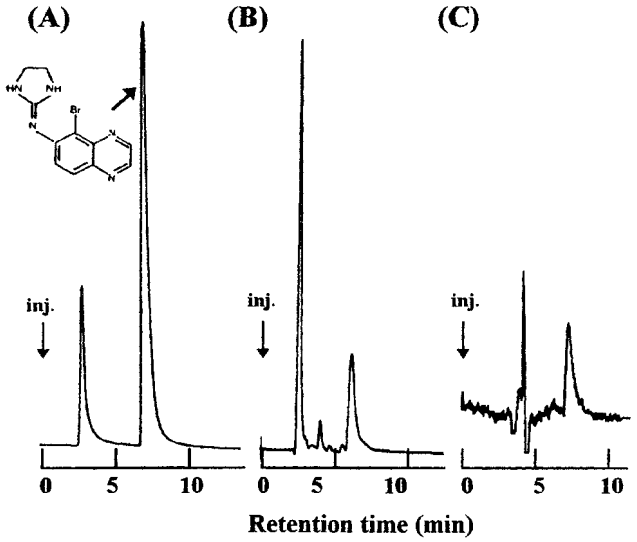

Figure 1. Typical RP-18 chromatogram of an Alphagan™ solution (13.2 ng brimonidine/mL) (A) and analysis of brimonidine (2.5 ng/mL) in aqueous humor (B) and blood serum (0.5 ng/mL) (C).

RESULTS AND DISCUSSION

Chromatographic separation and method's quality parameters

A typical chromatogram of brimonidine elution is given in Fig. 1(A). The retention time of brimonidine was reproducible under the chromatographic conditions used with a relative standard deviation (R. S. D.) of less than 3.5%. The majority of benzalkonium chloride used as a preservative in the ophthalmic solution and other substances used in clinical practice to cause mydriasis, such as cyclopentolate (1% solution obtained from Alcon Laboratories, Athens), phenylephine (5% solution from Cooper, Athens) and tropixal (0.5% solution of tropicamide obtained from Demo, Athens), have been eliminated by the solid-phase extraction. The remaining amounts of these compounds did not interfere with the determination as they are eluted earlier (3.5–4.5 min) than brimonidine. Alphagan™ ophthalmic solution was provided by ALLERGAN Inc. (Irvine, CA, USA) in aqueous solution and kept at 2–4°C until use, in the exclusion of light. Chromatographic analysis of Alphagan™ showed the absence of any contaminating substances which could interfere with the analysis of brimonidine [Fig. 1(A)]. The mobile phase used enabled good column performances over a long period of time.

Standard solutions (concentrations ranging from 30.0 pg/mL to 100.0 μg/mL) were prepared by serial dilutions of the stock solutions (1.32 mg brimonidine as free base per mL of aqueous solution). Calibration curves were constructed by plotting the peak area and peak height of brimonidine against its concentration expressed as ng/mL, and evaluated for their linearity according to the standard protocol of van Trijp and Roos as previously reported by Karamanos *et al.* (1996). Calibration graphs showed excellent linearity up to the entire interval tested. The detector response corresponds with the following equations: $y = -15601.67 + 38657.2\ x$ (SD = 35363.01) for the peak area and $y = -7.42 + 26.18\ x$ (SD = 19.79) for the peak height. The correlation coefficients of linearity graphs were 0.9997 and 0.9998, respectively. Twelve consecutive analyses of 15 μL injections from a 10.0 ng/mL standard solution were used to determine the precision of the method. The R. S. D.s obtained were in the range of 1.9–2.9%. The detection limit, based on the signal-to-noise ratio of 2:1, was 30 pg/mL.

Recovery of brimonidine during the sample preparation procedure on Sep-Pak C-18 was estimated by using either standard solutions of brimonidine, or blood serum enriched with a known amount of brimonidine (100 ng/mL). HPLC analyses showed that the recovery exceeded 98% (98.5–100.0 ng/mL), indicating a reproducible and precise procedure for sample preparation.

Brimonidine analysis in serum and aqueous humor

Samples of blood serum and aqueous humor from hospitalized healthy volunteers treated with Alphagan™, were collected over a period of 24 h. As shown in Fig. 1(B) and (C), brimonidine can be reliably separated and determined both in aqueous humor and serum, respectively, without any interference. Analysis of brimonidine levels in serum and aqueous humor (following ocular administration of 65 μg/mL as free base) from time 0–24 h after administration showed that maximal values were reached within 2–2.5 h, whereafter a declining pattern was obtained. The maximum value in serum was found to be 55–77 pg/mL. Approximately half of the brimonidine level was identified in the serum after 12 h, and in the aqueous humor after 4–5 h from the administration. These results show agreement with brimonidine declining profiles obtained by fluorometric and radioactivity measurements (Chien *et al.*, 1992; Acheampong *et al.*, 1995), indicating that the described HPLC method can be reliably used for rapid and accurate profiling of brimonidine levels during a patient's treatment.

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