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ORIGINAL RESEARCH

Development, validation and analytical error function of two chromatographic methods with fluorimetric detection for the determination of bisoprolol and metoprolol in human plasma

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ABSTRACT: This work describes two high-performance liquid chromatographic methods for the individual determination of bisoprolol and metoprolol in human plasma. Analytical methods involve two different liquid–liquid extractions of human plasma, with diethyl ether for bisoprolol and with dichloromethane for metoprolol, coupled with a similar Nucleosil C_{18} reversed-phase HPLC column. Fluorimetric detection was used to identify both β-blockers. Retention times for bisoprolol and metoprolol were 8.7 and 3.2 min, respectively. Linear regressions for the calibration curves were linear at a concentration range of 6.25–200 ng/mL. Intra- and inter-day precision coefficients of variations and accuracy bias were acceptable (within 15%) over the entire range for both drugs. Average recovery was 89% for metoprolol and 98% for bisoprolol. Once the methods had been validated, analytical error functions were established as standard deviation (SD; ng/mL) = $2.216 + 3.608 \times 10^{-4}C^2$ (C = theoretical concentration value) and SD (ng/mL) = $0.408 + 0.378 \times 10^{-1}C$ for bisoprolol and metoprolol, respectively. The methods developed and their associated analytical error functions will be suitable for pharmacokinetic studies and for determination of plasma concentration if posology individualization of these drugs is needed. Copyright © 2002 John Wiley & Sons, Ltd.

INTRODUCTION

Bisoprolol and metoprolol, shown in Fig. 1, are clinically important β-blockers used for the management of cardiovascular disorders such as essential hypertension, angina pectoris and arrhythmia. Besides, results from clinical trials have suggested that patients with heart failure may benefit from β-blocker therapy (Lechat *et al.*, 1998; Krumholz, 1999). Bisoprolol and metoprolol have similar pharmacologic properties, their main differences lying in molar activity level and pharmacokinetics. Thus, the usual dosage of bisoprolol is 10 mg given once daily in normal release forms. The dosing rate for metoprolol is 100 mg once or twice daily, formulated into sustained release presentations (Deroubaix *et al.*, 1996).

Recently, different high-performance liquid chromatographic assays for the determination of these non-enantiomer β-blockers in biological matrices have been reported (Deroubaix *et al.*, 1996; Basci *et al.*, 1998; Mistry *et al.*, 1998). Most methods used fluorescence detection after solid phase or liquid–liquid extraction. However, while some authors have found a linear relationship between detector response and concentration data, others resorted to a weighting factor of 1/concentration^2. In others, to our knowledge, the error function associated with the analytical method developed was not determined. Thus, it would be useful to have a practical way of obtaining the estimated standard deviation with which a single determination of a serum drug concentration is measured.

The objectives of the present work were to develop and validate two reverse-phase high-performance liquid chromatographic methods with fluorimetric detection for the quantification of bisoprolol and metoprolol in human plasma that cover a range of concentration from 6.25 to 200 ng/mL and after that, to determine their associated analytical error functions in order to provide a suitable data-weighting method covering the working concentration range.

EXPERIMENTAL

Chemicals and reagents. Bisoprolol fumarate was generously provided by Merck-Igoda (Mollet del Vallés, Spain) and metoprolol tartrate by Novartis Farmacéutica (Barcelona, Spain).

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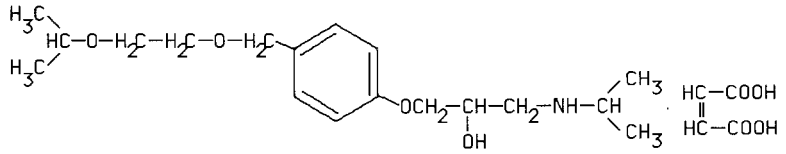
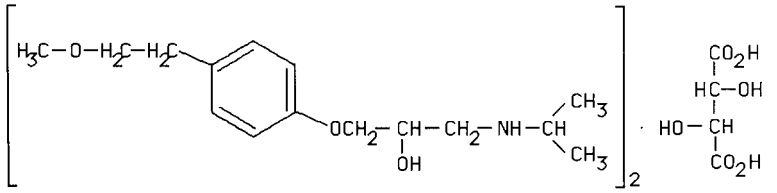


Bisoprolol fumarate



Metoprolol tartrate

Figure 1. Chemical structure of β-blockers studied.

