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Automated determination of total captopril in urine by liquid chromatography with post-column derivatization coupled to on-line solid phase extraction in a sequential injection manifold

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ABSTRACT

The present study reports a new liquid chromatographic (HPLC) method for the determination of the anti-hypertension drug captopril (CAP) in human urine. After its separation from the sample matrix in a reversed phase HPLC column, CAP reacts with the thiol-selective reagent ethyl-propiolate (EP) in a post-column configuration and the formed thioacrylate derivative is detected at 285 nm. Automated 4-fold preconcentration of the analyte prior to analysis was achieved by an on-line solid phase extraction (SPE) step using a sequential injection (SI) manifold. The Oasis HLB SPE cartridges offered quantitative recoveries and effective sample cleaning by applying a simple SPE protocol. The limits of detection and quantitation were 10 $\mu g\,L^{-1}$ and 35 $\mu g\,L^{-1}$ respectively. The percent recoveries for the analysis of human urine samples ranged between 90 and 96% and 95 and 104% using aqueous and matrix matched calibration curves respectively.

1. Introduction

Captopril (CAP) is the first angiotensin-converting enzyme inhibitor (ACE inhibitor) that was used primarily for the treatment of hypertension and was also expanded for some types of congestive heart failure. During the early stages of its development and commercial marketing, CAP was considered as a breakthrough in the pharmaceutical industry both because of its novel mechanism of action and also because of the revolutionary development process [1]. The most common side-effect of CAP is cough and this is attributed to elevated levels of bradykinin. Other typical adverse effects – most of them shared by all ACE inhibitors – include angioedema, agranulocystosis, proteinuria, hyperkalemia, taste alteration, teratogenicity, postural hypotension, acute renal failure and leucopenia [2].

The international literature confirms the continuous interest for the development of new analytical protocols for the determination of CAP, mainly in biological material and pharmaceutical formulations. Representative recent methods include a variety of techniques: voltammetric sensors based on carbon nanotubes (human urine [3]); flow-based spectrophotometry (dissolution studies of pharmaceuticals [4]); Raman spectroscopy (CAP solutions [5]); liquid chromatography coupled to electrochemical detection (tablets [6]); surface-assisted laser desorption/ionization mass spectrometry (urine [7]); LC–MS/MS (human plasma [8]) and LC–MS (human plasma [9]); headspace solid phase microextraction coupled to ion mobility spectrometry (human plasma and formulations [10]).

A particularly interesting and useful approach for the determination of CAP in biological material seems to be based on the derivatization of the analyte prior (pre-column) or after a separation step (post-column) [8,9,11–14]. The main goals of such procedures are (i) to increase the stability of CAP in the samples [1,8], (ii) to enhance its detectability by attaching chromophore or fluorophore moieties or its ionization for mass spectrometric detection [8,9] and (iii) to improve its chromatographic properties. Almost all reported derivatization methods are based on pre-column protocols using numerous suitable reagents such as monobromobimane (MS/MS [8] and FL [13]), p-bromo-phenacyl-bromide (MS [9]), 2-chloro-1-methylquinolinium tetrafluoroborate (UV–vis [11]); 2,4′-dibromoacetophenone (UV–vis [12]), ThioGlo^{TM}-3 (FL [14]). As mentioned above, one of the most significant advantages of pre-column derivatization when it comes to the analysis of thiols is the stabilization of the analytes in the biological samples. On the other hand, pre-column derivatization is prone to inaccuracies when complicated matrices have to be analyzed, due to incomplete reactions and competition phenomena by endogenous substances.

An interesting alternative seems to be post-column derivatization (PCD). In PCD each analyte is derivatized "in isolation", providing sufficient excess and activity of the reagent and uniform

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conversion across the suite of analytes. This feature is particularly useful for complicated samples such as biological material, food and environmental applications. Additionally, due to the on-line character of the procedure the stability of the derivatives is not a prerequisite. Among the drawbacks of PCD someone can point out the extra instrumentation for the propulsion of the reagents, the necessity for fast reactions and the higher consumption. To the best of our knowledge there is only one previous HPLC-PCD method for the determination of CAP in biological samples. The method is based on the reaction of the analyte with o-phthalaldehyde (OPA)/glycine followed by fluorimetric detection [15] and contrary to most pre-column protocols, it is suitable for the analysis of total CAP.

