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# Chiral separation of oxprenolol by affinity electrokinetic chromatography-partial filling technique using human serum albumin as chiral selector

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**Abstract**

The intrinsic characteristics of capillary electrophoresis have made this technique a powerful tool in the chiral separation field. The present paper deals with the enantiomeric separation of oxprenolol enantiomers by affinity electrokinetic chromatography-partial filling technique using human serum albumin (HSA) as chiral selector. Several experimental conditions and variables affecting the separation such as pH, HSA concentration and plug length, background electrolyte concentration, temperature and voltage were studied. Baseline separation of oxprenolol enantiomers was obtained in less than 8 min under the following selected conditions: electrophoretic buffer composed of 50 mM Tris–(hydroximethyl)-aminomethane (Tris) at pH 8.5; 190 μM HSA solution applied at 50 mbar for 225 s as chiral selector; oxprenolol samples contained 190 μM HSA solution injected hydrodynamically at 30 mbar for 2 s and the electrophoretic runs performed at 30 °C applying 15 kV voltage. The proposed methodology was applied for the analysis of two pharmaceutical preparations. Resolution, accuracy, reproducibility, speed and cost of the proposed method make it suitable for quality control of the enantiomeric composition of oxprenolol in drugs. The results show that a different affinity between oxprenolol enantiomers and HSA exists and can contribute to the pharmacokinetic differentiation of these enantiomers.
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*Keywords:* Affinity electrokinetic chromatography; Partial filling technique; Human serum albumin; Oxprenolol enantiomers

## 1. Introduction

The chiral nature of the living systems has evident implications in the activity of xenobiotics. Many pharmaceutical drugs are chiral and pharmacological characteristics often vary between enantiomers. In fact, very often one of them is the most active isomer while the other may produce side-effects and/or toxicity. In the last two decades, there has been a special interest in pharmaceutical laboratories to develop single enantiomer formulations. Chiral considerations are an integral part of drug research and development and of regulatory process. Consequently rapid and efficient analytical methods to control the enantiomeric purity of drugs are particularly required [1].

Different analytical techniques have been proposed for chiral separation of drugs being the chromatographic techniques the most widely used in the routine analysis of chiral compounds [1]. In the past few years, capillary electrophoresis (CE) has become a powerful analytical technique for enantiomeric separations due to its intrinsic properties: high separation efficiency, short analysis time, its capability of rapid optimization and low reagent consumption and small sample requirements compared to HPLC [2–3].

Among the chiral selectors that have been used in capillary electrophoresis for the enantiomeric separation of

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different classes of drugs, cyclodextrins and their derivatives are the most popular [4]. More recently, proteins have also received increased attention for use as stereoselective binding agents in capillary electrophoresis [3,5–7]. Proteins can be used as additives to the running buffer (affinity electrokinetic chromatography, AEKC) or as immobilized selectors (affinity capillary electrochromatography, ACEC). AECK, has, indeed, several advantages over ACEC. Methodological developments in AEKC are easily performed since this technique does not require protein immobilization. In addition the use of soluble proteins eliminates the possibility of altering both the structure and the binding properties of the protein. Furthermore, higher peak efficiency is obtained in AEKC [7]. In this technique, if the analyte and the analyte–protein complex have different mobility, the interaction of the compound with the protein results in a change in the net mobility of the analyte [5].

In spite of these advantages, the addition of proteins in the running buffer has some drawbacks. One of them is related to the possible adsorption of proteins onto the capillary wall. In order to minimize the adsorption of proteins the use of coating capillaries or adequate conditioning steps has been proposed [6]. The background UV absorption of a protein solution is a critical problem in AEKC. The most useful approach to overcome low detection sensitivity is the partial filling technique. This technique was introduced by Valtcheva et al. [8] and was later modified by Tanaka and Terabe [5], In this technique, the capillary is partially filled with the protein solution, while the rest of the capillary, including the detection window, contains the electrophoretic buffer. The experimental conditions such as pH, voltage, temperature, buffer concentration, chiral selector concentration and plug length are optimized so that the analyte migrates through the selector plug and enantiomers could separate and be detected out of the protein zone. Another additional advantage is that protein is hydrodynamically introduced into the capillary, and therefore the protein solution is not electrolyzed and can be used for several times so the analysis cost is low [9]. Because every protein is inherently chiral and AEKC has numerous advantages over HPLC, the development of new practical applications of this methodology is desirable.

