Solid phase extraction prior to non-aqueous capillary electrophoresis with ultraviolet/visible detection as a valuable strategy for therapeutic drug monitoring of cabozantinib

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ABSTRACT

Solid phase extraction (SPE) prior to non-aqueous capillary electrophoresis (NACE) with ultraviolet and visible detection was used for the first time for the determination of cabozantinib (CBZ), the main drug used for the treatment of metastatic renal cancer, in urine. SPE was carried out in commercial C_{18} cartridges loaded with 8 mL of urine. Tamoxifen (TAM) was added as internal standard. After removal of the matrix with 8 mL of phosphate buffer (pH 7.0; 10 mM), 7 mL of MeOH:H_2O (30:70; v/v) and 0.5 mL of MeOH, CBZ and TAM were eluted with 1.5 mL of methanol. The eluate was evaporated to dryness and reconstituted in background electrolyte (BGE). The CE method was carried out in a fused silica capillary (30 cm × 75 µm i.d.) at 19 ºC at 10 kV with a BGE consisting of ammonium acetate (pH 7.0; 8 mM) containing 3 % acetic acid (v/v) in methanol.

The preconcentration factor achieved was 5.3, and the limits of detection and quantification were 3.7 and 12 µg L^{-1} in urine, respectively. Linearity was observed up to 950 µg L^{-1}. Intra-day precision at 20, 400, and 850 µg L^{-1} of CBZ (*n*=8) was below 10 % in peak areas. As for inter-day precision, no statistical differences were found in two consecutive days, according to the Snedecor "F" test ($p>0.05$). Recovery studies in spiked urine samples ranged from 91.4 to 116.6 %.

The present method was applied to samples from a patient under treatment with CBZ for metastatic renal cancer as an alternative to the current strategies for therapeutic drug monitoring.

Keywords: Cabozantinib, renal cancer, urine, capillary electrophoresis, therapeutic drug monitoring.

1. INTRODUCTION

The appearance of oral tyrosine kinase inhibitors back in 2001 was an important milestone in the fight against cancer through targeted therapy. Since then, a number of drugs that inhibit the signalling pathways related to tumor cell growth, proliferation and metastasis have been approved [1].

One of these drugs is Cabozantinib (CBZ), a small molecule that inhibits a number of tyrosine kinase receptors, such as MET, RET, VEGFR-2, KIT, FLT-3, TIE-2, TRKB and AXL [2]. Its performance is excellent in the inhibition of VEGF, thus hindering angiogenesis in the tumor and, eventually, regression [3–5]. CBZ has shown antitumoral activity several cancers, i.e. breast, lung, prostate, and pancreas, and has been recently approved in the European Union and the USA for the treatment of metastatic renal cancer [6]. The recommended dose of CBZ is 60 mg a day for this kind of cancer and the maximum concentration in blood is reached 3 – 5 h after intake, but successive peaks can be observed later due to the enterohepatic recirculation. The drug is metabolized to its N-oxide derivative mostly by CYP3A4 and, to a minor extent, by CYP2C9. However, the parent compound appears to be the principal, pharmacologically active circulating analyte [7]. The elimination is via the hepatic (54% of the administered dose) and renal route (27%) [8].

Despite the positive features of CBZ in the treatment of renal cancer, safety is still a matter of concern due to side-effects. Therefore, a monitoring of the patient is required in case unacceptable toxicity occurs and dose reduction or temporary interruption of the treatment is necessary [9]. In this way, there is a sore need of analytical methods that provide information on therapeutic drug monitoring (TDM) in a fast, simple, cost-effective and reliable way in routine laboratories so that dosing can be promptly and safely adjusted to each patient.

Due to the recent launch and approval of CBZ, the related analytical literature is scarce. All of the published analytical methods for the determination of CBZ are based on liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) after a simple step of sample preparation, and applied to plasma samples. Due to the high selectivity and sensitivity of MS detection, there is no need for preconcentration in the sample preparation. The earliest two works reported information on pharmacokinetics of CBZ [10] and its main metabolite, the N-oxide derivative [11]. Liquid-liquid extraction with ethyl acetate and protein precipitation with an acetonitrile:methanol (MeCN:MeOH) mixture were carried out for sample preparation, respectively. The authors used rat serum and found lower limits of quantification (LLOQ) of 0.5 ng mL^{-1} in both studies. Later on, another work aimed at studying pharmacokinetics of CBZ and other oral oncolytics in human plasma reported a LOD of 100 ng mL^{-1}. More recently, a method to monitor the concentration of this drug in plasma was developed and validated after a single precipitation step with MeCN [12]. This method was set up specifically for TDM of

CBZ in patients of renal cancer treated with the standard 60 mg dose. In this case, the LOQ was 25 ng mL^{-1}, which is adequate for the determination of therapeutical levels in plasma, ranging from 200 to 2100 ng mL^{-1}.

