ELSEVIER

Journal of Chromatography B, 772 (2002) 179–183

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Short communication

Determination of betaxolol in human aqueous humour by high-performance liquid chromatography with fluorescence detection

Berrak Dulger[a,b,*], Nursabah E. Basci[a,b], Ilgaz Sagdic-Yalvac[c], Aytekin Temizer[a,b]

[a]*Hacettepe University, Faculty of Pharmacy, Department of Analytical Chemistry, 06100 Ankara, Turkey*
[b]*Turkish Doping Control Center, Hacettepe University, Faculty of Pharmacy, 06100 Ankara, Turkey*
[c]*Department of Ophthalmology, Ankara Hospital, Ankara, Turkey*

Received 30 July 2001; received in revised form 4 January 2002; accepted 8 January 2002

Abstract

A reversed-phase high-performance liquid chromatographic method is described for the determination of betaxolol in human aqueous humour. Betaxolol and the internal standard metoprolol were extracted with cyclohexane and separated on a reversed-phase column (Luna C_{18}, 250×4.6 mm, 5 μm) with a mobile phase containing acetonitrile–phosphate buffer (40:60, v/v) at a flow-rate of 0.8 ml/min. The column effluent was monitored with a fluorescence detector at 227 nm (excitation) and 301 nm (emission). The retention times for metoprolol and betaxolol were 3.55 and 5.63 min, respectively. The recovery from aqueous humour was found to be 71.6% for betaxolol at 1.25 μg/ml. The within-day and day-to-day accuracy values were in the range of 96.17–105.2% for betaxolol at 0.1, 4 and 12 μg/ml (n=6), within-day and day-to-day precision values were less than 10% for betaxolol at the concentrations given above. The detection limit corresponding to the signal-to-noise ratio of 3:1 was 15 ng/ml. The presented method was suitable for measuring betaxolol levels in human aqueous humour samples obtained from patients after topical administration.

Keywords: Fluorescence detection; Betaxolol; β-blocker

1. Introduction

Betaxolol, DL-[4-{2-cycloprophylmethoxyethyl}-1-phenoxy-3-isoprophylamino-2-propanol] (Fig. 1), is a cardioselective β-adrenergic blocking agent characterized by a high bioavailability (90%) and a long half-life in man of about 16–22 h [1]. Betaxolol is used for the treatment of hypertension and glaucoma [2]. Betaxolol is abused for doping purposes in some sports, therefore banned by International Olympic Committee like other β-blockers [3]. This drug is also used for the treatment of glaucoma and marketed under the trade name of Betoptic. In glaucoma

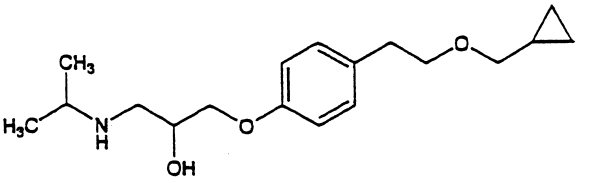

Fig. 1. Structure of betaxolol.

*Corresponding author. Hacettepe University, Faculty of Pharmacy, Department of Analytical Chemistry, 06100 Ankara, Turkey. Tel.: +90-312-3051-499; fax: +90-312-3106-776.

E-mail address: berrakdulger@hotmail.com (B. Dulger).

1570-0232/02/$ – see front matter
PII: S1570-0232(02)00023-5

patients, betaxolol is used due to its high efficiency in intra-ocular pressure decreasing effect; the adequate therapy of the patients is dependent on betaxolol levels in aqueous humor. Accordingly, to estimate the betaxolol levels in aqueous humour is very important for the decision of optimal betaxolol dosage regime given to the patients.

The assay of betaxolol by gas chromatography (GC) with electron-capture detection (ECD) and GC–mass spectrometry (MS) have been reported in the literature [4–7]. Chromatographic analysis of betaxolol in body fluids such as plasma and urine was mainly based on high-performance liquid chromatography (HPLC) combined with ultraviolet or fluorescence detection [8–12].

Betaxolol determination in ophthalmic solution and tenon capsule of the eye has been performed by HPLC–diode array detection (DAD) [13,14]. Betaxolol has been analyzed in human aqueous humour by a special method of radioreceptor assay (RRA) [15–18], because of its high specificity at low concentrations. But, RRA is an expensive, laborious, tedious, time-consuming method and is also not as common as HPLC. To the authors' knowledge, no method using HPLC has been reported for determination of betaxolol in human aqueous humour. The aim of the present study is to develop a HPLC method suitable for the analysis of betaxolol in human aqueous humour and to demonstrate its applicability to the samples obtained from patients.