In this paper, we focus our attention on the chiral separation of β-blocker oxprenolol using human serum albumin (HSA) as chiral selector by affinity electrokinetic chromatography and the partial filling technique. β-Blockers are widely used to treat cardiovascular disorders. Each of these drugs possesses at least one chiral center and an inherent high degree of enantioselectivity in binding to the β-adrenergic receptor. The enantiomers of β-blockers present markedly different pharmacodynamic and in some cases, pharmacokinetic properties [10].

Although different chiral selectors have been proposed for the resolution of oxprenolol enantiomers using chromatographic and electrophoretic [11–19] methods, the enantioseparation of oxprenolol using human serum albumin is of special interest. HSA is the major protein in blood; therefore enantiomeric drug separation can provide useful information on the pharmacokinetic differentiation of drugs. However the use of HSA as chiral selector in AEKC is reduced up to now [7,9,20,21].

## 2. Materials and methods

### 2.1. Chemicals and samples

All reagents were of analytical grade. Human serum albumin Fraction V (HSA) was purchased from Sigma (St. Louis, MO, USA); Tris–(hydroximethyl)-aminomethane (Tris) was from Scharlab (Barcelona, Spain); acetone was from Merck (Darmstadt, Germany); racemic oxprenolol standard was kindly donated by Ciba-Geigy. Barnstead E-pure deionized water (Sybron, Boston, MA, USA) was used to prepare solutions. All solutions were filtered prior to their use through 0.45 μm pore size nylon membranes (Micron Separation, Westboro, MA, USA).

### 2.2. Instruments and measurements

A Hewlett-Packard HP $^{3D}$CE capillary electrophoresis system (Hewlett-Packard, Waldbronn, Germany) equipped with a diode array detector (DAD) and HP $^{3D}$CE Chemstation software was used. Fused-silica capillaries (Polymicro Technologies, Phoneix, AZ, USA) of 50 μm i.d. and 363 μm o.d. with total and effective length of 65 and 56.5 cm, respectively, were used. The other selected CE conditions for oxprenolol enantiomers separation were: voltage, 10, 15, 20 and 25 kV; detection wavelength, 220 nm; capillary cassette temperature was set at 20, 25, 30, 35 and 40 °C. Electrolyte solutions were degassed in an ultrasonic bath (JP Selecta, Barcelona, Spain). A Crison Micro pH 2000 pHmeter from Crison Instruments (Barcelona, Spain) was employed to adjust the pH of the separation buffer.

### 2.3. Procedure

Buffers containing variable Tris concentration ranging from 10 to 100 mM were prepared with Barnstead E-pure water and the pH was adjusted with 1.5 M HCl. Human serum (500 μM) albumin stock solution was daily prepared in Tris buffer at the corresponding pH. Working protein solutions were obtained by dilution of filtered HSA stock solution with Tris buffer to a final concentration ranged between 140 and 190 μM. A 1000 mg/l stock standard oxprenolol solution was prepared in 50 mM Tris buffer at pH 8. Working solutions were obtained by dilution of oxprenolol stock solution with Tris buffer at the selected pH to a final concentration of 200 mg/l. A small amount of acetone was also added to the sample solutions to monitor electroosmotic flow.

In order to obtain good peak shapes and reproducible migration data, the capillary was conditioned at the beginning of the day with the following sequence: (i) 2 min rinse with

deionised water, (ii) 2 min rinse with 1 M NaOH, (iii) 2 min rinse with deionised water, and (iv) 15 min rinse with the running buffer at 1000 mbar. Between runs, the capillary was conditioned with deionised water for 2 min, 2 min 1 M NaOH, water for 2 min and running buffer for 2 min. Before sample injection, the capillary was partially filled with the chiral selector, HSA concentration in the range 140 to 190 μM, by applying 50 mbar pressure at different times in the range 0–240 s (equivalent 0–16 cm capillary length). Injection of oxprenolol solutions were performed hydrodynamically at 30 mbar for 2 s.

### 2.4. Sample preparation

For the analysis of the pharmaceutical preparations, five tablets were weighed, ground in a mortar. In order to guarantee the total dissolution of oxprenolol, the solid was dissolved in Tris buffer (50 mM) at pH 5.5 to a final concentration of 1000 mg/l of oxprenolol. Finally, the pH of injected samples was adjusted to 8.5. All the solutions were filtered through a 0.45 μm pore size disposable nylon filter from Micron Separation (Westboro, MA, USA). Three independent sample solutions were prepared and triplicate determinations were performed of each sample.