LC-MS/MS meets the requirements of selectivity and sensitivity for the determination of CBZ in plasma form patients under treatment for renal cancer. However, it requires a sophisticated and expensive instrument and also expensive consumables, i.e. LC-MS grade solvents and, in some cases, isotopically modified internal standards. All these are important shortcomings when it comes to implement this approach in clinical and/or routine laboratories, up to the point of making it unaffordable for most of them. Capillary electrophoresis with ultraviolet/visible detection (CE-UV/Vis) is a good alternative because it combines excellent separation efficiency, with high speed, selectivity, simplicity and economy of use [13]. Another major advantage is that the consumption of reagents and samples is typically in the range of nanoliters, which makes a big difference when only a little amount of sample is available. In case the analytes are little soluble in water, the non-aqueous capillary electrophoresis (NACE) mode can be used to sort this out, just by dissolving the analytes in an organic but conductive solvent, such as MeOH. The main limitation is sensitivity due to the reduced optical path, typically the inner diameter of the capillary but preconcentration techniques can be used to achieve the required levels. The most popular is solid phase extraction (SPE), a consolidated and cost-effective technique. Manufacturers offer a wide variety of SPE devices with high quality standards that ensure a high reproducibility of results.

As for specimens, only plasma samples have been studied and other biological fluids have been ignored. The case of urine is particularly interesting because it is an important way of excretion and this fluid can be easily obtained from patients in a non-invasive manner.

Therefore, the aim of this work is to develop and validate a method for the determination of CBZ in urine using SPE prior to CE-UV/Vis as a valuable alternative for TDM of patients undergoing treatment in terms of simplicity of handling, economy of use and reproducibility in comparison to previously reported LC-MS methods.

2. MATERIALS AND METHODS

2.1 Apparatus

A Beckman P/ACE System MDQ CE equipment with diode-array detection (DAD) and controlled by a P/ACE System MDQ CE software was used. Separations were carried out in a 30 cm (20 cm to the detector) × 75 µm i.d. fused-silica capillary housed in a cartridge with an 800 × 100 µm detection window.

A Crison micro-pH 2002 instrument was used for pH measurements. A Supelco (Bellefonte, PA, USA) vacuum manifold coupled to a Millipore (Milford, USA) vacuum pump was used for SPE.

A Selecta centrifuge (S-240, Barcelona, Spain) was used.

2.2 Standards, reagents and solutions

All solvents and reagents were of analytical grade or higher. MeOH was purchased from Panreac HPLC quality (Barcelona, Spain) and acetic acid was from Merck (Darmstadt, Germany). Ultrapure water of Milli-Q quality (Millipore) was used throughout the study.

CBZ (99% of purity) was purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA). Tamoxifen (TAM) (99% of purity), used as internal standard, was supplied by Sigma Life Science. Stock solutions 1 g L^{-1} of CBZ and TAM were prepared in MeOH and stored in amber glass vials at 5 ºC. The stock solutions were stable for at least 3 months. Intermediate solutions of 0.1 g L^{-1} were obtained by dilution in MeOH and stored at 5 ºC. Working solutions at 2 and 0.5 mg L^{-1} of CBZ and TAM, respectively, were obtained by the appropriate dilutions in MeOH.

Sep-Pak t_{C18} Plus SPE cartridges (500 mg) were from Waters (Milford, USA). Ammonium acetate (99 % of purity) was from Fluka Analytical (Netherlands).

2.3 Separation by NACE-UV/Vis

Before using for the first time, the capillary was conditioned by flushing water for 10 min, 0.5 M NaOH for 10 min and water for 3 min. The capillary was rinsed with 0.5 M NaOH for 1 min, water for 0.5 min and background electrolyte (BGE) for 2 min between consecutive injections.

The BGE was an ammonium acetate solution (pH 7.0; 8 mM) containing 3 % acetic acid (v/v) in MeOH. Different sets of vials were filled with BGE to be specifically used either for rinsing or separating to avoid siphoning, and all were refilled regularly with fresh volumes of it.