Acetonitrile was HPLC grade and was purchased from Scharlau (Barcelona, Spain). Triethylamine, 85% phosphoric acid and the rest of the chemicals (diethyl ether, dichloromethane, 97% sodium hydroxide and acetic acid) were purchased from Fluka Chemika-Biochemika (Buchs, Switzerland), Probus (Badalona, Barcelona, Spain) and Panreac (Montcada i Reixach, Spain), respectively. All of them were analytical grade and used without further purification. All aqueous solutions including the HPLC mobile phase were prepared with purified deionized water (Milli-Q Plus, Millipore, Barcelona, Spain).

Apparatus and chromatographic conditions. The HPLC system consisted of a 422 Kontron (Kontron Instruments, Barcelona, Spain) equipped with two 422 pumps, a Rheodyne 7161 injector with a 100 µL sample loop, a 491 mixer, a spectrofluorometer 25 variable wavelength detector and an INT-450 computerized integration system data output.

Analyses were performed at room temperature on a column packed with 5 µm Nucleosil RP_{18} (125 × 4 mm i.d.; Teknokroma, Barcelona, Spain). The mobile phase for both drugs consisted of an isocratic mixture of acetonitrile–HPLC water with 1.2% (w/v) of triethylamine and the pH adjusted to 3 with 85% orthophosphoric acid (18:82, 20:80, v/v), for bisoprolol and metoprolol, respectively. The mobile phase was prepared daily and filtered under vacuum through a 0.45 µm pore-size membrane filter before use and was delivered at a flow rate of 1 mL/min. The injection volume was 100 µL. Fluorescence detection was set at an excitation wavelength of 232 nm for bisoprolol and 280 nm for metoprolol, an emission wavelength of 300 nm for both drugs and a single sensitivity factor of 500 nm.

Preparation of standards. Standard solutions of β-blockers dissolved in drug-free human plasma were obtained by suitable dilution from stock solutions prepared in HPLC water at 300 µg/mL. The concentration range for the calibration curve in human plasma selected was 6.25–200 ng/mL for both bisoprolol and metoprolol. The stock and standards solutions of β-blockers were stored at approximately −20°C until use.

Sample preparation. The liquid–liquid extraction procedure was different for each drug. To each millilitre of human plasma treated with 100 µL of 1 M sodium hydroxide were added 6 mL of organic layer (dichloromethane for metoprolol and diethylether for bisoprolol). The tube was capped and the content mixed for 10 min on a rotary mixer and centrifuged for 5 min at 2000***g***. After this the upper aqueous layer was discarded.

In the case of metoprolol, 5 mL of the organic layer were removed to a conical glass tube and evaporated to dryness at 50 ± 1°C. Finally, the dry residue was dissolved in 150 µL of mobile phase and 100 µL were injected into the HPLC system, using a 100 µL Hamilton syringe.

As for bisoprolol, the organic layer (5 mL) was transferred to a conical glass tube containing 150 µL of 1 M acetic acid. The tube was capped and the content was back-extracted by mixing again, then centrifuged briefly to separate the layers. The organic phase was evaporated to dryness at 50 ± 1°C and 100 µL of the acid layer was injected into the HPLC system, using a 100 µL Hamilton syringe.

Analytical method validation. Evaluation of the HPLC methods was based on proportionality (linearity assay), precision and accuracy (Causon, 1997; Huber, 1998; US Department of Health and Human Services *et al.*, 2000).

The methods were validated by analysis of human plasma quality control samples. Linearity consisted of the determination of the same concentration range as the calibration curve, covering six concentrations, which were 200, 100, 50, 25, 12.50 and 6.25 ng/mL for both bisoprolol and metoprolol. Analyses were made in triplicate.

Precision and accuracy of the methods were performed by analysing three concentrations within the linearity range (low, medium and high), which were 200, 50 and 12.5 ng/mL for bisoprolol and 200, 50 and 6.25 ng/mL for metoprolol. Five standard solutions of each concentration were spiked to drug-free

