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Determination of the β-blocker atenolol in plasma by capillary zone electrophoresis

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Abstract

A capillary zone electrophoretic method was optimised for the determination of the β-blocker atenolol in plasma. Separation was performed in an uncoated silica capillary of 58.5 cm (effective length 50 cm)×75 μm I.D., and detection was at 194 nm. The effects of the buffer (concentration and pH), the injection time, the voltage applied and the plasma clean-up procedure were studied. The determination of atenolol was achieved in less than 3 min, using an electrolyte of 50 m*M* H_3BO_3–50 m*M* $Na_2B_4O_7$ (50:50, v/v) pH 9, injected hydrodynamically for 4 s at 50 mbar and applying a voltage of +25 kV. This method was applied to the determination of atenolol in plasma of nine hypertensive patients (male and female, aged from 39 to 73 years). Atenolol concentrations found vary from 30 to 585 ng/ml.

Keywords: Beta-blockers; Atenolol

1. Introduction

There is a growing interest in the use of capillary electrophoresis (CE) for the analysis of bulk drugs and pharmaceutical preparations. High separation efficiency, selectivity, large separation capacity, flexibility and relatively low operational cost are the attractions of this technique [1].

β-Adrenergic blocking agents are one of the different kinds of drugs that could be determined by this technique [2]. These are clinically used to treat angina pectoris, cardiac arrythmia, hypertension, anxiety attacks, thyrotoxicosis, migraine and glaucoma [3].

Some β-blockers are hydrophilic and others lipophilic. In addition, their relatively high pK_a values (pK_a 9.2–9.8) complicate the sample pretreatment and analysis. At physiological pH (pH 7.4), β-blockers exist as single cations, which enables their separation and determination by methods exploiting the different mobilities of analytes in an electrical field [2].

Atenolol [4-(2-hydroxy-3-isopropylaminopropoxy)phenylacetamide] is a cardioselective β_1-adren-

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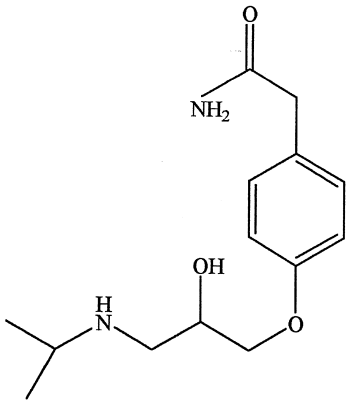

Fig. 1. Structure of the β-blocker atenolol.

ergic receptor-blocking agent prescribed for the treatment of hypertension (Fig. 1). The antihypertensive effects of atenolol means that a single dose of 50 or 100 mg needs to be administered daily [4], which gives plasma peak concentrations of 200–300 [5] and 500–600 ng/ml [4], respectively, obtained between 2 and 4 h after the intake of the pharmaceutical formulation.

Atenolol is a weak base (pK_a 9.6), with a partition coefficient of 0.015 (*n*-octanol–buffer, pH 7.4, 37°C). After an oral dose administration, atenolol is incompletely absorbed (50% of the drug is bioavailable). It is poorly bound to plasma proteins and only about 5% is metabolised by the liver [5].

As a consequence of the widespread use of β-blockers, there are many methods published for their determination in plasma incorporating a variety of analytical techniques. In the last decade, gas chromatography with mass spectrometry [6] or high-performance liquid chromatography (HPLC), using reversed-phase columns and UV [7,8] or fluorometric [9–12] detection have been the techniques generally used for the determination of atenolol in plasma. With these methods, sensitivity levels ranging from 5 to 25 ng/ml of atenolol in plasma were obtained. Since there is no previous study of the determination of atenolol in plasma by capillary zone electrophoresis (CZE), the aim of this work is to show the suitability of this technique for this purpose. The electrophoretic method and the extraction procedure for the plasma were optimised.

2. Experimental

2.1. Chemicals and stock solutions

Atenolol was kindly supplied by Sigma (Bilbao, Spain). Methanol and *n*-hexane were Lab-Scan HPLC grade (Dublin, Ireland). 2-Propanol was Fluka HPLC grade (Steinheim, Switzerland) and the other chemicals were of analytical grade and were supplied by Merck (Darmstadt, Germany). Water was obtained from Milli-RO and Milli-Q systems (Waters).