Samples were injected hydrodynamically for 3 s. The separation temperature was 19 ºC and the voltage was set up at 10 kV for 9 minutes. The DAD signal was acquired at 243 nm for CBZ and 202 nm for TAM, which are the wavelengths of maximum absorbance of each compound.

2.4 Sample preparation

Urine samples from a patient treated with 60 mg of CBZ a day in the Oncology Department of Hospital General of Ciudad Real (Spain) were obtained after their informed consent. Urine samples from healthy volunteers were used as controls.

SPE cartridges were conditioned with 5 mL of MeOH and 5 mL of phosphate buffer (pH 7.0; 10 mM). A volume of 8 mL of urine spiked with TAM at 500 μg L^{-1} was loaded and allowed to contact with the sorbent for 2 min. The matrix was removed from the cartridge with 8 mL of phosphate buffer (pH 7.0; 10 mM), 7 mL of MeOH:H_2O (30:70; v/v) and 0.5 mL of MeOH. Later, CBZ and TAM were eluted with 1.5 mL of MeOH, evaporated to dryness and reconstituted with 1.5 mL of 8 mM ammonium acetate containing 2 % of acetic acid.

This sample treatment allows to preconcentrate the sample up to 5.3 times. Once the treatment of the sample was done, it was ready to be analyzed by CE-UV/Vis.

3. RESULTS AND DISCUSSION

3.1 Optimization of the electrophoretic method

Preliminary experiments were carried out in the capillary zone electrophoresis (CZE) mode in aqueous media. Regardless of the composition of the BGE, ca. pH and organic modifiers, power cuts and elevated noise occurred, and more importantly, no peaks in the corresponding electropherograms were found, even for concentrations of up to 2 mg L^{-1} of CBZ. A similar situation was found when micellar electrokinetic chromatography was used as electrophoretic mode, in the presence of anionic micelles of sodium dodecylsulphate. This behavior was attributed to the poor solubility of CBZ in water, so the selected mode for further experiments was NACE. In this mode, the analytes are dissolved in a non aqueous but conductive solvent, such as MeOH. Doing so, the obtained electropherograms showed little noise, no power cuts occurred and the peak corresponding to CBZ was clearly identified.

The initial chemical conditions of the method were a BGE consisting of an ammonium acetate solution (10 mM) containing 3 % acetic acid (v/v) in MeOH. The initial instrumental conditions were 22 ºC of temperature, 10 kV of voltage and 3 s of hydrodynamic injection at 0.5 psi. These conditions were selected carefully in order not to be over 20 μA of current, which would lead to formation of bubbles in the capillary due to solvent boiling and, as a consequence, result in power cuts. A standard solution of CBZ at 2 mg L^{-1} in BGE was used throughout the optimization process. The optimization of the chemical and instrumental parameters of the NACE method was carried out by varying one by one, whilst keeping the rest constant.

3.1.1. Optimization of the chemical parameters

The influence of the concentration of ammonium acetate in the BGE was studied from 5 to 20 mM. Lower concentrations were not considered for this study because they might not buffer adequately. The results showed an increase in migration time of CBZ with the concentration of this parameter (Fig 1a). This is due to the compression of the diffuse double layer in the capillary wall, which results in a decrease of the electroosmotic flow (EOF) as the ionic strength increases. The current generated was

from 7.2 to 26.6 µA. Although the shortest migration time was obtained for 5 mM, the current generated at that concentration was too low (7.2 µA). Therefore, the optimum concentration was 8 mM because the migration time was still short and enough current (11.4 µA) was ensured.

The other chemical parameter optimized in the BGE was the percentage of acetic acid, which was studied up to 4 % (Fig 1b). The migration time of CBZ decreased as the percentage increased because CBZ seems to acquire higher positive charge density as the percentage of acetic acid increases, and thereby the electrophoretic mobility increases, too. Moreover, the peak width and tailing decreased as the percentage of acetic acid increased. The optimum percentage was 3 % because peak width was enough to achieve an adequate resolution from the EOF and peak tailing was negligible. Plus, the current was lower than for 4 %.

Apart from the composition of BGE, the medium in which the analytes are dissolved can affect diffusion of analytes in the sample zone and sometimes it makes a difference in terms of peak tailing. In this case, CBZ was dissolved in a methanolic solution of 8 mM ammonium acetate containing percentages of acetic acid from 0 to 3 %. The results showed that a 2 % of acetic acid in the sample provided no peak tailing, so this concentration was selected as optimum. This is due to the the fact that CBZ migrates faster in the sample zone than in the BGE, which has a higher ionic strength, and stacks in its front border. This stacking effect greatly avoids diffusion of the analytes and thereby peak tailing.