## 3. Results and discussion

### 3.1. Enantiomeric separation of oxprenolol

In order to estimate oxprenolol-HSA interaction at near-physiological conditions, the high-throughput capillary electrophoresis/frontal analysis method previously proposed was used [22]. For this purpose series keeping a constant concentration of oxprenolol 100 μM and increasing HSA concentration (from 0 up to 500 μM) were used .The binding parameters obtained for the first site of interaction were $n_1 = 1.0$, $K_1 = (2.6 \pm 0.4)\ 10^3$ and protein binding percentage $56 \pm 7$. Due to the moderate affinity of oxprenolol towards HSA, the possibility of using this protein as chiral selector for the separation of oxprenolol using the partial filling technique was evaluated

The influence of several experimental variables such as pH, temperature, chiral selector concentration and plug length, buffer concentration and voltage on the resolution of oxprenolol enantiomers was studied. The range of experimental variables was selected taking into account the physicochemical properties of oxprenolol and to assure the stability of the HSA solutions.

The pH range studied was 7.75–9.3; at theses pH values the protein is negatively charged (p$\underline{I}$ of 4.9 [23]) and oxprenolol is positively charged (aqueous p$K_a$ value 9.5 [24]). The increase in the buffer pH produces a decrease in the effective mobility of oxprenolol due to the diminution of the ionization degree and an increase in the mobility of HSA as a consequence of the increase in EOF hindering the analyte detection out of the HSA plug at higher pH values. The separation of oxprenolol enantiomers was only observed in the pH range 8.5–8.75 and pH 8.5 was selected for further studies.

So as to study the effect of the temperature on the enantioresolution of oxprenolol, series of runs applying 50 mbar for 200 s on a 160 μM HSA solution just before the racemic oxprenolol injection, were performed at 20, 25, 30, 35 and 40 °C. Electrophoresis was carried out using plain 50 mM Tris buffer at pH 8.5 and applying 15 kV. Although resolution increased with increasing temperature a temperature of 30 °C was kept for further studies in order to prevent possible denaturation of HSA.

The effect of Tris concentration in the electrophoretic buffer in the range 10 to 100 mM at pH 8.5 on the resolution was evaluated. Tris is a zwitter ionic buffer which, may minimize interactions of solute and protein with the capillary wall by shielding the capillary surface charge and by reducing sample adsorption [25]. The increase of Tris concentration produces more effective charge shielding; therefore a decrease in EOF is observed. As it can be observed in Fig. 1, the maximum resolution was achieved at 50 mM Tris buffer. The use of higher Tris concentrations produces a decrease in resolution, as a consequence of the increase in current and subsequent Joule heating that leads to peak broadening [25]. Therefore, a 50 mM Tris buffer was selected for further studies.

The effect of the HSA concentration on the enantioresolution was also studied. HSA solutions from 140 to 190 μM were prepared using 50 mM Tris solution at pH 8.5 and were applied at 50 mbar for 225 s. As it can be observed in Fig. 2, regardless of the HSA concentration in the electrophoretic buffer, the resolution achieved was constant and close to 1.14 in all cases. In order to increase the interaction time between oxprenolol and the protein, the same concentration of HSA

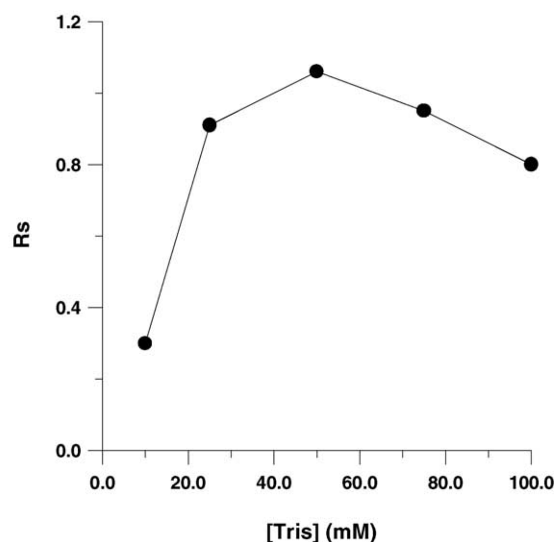


Fig. 1. Effect of Tris concentration on the resolution of oxprenolol enantiomers. Experimental conditions: 190 μM HSA solution applied at 50 mbar for 200 s; electrophoretic buffer pH 8.5, temperature 30 °C; voltage applied 15 kV; UV detection at 220 nm.

