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High sensitivity analysis of oxprenolol in urine by capillary electrophoresis with C18 packed on-line preconcentrator☆

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Abstract

High sensitivity analysis of oxprenolol in spiked human urine has been performed by capillary zone electrophoresis (CZE) in ammonium formate buffer pH 2.5 using an uncoated capillary with 1 cm length C18 on-capillary preconcentrator at the inlet side. The preconcentrator was fabricated in laboratory using the packing method and not encapped C18 5 μm particles as stationary phase material. The packed path was retained into the capillary by sintered stationary phase frits. Before running the CZE analysis, the oxprenolol was eluted from the preconcentrator by injecting a short plug of acetonitrile/water mixtures. With respect to classical CZE, the use of on-line preconcentrator widely increased the method sensitivity allowing the detection of the drug at 0.5 ng/mL (injected concentration). The method showed a linear response in the range of 1–150 ng/mL oxprenolol standard compound. The intra-day repeatability ($n = 11$) R.S.D. values for migration time, peak area and normalized peak area were 0.72%, 3.96% and 3.66%, respectively, while inter-day repeatability ($n = 5$ days) R.S.D. values were 2.74%, 9.41% and 9.83%, respectively. The method was successfully applied to the analysis of oxprenolol in extracted urine spiked at 250 pg/mL (oxprenolol LOQ concentration in urine).

Keywords: Capillary zone electrophoresis; On-line preconcentrator; Oxprenolol; Urine; High sensitivity

1. Introduction

Capillary electrophoresis (CE) is one of the most promising analytical methodology in the field of biomedical applications for the high separation efficiency and resolution power, the use of minimum quantities of reagents and samples, the short analysis time and the reduced sample pretreatment. Unfortunately its diffusion and applicability can be strongly limited by the low concentration detection sensitivity due to the very small volume injected (few nanolitres) and the reduced detection path length (few tens of micrometers). This can be particularly relevant when UV detection is used or medium or low molar absorbing compounds are analysed. In order to overcoming this drawback, several strategies can be applied such as on-line and off-line preconcentration techniques, use of special designed detection cell and capillaries, high sensitivity and specificity detectors (e.g. laser induced fluorescence detector, conductivity detector, mass spectrometry) [1].

As interestingly reviewed [2–4], the on-line preconcentration in CE involves the use of electrokinetic or chromatographic methodologies or their combination. This methodology is particularly advantageous in biomedical and pharmacological applications of CE where the analytes are often present at trace concentrations in the biological matrices. Solid phase extraction (SPE) on-line preconcentration in CE is performed by inserting a short plug of stationary phase material at the inlet side of the capillary providing the sample enrichment during the injection step on the basis of the chromatographic partition mechanism. In addition to the preconcentration, a sample clean-up can also be obtained [5,6].

Few papers show the successful application of CZE with SPE on-line preconcentration to the analysis of hypoglycemic drugs [7] and terbutaline enantiomers at low nanomolar level and untreated spiked plasma [8]. A 1 cm length packed C18 on-capillary preconcentrator was used for the CZE quantitative analysis of propranolol and doxepin in spiked urine at 500 ppb

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level [9]. Few papers report the use of CZE technique for the analysis of oxprenolol in urine in combination with off-line SPE extraction [10] and of its enantiomeric forms in presence of metabolites [11], however, both the methods showed a limit of quantification (LOQ) for oxprenolol in the range of 0.2–0.4 μg/mL.

The development of high selective analytical methods for the analysis of β-blockers in urine at low concentrations is relevant in different fields such as pharmaceutical and pharmacokinetic studies, toxicological, forensic analysis and doping control.

We developed a rapid and simple procedure for the fabrication of C18 packed on-line preconcentrator capillary that can be used in commercially available automated apparatus for increasing the CE detection sensitivity. This capillary was tested in CZE mode for the analysis of low concentrations of oxprenolol in spiked human urine. The oxprenolol lipophilic β-blocker was selected as test compound due its relatively hydrophobicity, the positive charge and the rapid and extensive metabolism, determining only the 5% of the administered dose found unchanged in urine [11]. The method development required the careful optimisation of the operating parameters providing the best performance of the preconcentrator for oxprenolol injection, adsorption and elution before the CZE analysis. The method was tested for linearity and repeatability with standard solution and applied to the analysis of oxprenolol in spiked human urine.