3.1.2. Optimization of the instrumental parameters

Instrumental variables such as temperature and voltage affect the electroosmotic mobility of the analytes and, consequently, have to be optimized in a method set up.

The influence of temperature was studied from 18 and 22 ºC. No values beyond this point were considered because of the high risk of bubble generation inside the capillary, taking into account that the BGE is a methanolic solution. The results showed a low decrease in migration time of CBZ and a moderate increase in peak width as temperature increased (Fig 1c). This is consistent with the fact that an increase in temperature produces a decrease in viscosity. This increases the EOF and makes analytes migrate faster, but also increases diffusion and, consequently, peak broadening occurs. In this case, 19 ºC was selected as optimum temperature because it provided the lowest peak width with an acceptable increase in migration time.

As for voltage, the range studied in the optimization was from 5 to 20 kV. Firstly, it was tested if the heat generated by the Joule effect due to the increase of the voltage, was properly dissipated by the capillary. That was demonstrated in the plot of current vs. voltage, which was linear along the studied range (Fig S1). Moreover, the maximum current obtained, for 20 kV, was right below 20 µA, which would result in formation of

bubbles inside the capillary, as explained is section 3.1. Regarding migration time, it decreased as voltage increased (Fig 1d). A compromise between migration time and current generated was found at 10 kV, which was selected as optimum.

Finally, the influence of injection time was studied from 3 to 10 s at 0.5 psi. On one hand, increasing injection time improved the intensity of the signal but, on the other hand, it led to base line distortion and even to power cuts. A time of 3 s was selected because none of these negative effects was reported, yet signal intensity was sacrificed.

3.2 Optimization of sample preparation

The objectives of sample preparation are to remove interferents from the matrix and to achieve a suitable preconcentration of the analytes. In the case of SPE, there are four steps to achieve this goals, namely, conditioning, loading, clean up and elution. The internal standard, TAM, was spiked to the urine at 500 μg L^{-1} to correct posible losses of analyte along the process. TAM was selected because the elution in the SPE was the same as CBZ and the migration times in NACE are sufficiently different from each other.

The conditioning of the cartridge was carried out following the recommendations of the manufacturer with 5 mL of MeOH and 5 mL of phosphate buffer (pH 7.0, 10 mM), successively. Sample loading is the maximum volume of sample that a cartridge can take without overloading. Following the experience aquired with urine samples in these kind of SPE devices, this volume was 8 mL [14]. In the clean up step, interferents of different polarities need to be removed. Phosphate buffer (pH 7.0, 10 mM) was selected to remove highly polar interferents, while MeOH:H_2O (70:30, v/v) was used for low polar ones. Most interferents of urine were removed with 8 mL of phosphate buffer (pH 7.0, 10 mM), 7 mL of MeOH:H_2O (70:30, v/v), and 0.5 mL of MeOH, successively. Finally, CBZ and TAM were eluted from the cartridge with MeOH in fractions of 0.5 mL. The elution profile indicated that CBZ and TAM were in the first three fractions, so 1.5 mL of MeOH was selected as the optimum elution volume.

The preconcentration factor (PF), defined as the urine volume over the elution volume, is 5.3.

As an example, Figure 2 shows the electropherogram of a urine sample from a healthy volunteer spiked with CBZ at 70 μg L^{-1} and TAM at 500 μg L^{-1} under the optimum extraction and electrophoretic conditions. The electropherogram is shown at 238 nm for illustration purposes.

3.3 Validation

The validation was carried out in spiked urine samples and the figures of merit are in Table 1. The limits of detection (LOD) and quantification (LOQ) were estimated in accordance with the baseline noise of a blank urine after being submitted to the

method. The baseline noise was evaluated by recording the detector response over a period of about ten times the peak width. The LOD and LOQ were estimated as the concentration of CBZ in sample that provided a peak with a height three and ten times the baseline noise level, respectively. Thanks to the preconcentration factor of 5.3, the values were 3.7 and 12 μg L^{-1} in urine, that is, in the range of previously reported methods based on LC-MS/MS [10–12,15].

A calibration curve was prepared by spiking urine at nine levels from 15 to 950 μg L^{-1} of CBZ and 500 μg L^{-1} of TAM as internal standard. These were submitted to the whole method and injected by triplicate. The analytical signal was the average relative peak area (A_{CBZ}/A_{TAM}) at the wavelengths of maximum absorbance of each compound. The response of the detector was linear within the whole concentration range, with a determination coefficient close to 1 and a negligible intercept according to Student's t-test (p>0.05).

