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Two reproducible and sensitive liquid chromatographic methods to quantify atenolol and propranolol in human plasma and determination of their associated analytical error functions ☆

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Abstract

Two liquid chromatography (LC) methods with fluorimetric detection have been developed to measure atenolol and propranolol in human plasma. The same 5 μm Nucleosil RP-18 column, extraction procedure and mobile phase (containing acetonitrile, water, triethylamine and phosphoric acid, pH 3) were used. The linearity ranges were 25–800 ng/ml for atenolol and 3.13–100 ng/ml for propranolol. The coefficients of variation for validation assays were lower than 15% at the concentration assayed. The functions of the analytical error were linear: SD (ng/ml)=7.698+0.037C for atenolol and SD (ng/ml)=0.126+0.036C for propranolol.

Keywords: Atenolol; Propranolol; β-Blockers

1. Introduction

Atenolol and propranolol are two β-blockers widely used as standard therapies in the treatment of high blood pressure, arrhythmias and angina pectoris. Besides, when administered chronically, they reduce the mortality due to hypertension and lengthen survival in patients with coronary heart disease [1,2].

Several liquid chromatography methods for the determination of atenolol and propranolol in human plasma using both fluorimetric [3–12] and UV detection [13–15] have been described. However, on one hand, few methods describe the quantitative analysis of more than one β-blocker of similar molecular structure (aryloxypropanolamines), but different lipophilicity. On the other, the error function associated to the analytical method is not usually established, although the measurement of this error can be essential in some applications. One of them, is an alternative to the weighting methods used in experimental data parameter estimation by non-linear regression analysis [16–18].

Thus, this study describes two analytical methods based on reversed-phase liquid chromatography (LC) for the quantification of atenolol and propranolol in human plasma that use the same column, mobile phase and extraction procedure, in order to reduce

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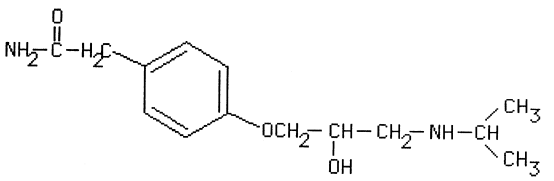
sample preparation and analysis time. After the validation of these methods, we determine the analytical error functions to estimate the uncertainty and to provide a suitable data-weighting method that would cover the concentration range of interest. Finally, error functions obtained for the same β-blockers but quantified by different analytical methods were compared, in an attempt to make this methodology useful for weighting procedures.

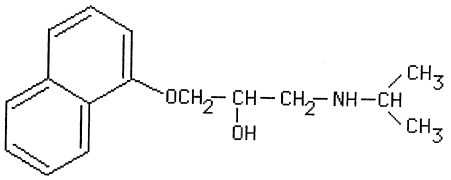
2. Experimental

2.1. Reagents and materials

Both, propranolol chlorhydrate and atenolol base were provided by ICI-Farma (Madrid, Spain). Their chemical structures are represented in Fig. 1. Acetonitrile was LC grade and was purchased from Scharlau (Barcelona, Spain). Triethylamine was purchased from Fluka Chemika-Biochemika (Buchs, Switzerland), 85% phosphoric acid from Probus (Badalona, Spain), 97% sodium hydroxide and dichloromethane from Panreac (Montcada i Reixach, Spain). All of them were analytical grade chemicals and used without further purification.



Atenolol



Propranolol

Fig. 1. Chemical structure of β-blockers assayed.

2.2. Equipment

The LC 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 computerised integration system data output.

2.3. Sample preparation

To each ml of human plasma treated with 0.1 ml of 1 *M* sodium hydroxide were added 6 ml of dichloromethane. The tube was capped and the contents were mixed for 10 min on a rotary mixer and centrifuged for 5 min at 2000 *g*. The upper aqueous layer was discarded and 5 ml of the organic layer was removed to a conical glass tube and evaporated to dryness at 50±1°C. The dry residue was redissolved in 150 μl of mobile phase and 100 μl was injected into the LC system, using a 100-μl Hamilton syringe.

2.4. Chromatography

Liquid chromatographic 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–LC water with 1.2% (w/v) of triethylamine and the pH adjusted to 3 with 85% orthophosphoric acid (5:95, 30:70, v/v, for atenolol and propranolol, respectively). The flow rate was 1 ml/min. The injection volume was 100 μl. Fluorescence detection was set at an excitation wavelength of 280 nm for both drugs, an emission wavelength of 300 nm for atenolol and 333 nm for propranolol and a single sensitivity factor of 500 nm.