A stock solution of atenolol (1000 μg/ml) was prepared in water and kept in an amber glass volumetric flask due to its easy photodegradation. It was stored in the dark under refrigeration to avoid possible decomposition. Working solutions were also prepared in amber glass volumetric flasks by appropriate dilution just before use.

2.2. Apparatus and electrophoretic conditions

This work was performed on a Hewlett-Packard HP ^{3D}CE capillary electrophoresis system (Waldbronn, Germany) equipped with a diode array detector. The tray samples were refrigerated at 23°C with a Selecta Frigiterm-10 external bath (Barcelona, Spain). The fused-silica capillaries were 58.5 cm×75 μm I.D.×375 μm O.D. with the detection window at 50 cm. The samples were introduced hydrodynamically for 4 s at a 50 mbar injection pressure and the applied voltage was +25 kV. The detection wavelength was 194 nm. The capillary temperature was set at 25±0.1°C. The separation buffer was prepared as follows: 50 m*M* sodium tetraborate and 50 m*M* boric acid were mixed at 50:50 (v/v) which gives rise to a pH 9 solution. The buffer used in the clean-up procedure was prepared in the same way, but the concentration of the salt and the acid was 0.5 *M*, instead of 50 m*M*.

Solid-phase extraction (SPE) was performed using Bond Elut Certify LRC 10 ml/130 mg cartridges (Varian, Harbor City, CA, USA) which were placed on a vacuum manifold system (Supelco, Bellefonte, PA, USA). The extracted plasma samples were evaporated to dryness under a gentle stream of nitrogen using a Zymark Turbovap LV evaporator (Hopkinton, MS, USA).

2.3. Capillary conditioning

The capillary was conditioned every day with an initial wash cycle consisting of 1 *M* NaOH for 15 min, deionized water for 10 min and running electrolyte for 5 min. Between injections, the capillary was washed with 0.1 *M* NaOH for 2 min, deionized water for 1 min and running electrolyte for 3 min. The separation buffer was refreshed after four analyses. Daily after finishing the experiments, the capillary was washed with 1 *M* NaOH for 10 min and deionized water for 10 min and purged with air for 3 min.

2.4. Sample collection

Blood was collected by syringe from healthy volunteers and immediately transferred into tripotassium EDTA tubes and gently mixed. Samples were immediately centrifuged at 5000 rpm at a temperature of 4°C. The drug-free plasma obtained was transferred to 5-ml tubes and stored at −20°C until analysis. Healthy volunteers were both male and female and with ages between 23 and 45 years, and were not under medical treatment.

Blood from patients under treatment with atenolol was collected between 2 and 4 h after the intake of the pharmaceutical formulation and treated in the same way as the one obtained from healthy volunteers.

2.5. Sample clean-up

Deep-frozen human plasma was thawed at room temperature. A volume of 1 ml of free of drug human plasma was spiked with a solution containing a known amount of atenolol. It was vortex-mixed and 20 μl of perchloric acid was added to precipitate proteins. This mixture was vortex-mixed again and centrifuged for 5 min at 5000 rpm and the pH was adjusted to 9 by addition of 1.5 ml of 0.5 *M* borate buffer, mixed for 30 s and finally it was centrifuged for 10 min to separate the precipitate.

A 2-ml volume of the buffered plasma solution (pH 9) was applied to a Bond-Elut Certify LRC cartridge, previously activated with 4 ml of methanol and 4 ml of deionized water. The sample was slowly drawn through the column by gravity. The cartridge was washed with 2 ml of water, 1 ml of 1 *M* acetate buffer (pH 4) and 1 ml of *n*-hexane. It was let dry for 30 min at full vacuum and then atenolol was eluted with 2 ml of chloroform–isopropanol (80:20, v/v)+2% of ammonium solution at a very low vacuum. The eluate was evaporated to dryness at 50°C under a gentle stream of nitrogen and the residue dissolved in 50 μl of deionized water.