2. Experimental

2.1. Chemicals

Ammonia solution (30%), phosphoric acid (85%), acetonitrile, methanol, *n*-hexane and ethyl acetate were purchased from Carlo Erba (Milan, Italy). All reagents were HPLC gradient grade. Double distilled water (Milli-Q, Millipore, Waters Milford, MA, USA.) was used for preparation of solutions and for CZE experiments. Oxprenolol was from Sigma (St. Louis, MO, USA). Analytes solutions (1 and 0.01 mg/mL) were prepared in methanol and stored at −18 °C. Further dilutions were daily made with BGE before injection.

2.2. Apparatus

Capillary zone electrophoretic experiments were performed on an Agilent Technologies Capillary Electrophoresis automated apparatus (Waldbronn, Germany) equipped with diode array UV detector and external nitrogen pressure. Fused silica uncoated capillaries of 75 μm i.d., 375 μm o.d. (Composite Metal Services, Hallow, Worcs., UK) and total length of 64.5 or 56 cm, corresponding to 56 and 48.5 effective lengths, were packed for 1 cm at the inlet side with Lichrospher 100 RP_{18} (5 μm particles) stationary phase (Merck, Darmstadt, Germany), following the procedure previously described [12] with the below described modifications.

Methanol was used as delivering solvent for packing and frits were made by sintering the C18 stationary phase (30 s × 400 °C followed by 30 s at 600 °C) after replacing the methanol with pure water. The use of an heating wire and relatively low temperature for short times for frit fabrication allowed to obtained very thick and resistant frit paths without burning the external polyimide layer.

After the packing and the frit formation, the capillaries were reversed in order to remove the unretained stationary phase. The empty capillary path was then cut to the desired total length and the detection cell performed at 8.5 cm from the outlet side by burning out the external polyimide material. The capillary was inserted into the CE automated instrument and tested for permeability by injecting an aqueous solution of the unretained compound thiourea and recording the time necessary to reach the detection cell. This parameter was used to adjust all the rinsing, preconditioning and injecting procedures of the method. This procedure was performed each time a new capillary was prepared. Before packing, all the fused silica capillaries were rinsed with water (12 bar × 1 min) then with 0.1 M sodium hydroxide (6 bar × 15 min) and finally rinsed with water (12 bar × 5 min).

Between the CZE runs the capillary was rinsed with water/acetonitrile solution (10:90) and BGE by applying at the outlet side −9 bar × 1.0 min and −9 bar × 3.0 min, respectively. The analytes were injected dissolved in the BGE solution at the inlet side by applying 9 bar × 6 min followed by counterdirection injections at the outlet side of water/acetonitrile solution (70:30) (−7 bar × 0.05 min) followed by the BGE (−9 bar × 3.0 min). Finally before running the CZE analysis the drug was eluted from the C18 preconcentrator with a short plug of water/acetonitrile mixture (30:70, v/v) (see Section 3 for eluent optimisation) by applying 7 bar × 0.04 min followed by a short injection plug of BGE, 9 bar × 0.1 min. The running voltage was 25 kV. The BGE, 50 mM sodium phosphate pH 2.5 and pH 3.0 were prepared by titrating a solution of 50 mM orthophosphoric acid to the final pH value with sodium hydroxide aqueous solution. The BGE was weekly prepared and stored at +4 °C.

During the run the capillary was air thermostated at 25 °C and pressurised at both ends by applying 8 bar from the external pressure to prevent the formation of bubbles at the interface between the packed and the empty capillary paths. The electropherograms were recorded at 195 nm output UV wavelength.

2.3. Urine samples extraction procedure

A volume of 200 μL of urine was basified (pH 11.6) by the addition of 10 μL of ammonium hydroxide solution (30%, v/v) and vortex extracted with 1 mL of *n*-hexane/ethyl acetate mixture (8:2) for 10 min. After 5 min centrifugation at 200 × *g* the organic phase was recovered and dried under a gentle nitrogen stream. The sample was then reconstituted in 50 μL BGE.