Standard solutions of β-blockers dissolved in drug-free human plasma were obtained by suitable dilution from stock solutions prepared in water at 300 μg/ml. The concentration range for the calibration curve in human plasma was 25–800 for atenolol and 3.13–100 ng/ml for propranolol. The

limits of quantification and detection were also determined.

2.5. Validation

Evaluation of the reversed-phase LC methods was based on proportionality (linearity assay), precision and accuracy [19–21].

2.5.1. Linearity

Linearity consisted in the determination of the same concentration range as the calibration curve, covering six concentrations, which were: 800, 400, 200, 100, 50, and 25 ng/ml for atenolol and 100, 50, 25, 12.5, 6.25 and 3.13 ng/ml for propranolol. This analysis was made in triplicate.

2.5.2. Precision and accuracy

Three concentrations within the linearity range (low, medium and high) were selected: 400, 100 and 25 ng/ml for atenolol and 100, 25 and 6.25 ng/ml for propranolol. Five standard solutions of each concentration were spiked to drug-free human plasma and analysed (intra-day assay). The assay was repeated on 5 days (inter-day assay).

2.5.3. Recovery (extraction efficiency)

The absolute recovery 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.

2.6. Analytical error function

The study of the analytical error function was carried out using the same ranges and concentrations of the calibration curves for each drug. The procedure used to obtain the error function of each validated analytical method was the same as previously described [22,23]. The best functionalization between the standard deviation (SD) obtained for each concentration of the calibration curve, and the theoretical concentration values (C), were calculated using multiple regression, applying the stepwise forward selection method [24].

3. Results and discussion

3.1. Chromatogram

Chromatographic conditions of the β-blockers studied are shown in Table 1. Fig. 2a–d shows blank and drug spiked chromatograms: (a) correspond to the blank plasma in condition of atenolol method, (b) to the blank plasma spiked with 200 ng/ml of atenolol, (c) to the blank plasma in condition of propranolol method, and (d) to the blank plasma spiked with 50 ng/ml of propranolol. Fluorescence detection improved sensitivity and removed endogenous chromatographic interferences.

By these reversed-phase LC methods, both drugs were resolved and quantified acceptably, with approximate retention times of 3.4 and 3.8 min for atenolol and propranolol, respectively.

A minimum signal-to-noise ratio of 3:1 was obtained with the lowest concentrations, allowing a detection limit of 12.5 ng/ml for atenolol and 1.56 ng/ml for propranolol. Thus, the limits of quantifica-

Table 1
Chromatographic conditions of drugs studied

β-Blocker	Mobile phase (% acetonitrile)	Injection volume (μl)	Wavelength		Response time (min)	Limit of quantification (ng/ml)
			$\lambda_{excit.}$ (nm)	$\lambda_{emis.}$ (nm)		
Atenolol	5	100	280	300	3.4	12.5
Propranolol	30	100	280	333	3.8	1.56