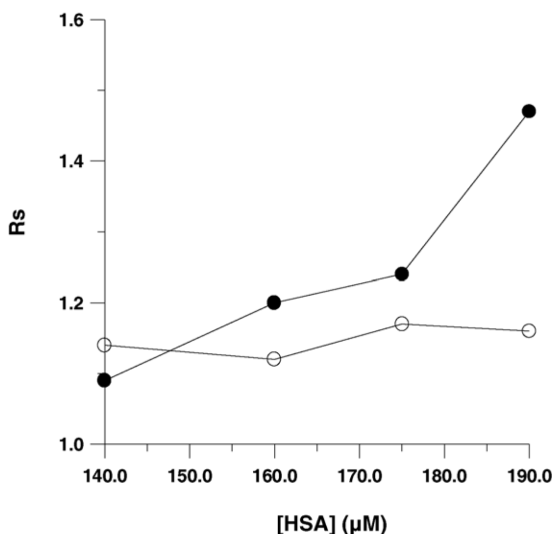


Fig. 2. Influence of HSA concentration on the resolution of oxprenolol enantiomers: (◯) without HSA solution in sample; (●) by adding the same concentration of HSA solution to the sample as in the chiral selector plug. Experimental conditions: 50 mM Tris at pH 8.5 as electrophoretic buffer, HSA solution applied at 50 mbar for 225 s; temperature 30 °C; voltage applied 15 kV; UV detection at 220 nm.

applied for each running was also added to the sample solution. As it can be observed in Fig. 2, resolution increased as the HSA concentration in sample increased. For further studies, a 190 μM HSA solution was added to the buffer and sample solutions.

In order to study the effect of the chiral selector plug length, experiments using a 190 μM HSA solution applied for variable times from 0 to 240 s (capillary equivalent length 0–16 cm) before racemic oxprenolol solution injection were

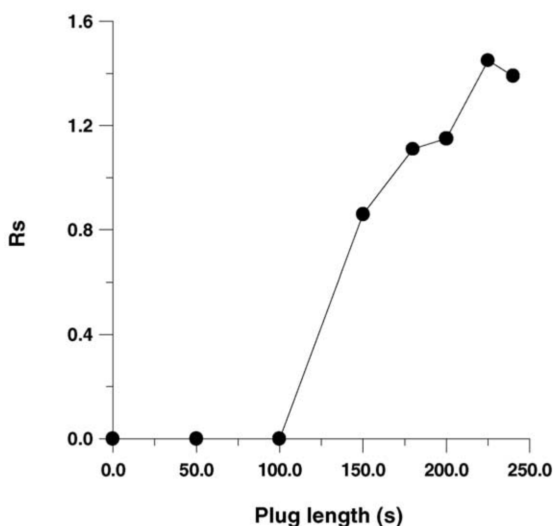


Fig. 3. Influence of the chiral selector plug length on the resolution of racemic oxprenolol. 190 μM HSA solution applied at 50 mbar for different times from 0 to 240 s. Experimental conditions: 190 μM HSA solution added to the sample solution; 50 mM Tris at pH 8.5 as electrophoretic buffer, temperature 30 °C; voltage applied 15 kV.

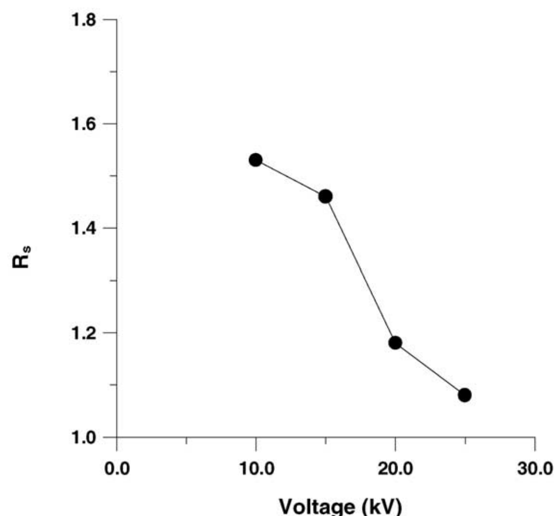


Fig. 4. Influence of voltage applied on the resolution of oxprenolol enantiomers. Experimental conditions: 50 mM Tris at pH 8.5 as electrophoretic buffer; 190 μM HSA solution applied at 50 mbar for 225 s; 190 μM HSA solution added to the sample solution; temperature 30 °C.