The plasma samples obtained from patients under treatment with atenolol were treated in the same way.

2.6. Quantitative parameters

The calibration curve for atenolol was made by spiking blank plasma samples with known amounts of atenolol in the range of 50–400 ng/ml, extracting them in the same way as the unknown samples. This range was chosen taking into account the concentrations expected in plasma samples.

The calibration equation was obtained by least-squares linear regression of the data peak area vs. concentration of atenolol. Unknown concentrations were calculated by interpolation.

Detection and quantitation limits were calculated as the analyte concentrations that produced a peak height with signal-to-noise ratios of 3 and 10, respectively [13].

The reproducibility of the method was calculated at three concentration levels: 100, 200 and 400 ng/ml. Five extractions were performed of five spiked blank plasma sample solutions at each concentration level to calculate the intra-day reproducibility. This process was repeated three times in 3 different weeks to calculate the inter-day reproducibility.

Accuracy was estimated at two concentration levels, extracting plasma samples spiked with atenolol and comparing the added concentrations with the obtained using the calibration plot. Two replicates were done.

The recovery was calculated comparing the peak areas of atenolol obtained after extraction with the peak areas of standard solutions of the same concentration in water. In these calculations a preconcentration factor must be taken into account, and was calculated on the basis of the following equation:

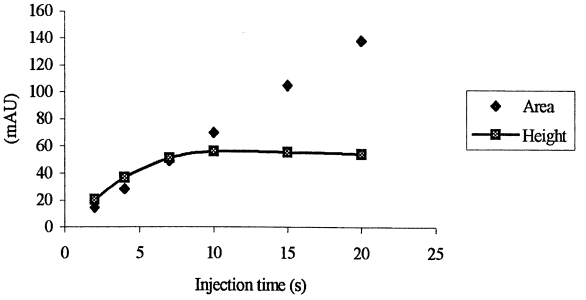

Fig. 2. Effect of the injection time on peak height and area of atenolol (10 μg/ml): 50 m*M* H_3BO_3–50 m*M* $Na_2B_4O_7$ (50:50), pH 9.0; *V*=25 kV, λ=194 nm.

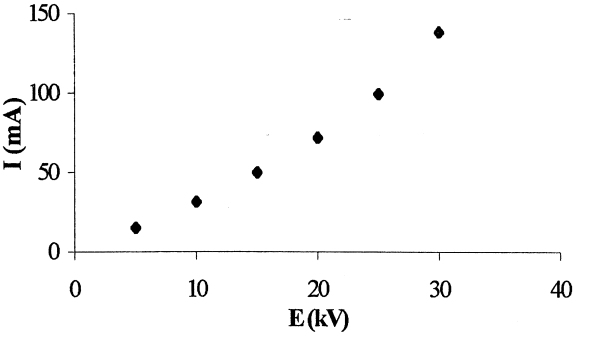

Fig. 3. Ohm's plot: electrolyte, 50 m*M* H_3BO_3–50 m*M* $Na_2B_4O_7$ (50:50), pH 9.0; t_{inj}=4 s, *V*=25 kV, λ=194 nm.

$$C_F = C \cdot \frac{2 \times 10^{-3}(1 + x \cdot 10^{-3})}{y \cdot 10^{-3} \cdot (2.52 + x \cdot 10^{-3})}$$

where: C_F is the final concentration of atenolol after the extraction, *C* the concentration of atenolol used to spiked the plasma, *x* the volume (μl) of atenolol added to the sample and *y* the volume (μl) of deionized water used to reconstitute.

The values obtained for the preconcentration factor were between 16 and 18 for the concentrations used in the calibration curve.

3. Results

3.1. Optimisation of the electrophoretic method

Upon the basis of the electrophoretic method developed in our laboratory for the determination of atenolol in urine using a Quanta 4000 (Waters Chromatography Division, Barcelona, Spain) [14], a study of optimisation of several electrophoretic parameters was carried out for the determination of atenolol in plasma.