2. Materials and methods

2.1. Chemicals

Metoprolol and betaxolol were purchased from Sigma (St. Louis, MO, USA). HLPC-grade quality acetonitrile (Riedel-de Haën, Seelze, Germany) was used and other chemicals were of analytical grade.

2.2. Chromatography

The HPLC equipment comprised of a solvent delivery system (Hewlett-Packard 1090 M) and a fluorescence detector (Hewlett-Packard 1046 A) or a UV–visible detector (Hewlett-Packard 79880AX). The analytical column was a stainless steel column packed with 5 μm Luna C_{18} (250 mm×4.6 mm I.D., Phenomenex). The mobile phase was consisted of acetonitrile–10 m*M* phosphate buffer (40:60, v/v) with a flow-rate of 0.8 ml/min at ambient temperature. The UV–visible detector was set to 220 nm; fluorescence excitation and emission wavelengths were 227 and 301 nm, respectively.

2.3. Preparation of standards

Stock solutions of betaxolol (1 mg/ml) and metoprolol (1 mg/ml) were prepared in deionized water. Standard solutions were daily prepared by diluting stock solutions in mobile phase to the betaxolol concentrations of 0.05, 0.25, 0.5, 1, 2, 5, 8, 10 and 15 μg/ml containing metoprolol (1 μg/ml) as internal standard. Labetolol was an internal standard for UV–visible detector trials at a concentration of 2 μg/ml. Stock solutions and samples were stored at −25 °C until analysis and protected from light because of the light sensitivity of metoprolol.

2.4. Calibration curve

The calibration curve is prepared by plotting the peak area ratio of betaxolol to metoprolol against to the concentration of betaxolol and used for the determination of betaxolol levels in samples. Calibration curve standards and aqueous humor samples are prepared by the same procedure given in Section 2.5. Calibration curve in drug-free aqueous humour was not possible because of the lack of adequate blank aqueous humour. Thus the calibration curve standards were prepared in distilled water.

2.5. Sample preparation

Aqueous humour samples (30 μl) were diluted with distilled water (1:8, v/v) and metoprolol (1 μg/ml) was added as an internal standard. The mixture was alkalinized (pH 9–10) with 60 μl of 0.5 *M* Na_2CO_3 and extracted with 1.5 ml of cyclohexane by mixing on a vortex-mixer for 15 s. After centrifu-

gation (1000 *g*, 5 min), the organic phase was transferred to a clean tube and evaporated to the dryness on a rotavapor at ambient temperature. The residue was dissolved in 240 μl of mobile phase and 20 μl of the solution was injected onto the column.

2.6. Sample collection

Patients were under topical betaxolol therapy (0.5%; 2×1 daily, Betoptic; Alcon Laboratory, Fort Worth, TX, USA) and aqueous humor samples were drawn by parcentesis during the cataract surgery. None of the patients had any ocular pathology other than cataracts. Local ethical committee approved this study and all patients gave written informed consent.

2.7. Validation

For validation of the method, the parameters of accuracy, precision, limit of quantification (LOQ), limit of detection (LOD), linearity and selectivity were investigated.

The recovery of betaxolol was determined by comparison of peak area values of a spiked aqueous humour standard (1.25 μg/ml) with a standard solution in water at the same concentration ($n=1$). Because of ethical restrictions, the amount of blank aqueous humour was not adequate and recovery of betaxolol was carried out only in one spiked aqueous humour standard.

To evaluate accuracy and precision, standard solutions in water at three concentration levels over the calibration curve were analyzed. The concentration levels were 0.1, 4.0 and 12.0 μg/ml for betaxolol. Each of these samples was analyzed six times within day and in 6 different days, to determine the intra-day and inter-day accuracy and precision.

Precision was calculated as relative standard deviation (RSD), the accuracy was percentage deviation of found concentration from added concentration.

The selectivity was described as the ability of the method to resolve betaxolol from baseline and other peaks.