performed. All the samples contained 190 μM HSA solution. The results are shown in Fig. 3. As it can be seen, no chiral recognition was obtained when HSA solution was applied for less than 100 s (7 cm). Resolution and efficiency increased as the chiral selector plug time increased. However, times over 240 s did not provide an improvement in resolution and the peaks became asymmetric and overlapped with the HSA zone. Therefore applications of HSA solutions at 50 mbar for 225 s were selected for further studies.

Finally a study of the applied voltage from 10 to 25 kV was carried out. Migration times decreased from 11 to 4 min and resolution decreased from 1.53 to 1.08, with increasing voltage (see Fig. 4). An applied voltage of 15 kV was selected because voltages over 20 kV provokes partial overlapping of the analyte peaks with the HSA zone and a loss of efficiency due to a Joule heating. The use of an applied voltage of 10 kV produces longer migration times and a similar resolution (10 kV, Rs = 1.53; 15 kV, Rs = 1.46).

From the results obtained the following experimental conditions were selected: electrophoretic buffer composed of 50 mM Tris at pH 8.5; 190 μM HSA solution applied at 50 mbar for 225 s as chiral selector; oxprenolol samples contained 190 μM HSA solution injected hydrodynamically at 30 mbar for 2 s; the electrophoretic runs performed at 30 °C applying 15 kV voltage. Under these experimental

Table 1
Statistical features of the calibration graphs for the first ($E_1$) and second ($E_2$) migrating oxprenolol enantiomer

| | $n$ | $b_0 \pm ts_{b_0}$ | $b_1 \pm ts_{b_1}$ | $r$ |
|---|---|---|---|---|
| $E_1$ | 5 | 0.4 ± 1.2 | 0.092 ± 0.012 | 0.998 |
| $E_2$ | 5 | 0.6 ± 1.4 | 0.088 ± 0.014 | 0.996 |

$n$: Number of standard solutions; $b_0$ intercept; $b_1$ slope; ts, confidence interval at the 95% probability level; $r$: correlation coefficient.

Table 2
Intra-day and inter-day precision

| | Intra-day R.S.D. | | | Inter-day R.S.D. | | |
|---|---|---|---|---|---|---|
| | 26.7 $mg\,l^{-1}$ | 95.3 $mg\,l^{-1}$ | 152.4 $mg\,l^{-1}$ | 26.7 $mg\,l^{-1}$ | 95.3 $mg\,l^{-1}$ | 152.4 $mg\,l^{-1}$ |
| $E_1$ | 3 | 2 | 2 | 5 | 2 | 1 |
| $E_2$ | 6 | 3 | 2 | 6 | 5 | 4 |

R.S.D.: relative standard deviation.

conditions, oxprenolol enantiomers were resolved in 7.5 min, being the resolution value 1.47.

When a new capillary was used, a gradual and slight increase in the retention times of oxprenolol enantiomers and also a slight increase of resolution in the first ten runs were observed. This effect can be due to the irreversible protein adsorption to the capillary wall that also contributes to the oxprenolol enantiomers resolution. This phenomenon has been previously described when using HSA at neutral or slightly basic pH [20,26]. In the following runs the retention times and resolution values remained constant.

### 3.2. *Enantiomeric determination of oxprenolol in pharmaceutical preparations*

The proposed method was applied to determine the content of oxprenolol enantiomers in two pharmaceutical preparations containing this β-blocking agent. All of them are commercialized as racemic mixtures of oxprenolol hydrochloride and presented as tablets.

The linearity, intra- and inter-day precision, recovery and accuracy were evaluated by using peak areas as dependent variable. Calibration graphs were obtained by triplicate injections of standard solutions containing 25 to 152 $mg\,l^{-1}$ of each oxprenolol enantiomer. Table 1 summarizes the statistical features of the calibration graphs obtained for the first ($E_1$) and the second ($E_2$) migrating enantiomer. In all cases, the calibration curves showed adequate regression coefficients ($R > 0.99$). The slope values for both enantiomers were statistically significant at 95% confidence level. The intercept values were not statistically significant at 95% confidence level.