The injection of samples was made hydrodynamically at 50 mbar. The effect of the injection time on the peak area and height was studied. In Fig. 2 the variation of both parameters with the injection time is shown. A value of 4 s was chosen as optimum since the peak height saturation occurs at higher injection times.

The effect of the pH of the running electrolyte was studied preparing a 50 m*M* boric acid solution and adding different volumes of 3 *M* NaOH solution to get pH values between 9 and 10. An increase in pH produces an increase in current intensity, migration time and peak area. Taking this fact into account, 9.7 would be the best value, but at this pH the peak was tailed and with a rounded point, so pH 9 was considered as the appropriate value for the running electrolyte.

In Table 1 the effect of borate buffer concentration is shown. An increase in the borate buffer concentration produces an increase of the migration time and the current intensity, so a 50 m*M* borate buffer was used due to its relative low migration time and current value.

In order to determine the optimal voltage to be applied, an Ohm's plot was made (Fig. 3). Values

Table 1
Effect of the electrolyte concentration: H_3BO_3–$Na_2B_4O_7$ (50:50), pH 9.0; t_{inj}=4 s, *V*=25 kV, λ=194 nm

Concentration (m*M*)	Intensity (mAU)	Area (mAU s)	Height (mAU)	Width (min)	t_m (min)
10	21	9.4	7.4	0.0179	2.173
25	34	12.0	9.8	0.0175	2.375
50	85	11.1	10.7	0.0151	2.574
75	135	11.3	11.7	0.0137	2.688
100	195	10.3	11.4	0.0140	2.628

over +25 kV induced an increase on the current intensity; thus that voltage was selected as the optimum.

The detection wavelength was obtained from the spectra of a sample of atenolol in the running electrolyte using the optimised conditions. As could be seen in Fig. 4, at a wavelength of 194 nm there is a absorption maximum, so this is the value used in all the determinations.

Under the optimal experimental conditions (50 m*M* borate buffer, pH 9, voltage of 25 kV, 4 s injection time) the determination of atenolol occurs in less than 3 min (2.6±0.08 min).

3.2. Optimisation of the extraction procedure

The SPE procedure used in the determination of atenolol in urine by Maguregui et al. [14] was initially utilised for the plasma clean-up but interferences found made necessary to optimise the clean-up procedure.

Firstly, the proteins of plasma matrix were precipitated with perchloric acid to avoid interferences. Several experiments were developed to optimise the quantity of perchloric acid to be used. Different amounts of perchloric acid, between 10 and 200 μl, were added to a 1-ml volume of plasma spiked with 740 ng/ml of atenolol. After that, the extraction procedure was followed. The samples were injected into the electrophoresis system and the peak areas were plotted versus the quantity of perchloric acid added (Fig. 5). It could be seen that the quantity of perchloric acid that gave the higher area is 20 μl, so this amount of perchloric acid was used in all the experiments.

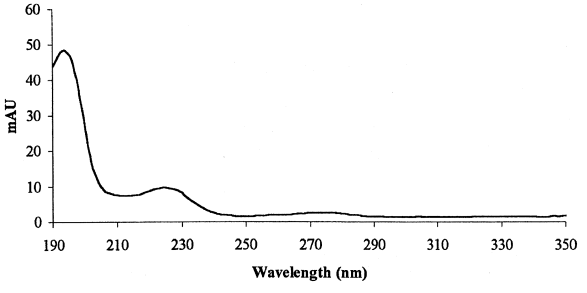

Fig. 4. Atenolol spectra (10 μg/ml): 50 m*M* H_3BO_3–50 m*M* $Na_2B_4O_7$ (50:50), pH 9.0; t_{inj}=4 s, *V*=25 kV.

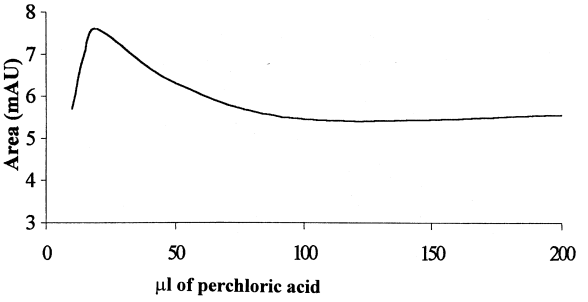

Fig. 5. Optimisation of the perchloric acid quantity (740 ng/ml of atenolol): 50 m*M* H_3BO_3–50 m*M* $Na_2B_4O_7$ (50:50), pH 9.0; t_{inj}=4 s, *V*=25 kV, λ=194 nm.