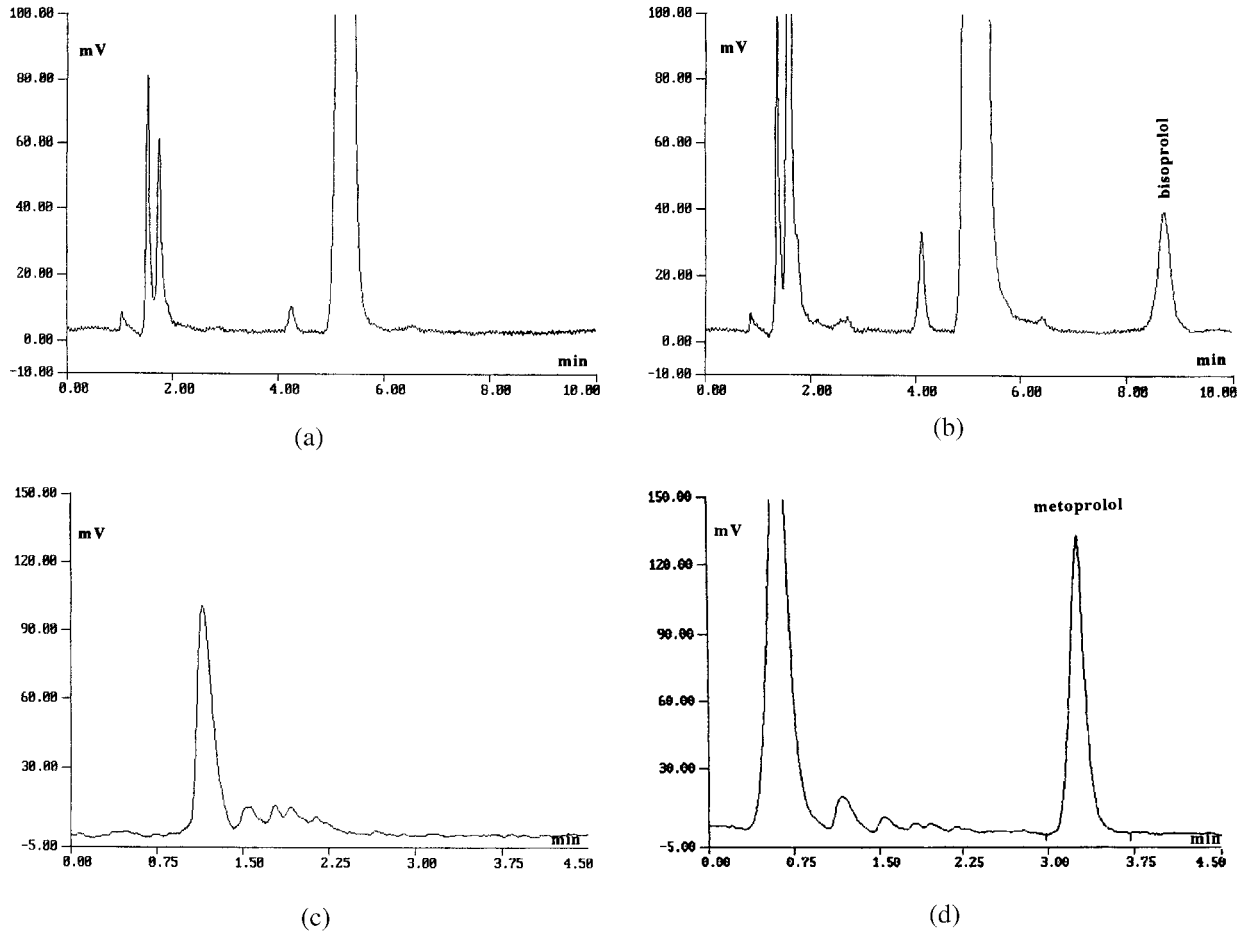


Figure 2. Representative chromatograms of (a) blank plasma in condition of bisoprolol method; (b) blank plasma spiked with 50 ng/mL of bisoprolol; (c) blank plasma in condition of metoprolol method; (d) blank plasma spiked with 50 ng/mL of metoprolol.

human plasma and analysed (intra-day assay). The assay was repeated on 5 days (inter-day assay).

The absolute recovery (extraction efficiency) of both β-blockers from human plasma was performed in the full concentration range and was established by comparing the peak area responses obtained from the standard solutions of drug-free plasma spiked with the drug in replicated of three with those of non-extracted standards, which represent 100% recovery. The limits of quantification and detection were also determined.

To study the stability, human plasma quality control samples of each drug were prepared, stored at -20°C and used during routine analysis. The results obtained confirmed the stability of each drug in human plasma during the period of the study and during the sample preparation and analysis time.