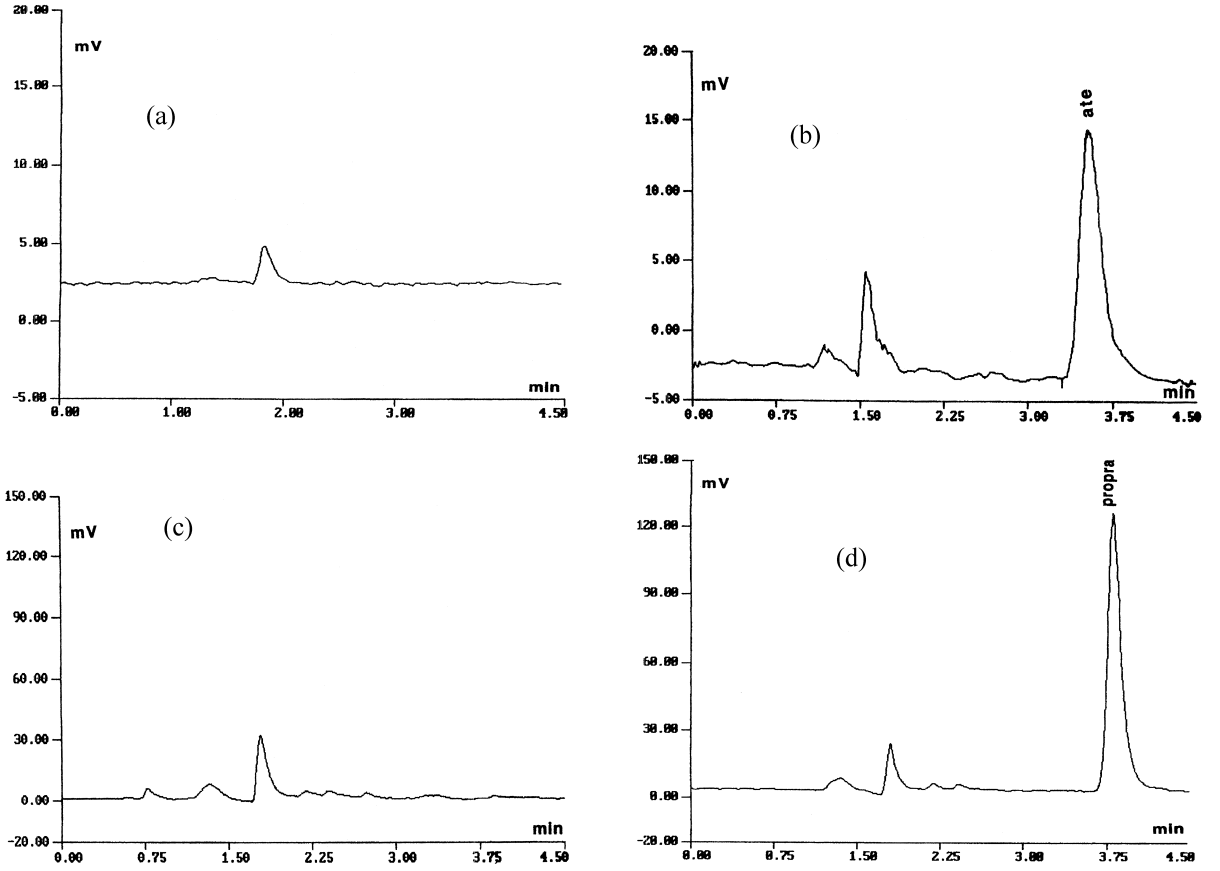


Fig. 2. Representative chromatograms of (a) blank plasma in condition of atenolol method; (b) blank plasma spiked with 200 ng/ml of atenolol; (c) blank plasma in condition of propranolol method; (d) blank plasma spiked with 50 ng/ml of propranolol.

tion used (25 and 3.13 ng/ml) were higher than the absolute limits of the assays. The injection volume was the same for both drugs (Table 1).

3.2. Validation

In the linearity assay, the response factors expressed by the coefficient of variation (C.V.) were 8 and 12% for atenolol and propranolol, respectively. The regression equations obtained from unweighed least-squares linear regression were $y=-0.2226+0.0229x$, $r=0.9986$, and $y=-0.5528+0.7964x$, $r=0.9986$, where y is peak area and x is concentration. So, 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 summarised in Table 2.

Maximum C.V. values were 12 and 5% in intra-day and 9 and 15% in inter-day precision, for atenolol and propranolol, respectively and corresponded to the lowest concentrations for both drugs.

The biggest %bias were 12 and 7% in intra-day and 14 and 3% in inter-day accuracy for atenolol and propranolol, respectively and also corresponded to the lowest concentration for both drugs.

In the range of calibration standards, the analytical

Table 2
Intra-day (1 representative day) and inter-day precision and accuracy for atenolol and propranolol in human plasma[a]

β-Blocker	Concentration added (ng/ml)	Intra-day (n=5)		Inter-day (n=5)	
		Concentration measured (ng/ml)	% Bias	Concentration measured (ng/ml)	% Bias
Atenolol	400	401 SD 9 C.V.% 2	0.3	401 SD 0.9 C.V.% 0.2	0.3
	100	95 SD 7 C.V.% 8	−5	95 SD 3 C.V.% 3	−5
	25	28 SD 3 C.V.% 12	12	29 SD 2 C.V.% 9	14
Propranolol	100	100 SD 3 C.V.% 3	−0.5	97 SD 6 C.V.% 6	−3
	25	24 SD 0.9 C.V.% 4	−6	25 SD 2 C.V.% 8	2
	6.25	7 SD 0.3 C.V.% 5	7	7 SD 0.9 C.V.% 15	3

[a] % Bias=100×(measured concentration−added concentration)/added concentration, SD=Standard deviation, C.V.=Coefficient of variation.

recovery in the plasma sample averaged 56% for atenolol and 31% for propranolol.