Intra-day precision was studied on spiked urine samples at three different concentrations of CBZ 20, 400, and 850 μg L^{-1}. After processing, the corresponding extracts were injected eight times in the same day. As for the assessment of inter-day precision, a different operator in a different day the same experiments. As shown in Table 1, the highest relative standard deviations (RSDs) in peak area were found for the lowest concentrations in both intra-day and inter-day precision. Moreover, no statistical differences were found in the precision obtained in the two days, according to the Snedecor "F" test (p>0.05).

Recovery studies on spiked urine samples at seven levels from 150 to 800 μg L^{-1} were carried out to evaluate accuracy. The obtained extracts were injected in the CE-UV/Vis system in duplicate and the recoveries obtained ranged from 91.4 to 116.6 %.

3.4 Application

The present method was applied to urine samples from a patient with metastatic renal cancer treated with Cabometyx® (60 mg of CBZ) once a day. The urine was sampled at seven different times after the drug intake. The extracts were injected in duplicate and the concentrations were calculated using the calibration curve.

The results are in Table 2 and show that the concentraton of CBZ increases up to 7 h, where the maximum occurs, followed by a decrease below LOD for longer times until it is detectable again at t = 23 h. This pattern is consistent with the enterohepatic recirculation of CBZ reported elsewhere [11]. An electropherogram corresponding to the analysis of the urine at t = 7 h is shown in Figure 3.

The indentification of CBZ was confirmed in this sample by adding an equivalent amount of the standard to the extract, which doubled the peak area. This shows that detectable levels of CBZ as parent compound can be found in urine.

3.5 Comparison with literature

Since this is the first work reporting determination of CBZ in urine, it is no possible to compare the concentrations found in the analysed sample with other reports. As general information, the levels found in urine are lower than the ones reported previously in plasma of patients undergoing the same treatment, ca. 203 – 2100 μg L^{-1} [12].

Nonetheless, a comparison in analytical terms of the proposed approach with previous methods is shown in Table 3. As can be seen, this is the first method based on NACE-UV/Vis. It is also the only one with SPE as sample preparation technique, which enables a preconcentration factor of 5.3 in this case. This leads to a LOQ in the range of μg L^{-1}, as in those reported by LC-MS/MS, which is inherently more sensitive than NACE-UV/Vis.

Intraday precision in the present method showed the lowest RSD (3.7 %) in comparison with the previous literature and intraday precision was the second lowest (9.3 %). It should be noted that these figures are better than the ones obtained with the use of isotopically modified internal standards, which theoretically are the best option in method validation.

4. CONCLUSION

SPE combined with NACE-UV/Vis has been used for the first time for the determination of CBZ in urine. This conventional approach provides an adequate answer to the analytical problem concerned in this article without the need of other sophisticated and expensive thechniques. Moreover, the use of urine as specimen brings the opportunity of monitoring CBZ after intake in a non invasive way, which is most valuable for TDM.

Unlike LC-MS/MS, the most widely used technique for this kind of analysis, NACE-UV/Vis is simple to operate, economical in terms of acquisition and consumption of reagents, suitable for samples with limited or poor availability, and environmentally friendly as it generates little waste. The present method provides high selectivity, precision, quantitative recoveries and LOD/LOQs in the range of those obtained with more sophisticated and expensive LC-MS/MS, which makes it competitive in comparison to them.

5. ACKNOWLEDGEMENTS

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Legends to Figures

Figure 1. Influence of a) the amount of ammonium acetate; b) acetic acid percentage; c) temperature; d) voltage on migration time of CBZ.

Figure 2. Electropherograms of a urine spiked at 70 µg L^{-1} of CBZ and 500 µg L^{-1} of TAM at 238 nm.

Figure 3. Electropherogram of a urine sample under treatment with 60 mg of CBZ once a day recorded at 238 nm, 7 h after intake.