Analytical error function. The study of the analytical error function for the HPLC analysis was carried out using the same range and concentrations of the calibration curve for each drug. The procedure used by us to obtain the error function of each validated analytical method has already been reported (Modamio *et al.*, 1996, 1998) and was as follows: three calibration curves (three replicates) were obtained every day during four different days. Each day, the mean and standard deviation (SD) of each standard concentration were obtained from the calibration curve. After that, the best function between the SD obtained for each concentration of the calibration curve as dependent variable, and the theoretical concentration values (C), as independent variable, were calculated using multiple regression, applying the stepwise forward selection method (Kleinbaum *et al.*, 1988). The final function obtained explains better the relationship between the standard deviation and the concentration and, therefore, the weights obtained for each experimental concentration value are more appropriate.

RESULTS AND DISCUSSION

Figure 2(a–d) shows blank and drug spiked chromatograms: (a) correspond to the blank plasma in condition of bisoprolol method; (b) to the blank plasma spiked with 50 ng/mL of bisoprolol; (c) to the blank plasma in condition of metoprolol method; and (d) to the blank plasma spiked with 50 ng/mL of metoprolol. Fluores-

Table 1. Intra-day (1 representative day) and inter-day precision and accuracy for bisoprolol and metoprolol in human plasma

β-Blocker	Spiked concentration (ng/mL)	Intra-day ($n = 5$)			Inter-day ($n = 5$)		
		Data (ng/mL)	CV (%)	Bias (%)	Data (ng/mL)	CV (%)	Bias (%)
Bisoprolol	100	98 ± 7	7	−2	99 ± 2	2	−1
	50	52 ± 6	11	3	48 ± 4	8	−3
	12.5	13 ± 2	13	4	14 ± 2	13	11
Metoprolol	200	200 ± 9	4	0	200 ± 0	0.1	0
	50	49 ± 2	4	−1	50 ± 1	2	−1
	6.25	7 ± 0	4	9	6 ± 1	13	4

Data are means ± SD. CV(%) = (SD/mean) × 100; Bias (%) = [(measured concentration − spiked concentration)/spiked concentration] × 100.

cence detection improved sensitivity and removed endogenous chromatographic interferences.

By these reversed-phase HPLC methods, both drugs were resolved and quantified acceptably, with approximate retention times of 8.7 and 3.2 min for bisoprolol and metoprolol, respectively.

A minimum signal-to-noise ratio of 3:1 was obtained with the lowest concentrations, allowing a detection limit of 3.13 ng/mL for both β-blockers. Thus, the limits of quantification used (6.25 ng/mL) were higher than the absolute limits of the assays. The injection volume used was the same for both drugs.

Assay validation

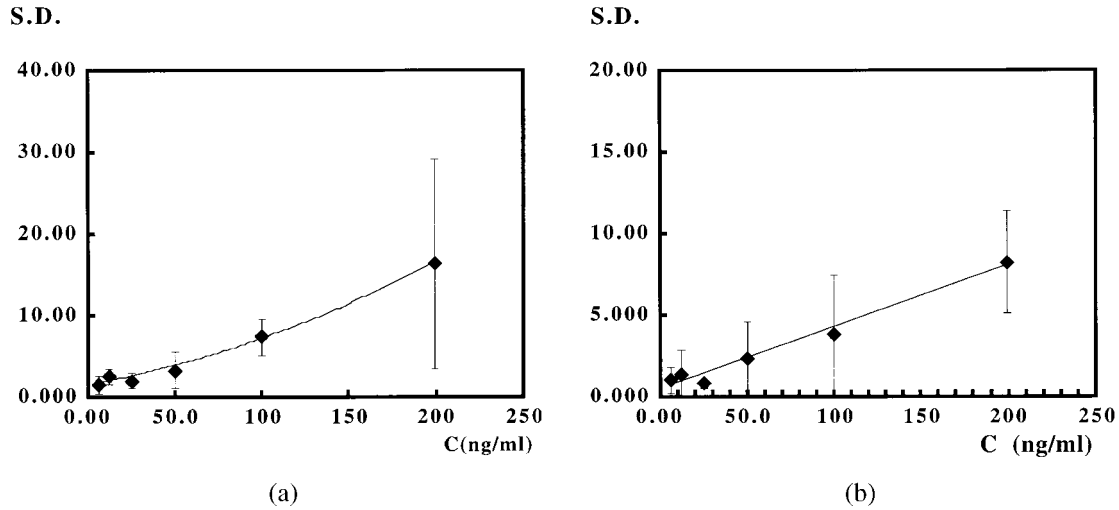
In the linearity assay, the response factors expressed by coefficient of variation (CV) were 14 and 8% for both bisoprolol and metoprolol, respectively. The regression equations obtained from unweighed least-squares linear regression were $y = 0.338 + 0.164x$, $r^2 = 0.9857$, and $y = -0.205 + 0.401x$, $r^2 = 0.9959$, where y is peak area and x is concentration. Thus, a good linear relationship between the peak area and concentration was observed over the entire range for both drugs.