3.3. Analytical error procedure

The best analytical error functions obtained from the stepwise forward selection method were the following: SD (ng/ml)=7.6978+0.0369C for atenolol and SD (ng/ml)=0.1259+0.0362C for propranolol. Fig. 3a 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 atenolol and propranolol, respectively.

The analytical error function may be linear or not. Here, the error corresponding to both atenolol and propranolol is described by linear functions. Although the same model of error function is found, there are differences between drugs, due to the analytical method (Table 1) and the range of concentration chosen.

However, in a previous study [23], the analytical error functions of the same active principles were linear for atenolol and non-linear for propranolol. As in the present work, there were differences between the drugs in analytical methods, although the concentration range used was the same for both drugs (25–1.56 μg/ml).

On the other hand, if we compare the analytical error functions of the drugs in each of the studies, there are many more differences that in the previous comparison. Analytical methods, mobile phase, injection volume, detection system (UV vs. fluorescence detector), calibration curve ranges and plasma drug extraction (this error source was not considered in the previous study since it dealt with simple matrixes) showed differences.

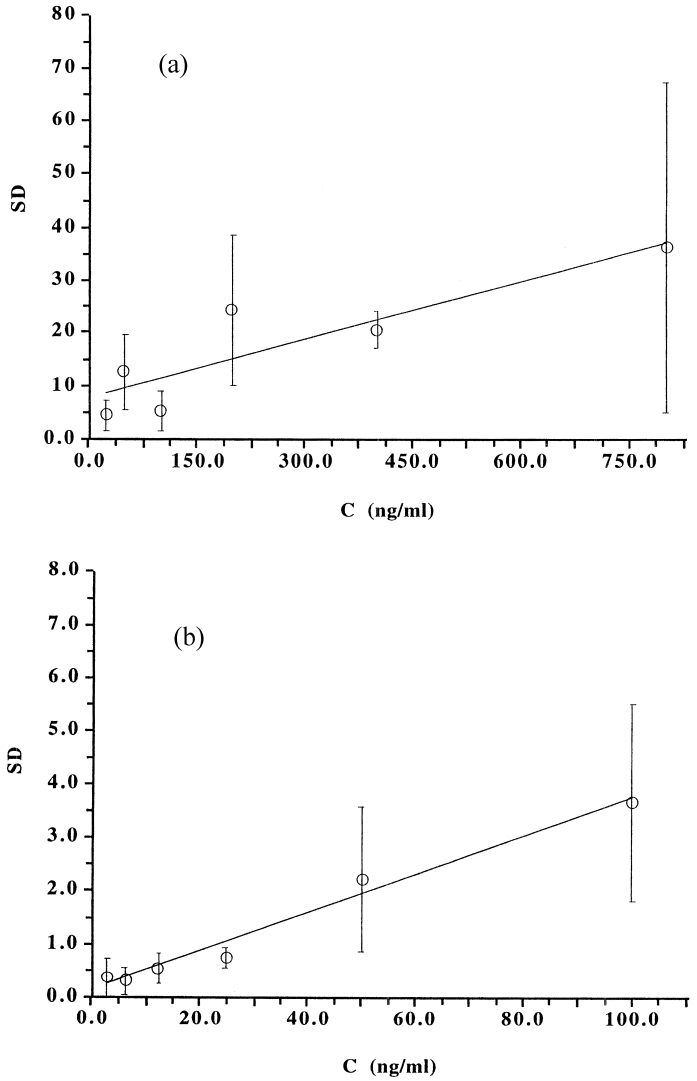


Fig. 3. Mean values and standard deviations obtained in the study of the analytical error function vs. theoretical concentrations from the calibration curves for (a) atenolol and (b) propranolol.

Therefore, the analytical error function does not depend on whether there are significant differences between drugs or between analytical methods, which could be but is not the reason of why different error functions are found for the same drug. Again, we can state, that analytical errors do not fit any expected pattern, but have to be determined individually for each drug and its analytical method [17].

Once a method is developed and validated and its analytical error function determined, we could discriminate among analytical techniques and analytical methods, in order to know which of them produce the highest or lowest error.

As already mentioned, the error function (the reciprocal of the square of the standard deviation of any concentration value) could be used also as a weighting method in non-linear parameter estimation and, to that end, is considered a proper choice.

4. Conclusions

The data demonstrate that the analytical methods we have developed showed acceptable linearity, precision and accuracy over the concentration range. The maximum C.V. values are within 15% for the linearity, intra-day and inter-day precision and accuracy assays.