In the present study we report a fully automated analytical scheme for the determination of total CAP in human urine. Analysis of urine-excreted CAP is important not only to investigate the pharmacokinetic behaviour of the drug, but also to avoid toxicological side-effects in patients with renal failure. CAP is isolated quantitatively from the sample matrix and preconcentrated using an automated flow-through sequential injection (SI) setup and the advantageous Oasis HLB solid phase extraction (SPE) cartridges. The SPE configuration is interfaced to the HPLC-PCD system where CAP is detected at 285 nm after post-column derivatization with the analytical reagent ethyl-propiolate (EP). Compared to the earlier PCD method [15], the proposed system offers ca. 8-times lower quantitation limits for urine analysis and the use of a single reagent (EP versus OPA/glycine).

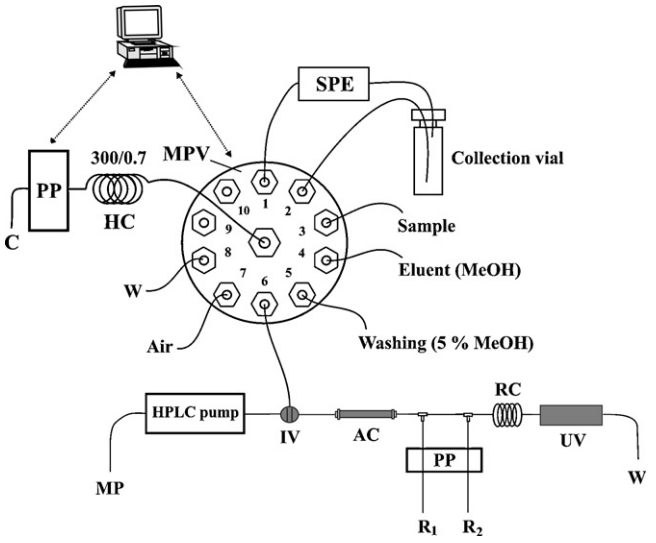

Fig. 1. Schematic diagram of the SI-SPE-HPLC–PCD setup for the determination of CAP: C, carrier (water); PP, peristaltic pump; HC, holding coil; MPV, multiposition valve; SPE, Oasis HLB SPE cartridge; W, waste; MP, mobile phase (MeOH:Na_2HPO_4 (20 mmol L^{-1}, pH = 2.5); 15:85 v/v, Q_V = 1.0 mL min^{-1}); IV = injection valve (V = 20 μL); AC = analytical column (Hypersil ODS, 100 mm × 4.0 mm i.d., 3 μm); R_1 = B–R buffer (c = 0.1 mol L^{-1}, pH = 11.5, Q_V = 0.25 mL min^{-1}); R_2 = EP (c = 10 mmol L^{-1}, Q_V = 0.25 mL min^{-1}); RC = reaction coil (90 cm/0.5 mm i.d.); UV = UV–vis detector (λ_{max} = 285 nm).

2. Experimental

2.1. Instrumentation and materials

The HPLC setup comprised the following parts: a AS3000 autosampler including a column oven (Thermo Scientific); a 7010 injection valve (Rheodyne, US) with a 20-μL sample injection volume; a LC-9A binary pump (Shimadzu); a SPD-10A UV–vis detector (Shimadzu) and an Elite™ vacuum degasser (Alltech). Data acquisition was carried out via the Clarity® software (DataApex, Czech Republic). The analytical column was a Hypersil ODS reversed phase column (100 mm × 4.6 mm i.d., 3 μm). The post-column reagents were propelled by a Minipuls3 peristaltic pump (Gilson, France). All post-column connections including reaction coils were made of PTFE tubing (i.d. = 0.5 mm).

The SI setup used in the automated solid phase extraction step was comprised: (i) a micro-electrically actuated 10-port valve (Valco, Switzerland); (ii) a Minipuls3 (Gilson, France) peristaltic pump equipped with Tygon tubes; (iii) PTFE tubing was used throughout the flow lines; (iv) a control program developed by the LabVIEW 5.1.1 instrumentation software package (National Instrument, U.S.). SI was interfaced to HPLC by direct connection of port 6 of the low pressure multiposition valve to port 1 of the high pressure injection valve (Fig. 1). The SPE cartridges (3 cc/60 mg, Oasis HLB, Waters) were incorporated in the SI manifold by a home-made push-fit interface [16].