After several experiments, it was demonstrated that the SPE method used in the determination of atenolol in urine samples could be used also to determine atenolol in plasma samples employing previously perchloric acid to precipitate proteins. The SPE method was optimised and the time necessary to dry the cartridge was the only factor necessary to modify. Several samples of plasma spiked with 740 ng/ml of atenolol were extracted following the method used in the determination of atenolol in urine, and after the washing step the cartridges were allowed to dry for different intervals of time, from 5 to 35 min. The time that gives the biggest peak area was 30 min, so this was the time used to let the cartridge dry. Using this time in the drying step, the average recovery was approx. 75%.

In order to improve the recovery obtained a liquid–liquid extraction was checked as an alternative clean-up procedure. Taking into account that atenolol is a very hydrophilic compound, a very polar solvent was needed in the liquid–liquid extraction. Ethyl acetate was chosen as the most suitable solvent. The method consisted of adding 50 μl of 1 *M* NaOH to 1 ml of spiked plasma. After this, 3 ml of ethyl acetate was added, the mixture was shaken for 10 min, centrifuged for 10 min at 5000 rpm and with a Pasteur pipette the organic layer was separated from the aqueous one. This process was repeated twice and the two volumes of solvent were joined and evaporated to dryness at 50°C under a gentle stream of nitrogen and the residue dissolved in 200 μl of deionized water, shaking vigorously with a vortex-mixer. This final solution was filtered with a 0.45 μm filter and then

Table 2
Quantitative parameters obtained for the analysis of atenolol by CZE in plasma: 50 m*M* H_3BO_3–50 m*M* $Na_2B_4O_7$ (50:50), pH 9.0; $t_{inj}=4$ s, $V=25$ kV, $\lambda=194$ nm

Calibration range (ng/ml)	50–400
Slope of the calibration	0.043±0.001
Intercept	−0.4±0.2
Regression coefficient (r^2)	0.995
Reproducibility intra-day (RSD, %)	5.05[a]; 2.42[b]; 2.27[c]
Reproducibility inter-day (RSD, %)	2.50[a]; 6.91[b]; 8.22[c]
Accuracy (spiked, ng/ml; relative error, %)	76.92; 6.1 251.75; 1.9
Detection limit (ng/ml) ($S/N=3$)	27
Quantitation limit (ng/ml) ($S/N=10$)	90
Recovery (%)	82.3[a]; 73.5[b]; 67.8[c]

[a] Concentration of atenolol 100 ng/ml.
[b] Concentration of atenolol 200 ng/ml.
[c] Concentration of atenolol 400 ng/ml.

was injected directly into the electrophoretic system. The recovery of atenolol obtained was very low (ca. 23%), so the method was not considered adequate.

3.3. Quantitative determination

A calibration curve for atenolol in plasma was made using spiked blank plasma samples and extracting them using the developed SPE method. The samples were calculated by interpolation in the calibration curve. The quantitative parameters calculated for this system are collected in Table 2.

The recoveries of the extraction were calculated comparing the areas of extracted spiked samples with those of standard solutions and taking into account the preconcentration factor of each case. The recoveries values at three concentration levels are also shown in Table 2.

3.4. Analytical applications

The developed method was applied to the determination of atenolol in plasma samples obtained from hypertensive patients of different age, sex and treatment duration. Most of the patients follow a therapy with different amounts of atenolol, while the rest of patients were under treatment with two or three antihypertensive agents: atenolol-diuretic (Table 3).

The atenolol was determined without interference from endogenous compounds of plasma, after the SPE procedure described.