3. Results

On-line preconcentration in CE significantly increases the method sensitivity allowing the injection of large sample volumes without diffusion and band broadening effects. The efficacy of the preconcentration effect is depending on the operating condition used for the analytes adsorbtion and elution and required a careful optimisation.

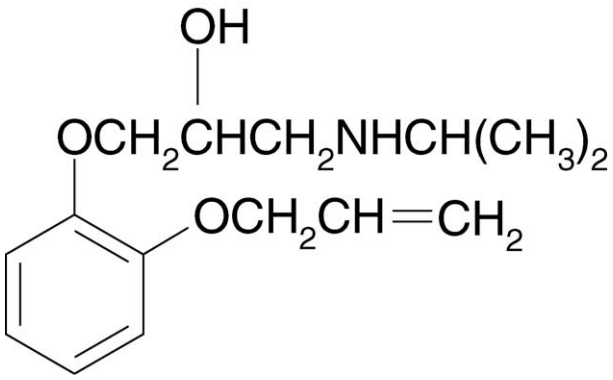

Fig. 1. Chemical structure of oxprenolol.

In this paper a simple and rapid procedure was used for the fabrication of a CE capillary containing a C18 packed preconcentrator (1 cm length at the inlet side) providing the sample enrichment using the solid phase extraction method. It is noteworthy that these capillaries can be used in commercially available CE instruments.

The presence of reversed phase in the preconcentrator produced the analyte adsorption mainly based on hydrophobic interactions. Under the suitable operating conditions, the analyte adsorbed on the preconcentrator during a prolonged sample injection step. A short plug of eluent was then injected in the capillary to desorb the analyte from the preconcentrator into a small zone immediately before the CZE analysis.

The fabricated on-line preconcentrator capillary was tested for the high sensitivity analysis of oxprenolol in human urine. The oxprenolol was selected as test drug on the basis of its physico-chemical properties, e.g. the relative hydrophobicity and the positive charge (see Fig. 1 for its molecular structure). In addition, being subjected to an extensive metabolism, only very low concentrations of the unchanged drug are present in urine requiring the availability of sensitive analytical methods for their determination.

3.1. CZE analysis of oxprenolol

Before testing the on-line preconcentrator capillary, the analysis of oxprenolol was first optimised in classical CZE mode by studying the optimum conditions of injection providing the highest sensitivity. The experiments were performed in fused silica uncoated capillary of 75 μm i.d. and 64.5 cm of total length using 50 mM sodium phosphate buffer pH 3.0 as background electrolyte (BGE). The oxprenolol (aqueous solution) was injected at different pressure values, namely 2 or 3 bar, and injection times in the range of 0.01–0.03 min. The application of 2 bar × 0.02 min was selected as the optimum value, a further increase of the applied pressure or of the injection time produced a deterioration of the peak shape. Under these conditions the CZE method provided a LOD value for oxprenolol of 100 ng/mL using 195 nm as the output UV wavelength.

The same capillary was then packed with C18 5 um stationary phase particles for 1 cm length at the inlet side using methanol as delivering solvent. After the methanol was replaced by water,

