Environmental Pollution 213 (2016) 308–313

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Environmental exposure to pharmaceuticals: A new technique for trace analysis of carbamazepine and its metabolites in human urine☆

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ARTICLE INFO

Article history:
Received 15 December 2015
Received in revised form
11 February 2016
Accepted 13 February 2016
Available online xxx

Keywords:
Recovery
Treated wastewater
Exposure
LC/MS/MS

ABSTRACT

Pharmaceutically active compounds are taken up and accumulate in crops irrigated with treated wastewater. This raises the concern of chronic human exposure to pharmaceuticals via food consumption. Thus, there is a need to develop a reliable technique to detect and quantify pharmaceuticals at environmentally relevant concentrations in human biological matrices, particularly urine. In this study, we focus on carbamazepine, an antiepileptic drug and recalcitrant compound that is taken up by crops—making it an excellent model compound for this study. This paper presents a new analytical technique enabling quantification of trace concentrations of carbamazepine and its metabolites in the urine of individuals who have been environmentally exposed. Sample preparation included extraction with acetonitrile followed by clean-up through mixed-mode ion-exchange cartridges and analysis using LC/MS/MS. This technique, which was validated for a wide range of concentrations (5–2000 ng L^{-1}), exhibits low limits of quantification (3.0–7.2 ng L^{-1}), acceptable recovery levels (70–120%), and low relative standard deviation (<20%). Unlike currently available methods for the analysis of water or treated wastewater that require large volumes (up to 1 L), the new method uses only 10 mL of urine. Moreover, relative to available methods for carbamazepine detection in the urine of individuals who are chronically treated with this drug, the limit of quantification values with our method are six orders of magnitude lower. The newly developed method has been successfully applied for the quantification of carbamazepine and its metabolites in the urine of healthy people exposed to this pharmaceutical through their diet. Our analytical protocol can provide the scientific community and stakeholders with real data for risk assessments and the design of policies ensuring safe use of wastewater for crop irrigation.

1. Introduction

To meet the mounting water demands by municipal and industrial sectors, treated wastewater is used as an alternative water source in the agricultural sector. In arid and semi-arid zones such as the Middle East, parts of India and China, Mexico and southern regions of the United States, treated wastewater is used as a valuable source for crop irrigation (Sato et al., 2013). However, even when treated wastewater meets irrigation standards, it contains a wide array of organic pollutants, including active pharmaceutical compounds and personal care products, hormones, endocrine-disrupting chemicals, plasticizers, surfactants, fire retardants, perfluorinated compounds, synthetic musks and pesticides (Al-Odaini et al., 2010; Cahill et al., 2011; Ghosh et al., 2010; Kasprzyk-Hordern et al., 2009; Lee et al., 2010; Pan et al., 2011; Robert-Peillard et al., 2015; Woudneh et al., 2015). The presence of these organic pollutants in the irrigation water raises safety concerns regarding

☆ This paper has been recommended for acceptance by Charles Wong.
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http://dx.doi.org/10.1016/j.envpol.2016.02.027
0269-7491/

possible exposure of the population to them through consumption of wastewater-irrigated crops.

Pollutants of emerging concern, such as pharmaceutical compounds and personal care products, have been the focus of many studies (Nakada et al., 2007; Oliveira et al., 2015; Pennington et al., 2015; Qin et al., 2015). These compounds are not effectively removed by wastewater treatment; thus they are ubiquitous in effluents (Bartelt-Hunt et al., 2009; Jones-Lepp et al., 2012; Yargeau et al., 2014; Zhang et al., 2008), as well as in the receiving environment (Duan et al., 2013). The antiepileptic drug carbamazepine (CBZ) is one of the most frequently detected pharmaceuticals in aquatic environments, including effluent wastewater (Bahlmann et al., 2014), surface water (Zhang et al., 2007), groundwater (Ternes et al., 2007) and even drinking water (Guo and Krasner, 2009). In addition to the parent compound, metabolites of CBZ are also found in wastewater effluents (Bahlmann et al., 2014). Negative effects of CBZ on certain aquatic species have already been shown (Ferrari et al., 2003; Li et al., 2010) and in recent years, a growing number of reports have suggested that CBZ is taken up by crops irrigated with treated wastewater (Goldstein et al., 2014; Malchi et al., 2014; Wu et al., 2013). CBZ is known to be highly stable in the environment (Grossberger et al., 2014; Tixier et al., 2003) and thus can be a suitable marker for anthropogenic pollution, as well as for human exposure to pharmaceuticals via consumption of crops irrigated with treated wastewater.