The results obtained in intra-day and inter-day precision and accuracy at the three different concentrations in plasma are summarized in Table 1. Precision was reported by the CV and accuracy was by bias. Maximum CV values were 13 and 4% in intra-day precision for bisoprolol and metoprolol, respectively and 13% in inter-day precision for both. These high values corresponded to the lowest concentrations, except to metoprolol intra-day precision. In this case the same CV values were obtained for the three assayed concentrations. Intra- and inter-day accuracy bias were below 10 and 12% for all concentrations and for both drugs.

In the range of calibration standards, the analytical recovery in the plasma sample averaged 89% for bisoprolol and 98% for metoprolol.

Analytical error procedure

The best analytical error functions obtained from the stepwise forward selection method were the following:



(a) (b)

Figure 3. Mean values and standard deviations obtained in the study of the analytical error function vs theoretical concentrations from the calibration curves for (a) bisoprolol and (b) metoprolol.

Table 2. Analytical error functions corresponding to β-blockers assayed

β-Blocker	Analytical error function
Oxprenolol[a]	SD (ng/mL) = $8.906 + 0.81 \times 10^{-7}\ C^3$
Bisoprolol	SD (ng/mL) = $2.216 + 0.36 \times 10^{-3}\ C^2$
Atenolol[b]	SD (ng/mL) = $7.698 + 0.37 \times 10^{-1}\ C$
Celiprolol[a]	SD (ng/mL) = $3.096 + 0.41 \times 10^{-1}\ C$
Metoprolol	SD (ng/mL) = $0{,}408 + 0.38 \times 10^{-1}\ C$
Propranolol[b]	SD (ng/mL) = $0.126 + 0.36 \times 10^{-1}\ C$

[a] Obtained from Braza *et al.* (1998).
[b] Obtained from Braza *et al.* (2000).

SD (ng/mL) = $2.216 + 3.608 \times 10^{-4}C^2$ for bisoprolol and SD (ng/mL) = $0.408 + 0.378 \times 10^{-1}C$ for metoprolol. Figure 3(a and b) show the fit of these functions to the mean values of SD obtained for each theoretical concentration (error bars represent the SD of the mean values on the four analysis days) for bisoprolol and metoprolol, respectively.

Although the HPLC methods developed used similar reversed-phase column, mobile phase, detection system and, even, the concentration range selected was the same (6.25–200 ng/mL) for both drugs, different models of error function were obtained: linear in the case of metoprolol and nonlinear in bisoprolol. Previous studies reported by us (Braza *et al.*, 1998, 2000) have let different analytical error functions be found for other β-blockers, as it is shown in Table 2 together with those found in the present study. So, the determination of analytical error function has to do it for each drug individually, in spite of similarities or small differences found in the analytical methods developed or in drugs assayed.

Since one of the error source known comes from the quantification of drug concentrations, the measurement of it by means of analytical error function previously determined can be useful, among others, in parametric approach. For example, in pharmacokinetic analysis of experimental data performed by weighted least squares nonlinear regression, the inverse of the concentration variance calculated through the analytical error function (1/variance) can be a correct weighting method since is not always adequate to use constant weighting such as $1/C$ or $1/C^2$ (Jelliffe, 1989; Proost, 1995; Mariño *et al.*, 1996; Jansat *et al.*, 1998; Jelliffe *et al.*, 1998).

CONCLUSION

Results obtained from standard operating procedures used at our laboratory for the validation of HPLC methods demonstrate that these analytical methods have acceptable linearity, precision and accuracy between the peak area and concentration range. The CV and bias values do not surpass the 15% permitted, the maximum values found being 14, 13 and 14% for the linearity, intra- and inter-day precision and accuracy assays, respectively. Moreover, these methods can be used for analysis of a large number of samples daily, since similar type of reversed-phase HPLC column, mobile phase and detection procedure are used in the determination of both drugs and also because of their relative short chromatographic run time (about 10 min for bisoprolol and 4 min for metoprolol).

Besides, the analytical error function established for each β-blocker could be a useful choice as a weighting method in nonlinear regression analysis, which would reduce part of the total variability in pharmacokinetic studies and in hospital laboratory for determination of plasma concentration if posology individualization of these drugs is needed.

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