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Error structure for the HPLC analysis for atenolol, metoprolol and propranolol: a useful weighting method in parameter estimation[1]

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Abstract

Three reversed-phase high performance liquid chromatography (HPLC) methods with UV detection were developed and fully validated for the quantification of three β-blockers: atenolol, metoprolol and propranolol. After validation, error structures for the HPLC analysis were established using a convenient and practical procedure. The mean percentage of relative standard deviation (RSD) of the experimental concentrations (C), were less than 4.29% for proportionality and less than 3.68% for precision for any of the drugs, which allowed the quantitation of β-blockers assayed at concentrations in the range 25–0.78 $\mu g \cdot ml^{-1}$. The error structures for the HPLC analysis were: SD ($\mu g \cdot ml^{-1}$) $= 5.02 \times 10^{-2} + 0.65 \times 10^{-2}\ C$ for atenolol, SD ($\mu g \cdot ml^{-1}$) $= 4.55 \times 10^{-2} + 0.63 \times 10^{-2}\ C - 7.58 \times 10^{-6}\ C^3$ for metoprolol and SD ($\mu g \cdot ml^{-1}$) $= 2.73 \times 10^{-2} + 1.46 \times 10^{-2}\ C - 3.49 \times 10^{-4}\ C^2$ for propranolol. The reciprocal of the square of the SD of the drug concentrations measured within the calibration curve could be used as weighting methods in parameter estimation by non-linear regression.

Keywords: β-Blockers; Reversed-phase HPLC; Error structure; Weighting method; Variance model; Non-linear regression

1. Introduction

In pharmaceutical studies, estimation techniques such as non-linear regression methods, usually require the incorporation of a weighting scheme to select values for parameters that best fit the observed data. Since there is normally no way of knowing in advance the data analysis for which weighting scheme to choose, selection is often subjective and somewhat arbitrary [1,2]. Because of this, many studies have investigated the effect of using different types of weight, showing a wide discrepancy in the regression parameters estimated and, therefore, the importance the selection of the right weighting method has in regression [3–5].

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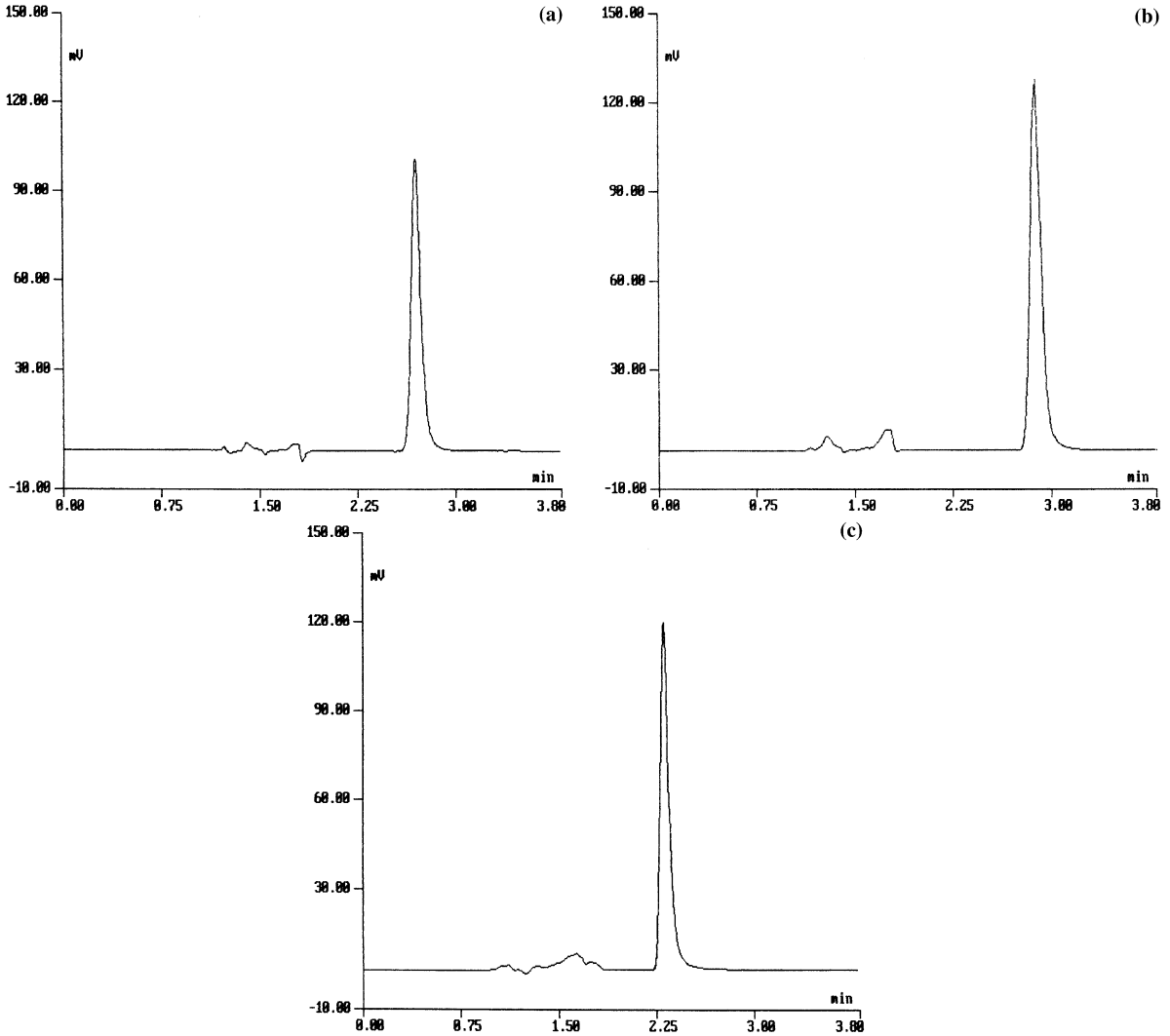