3. Results and discussion

3.1. Analysis of betaxolol in aqueous humour

In the presented method, betaxolol and metoprolol (internal standard) are accurately separated on a Luna C_{18} column (250×4.6 mm, 5 μm) using a mobile phase consisting of acetonitrile–10 m*M* phosphate buffer (40:60, v/v) after optimization of chromatographic conditions. The optimum flow-rate was found to be 0.8 ml/min. The selection of a

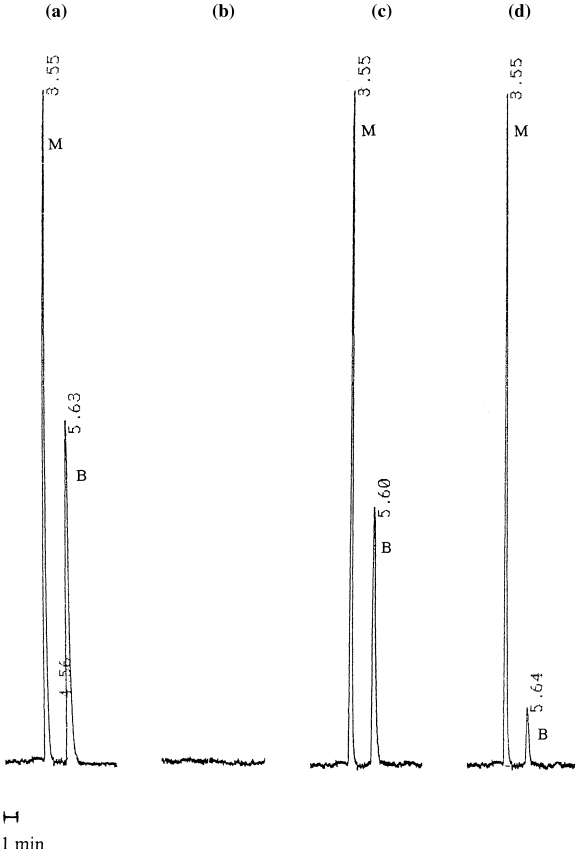

Fig. 2. Representative chromatograms of (a) betaxolol standard (1.25 μg/ml) with metoprolol (1 μg/ml) in mobile phase, (b) after extraction of drug-free aqueous humour; (c) after extraction of aqueous humour sample spiked with betaxolol (1.25 μg/ml) and metoprolol (1 μg/ml) and (d) aqueous humor sample containing betaxolol (0.25 μg/ml) obtained from a patient under treatment injected into the HPLC–fluorescence detection system. **B**: Betaxolol, **M**: metoprolol (internal standard). The fluorescence detector was at an attenuation value of 4.

Table 1
Intra-assay accuracy and precision data for the determination of betaxolol

Added concentration (μg/ml)	Found concentration (μg/ml)[a]	Accuracy (%)	Precision (%)
0.1	0.11±0.03	105.20	9.50
4.0	4.17±0.03	104.30	1.92
12.0	11.54±0.38	96.17	8.15

[a] Values are given as mean±standard error.

Table 2
Inter-assay accuracy and precision data for the determination of betaxolol

Added concentration (μg/ml)	Found concentration (μg/ml)[a]	Accuracy (%)	Precision (%)
0.1	0.11±0.004	105.20	9.10
4.0	4.08±0.020	102.00	1.47
12.0	12.21±0.230	101.75	4.59

[a] Values are given as mean±standard error.

suitable detection procedure is also important for optimizing a HPLC method. For betaxolol, essentially two types of detection can be used: UV–visible spectrometry and fluorescence. The optimum wavelengths of betaxolol at its the maximum responses in UV–visible and fluorescence detectors were λ_{max}= 220 nm, λ_{ex}=227 nm and λ_{em}=301 nm. After direct injection of betaxolol standard into the HPLC system with UV–visible or fluorescence detectors, detection limits with a signal-to-noise ratio of 3 were 200 and 15 ng/ml, respectively.

In HPLC–UV–visible method, the retention times of labetolol (internal standard) and betaxolol were, respectively, 3.7 and 5.1 min. Additionally, interfering peaks from matrix occurred in the chromatogram of a drug-free aqueous humour sample with UV–visible detection. Although, betaxolol and labetolol (internal standard) were well separated from interfering peaks, UV–visible detection response was about 13-fold lower than fluorescence detection response. Because of these observations fluorescence detection was used in the study.

In HPLC–fluorescence detection, the retention times of metoprolol and betaxolol were 3.55 and 5.63 min, respectively (Fig. 2a). After extraction of drug-free aqueous humour sample with cyclohexane, all extra peaks are eliminated (Fig. 2b). Metoprolol and betaxolol peaks are well-resolved in the standard aqueous humour sample and an aqueous humor sample obtained from a patient under treatment (Fig. 2c and d).

3.2. Assay validation

The calibration curve of the method was linear over the range of 0.05–15 μg/ml (y=0.5007x−0.0363, r=0.9971) and the method was validated in this range. The lower LOD was 15 ng/ml at S/N=3 and the LOQ was 50 ng/ml (RSD=14.8%, n=6).