2.2. Reagents and solutions

All chemicals used throughout this study were of analytical-reagent grade and commercially available (Merck, Sigma or Fluka). Ultra-pure quality water was produced by a Milli-Q system (Millipore).

The standard stock solution of CAP (γ = 500 mg L^{-1}, Sigma) was prepared in 0.01 mol L^{-1} EDTA (Merck) to prevent oxidation by metallic ions and was found to be stable for one week at 4 °C. Working solutions of ethyl-propiolate (c(EP) = 10 mmol L^{-1}, Sigma) were prepared daily by suspending the appropriate volume of the reagent in water and ultrasonicating for 5 min to facilitate complete homogenization. The Britton–Robinson (B–R) buffer consisted of a mixture of H_3PO_4, CH_3COOH and H_3BO_3 (0.1 mol L^{-1} each) and the pH was adjusted to the desired values by adding appropriate volumes of a NaOH solution (c = 2.0 mol L^{-1}). A mixture of 0.01 mol L^{-1} tris-(2-carboxyethyl)phosphine (TCEP, Sigma) and 0.01 mol L^{-1} EDTA was used as sample diluent.

2.3. SI-HPLC-PCD procedure

The SI-HPLC-PCD setup is depicted in Fig. 1. The SI sequence comprised the following steps: (i) activation of the column by 1000 μL MeOH followed by washing with 1000 μL water at a flow rate of 0.5 mL min^{-1}; (ii) loading of 2000 μL of sample (in 4 × 500-μL aliquots) in the Oasis HLB SPE column at a flow rate of 0.5 mL min^{-1}; (iii) washing the SPE column with 1000 μL of a 5% MeOH solution at a flow rate of 0.5 mL min^{-1}; (iv) elution of the retained CAP by 500 μL MeOH at a flow rate of 0.25 mL min^{-1} and collection to a suitable vial; (v) aspiration of 300 μL of the eluent and loading of the HPLC injection valve for HPLC-PCD analysis. In order to avoid dilution of the washing, elution and sampling solutions by the carrier stream, they were isolated by two segments of air (500 μL each).

Twenty microliters of standards and/or samples were injected via the autosampler to the analytical column. The mobile phase was a mixture of MeOH:phosphate buffer (20 mmol L^{-1}, pH = 2.5) at a ratio of 15:85. The flow rate was 1.0 mL min^{-1} and the column temperature 60 °C. The column eluent was merged downstream successively with reagent R_1 (B–R buffer, pH = 11.5) and reagent R_2 (EP, 10 mmol L^{-1}). The flow rate of each PCD reagent was 0.25 mL min^{-1}. The EP-CAP derivative was formed on passage through a 90-cm long reaction coil (RC, 0.5 mm i.d.) and monitored at 285 nm.

Under the above-mentioned conditions CAP was eluted at a t_R of 7.1 min. When real urine samples were analyzed the chromatograms were recorded for 15 min in order to exclude elution of strongly retained compounds. Since SI and HPLC-PCD operated independently the total analysis time was determined solely by the

HPLC cycle time of 15 min. Each sample or standard was injected in triplicate.

2.4. Preparation of samples

24-h urine samples from twelve healthy volunteers were collected for this study. For validation purposes, a pooled sample was prepared, filtered under vacuum through 0.45 membrane filters (Whatman) and kept at −18 °C. Prior to analysis, aliquots of the pooled urine sample were spiked with known amounts of the analyte and diluted 1:1 with the TCEP/EDTA solution. TCEP is a very effective reducing agent for thiols and is used to recover the oxidized form of the drug (CAP disulfide or mixed disulfides) in the samples, while EDTA prohibits catalytic oxidation of the analyte by trace metals. Individual urine samples were treated similarly.

3. Results and discussion

3.1. Development of the HPLC conditions

The investigation of the HPLC conditions was carried out by direct UV detection of CAP (γ = 100 mg L^{-1}) at 220 nm. A Hypersil ODS column (125 mm × 4.6 mm i.d., 5 μm) thermostated at 50 °C was used as starting column.