To assess human exposure to CBZ and other xenobiotics via dietary consumption of fruits and vegetables, it is important to develop a reliable technique for the detection and quantification of trace concentrations of these compounds in urine samples. Several analytical methods for the quantification of different pharmaceuticals in environmental samples have been recently developed (Chen et al., 2015; Fick et al., 2010; Huerta et al., 2013; Miao et al., 2005; Tanoue et al., 2014). Environmentally relevant limits of quantification (low ng g^{-1} and ng L^{-1} ranges) have been reported. However, those methods have been applied for analysis of CBZ in water, biosolids, fish muscle, liver and bile; none have been—nor can they be—used to analyze urine. Moreover, most of the available methods for analysis of pharmaceutical compounds, including CBZ, in water require large sample volumes (100–1000 mL) to ensure a sufficient level of the analyte. This cannot be applied to urine which is obtained in limited volumes (typically <100 mL). On the other hand, available methods for analysis of CBZ in the urine (Rani et al., 2012; Rezaee and Mashayekhi, 2012) of patients who are being chronically treated with this drug (daily doses of 400 to 1000 mg) are not applicable because they deal with concentrations that are six orders of magnitude higher than what is expected for environmental exposure. Therefore, the objective of this study was to develop a reliable technique for the detection and quantification of CBZ and its metabolites in human urine at trace (i.e., nanogram per liter) levels to quantify environmental exposure of this drug.

2. Methodology

Technique development included the following steps: 1) sample extraction and clean-up procedure; 2) analysis of CBZ and its metabolites by LC/MS/MS; 3) method validation, and 4) applicability of the developed technique to real samples.

2.1. Materials and analytical standards

Analytical standards of 10,11-epoxy-10,11-dihydro-carbamazepine (EP-CBZ; 96% purity), 2-hydroxy-carbamazepine (2-OH-CBZ; 98%), 3-hydroxy-carbamazepine (3-OH-CBZ; 98%), 10,11-dihydro-10-hydroxy-carbamazepine (10-OH-CBZ; 98%), 10,11-dihydro-trans-10,11-dihydroxy-carbamazepine (DiOH-CBZ; 97%), 10,11-dihydro-10-hydroxy-CBZ glucuronide (CBZ-O-glucuronide, 98%), 10-OH-CBZ-D3 (99.4%), EP-CBZ-D8 (98%) and CBZ-D2-^{13}C (99%) were obtained from Toronto Research Chemicals Inc. (North York, Canada). CBZ (97% purity) was purchased from Sigma–Aldrich Israel Ltd. (Rehovot, Israel). Weak anion exchange and weak cation exchange Strata X anion weak (AW) and cation weak (CW), respectively (33 μm, 200 mg) and Strata X polymeric reversed-phase sorbent (33 μm, 500 mg) were purchased from Phenomenex Inc. (Torrance, CA). β-Glucuronidase (*Escherichia coli* type IX-A, 125 kU) for deconjugation of CBZ glucuronide was purchased from Sigma–Aldrich Israel Ltd.

2.2. Sample extraction and clean-up procedure

For the sample extraction and clean-up procedure, blank samples (deionized water and urine free of CBZ and its metabolites) and urine samples fortified to 10 and 200 ng L^{-1} (native compound and labeled compound, respectively) were used. Urine samples (10 mL) were frozen in liquid nitrogen and then freeze–dried. Acetonitrile (3 mL) was added to the freeze–dried urine samples followed by 5 min sonication. The liquid phase (extract) was transferred to a 10 mL glass tube containing 1 mL of 20 mM ammonium acetate buffer (pH 6–7). Further clean-up of extracts was carried out using Strata X CW and Strata X AW cartridges. Before use, the cartridges were washed with 2 mL of MeOH and pre-conditioned with 2 mL of acetonitrile/buffer (75/25 v/v). The extracts were loaded onto the solid-phase extraction (SPE) cartridges: Strata X CW was connected with an SPE tube adapter to Strata X AW. The eluents were evaporated to dryness and then re-dissolved in 100 μL of mobile phase (water/acetonitrile, 80/20) acidified with 1% acetic acid and analyzed by LC/MS/MS.

We also quantified the level of CBZ-O-glucuronide in urine samples as it is one of the most abundant metabolites of CBZ excreted in the urine. A de-conjugation test was conducted by incubating urine samples with β-glucuronidase (5 kU of enzyme per 10 mL sample) at 37°C overnight. Thereafter the samples were treated as described above.

2.3. LC/MS/MS analysis

Analysis of CBZ and its metabolites was carried out using an Agilent 1200 Rapid Resolution LC system coupled to an Agilent 6410 triple quadruple mass spectrometer (Agilent Technologies Inc., Santa Clara, CA). Target analytes were separated on an Acclaim C18 RSLC column (2.1 × 150 mm, particle size 2.2 μm, Dionex). A gradient of acetonitrile and water (acidified with 0.1% acetic acid) was used for the separation of target compounds (see Table S1, Supplementary material). All analytes were ionized using an electrospray interface in positive ion mode. The following parameters were used for the mass spectrometer: capillary voltage 4000 V; drying gas (nitrogen) temperature and flow 350 °C and 10 L min^{-1}, respectively; nebulizer pressure 35 psi; nitrogen (99.999%) was used as a collision gas. The m/z ratios for precursor and product ions of target analytes, as well as collision energies and retention times, are presented in Table S2, Supplementary material.