The plasma was collected between 2 and 4 h after the intake of the pharmaceutical formulation. Each plasma sample was analysed at least three times and

Table 3
Determination of atenolol in the plasma of hypertensive patients after the ingestion of a dose of different pharmaceutical formulations[a]

Patient	Formulation	Composition	Duration of the treatment (years)	Sex[b]	Age (years)	Concentration atenolol (ng/ml)
1	Blokium 50	Atenolol 50 mg	5	M	39	30.3
2	Blokium 50	Atenolol 50 mg	6	M	50	37.7
3	Tenormin 50	Atenolol 50 mg	6	M	72	94.1
4	Blokium 50	Atenolol 50 mg	8	F	67	305.9
5	Tenormin 100	Atenolol 100 mg	2	M	73	335.4
6	Blokium 50	Atenolol 50 mg	5	F	56	361.0
7	Tenormin 50	Atenolol 50 mg	3	M	55	133.7
8	Tenormin 50	Atenolol 50 mg	5	F	64	248.8
9	Tenoretic	Atenolol 100 mg Chlorthalidone 25 mg	3	M	43	344.1
10	Kalten	Atenolol 50 mg Hydrochlorothiazide 25 mg Amiloride 2.5 mg	4	F	70	92.4
11	Blokium-DIU[c]	Atenolol 100 mg Chlorthalidone 25 mg	1	M	65	584.9

[a] Mean value of three extractions, two injections of each sample: 50 m*M* H_3BO_3–50 m*M* $Na_2B_4O_7$ (50:50), pH 9.0; $t_{inj}=4$ s, $V=25$ kV, $\lambda=194$ nm.
[b] Sex: M=male; F=female.
[c] Doses: two tablets daily.

each final extract injected twice in the electrophoretic system. Mean values are given.

As can be seen, great differences in terms of concentration were found among the patients under treatment with atenolol. The drug concentration obtained ranges from 30 to 585 ng/ml. A preconcentration step was necessary in all cases.

Hydrochlorothiazide and amiloride do not interfere in the analysis of atenolol since both were not extracted under the working conditions. However, chlorthalidone could be simultaneously determined with atenolol, as can be seen in Fig. 6.

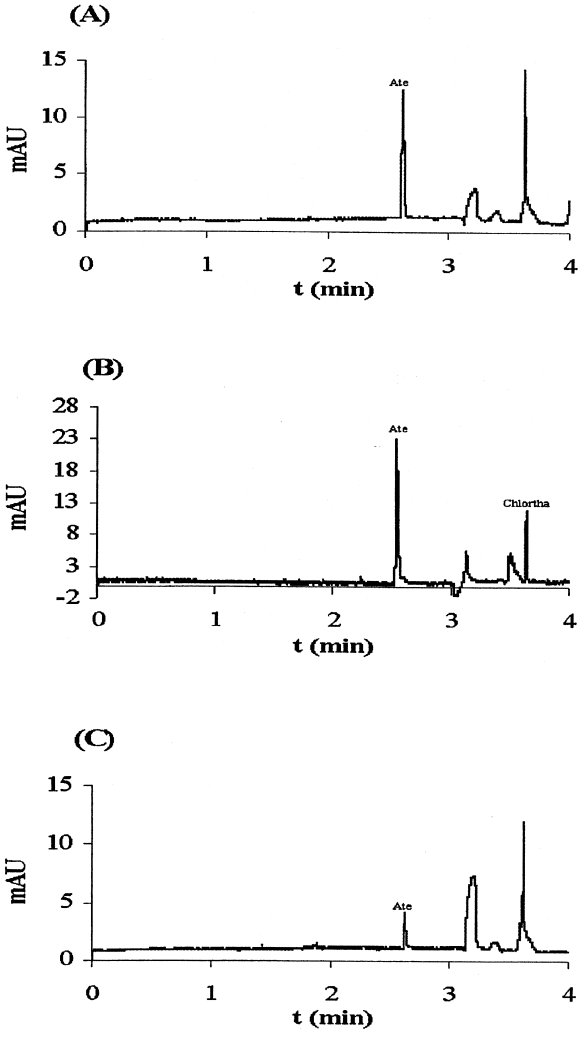

Fig. 6. Electropherograms of three different patients under treatment of atenolol: (A) patient 4 (Blokium 50: atenolol 50 mg), (B) patient 11 (Blokium-DIU: atenolol 100 mg, chlorthalidone 25 mg) and (C) patient 10 (Kalten: atenolol 50 mg, hydrochlorothiazide 25 mg, amiloride 2.5 mg): 50 m*M* H_3BO_3–50 m*M* $Na_2B_4O_7$ (50:50), pH 9.0; t_{inj} = 4 s, V = 25 kV, λ = 194 nm.