Initial experiments were carried out in order to select a satisfactory mobile phase additive for pH adjustment. Acetonitrile at a volume fraction of 15% was used as organic modifier and the pH was adjusted to 2.5 in all cases. Na_2HPO_4 (20 mmol L^{-1}), trifluoroacetic acid (0.05%, TFA) and triethanolamine (20 mmol L^{-1}, TEA) showed similar behaviour with the retention times being in the range of 4.4–4.6 min the peak symmetry between 1.05 and 1.15 and the theoretical plate number (per column meter) in the range of 8900–10,500. Acetic acid (0.1%) was less effective in terms of both peak symmetry and separation efficiency. Na_2HPO_4 at an amount concentration of 20 mmol L^{-1} was selected as mobile phase additive for simplicity and handling/safety reasons.

Column temperature and the pH of the mobile phase are expected to have the most significant impact on the chromatographic behaviour of CAP under the reversed phase mechanism. Previous studies have shown that slow isomerization kinetics of L-proline-containing compounds yield de-formed or even multiple peaks that are influenced dramatically by the temperature of the column and the pH [17]. CAP is an L-proline derivative and is known to undergo *cis* and *trans* isomerization on the time order of minutes [18]. The experiments confirmed the expected significant effect of the temperature. As can be seen in the overlaid chromatograms of Fig. 2, increase of the column temperature in the range of 25–70 °C resulted in a dramatic improvement of the chromatographic profile of CAP. For example, the peak width decreased by a factor of 3 and subsequently the number of theoretical plates increased by a factor of 5.5. The value of 60 °C was selected for further studies taking in mind the specifications of the column regarding its thermal stability under pro-longed operation. The effect of the pH was investigated in the range of 2.5–5.0. Increase of the pH resulted in decrease on the retention of CAP on the column until CAP was practically not retained at pH = 5.0 (t_R = 1.6 min). A pH value of 2.5 ensured sufficient retention (t_R = 4.1 min), good peak symmetry (<1.2) and separation efficiency (N/m = 11,000).

Attempting to further improve the HPLC separation we examined the chromatographic behaviour of CAP using a similar Hypersil ODS column with smaller particle size (100 mm × 4.0 mm i.d., 3 μm). Under the same conditions described above, the number of theoretical plates was improved significantly (15,400 versus 11,000) and the peak width was reduced (0.170 versus 0.277 min). A final series of experiments involved the replacement of the initially used acetonitrile with the more cost effective MeOH as organic modifier. The effect of the volume fraction of MeOH in the mobile phase was examined in the range of 15–20%. The experiments showed that a volume fraction of 15% provided satisfactory efficiency (N/m = 15,061) and peak symmetry (1.18), while the retention time of CAP was acceptable in terms of throughput (t_R = 7.1 min).

3.2. Investigation of the post-column derivatization conditions

Under the above-mentioned HPLC conditions, the main instrumental and chemical variables for the PCD reaction were investigated using the univariate approach. These variables were the flow rate of the post-column reagents, the length of the reaction coil, the pH and the amount concentration of the derivatization reagent. As can be seen in Fig. 1, the derivatization reagent (EP) and the buffer were kept at separate containers since earlier studies have proven limited stability of EP at alkaline pH [19]. The starting values of the PCD variables were: Q_V = 0.5 mL min^{-1} (0.25 mL min^{-1} each stream), l(RC) = 60 cm (0.5 mm i.d.), pH = 11.0 and c(EP) = 10 mmol L^{-1}.

The effect of the total flow rate of the post-column streams was investigated in the range of 0.4–1.0 mL min^{-1}, keeping the individual flow rates of the streams equal. Increase of the total flow rate of the PCD streams up to 1.0 mL min^{-1} resulted in a ca. 26% decrease in the area of CAP indicating fast reaction kinetics. The value of 0.5 mL min^{-1} (0.25 mL min^{-1} for each R_1 and R_2) was selected, corresponding to a total flow rate of the LC system – including the mobile phase – of 1.5 mL min^{-1}.

The length of the reaction coil is an important parameter in PCD methods since it determines the time that the reaction step is allowed to proceed. In the present study, the effect of the reaction coil was studied in the range of 0 (no reaction coil except for the necessary connections) to 120 cm using 0.5 mm i.d. coiled PTFE tubing. A ca. 60% increase in sensitivity was achieved in the range of 0–90 cm, while no improvement was observed thereafter. A length of 90 cm was therefore selected for subsequent experiments.