Fig. 1. Representative chromatograms of (a) atenolol and (b) metoprolol at 12.5 $\mu g \cdot ml^{-1}$, and (c) propranolol at 25.0 $\mu g \cdot ml^{-1}$.

In a previous paper [6], we proposed, as a possible alternative, the use of the error structure for the HPLC analysis as a model of variance in regression. The different error functions found in this first study, where three β-blockers were quantified by similar isocratic high performance liquid chromatography (HPLC) methods, led us to perform the present study with three other β-blockers, namely atenolol, metoprolol and propranolol. They are frequently prescribed and have an accepted role, amongst others, in the treatment of hypertension, secondary prevention of myocardial infarction and arrhythmias [7,8].

Therefore, the objectives of this study were firstly, to develop and validate three analytical methods for the quantification of all three β-blockers assayed by HPLC using a suitable chromatographic column and mobile phase. Secondly, after the validation of the reversed-phase HPLC methods, to determine their error structures for the HPLC analysis in order to provide a data-weighting method throughout their working range.

Table 1
Chromatographic conditions of β-blockers assayed

Drug	Mobile phase (% acetonitrile)	Injection volume (μl)	Wavelength (nm)	Response time (min)	Limit of quantitation ($\mu g \cdot ml^{-1}$)
Atenolol	10	20	225	2.6	0.195
Metoprolol	25	40	227	2.8	0.195
Propranolol	40	80	294	2.4	0.098

Table 2
Results obtained from validation assays

Drug	Theoretical concentration ($\mu g \cdot ml^{-1}$)	Intra-day RSD (%)	Inter-day RSD (%)	Linear regression model	r
Atenolol	25.00	0.68	0.05		
	6.25	1.10	0.90	$PA = 0.380\ C - 0.029$	0.9999
	1.56	0.61	3.68		
Metoprolol	25.00	0.96	0.03		
	6.25	1.82	0.51	$PA = 0.521\ C - 0.127$	0.9999
	1.56	1.99	1.50		
Propranolol	25.00	2.27	0.03		
	6.25	0.87	0.77	$PA = 0.849\ C - 0.534$	0.9999
	1.56	1.97	1.16		

2. Material and methods

2.1. Reagents and materials

Both atenolol and propranolol were provided by ICI-Pharma (Madrid, Spain) and metoprolol by Ciba-Geigy (Barcelona, Spain).

2.2. Apparatus

The HPLC system consisted of a Kontron (model 420) (Kontron Instruments, Barcelona, Spain) equipped with an automatic sampling system with a variable volume injector (model 465), two pumps (model 420), a mixer (model 491), a capillary UV-visible detector with variable-wavelength (model 433) and a computerized integration system data output (model MT-450). Liquid chromatographic analyses were performed on a C_{18} column (12.5 cm $\times$ 4 mm i.d.) packed with 5 μm Nucleosil (Teknokroma, Barcelona, Spain) operating at room temperature.