The recovery of betaxolol from aqueous humour was 71.6% at a concentration of 1.25 μg/ml (n=1). Precision and accuracy values for within-day and day-to-day studies are given in Tables 1 and 2. Precision (RSD) was less than 10.0% at selected

Table 3
Betaxolol levels in human aqueous humour samples from patients after topical administration (0.5%; 2×1 daily)

Subject No.	Betaxolol levels (μg/ml)
1	0.41
2	0.21
3	0.33
4	0.21
5	0.29
6	0.25
Mean	0.28
SD	0.08

concentrations and accuracy values ranges from 96.17 to 105.20%. These values were acceptable according to the acceptance criteria's for accuracy and precision [19].

3.3. Application to aqueous humour samples

The method was applied to the analysis of aqueous humour samples obtained from six volunteers. Betaxolol levels after topical administrations of 0.5% betaxolol are given in Table 3. Betaxolol levels in aqueous humour after topical administration were found to be 0.28 ± 0.08 μg/ml (mean±SD, $n=6$) and all values were higher than the lower determination and quantitation levels of the developed method.

4. Conclusion

The HPLC–fluorescence method developed in this study has proved to be adequate for the analysis of the low-level betaxolol in less than 10 min. UV–visible detection is found to be less sensitive than fluorescence detection accordingly fluorescence detection is more preferable to UV–visible detection. Additional sample clean-up procedure provides an advantage to eliminate interfering peaks hence betaxolol can be analyzed without any concentrating step for the low amount of human aqueous humour sample (30 μl). The observations of this study are in agreement with previous studies [20,21] suggesting that HPLC–fluorescence detection can be utilized for drug analysis in aqueous humour samples.

In conclusion, a selective, sensitive, precise, reproducible method applicable to the samples of patients was developed for the determination of betaxolol in human aqueous humour.

References

[1] R. Beresford, R.J. Heel, Drugs 31 (1986) 6.
[2] J.L. Rait, Aust. N.Z. J. Ophtlalmol. 27 (1999) 57.
[3] International Olympic Committee (IOC) Medical Commission, List of Doping Classes and Methods, 2001.
[4] G. Bianchetti, J. Ganansia, P.L. Morselli, J. Chromatogr. 176 (1979) 134.
[5] J. Ganansia, G. Gillet, P. Padovani, G. Bianchetti, J. Chromatogr. 275 (1983) 183.
[6] M.S. Leloux, E.G.D. Jong, R.A.A. Maes, J. Chromatogr. B 488 (1989) 357.
[7] G.D. Branum, S. Sweeney, A. Palmeri, L. Haines, C. Huber, J. Anal. Toxicol. 22 (1998) 135.
[8] Y.W.J. Wong, T.M. Ludden, J. Chromatogr. B 534 (1990) 161.
[9] M. Canal, B. Flouvat, J. Chromatogr. B 342 (1985) 212.
[10] R.K. Bhamra, A.E. Ward, D.W. Holt, J. Chromatogr. B 417 (1987) 229.
[11] H. Caqueret, G. Bianchetti, J. Chromatogr. B 311 (1984) 199.
[12] A. Tracqui, P. Kintz, J. Himber, A.A. Lugnier, P. Mangin, Forensic Sci. Int. 38 (1988) 37.
[13] M.I.R.M. Santoro, H.S. Cho, E.R.M. Kedor-Hackmann, Anal. Lett. 28 (1995) 71.
[14] W.E. Sponsel, S. Terry, H.D. Khuu, K.W. Lam, H. Frenzel, Surv. Ophthalmol. 43 (1999) 210.
[15] E. Vainio-Jylha, M.L. Vuori, T. Kaila, R. Huupponen, Eur. J. Clin. Pharmacol. 54 (1998) 389.
[16] M.L. Vuori, T. Kaila, E. Iisalo, K.M. Saari, Acta Ophthalmol. 71 (1993) 667.
[17] M.L. Vuori, T. Ali-Melkkila, T. Kaila, E. Iisalo, K.M. Saari, Acta Ophthalmol. 71 (1993) 201.
[18] T.M. Phan, K.P. Nguyen, J.C. Giacomini, D.A. Lee, J. Ocul. Pharmacol. 7 (1991) 243.
[19] S. Braggio, R.J. Barnaby, P. Grossi, M. Cugola, J. Pharm. Biomed. Anal. 14 (1996) 375.
[20] N.E. Basci, A. Bozkurt, D. Kalayci, S.O. Kayaalp, J. Pharm. Biomed. Anal. 14 (1996) 353.
[21] N.E. Basci, S. Hanioglu-Kargi, H. Soysal, A. Bozkurt, S.O. Kayaalp, J. Pharm. Biomed. Anal. 15 (1997) 663.