The pH is expected to be an important parameter that affects the PCD reaction. Effective reaction between EP and the thiolic analyte premises dissociation of the –SH group since it is based on the nucleophilic attack of the thiolate ion to the α-carbon atom of the triple bond of the ester [20]. According to the literature the pK_a value of the –SH group of CAP is 9.8 [21] and pH values in the alkaline region should be more suitable. Additionally, the buffer should have adequate capacity in order to provide a sufficiently alkaline medium after mixing with the acidic HPLC mobile phase (pH = 2.5). A pH range between 11.0 and 12.0 provided the highest and constant sensitivity. The middle value of 11.5 was therefore selected.

Increase of the amount concentration of EP from 5 to 10 mmol L^{-1} resulted in a ca. 15% increase in the signals. Further variation of the amount concentration of the derivatizing reagent up to 40 mmol L^{-1} offered negligible improvements (<10%). Taking in mind the consumption of the reagent under continuous flow PCD conditions, an amount concentration of 10 mmol L^{-1} was selected for subsequent studies.

Under the selected HPLC and PCD conditions linearity was obeyed in the range of 0.5–25.0 mg L^{-1} CAP (n = 8) with a slope of 186.2 (±1.5), regression coefficient of 0.9999 and percent residuals between −1.5 and 3.0%. The within-day precision at 1.0 and 5.0 mg L^{-1} was better than 2% (n = 8) and the day-to-day precision better than 5% (n = 6). Compared to direct UV detection of CAP without derivatization at 220 nm, the adaptation of the PCD step improved the sensitivity by a factor of ca. 5.6 – the slope at 220 nm was 33.5 (±0.6) – and shifted the detection wavelength at 285 nm where enhanced selectivity is expected for the analysis of

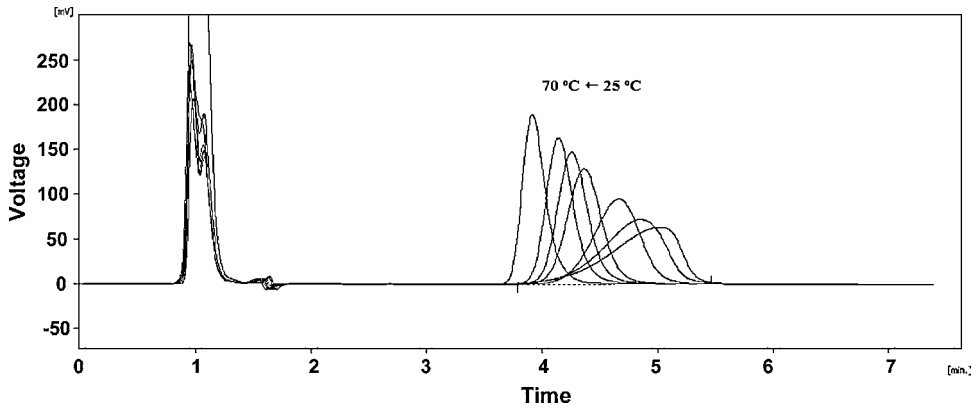

Fig. 2. Effect of the column temperature on the chromatographic behaviour of CAP (for experimental details see Section 3.1).

urine samples. The latter can be confirmed by the direct analysis of diluted (1:10) blank urine samples at 220 nm (Fig. 3A) and at 285 nm by the PCD protocol (Fig. 3B).

3.3. Development of the automated solid phase extraction step

The adaptation of a solid phase extraction step prior to HPLC analysis had two main goals: (i) the purification of the urine samples by removing potential interfering compounds and protecting the analytical column and (ii) the enhancement of the sensitivity by preconcentrating CAP. For this purpose the Oasis HLB copolymer SPE cartridges (Waters) were selected as this material offers some interesting features: (i) the hydrophilic–lipophilic character of the material enables retention of polar compounds as well; (ii) extreme pH tolerance; (iii) high recoveries using simple protocols and (iv) high extraction efficiency even if the material runs dry.

The suitability of the sorbent material to retain CAP was investigated by breakthrough studies [22]. In brief, a typical breakthrough experiment involved continuous pumping of an aqueous solution of CAP ($\gamma = 10\ mg\ L^{-1}$) through the SPE cartridge at a constant flow rate of 0.5 mL min^{-1}. Detection was carried out on-line at 220 nm. As can be seen in Fig. 4, the capacity of the Oasis HLB cartridge was satisfactory and was calculated to be 120 μg CAP (the inset diagram of Fig. 4 represents the same experiment in the absence of the SPE column). It should be noted that the criterion for breakthrough was set at a 5% increase of the UV absorbance at 220 nm.