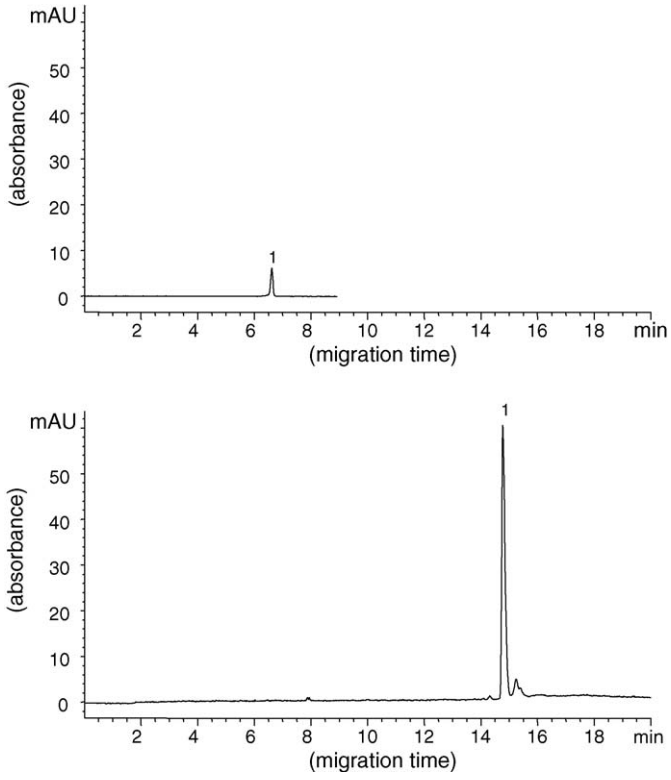

Fig. 2. Analysis of oxprenolol (1 μg/mL concentration) obtained in (a) classical CZE and (b) in CZE with on-line preconcentrator. BGE: 50 mM sodium phosphate buffer pH 3.0. Capillary: 64.5 cm total length (effective length 56 cm) × 75 μm i.d. For other experimental conditions see the text and Section 2. (1) Oxprenolol.

the retaining frits were made under pressure by sintering the C18 stationary phase. The capillary was therefore reversed to remove the unretained stationary phase and inserted into the CE cartridge for CZE preconcentrator analysis. Due to the very short length of the packed path, the capillary preconditioning did not require particular procedures and the system could immediately operate in CZE mode.

Fig. 2 shows the preliminary results obtained by injecting the same solution of oxprenolol at concentration of 1 μg/mL in classical CZE (panel a) and in CZE with on-line preconcentrator (panel b) using the same capillary before and after the packing, respectively, using 50 mM sodium phosphate buffer pH 3.0 as BGE. The obtained results immediately showed the advantages of using the on-line preconcentration in terms of analyte signal increase although no optimisation of the oxprenolol loading and eluting conditions were already performed.

3.2. Optimisation of the operating conditions in on-line preconcentrator CZE

The efficiency of the SPE on-line preconcentration methodology required the careful optimisation of the operating conditions providing the analyte adsorption on the packed path at the injection step and its total elution in a sharp zone before the CZE separation. They are strongly dependent on the stationary phase material used in the preconcentrator and the physico-chemical

properties of the analyte and determine the composition of the sample injection and eluting solutions.

Before starting the CZE experiments, the permeability of the packed capillary path was measured by injecting a solution of thiourea at 9 bar × 5 min and recording the time of the UV signal rising. This test was performed each time a new capillary was prepared and was used for measuring (pressure × time values) the back pressure generated by the preconcentrator and the enrichment capillary volume of the packed path. This parameter was useful for monitoring the degree of oxprenolol retention on the preconcentrator and for optimising both the rinsing and preconditioning procedures, being the counterpressure of the packed path determining the bulk solution velocity in the entire capillary.

The oxprenolol standard compound was injected dissolved in the same buffer used for running the CZE analysis. Under these conditions the injection of oxprenolol for a time (8 bar × 10 min) strongly longer than that of the unretained urea, did not provide any UV signal meaning its complete retention on the C18 phase.

The following step was to investigate the optimum conditions for oxprenolol elution from the preconcentrator. A short plug of eluent solutions were injected immediately after the sample injection by applying 7 bar × 0.06 min in order to elute the analyte in a small zone of solvent. The effect of different eluent mixtures, namely water/acetonitrile solutions in the range from 50:50 to 10:90 (v/v) and pure acetonitrile, were studied by comparing the analyte migration time (t_m), signal/noise ratio, peak area and efficiency data obtained after the CZE analysis. These experiments were performed in 50 mM sodium phosphate buffer pH 2.5 in order to reduce any adsorption phenomena of the basic drug on silica possibly affecting the studied effects. All the investigated solvent mixtures provided the elution of oxprenolol from the preconcentrator, however, the 50%, 90% and 100% (v/v) acetonitrile solutions showed lower values of signal/noise ratio and peak area than the 60%, 70% and 80% (v/v) contents (data not shown). The migration time was not remarkable affected by the eluent composition however a slight increase of t_m was recognised at rising content of organic modifier, particularly when pure acetonitrile was used. On the opposite the higher the acetonitrile content the higher the peak efficiency value (data not shown). The 60%, 70% and 80% acetonitrile produced comparable results. Among them 70% content was considered the best compromise and was selected for further experiments to study the influence of eluent injection time in the range 0.01–0.08 min at 7 bar fixed value of applied pressure. The injection time influenced the analyte t_m, the peak shape and peak height (data not shown). 7 bar × 0.04 min was the selected value providing the best compromise for analyte signal intensity, peak shape and migration time.