Intra- and inter-day precision, expressed as the relative standard deviation, were evaluated at three concentration levels, 26.7, 95.3 and 152.4 $mg\,l^{-1}$ of each enantiomer. The intra-day precision was evaluated by injecting three times each independent solution while the inter-day precision was evaluated by injecting each solution at least six times over 2 days. The results are shown in Table 2. In all cases the relative standard deviations were lower than 6%.

Table 3
Recoveries obtained for oxprenolol

| Pharmaceutical preparations | Recovery (%) ± $s_{n-1}$ | |
|---|---|---|
| | $E_1$ | $E_2$ |
| Trasitensín Retard® | 100 ± 1 | 98 ± 2 |
| Trasicor® | 101 ± 1 | 98 ± 1 |

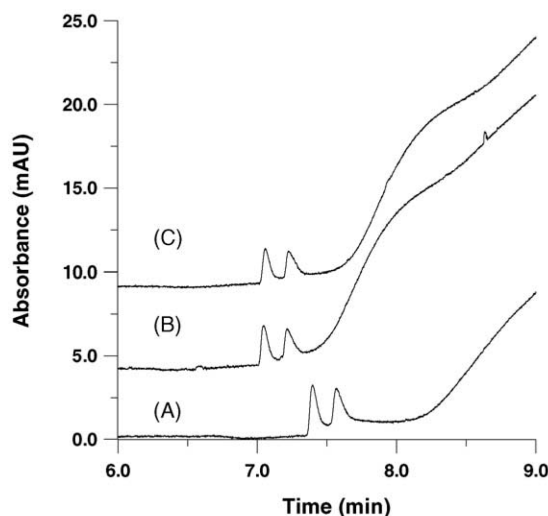


Fig. 5. Electropherograms obtained for (A) 200 $mg\,l^{-1}$ of racemic oxprenolol hydrochloride; (B) Trasitensín Retard and (C) Trasicor (after appropriate dilution). Experimental conditions: 50 mM Tris at pH 8.5 as electrophoretic buffer, HSA solution applied at 50 mbar for 225 s; 190 μM HSA solution added to the sample solution; temperature 30 °C; voltage applied 15 kV.

The accuracy estimation of the proposed method was also evaluated. Three sample solutions of the pharmaceutical preparations were spiked with 11.43 $mg\,l^{-1}$ of each oxprenolol enantiomer. These samples were analyzed following the proposed methodology and provided recoveries for both oxprenolol enantiomers ranged between 98 and 101% (see Table 3).

The proposed method was applied to the analysis of two pharmaceutical preparations containing oxprenolol hydrochloride. Fig. 5 shows the electropherograms obtained for the pharmaceutical preparations studied and for the racemic oxprenolol hydrochloride in the selected experimental conditions. As it can be seen adequate resolution was obtained

Table 4
Determination of oxprenolol enantiomers in pharmaceutical preparations

| Pharmaceutical preparations | Composition | R (%) ± $s_{n-1}$ | |
|---|---|---|---|
| | | $E_1$ | $E_2$ |
| Trasitensín Retard® | Oxprenolol HCl 160 mg, chlortalidone 20 mg and other excipients | 106 ± 1 | 102 ± 3 |
| Trasicor® | Oxprenolol HCl 80 mg and other excipients | 105 ± 2 | 101 ± 2 |

R, recovery with respect to the content declared by the manufacturer; $s_{n-1}$: relative standard deviation.

in all cases. The results expressed as percentage of recovery relating to the content declared by the manufactures were always ranged between 101 and 106% which agrees with the tolerance limits for racemic oxprenolol in this kind of samples (±5%) [27] (see Table 4).

## 4. Conclusions

This paper describes the application of the partial filling technique to the separation of oxprenolol enantiomers by capillary electrophoresis using HSA as chiral selector. The addition of HSA solution to the sample is the key factor to the enantioresolution of oxprenolol. Furthermore, the irreversible and stable protein adsorption to the capillary wall also improves resolution of oxprenolol enantiomers. The proposed methodology provides adequate results in terms of simplicity, cost, sample throughput, reproducibility and accuracy for quality control of oxprenolol enantiomers in pharmaceutical preparations. The main advantage of the proposed methodology compared to the methodologies proposed until now, is the low cost per analysis (0.006 euros/run), since the HSA solution does not become electrolyzed and can be reused for several runs. The results show that there exists a different affinity between oxprenolol enantiomers and HSA and it can contribute to the pharmacokinetic differentiation of these enantiomers.

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