In order to automate the SPE protocol all necessary steps (activation, loading, washing, elution) were carried out in a flow-through format using a sequential injection (SI) manifold (see Section 2.4 and Fig. 1). In first series of experiments we examined the behaviour of CAP on the Oasis HLB cartridge. The loading and elution (MeOH) volumes were equal to 1000 μL offering no preconcentration of the analyte. The experiments confirmed quantitative retention and elution of CAP from the SPE sorbent. A calibration curve using aqueous solutions in the range of 0.5–25 mg L^{-1} ($n = 8$) had excellent linearity ($r = 0.999$) and a slope that its value corresponded to 98% of the slope of the calibration curve without SPE. The precision of eight independent SPE experiments at the 5 mg L^{-1} level was 2.8%.

A second series of experiments was carried out in order to investigate the preconcentration capabilities of the automated SPE step. The sample volume was 2000 μL and the mass concentration of CAP 2500 μg L^{-1} (corresponding to an absolute amount of 5 μg CAP that is significantly lower than the capacity of the cartridge). Quantitative retention of CAP was observed in all cases. The volume of the elution solvent (MeOH) was varied between 250 and 2000 μL. Quantitative elution of CAP (95–98%) was observed in the range of 400–2000 μL. A MeOH volume of 500 μL offered a preconcentration factor of 4, linearity in the range of 150–2500 μg L^{-1} CAP ($r = 0.9991$, $n = 8$), LOD = 10 μg L^{-1} (S/N = 3) and LOQ = 35 μg L^{-1} (S/N = 10). A typical regression equation was:

$$A = [715.1(\pm 11.9) \times 10^{-3}] \times \gamma(\mathrm{CAP}) - 9.1(\pm 7.3)$$

A is the peak area and γ(CAP) the mass concentration of the analyte in μg L^{-1}. The day-to-day precision was validated for six days by constructing calibration curves in the range of 250–2000 μg L^{-1}. The relative standard deviation of the slopes was 2.9% ($n = 6$).

3.4. Application to urine samples

A pooled urine sample was treated according to section 2.4 and aliquots were spiked with elevated mass concentrations of CAP in the range of 250–2000 μg L^{-1}. After subjecting the samples to

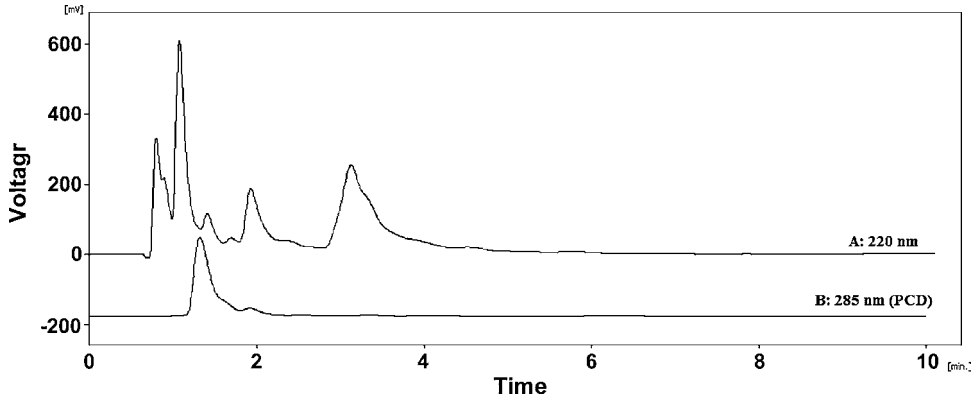

Fig. 3. Chromatographic profile of a 1:10 diluted blank pooled urine sample at (A) 220 nm using direct UV detection and (B) at 285 nm after PCD.