Being using an on-line preconcentrator, the analyte signal was supposed to be proportional to the analyte adsorption capacity of the sorbent material and to the duration of injection. To further maximise the analyte signal, the injection pressure was set at higher value, namely 9 bar, and the injection time increased from 2 to 6 min. At 9 bar pressure the analyte signal increased with the applied time. However, a further prolonging of the injection time was not evaluated to not considerably affect the total analysis time.

In order to better preserve the silica based material of the frits and the packing material and to speed the analysis time, further experiments were made in 50 mM sodium phosphate buffer at pH 3.0 and shorter capillary (75 um i.d., 56 cm of total length, 48.5 of effective length 1 cm C18 packed at the inlet side). Under

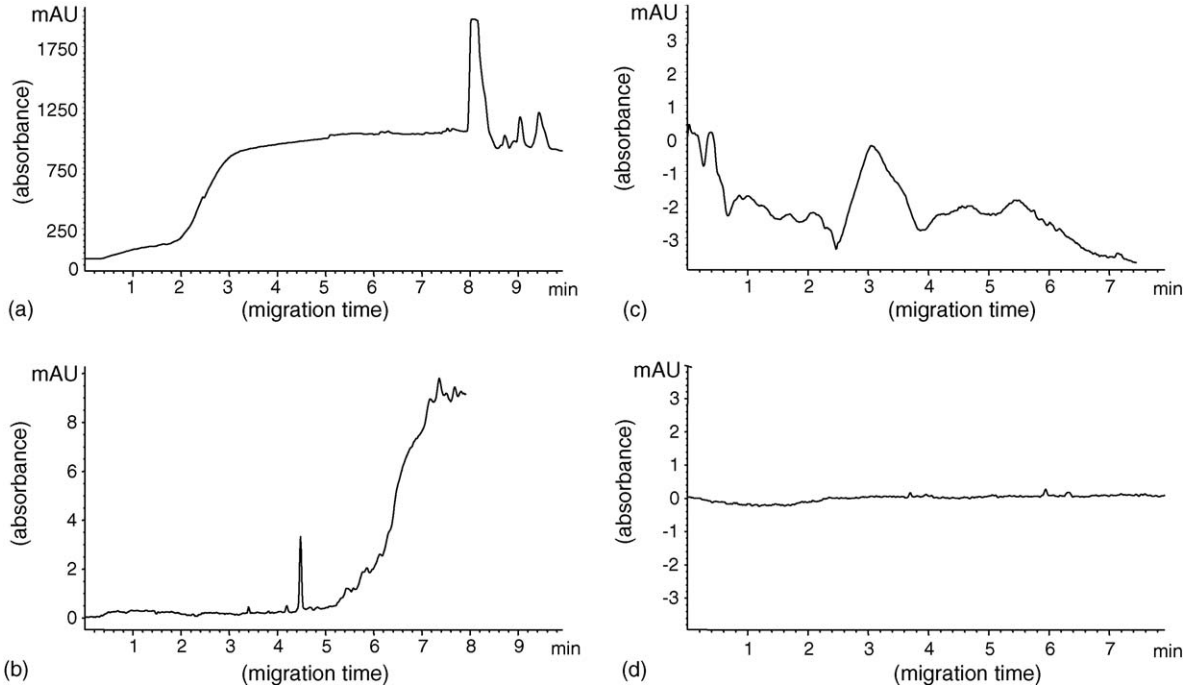

Fig. 3. Analysis untreated (panels a and b) and extracted (panels c and d) human urine spiked at 0.1 μg/mL oxprenolol concentration obtained without (upper panels) and with (lower panels) 30% (v/v) acetonitrile preclean-up step after sample injection. For other experimental conditions see the text and Section 2.

these conditions the oxprenolol migrated within 6.5 min with peak efficiency value of 113,280 theoretical plates per meter.