2.3. Chromatography

The mobile phase for all drugs was composed of acetonitrile (solvent A) and phosphate buffer (solvent B), with 0.2% (w/v) of triethylamine, with the pH adjusted to 3 with orthophosphoric acid 85% (0.067 M 10:70, 25:75, 40:60 v:v, pH 3, for atenolol, metoprolol and propranolol, respectively). The flow rate was 0.8 $ml \cdot min^{-1}$. The injection volume was 20, 40 and 80 μl for atenolol, metoprolol and propranolol, respectively. The UV detection was accomplished at 225, 227 and 294 nm for atenolol, metoprolol and propranolol, respectively, at 0.05 AUFS and 0.5 s response time.

Standard solutions of each active principle were obtained by suitable dilution from stock solutions prepared at 0.25 $mg \cdot ml^{-1}$ in phosphate buffer (pH 7.4, 0.067 M). The concentration range of the calibration curves was 25–0.78 $\mu g \cdot ml^{-1}$. The limits of quantitation were also determined by suitable dilution from the lowest concentration of the calibration curve range.

Table 3
Polynomial statistical analysis of the error structure for the HPLC analysis of atenolol

Variables used in regression	r^2	SE	F	P ($\times 10^{-2}$)
C	0.2155	0.1083	9.34	0.43
C^2	0.1745	0.1111	7.19	1.12
C^3	0.1491	0.1128	5.96	2.00
C, C^2	0.1865	0.1087	5.01	1.26
C, C^3	0.1867	0.1087	5.02	1.25
C^2, C^3	0.1822	0.1090	4.90	1.37
C, C^2, C^3	0.1613	0.1104	3.24	3.47

Table 4
Polynomial statistical analysis of the error structure for the HPLC analysis for metoprolol

Variables used in regression	r^2	SE	F	P
C	0.1603	0.0304	6.49	1.55×10^{-2}
C^2	0.0758	0.0319	2.79	1.04×10^{-1}
C^3	0.0411	0.0325	1.46	2.36×10^{-1}
C, C^2	0.3347	0.0267	9.80	4.55×10^{-4}
C, C^3	0.3529	0.0263	−10.54	2.88×10^{-4}
C^2, C^3	0.3736	0.0259	11.44	1.68×10^{-4}
C, C^2, C^3	0.3541	0.0263	7.40	6.73×10^{-4}

2.4. Validation

Evaluation of the reversed-phase HPLC methods was based on proportionality (linearity assay), precision (repeatability and reproducibility assays) and accuracy [9–12].

2.4.1. Linearity

Linearity involved the determination of the same concentration range as the calibration curve, covering six concentrations: 25, 12.5, 6.25, 3.125, 1.56 and 0.78 $\mu g \cdot ml^{-1}$. Each concentration was analysed in triplicate.

2.4.2. Precision and accuracy

Three concentrations within the linearity range (low, medium and high) were selected: 25, 6.25 and 1.56 $\mu g \cdot ml^{-1}$. Five standard solutions of each concentration were prepared and analysed in triplicate (repeatability assay). This assay was repeated for 5 days (reproducibility assay).

2.5. Analytical error

The study of the error structure for the HPLC analysis was carried out by preparing a calibration curve with six concentration levels: 25, 12.5, 6.25, 3.125, 1.56 and 0.78 $\mu g \cdot ml^{-1}$, for each β-blocker The procedure used to obtain the error function of each analytical method [6] validated previously was as follows: for each active principle, six calibration curves (6 replicates) were obtained every day during six different days. Each day, the mean and SD of each concentration level were obtained from the calibration curve ($n = 36$). After that, the SD (as dependent variable) and their theoretical concentration values (as independent variable) were regressed using polynomial analysis, in order to establish the best function that would relate both variables, whose general equation is:

$$SD = A_0 + A_1 \cdot C + A_2 \cdot C^2 + A_3 \cdot C^3$$

Table 5
Polynomial statistical analysis of the error structure for the HPLC analysis for propranolol

Variables used in regression	r^2	SE	F	P
C	0.5989	0.0403	50.77	3.10×10^{-8}
C^2	0.4553	0.0470	28.42	6.39×10^{-6}
C^3	0.3759	0.0503	20.48	7.03×10^{-5}
C, C^2	0.6760	0.0357	37.51	3.18×10^{-9}
C, C^3	0.6748	0.0358	37.31	3.38×10^{-9}
C^2, C^3	0.6499	0.0371	33.49	1.14×10^{-8}
C, C^2, C^3	0.6659	0.0362	24.25	2.24×10^{-8}

where SD corresponds to the SD associated with the measurement of each concentration value and C corresponds to theoretical concentration values.