4. Discussion

The CZE method described was adequate for the quantification of atenolol in human plasma samples at different concentration levels. The SPE procedure is very simple and effective, and the electrophoretic separation takes place in less than 3 min. Both the reproducibility and the linearity have good values, and it is worth mentioning the excellent selectivity of the method, since there is almost no presence of endogenous substances of the plasma in the electropherograms.

The poor sensitivity of the capillary electrophoresis with photometric detection makes a preconcentration of the samples necessary, which it is possible due to the small injection volume needed.

Due to the small number of patients under study the great differences of atenolol concentration found cannot be related to the different parameters studied: sex, age, duration of the treatment and dosage of other drugs co-administered. In previous work [15], the genotoxicity of atenolol could be predicted because of the significant enhancement in the frequency of micronucleus (MN) in these patients under chronic exposure to the drug. Because of the great variability in the concentration of atenolol found in the patients' plasma and the lack of relation between plasma levels and MN frequencies, it would be of interest to carry out a more detailed study in order to determine the plasmatic profile of each patient. The electrophoresis method developed would allow to obtain this plasmatic profile, necessary to elucidate if there is any relationship between the genotoxic potential of atenolol and the β-blocker plasma concentration.

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References

[1] D.N. Heiger, High Performance Capillary Electrophoresis, Hewlett-Packard, 1992.
[2] P. Lukkari, T. Nyman, M.-L. Riekkola, J. Chromatogr. A 674 (1994) 241.
[3] V. Marko, Determination of β-Blockers in Biological Material, Part C, Elsevier, Amsterdam, 1989.
[4] R. Brent Miller, J. Pharm. Biomed. Anal. 9 (1991) 849.
[5] M.L. Martins, M.A. Pierossi, L.A. Moraes, W. Ribeiro, E. Abbib Jr., G.B. Mendes, A. Poli, G. de Nucci, M.N. Muscará, Int. J. Clin. Pharmacol. Ther. 35 (8) (1997) 324.
[6] G. Meissner, K. Hartonen, M.-L. Riekkola, Fresenius J. Anal. Chem. 360 (5) (1998) 618.
[7] D.J. Chatterjee, W.Y. Li, A.K. Hurst, R.T. Koda, J. Liq. Chromatogr. 18 (4) (1995) 791.
[8] C. Giachetti, A. Tencoti, S. Canali, G. Zanolo, J. Chromatogr. B 698 (1997) 187.
[9] A.D. Dale, S.E. Turner, J. Pharm. Biomed. Anal. 8 (8) (1990) 1055.
[10] R.B. Miller, J. Pharm. Biomed. Anal. 9 (10–12) (1991) 849.
[11] R.G. Morris, N.C. Saccoia, B.C. Sallustio, R. Zacest, Ther. Drug Monit. 13 (1991) 345.
[12] F.C.K. Chiu, J.N. Zhang, R.C. Li, K. Raymond, J. Chromatogr. B 691 (1997) 473.
[13] J.C. Miller, J.N. Miller, Estadística Para Química Analítica, Addison–Wesley Iberoamericana, DE, 1993.
[14] M.I. Maguregui, R.M. Alonso, R.M. Jiménez, J. Liq. Chromatogr. Rel. Technol. 20 (20) (1997) 3377.
[15] M. Télez, B. Martínez, B. Criado, C.M. Lostao, O. Peñagarikano, B. Ortega, P. Flores, E. Ortiz-Lastra, R.M. Alonso, R.M. Jiménez, I. Arrieta, Mutagenesis 15 (2000) 195.