3.3. Method quantitation

The method was tested for linearity and injection repeatability on oxprenolol standard solutions using the external standard method. Under the used conditions the method provided an LOD value for oxprenolol of 0.25 ng/mL (signal/noise = 3) and a LOQ of 1.0 ng/mL (signal/noise = 10). The method was linear in the range of oxprenolol concentration 1–150 ng/mL (8 calibration points) showing squared correlation coefficients (r^2) of 0.9967 and 0.9998 for peak area and t_m normalized peak area, respectively. The intra-day repeatability ($n = 11$) showed R.S.D. value of 0.72%, 3.96% and 3.66% for migration time, peak area and normalized peak area, respectively. Inter-day repeatability ($n = 5$ days) showed R.S.D. values of 2.74%, 9.41% and 9.83% for migration time, peak area and normalized peak area, respectively. The method was therefore tested to be applied to the analysis of oxprenolol in spiked urine.

3.4. Analysis of spiked urine

When the method was tested for the analysis of oxprenolol in spiked human urine, a further optimisation was necessary due to the preconcentration effect simultaneously obtained for both the oxprenolol and the matrix compounds.

The analysis of spiked urine was preliminary performed in the longer capillary used (64.5 cm total length) by directly injecting untreated urine spiked at 100 ng/mL oxprenolol concentration. The spiked urine was simply diluted 1–10 with BGE before injection. In order to reduce the elution of unwanted matrix components from the preconcentrator, 60% of acetonitrile was used as eluent solution instead of 70%. As illustrated in Fig. 3a, the electropherograms exhibited a strong UV absorption starting from 2 min analysis time that made impossible the detection of the oxprenolol compound. In trying to purify the adsorbed sample on the preconcentrator and to remove or reduce the presence of interferences in the analysis, two different procedures were used. We first tried to introduce an additional preclean-up step after the sample injection by flushing 30% (v/v) acetonitrile aqueous solution in counterdirection flow (Fig. 3b). Alternatively, the urine was extracted before the analysis by a simple liquid/liquid extraction (Fig. 3c). Based on our previous experience on basic drug extraction from biological fluids [13–15], a similar procedure was used for the analysis of oxprenolol in urine as specified in the materials and methods section. With the aim of miniaturizing also this step of the method, the extraction procedure was optimised starting from as low urine volume as 200 μL.

Although both the treatments produced better electropherograms profiles, only by using a combination of them it was possible to obtain a clean baseline suitable for the detection of oxprenolol at very low concentrations (Fig. 3d).

Under these conditions the analysis of blank urine (Fig. 4a) and blank urine spiked with 0.25 ng/mL (Fig. 4b) and 12.5 ng/mL (Fig. 4c) oxprenolol concentration levels was successfully performed. These urine concentrations corresponded after the extraction procedure to 1.0, 50.0 and 100.0 ng/mL oxprenolol concentrations (injected values), respectively.