3. Results and discussion

3.1. Chromatograms

Fig. 1a–c shows representative chromatograms at 12.5 $\mu g\cdot ml^{-1}$ for atenolol and metoprolol and at 25 $\mu g\cdot ml^{-1}$ for propranolol. Chromatographic conditions of the drugs are shown in Table 1. They were resolved and quantified satisfactorily by these reversed-phase HPLC methods. Their retention times were 2.6, 2.8 and 2.4 min for atenolol, metoprolol and propranolol, respectively.

A minimum signal-to-noise ratio of 5:1 was obtained with the lowest concentrations, allowing a quantitation limit of 0.195 $\mu g\cdot ml^{-1}$ for both atenolol and metoprolol and 0.098 $\mu g\cdot ml^{-1}$ for propranolol. Thus, the limit of quantitation used (0.78 $\mu g\cdot ml^{-1}$) is higher than the absolute limit of the assays. The injection volume is double for propranolol relative to metoprolol and also for metoprolol relative to atenolol.

3.2. Validation

The results obtained in the validation assay procedure are summarised in Table 2. In the linearity assay, the response factors expressed as a percentage of the RSD were 2.24, 3.82 and 4.05% for atenolol, metoprolol and propranolol, respectively. The regression equations obtained by unweighted least-squares linear regression are represented by $PA = a + bC$, where PA is the peak area and C is concentration. Good linearity between the peak area and concentration was observed for all drugs with correlation coefficients of 0.9999. Maximum RSD values in the repeatability and reproducibility assays were 1.10 and 3.68% for atenolol, 1.99 and 1.50% for metoprolol and 2.27 and 1.16% for propranolol, respectively. Accuracy expressed as a percentage of the mean recovery was confirmed after applying a Student's t-test. No significant differences ($P > 0.05$) appeared between the mean recovery and 100% in any of the drugs.

3.3. Analytical error procedure

In order to identify the function that best fits the experimental data, the corresponding statistical study was performed. The results obtained are shown in Tables 3–5 for atenolol, metoprolol and propranolol, respectively.

From these tables, the most significant function for each active principle can be selected. The selection was made by a stepwise forward selection method [13], which permitted us to discriminate the best fitting when differences among functions in the coefficient of correlation, F, standard error of estimate and level of probability values did not differ significantly. Taking into account these results, the error structures for the HPLC analysis chosen are the following: SD $(\mu g\cdot ml^{-1}) = 0.0502 + 0.0065\ C$ for atenolol; SD $(\mu g\cdot ml^{-1}) = 0.0455 + 0.0063\ C - 7.58\times10^{-6}\ C^3$ for metoprolol; SD $(\mu g\cdot ml^{-1}) = 0.0273 + 0.0146$

$C - 3.49 \times 10^{-4}\ C^2$ for propranolol. Three illustrative plots (one for each β-blocker) of the mean value of the SD are shown in Fig. 2a–c. These figures were obtained every day for each concentration level of the calibration curve (6 replicates) as a function of the theoretical values (C).