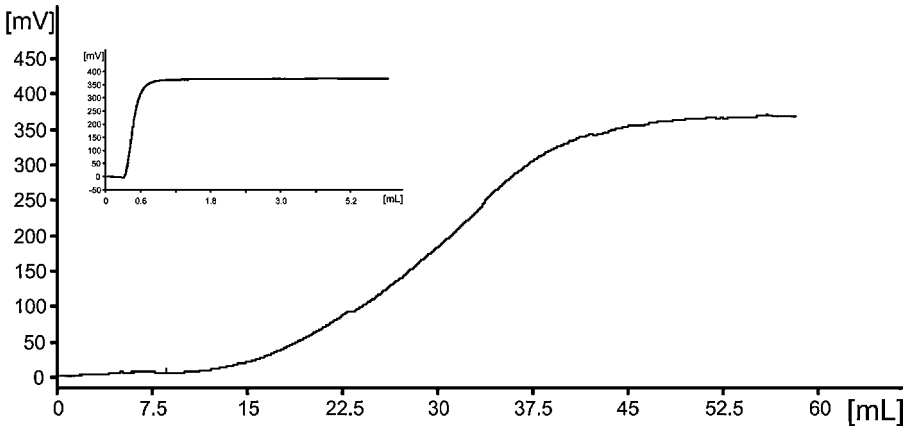

Fig. 4. Breakthrough studies of the retention of CAP on the Oasis HLB cartridge; γ(CAP) = 10 mg L^{-1}, Q_V = 0.5 mL min^{-1}, λ = 220 nm (inset diagram: same experiment without the SPE column).

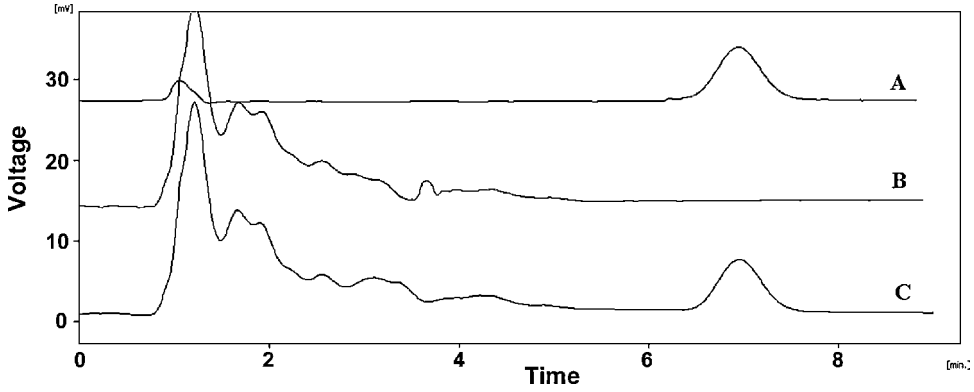

Fig. 5. Chromatogram of (A) an aqueous standard of CAP (250 μg L^{-1}), (B) a blank urine sample and (C) a spiked urine sample (γ(CAP) = 250 μg L^{-1}).

the automated SPE protocol they were analyzed by the proposed HPLC–PCD method. The results confirmed the suitability of the method since the linearity was excellent (r = 0.999) and the value of the slope of the resulted calibration curve was 97% of that of the respective curve using aqueous solutions. The within day precision of six independent SPE experiments was 4.2% (500 μg L^{-1} CAP). The day-to-day precision was also validated for six days. The relative standard deviation of the slopes was 4.8% (n = 6). Representative chromatograms of an aqueous standard of CAP (250 μg L^{-1}), a blank and a spiked urine sample (250 μg L^{-1} CAP) can be found in Fig. 5A–C respectively.

The proposed SI-SPE/HPLC-PCD method was applied to the analysis of individual urine samples spiked with CAP at three concentration levels (250, 500 and 750 μg L^{-1}). The percent recoveries are included in Table 1. The recoveries were calculated both by using the aqueous calibration curve and a "matrix-matched" curve using the pooled urine sample. Both approaches yielded satisfactory results with the recoveries being in the range of 90–96% and 95–104% respectively.

Table 1
Determination of CAP in human urine by the proposed method.

Sample	CAP added (μg L^{-1})	Recovery (%)	
		Aqueous curve	Matrix matched curve
Urine I	250	90	96
	500	96	103
	750	94	102
Urine II	250	94	97
	500	91	95
	750	93	98
Urine III	250	92	98
	500	95	104
	750	94	103

4. Conclusions

The proposed on-line SPE/HPLC–PCD method for the determination of CAP in human urine samples offers some interesting features: (i) it is more sensitive and simple compared to the only previously reported PCD method; (ii) sample preparation and analysis steps are fully automated by coupling SI and HPLC; (iii) the Oasis HLB SPE cartridges offer quantitative recoveries of the analyte from the biological matrix, preconcentration potentials and adequate capacity and stability; (iv) EP is an excellent reagent for CAP in terms of sensitivity and rapid reaction under flow conditions; (v) the method is capable of determining total CAP since the excess of the reducing reagent does not interfere in the PCD mode.

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