The methods described are rapid since preparation of plasma samples prior to chromatography is relatively simple and the total chromatographic run time is about 5 min, and sensitive with a limit of quantification of 25 and 3.13 ng/ml for atenolol and propranolol, respectively. Moreover, these chromatographic methods can be used to analyse a large number of human plasma samples from different patients each day in clinical and analytical laboratories, since they need the same reversed-phase column, mobile phase, detection system and drug extraction procedure.

The analytical error function has been established for each β-blocker and could be used as a possible weighting method, which would greatly reduce the overall variability in the pharmaceutical studies of these drugs.

In conclusion, we would like to emphasise some aspects to improve the use of the analytical error function in a weighting procedure application: first, it can only be determined if the analytical method has been previously validated; moreover, it has to be established for each drug and each analytical method of quantification; finally, the analytical error function can only be used provided the concentrations are within the calibration curve range where it has been determined, preventing extrapolations.

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References

[1] J.B. Soriano, A.W. Hoes, L. Meems, D.E. Grobbee, Prog. Cardiovasc. Dis. 39 (1997) 445.
[2] G. Emilien, J.M. Maloteaux, Eur. J. Clin. Pharmacol. 53 (1998) 389.
[3] M.T. Rosseel, M.G. Bogaert, J. Pharm. Sci. 70 (1981) 688.
[4] H. Winkler, W. Ried, B. Lemmer, J. Chromatogr. 228 (1982) 223.
[5] E.C. Kwong, D.D. Shen, J. Chromatogr. 414 (1987) 365.
[6] R.P. Koshakji, A.J.J. Wood, J. Chromatogr. 422 (1987) 294.
[7] S.M. Huang, H.S. Weintraub, T.B. Marriott, B. Marinan, R. Abels, J. Pharmacokin. Biopharm. 15 (1987) 557.
[8] M.A. Eldon, A.W. Kinkel, J.E. Daniel, J.R. Latts, Biopharm. Drug Dispos. 10 (1989) 69.
[9] R.B. Miller, J. Pharm. Biomed. Anal. 9 (1991) 849.
[10] G.S. Rekhi, S.S. Jambhekar, P.F. Souney, D.A. Williams, J. Pharm. Biomed. Anal. 13 (1995) 1499.
[11] Y.M. Irshaid, N.M. Rawashdeh, F.F. Awwadi, M.K. Kato, Int. J. Clin. Pharmacol. Ther. 34 (1996) 457.
[12] C. Giachetti, A. Tenconi, S. Canali, G. Zanolo, J. Chromatogr. B 698 (1997) 187.
[13] A. Wolf-Coporda, F. Plavsic, B. Vrhovac, Int. J. Clin. Pharmacol. Ther. Toxicol. 25 (1987) 567.
[14] F.F.T. Ververs, H.G. Schaefer, J.F. Lefevre, L.M. Lopez, H. Derendorf, J. Pharm. Biomed. Anal. 8 (1990) 535.
[15] M. Walshe, M.T. Kelly, M.R. Smyth, J. Pharm. Biomed. Anal. 14 (1996) 475.
[16] E.L. Mariño, J.M. Jansat, M.A. March, C.F. Lastra, Int. J. Clin. Pharmacol. Ther. 34 (1996) 546.
[17] A.J. Braza, P. Modamio, E.L. Mariño, J. Chromatogr. B 718 (1998) 267.
[18] J.M. Jansat, C.F. Lastra, E.L. Mariño, Int. J. Clin. Pharmacol. Ther. 36 (1998) 340.
[19] J. Vessman, J. Pharm. Biomed. Anal. 14 (1996) 867.
[20] R. Causon, J. Chromatogr. B 689 (1997) 175.
[21] L. Huber, LC·GC Int. 2 (1998) 96.
[22] P. Modamio, C. Fernández-Lastra, E.L. Mariño, J. Pharm. Biomed. Anal. 14 (1996) 401.
[23] P. Modamio, C. Fernández-Lastra, E.L. Mariño, J. Pharm. Biomed. Anal. 17 (1998) 507.
[24] D.G. Kleinbaum, L.K. Lawrence, K.E. Muller, in: D.G. Kleinbaum, L.L. Kupper, K.E. Muller (Eds.), Applied Regression Analysis and Other Multivariate Methods, 2nd Edition, Duxbury Press, Belmont, CA, 1988.