Figure 1a

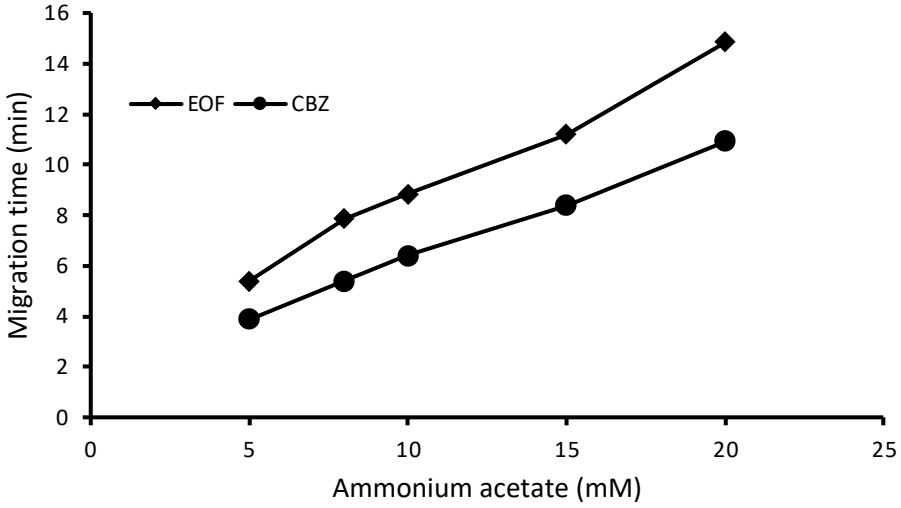


Figure 1b

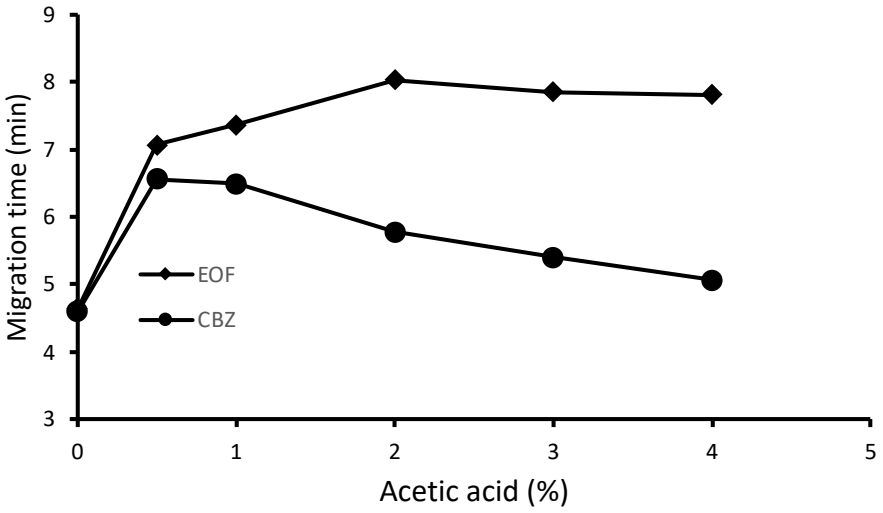


Figure 1c

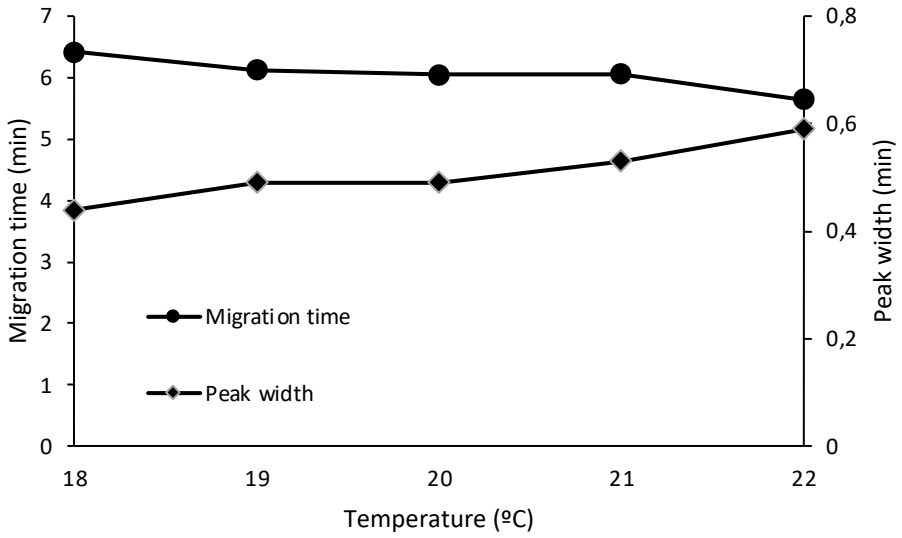


Figure 1d