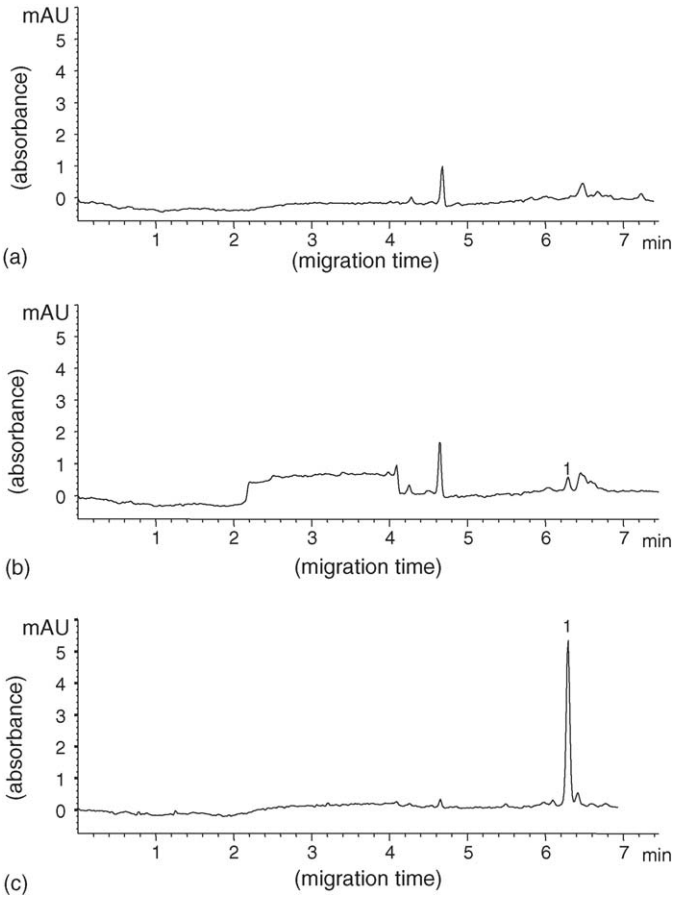

Fig. 4. Analysis of blank (a) and spiked extracted human urine at 0.25 ng/mL (b) and 12.5 ng/mL (c) oxprenolol spiked levels obtained under the optimum experimental conditions. BGE: 50 mM sodium phosphate buffer pH 3.0. For other experimental conditions see the text and Section 2. (1) Oxprenolol.

Although urine is an easily available biological sample, the miniaturisation also of the pretreatment step contributed to the development of a low cost and environmental pollution impact methodology with reduced solvent manipulation, consumption and disposal. The recoveries of the extraction procedure were performed by spiking in duplicate the urine with 0.25 and 25 ng/mL and in triplicate with 12.5 ng/mL oxprenolol concentration levels and considering the mean values of two separate runs. The recoveries values were in the range of 89.4–89.6%, 69–87% and 79–90% values for 0.25, 12.5 and 25 ng/mL concentrations, respectively.

It is important to consider that the analysis of the oxprenolol standard compound at 100 ppb level by preconcentrator-CZE with and without the 30% acetonitrile preclean-up step showed different peak area values, being the preclean-up step providing a decrease of 27% of the value that was however compensated by the cleaner urine baseline electropherogram profiles. Probably the use of a different stationary phase material and/or different organic solvent mixtures could be investigated to improve the efficiency of the preclean-up step, however, taking into account

that the use of organic solvent different from acetonitrile could produce bubbles in the system or/and a loss of peak efficiency.

4. Conclusions

The developed methodology can be advantageously used in commercially available CE instruments for increasing the detection sensitivity of CE technique and further expanding its applicability to the analysis of drug at ppt levels in complex biological matrix. This methodology allows the injection of large sample volumes avoiding diffusion phenomena and band broadening effects by maintaining the resolution and separation efficiency that strongly characterises the CZE technique. In the present paper the use of on-line preconcentrator produced a 400 times increase of oxprenolol limit of detection (LOD) in CZE. In addition to the analyte enrichment effect, the on-line preconcentrator can usefully operate a purification of the analyte from the biological sample matrix strongly reducing the presence of interfering compounds.

CZE capillaries equipped with on-line packed preconcentrator can be easily and rapidly fabricated using stationary phase materials with the packing method. The strong advantage in preparing on-line preconcentrator capillary in laboratory is the possibility of adjusting the capillary length, the preconcentrator stationary phase material and its length according to the biomedical application target. The fabrication procedure however required the careful optimisation of the operating conditions used for the frits fabrication, especially in terms of frit porosity and robustness. The application of low temperatures and short time for stationary phase sintering helps in producing a suitable frits permeability without removing the external polyimide layer confering resistance to the entire capillary. The resulting capillaries could be used for hundreds analysis and several months (3–4 months, considering at least an average value of 10 runs per day). The capillary to capillary fabrication repeatability was still not evaluated. However, although in this study on-line preconcentrator capillaries of different lengths were used, they provided similar performances and detection sensitivity increase capacity (data not shown).

The optimisation of the method of analysis of oxprenolol by CZE on-line preconcentrator required the careful investigation of the sample injection and eluting conditions providing the best performances according to the physico-chemical properties of the analyte and the stationary phase material of the preconcentrator. The method exhibited quantitative results on standard solution with repeatability and linearity and showed high detection sensitivity allowing the analysis of oxprenolol in spiked urine at ppt level (250 pg/mL spiked concentration).

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