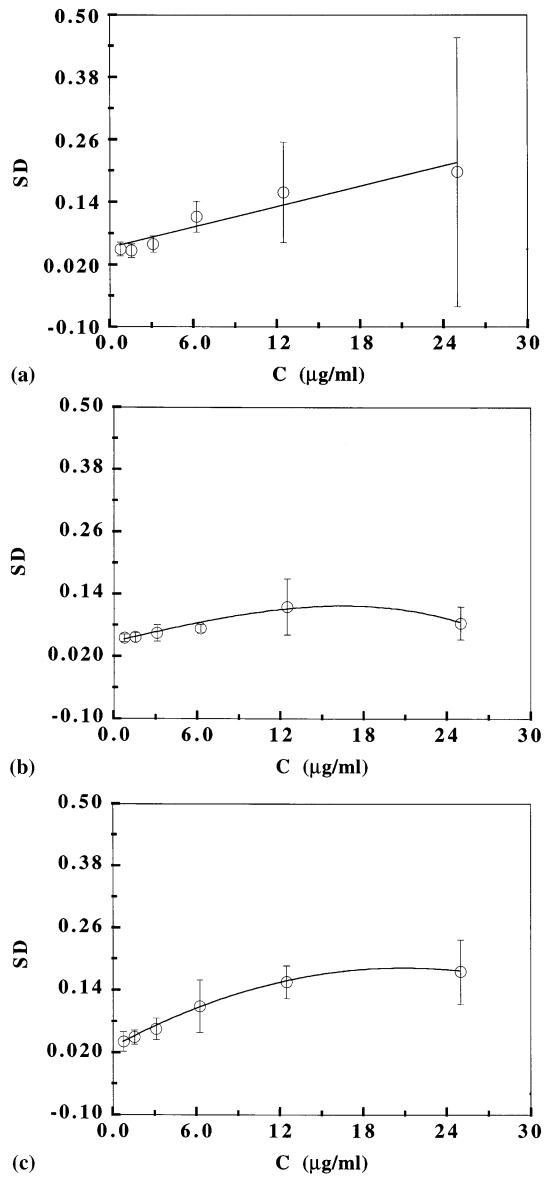

Fig. 2. Illustrative plots of the mean values and SD obtained in the study of the error structure for HPLC analysis vs. theoretical concentrations from the calibration curves for (a) atenolol, (b) metoprolol, and (c) propranolol.

Therefore, analytical errors do not fit any pattern foreseen a priori, but rather they can be described by several different functions. For example, only the error corresponding to atenolol is described by a linear function, whilst errors corresponding to metoprolol and propranolol are described by non-linear functions for the same range of concentrations. This variety of functions applicable to the description of errors is found in spite of the fact that the chemistry of all three drugs is based on the aryloxypropanolamine structure. In addition, they were quantified with the same analytical technique (reversed-phase HPLC). Finally, the analytical methods used have the same mobile phase but different aqueous:organic proportions (Table 1). From the above and from previous studies of error function for other β-blockers (celiprolol, bisoprolol and oxprenolol) [6], it is clear that the error structure for the HPLC analysis of each active principle should be determined individually. The different error functions found with all these β-blockers will be used, in the near future, as a weighting method in the determination by non-linear regression of parameters from a percutaneous absorption study recently started in our unit.

Another main application of the error structure for the HPLC analysis as a possible weighting method, is in pharmacokinetic data analysis, both in clinical studies where pharmacokinetic properties of a drug are studied, and in the fitting of serum drug concentration data for patients, i.e. in therapeutic drug monitoring [14–18]. The usual practice in most hospitals and laboratories is simply to make sure that the SD values of each assay (intra- and inter-day variability) are within acceptable limits. Once this is done, the actual analytical error is usually ignored, and not reported along with the concentration itself [17]. In this sense, it would be interesting to have a practical means to determine the standard deviation of each serum drug concentration as it is routinely measured. Thus, the method shown here (replicate measurements of the representative samples of the working range concentrations) would be a convenient and

practical way to obtain the estimated standard deviation with which a single determination of a serum drug concentration is measured.

In our study of the error structure for the HPLC analysis, the possible effect of the active principle extraction process from a biological matrix was not taken into account, and this effect should be considered in a possible pharmacokinetic data parametric estimation.

4. Conclusions

Results proved that these analytical methods have acceptable precision, accuracy and linearity between the peak area and concentration. None of the RSDs surpassed the maxima permitted of 5, 3 and 5% for linearity, repeatability and reproducibility assays, respectively. Moreover, these methods allow the quantification of a large number of samples daily, since a single mobile phase and a type of reversed-phase column are used for the determination of all three drugs.

The error function for each validated analytical method has been determined by a convenient and practical procedure. The error structure for the HPLC analysis, established for each active principle, will firstly, allow the determination of a variance associated with a concentration value within the working calibration curve range and secondly, to use it as a possible heteroscedastic weighting method (1/variance) of the parameter estimation in further studies.

Independent of other errors such as incorrect model specifications, inappropriate experimental designs and uncertainty (stochastic control), the use of this weighting method may lead to a better quantification of the drugs since it explains, at least, a part of the total error produced in parameter estimation.

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