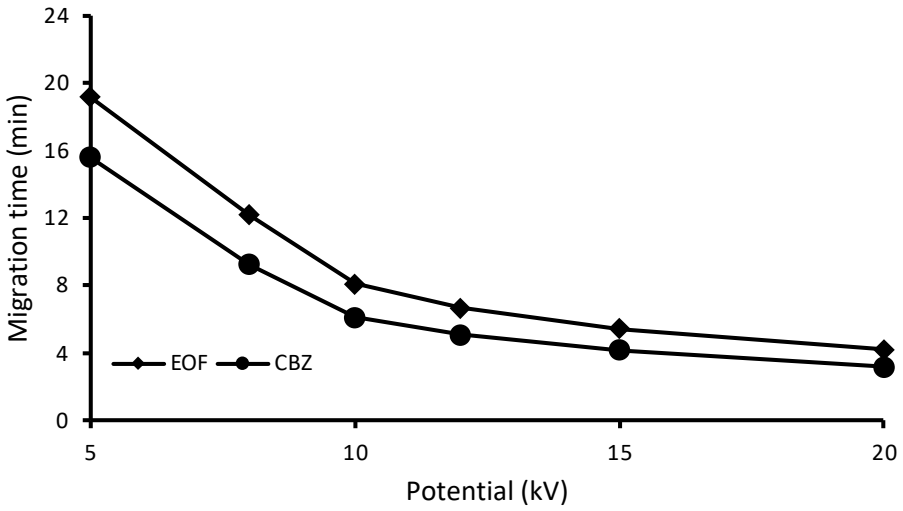
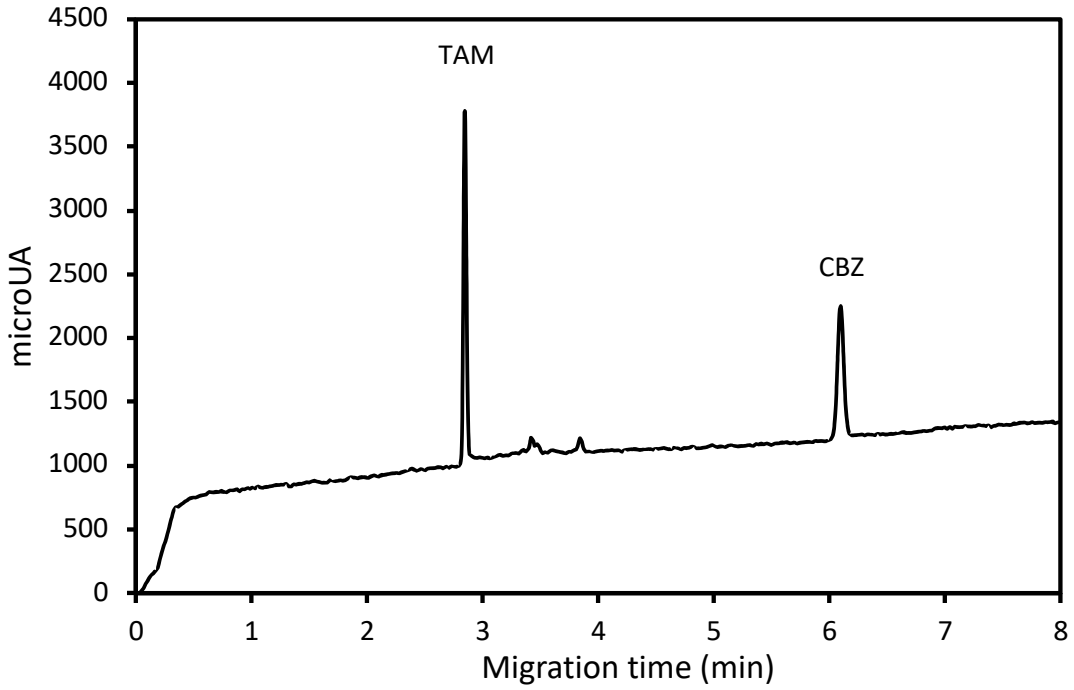


Figure 2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8
Migration time (min)

Figure 3

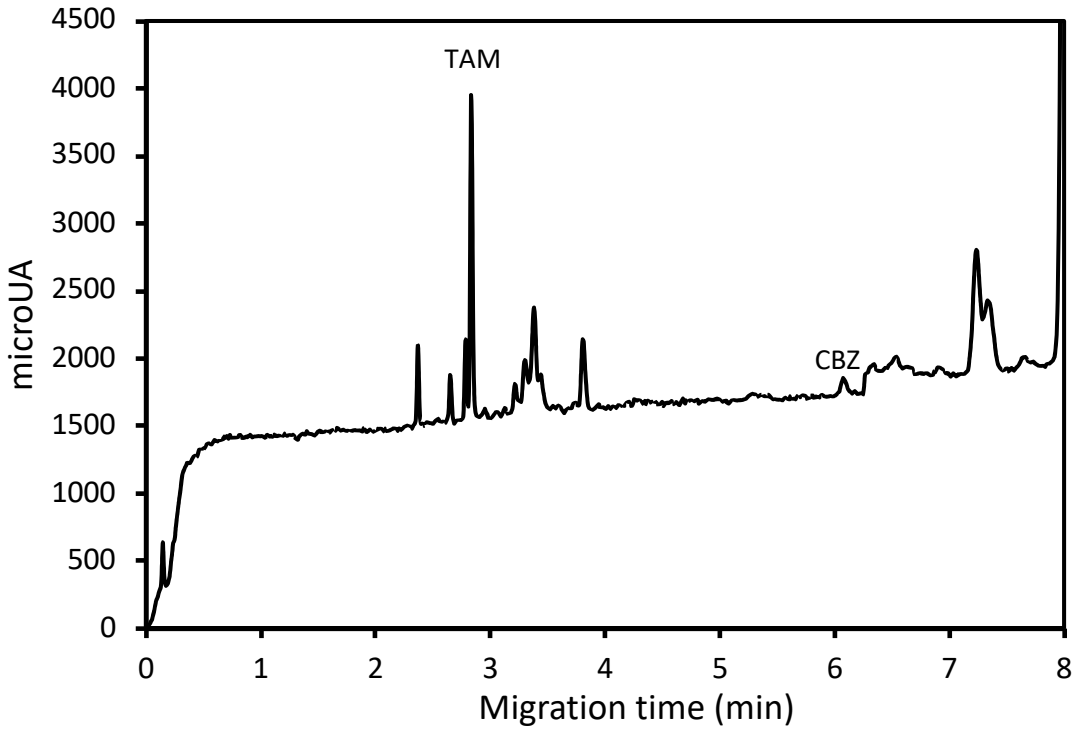
4500
4000
3500
3000
2500
2000
1500
1000
500
0
microUA
TAM
CBZ
0
1
2
3
4
5
6
7
8
Migration time (min)

Table 1. Analytical figures of merit. Standard deviation of intercept and slope in parenthesis. Precision expressed as day 1/day 2 for each concentration.

Calibration curve	r^2	LOD/LOQ ($\mu g\ L^{-1}$)	Precision (*n*=8, % RSD)			Accuracy (%)	
			20 $\mu g\ L^{-1}$	400 $\mu g\ L^{-1}$	850 $\mu g\ L^{-1}$	Mean ± SD (*n*=7)	Range
$A = 7.8x10^{-3}(\pm 0.1x10^{-3})\ c + 0.03(\pm 0.05)$	0.998	3.7/12	9.3/6.9	3.7/3.7	3.9/4.1	101.3 ± 8.9	91.4 – 116.6

A: Relative peak area (arbitrary units); c: concentration ($\mu g\ L^{-1}$).

Table 2. Concentration of CBZ (mean ± standard deviation, *n*=3) in urine of a patient under treatment for metastatic renal cancer.

Time after intake(h)	Concentraton of CBZ (µg L^{-1})
2	12 ± 1
4.5	13 ± 1
7	17.3 ± 0.7
9.5	<LOD
12	<LOD
20	<LOD
23	<LOQ

Table 3. Comparison with literature.

Sample preparation	Instrumental technique	LOD/LOQ ($\mu g\ L^{-1}$)	Precision (intra/interday) (% RSD)	Internal standard	Reference
LLE	LC-MS/MS	n.a./0.5	8.76/14.1	Erlotinib	[10]
Precipitation	LC-MS/MS	n.a./0.5	10.9/14.3	HZ08*	[11]
Precipitation	UPLC-MS/MS	n.a/100	10.4/7.4	$^{2}H_4$-CBZ	[15]
Precipitation	LC-MS/MS	n.a./25	6.47/7.2	$^{2}H_4$-CBZ	[12]
SPE	NACE-UV/Vis	3.7/12	3.7/7.3	Tamoxifen	This work

n.a.: not avaliable; *N-cyano-1-[(3,4-dimethoxyphenyl) methyl]-3,4-dihydro-6,7-dimethoxy-N'-octyl-2-(1H)-isoquinoline-carboximidamide.