2.4. Validation of the developed technique

The method was validated by considering its linearity, limit of quantification (LOQ), recovery and precision. Calibration curves for all analytes in the range 0.05–100 μg L^{-1} were prepared using water/acetonitrile (80/20) acidified with 1% acetic acid. The lowest standard concentration in the calibration curve was considered the LOQ. The LOQ response should be 10 times higher than that of the average noise in the chromatogram, and identifiable and

reproducible within a precision of 20%. Recovery was assessed at five concentrations (5, 10, 25, 100 and 2000 ng L^{-1}) in five replicates for each level.

To test the proposed method, a pilot study was performed to quantify CBZ and its main metabolites in the urine of healthy people that were potentially exposed to CBZ only via their diet (e.g., through consumption of crops irrigated with treated wastewater). We performed a single blind study which included 34 healthy volunteers, 20 women and 14 men aged 18–63 years, recruited from the campuses of the Hebrew University of Jerusalem. The following exclusion criteria were applied: therapeutic exposure to CBZ, pregnancy, and pure vegetarian, vegan or organic diets. The study was approved by the institutional review board of the Hebrew University–Hadassah Medical Center, and the protocol was registered at www.clinicaltrials.gov (NCT02101801). The urine samples were collected from all volunteers on the same date and then stored at −20 °C until analysis.

3. Results and discussion

The most commonly used procedures to analyze urine for active drugs at concentrations ranging from micrograms to milligrams per liter involve an initial sample preparation: dilution or protein precipitation. Thus, commonly used methods are only applicable for pharmacokinetics and pharmacodynamics studies which are performed with high drug concentrations. These methods are not feasible for the determination of the trace levels of target analytes that are relevant to humans under environmental exposure. To overcome these limitations, a new analytical approach that includes sample concentration is suggested. When the drug is present at lower concentrations, SPE and liquid–liquid extraction (LLE) are commonly used for sample concentration. However, magnified matrix effects have been reported for the SPE technique when applied to urine, while with LLE it is difficult to obtain high recovery rates for polar analytes (Dams et al., 2003). Therefore, the available methods for the analysis of CBZ in environmental samples, as well as those developed for the analysis of CBZ in biological matrices of treated patients cannot be applied for the urine of people who have been environmentally exposed to the drug.

3.1. Optimization of sample preparation procedure

While target analytes need to be extracted as efficiently as possible, co-extraction of matrix components is undesirable because co-extracts can affect ionization of target analytes by interfering with them. Therefore, method selectivity was assessed during the extraction-optimization step. The sample preparation procedure was optimized for CBZ and its metabolite EP-CBZ. SPE was performed using Strata X polymeric reversed-phase sorbent pre-conditioned with 5 mL MeOH followed by 5 mL deionized water. Samples (10 mL urine) were loaded on the cartridges, and the cartridges were washed with 5 mL deionized water; then the target analytes were eluted with 5 mL acetonitrile. Collected eluents were fully evaporated under a gentle stream of nitrogen (~40 °C), then re-dissolved in 100 μL mobile phase, and analyzed by LC/MS/MS. LLE was carried out using diethyl ether as the extraction solvent. While both SPE and LLE were suitable in terms of CBZ selectivity and recovery (93% and 95% recovery for SPE and LLE, respectively), EP-CBZ could not be quantified due to interference from other extract components. For EP-CBZ, low quality peak shape and low sensitivity were observed (Fig. 1). Concentration of the target compounds led to concentration of the matrix components as well. Hydrophilic residual components are considered to be the main interfering compounds in urine (Dams et al., 2003). The presence of uric acid and creatinine can also contribute to the interference.

To improve the extraction procedure, we freeze–dried the liquid urine samples and then reconstituted them with acetonitrile to remove some of the interfering components, which are less likely to dissolve in acetonitrile. Then clean-up was carried out using Strata X polymer-based cartridges (CW followed by AW) to remove additional basic and acidic interfering compounds, respectively. Fig. 2 shows a comparison of chromatograms for the urine samples: (1) freeze–dried and extracted with acetonitrile only followed by analysis without further purification, and (2) freeze–dried, extracted with acetonitrile and then subjected to a clean-up stage using Strata X polymer-based cartridges. As can be seen in Fig. 2, optimized extraction procedure and cleanup using the two ion exchange SPE cartridges successfully allowed to quantify not only CBZ but also EP-CBZ.

Considering these data, to determine CBZ and its metabolites at low nanogram per liter levels in the urine, sample preparation, including freeze–drying, acetonitrile extraction and clean-up through two ion-exchange cartridges is essential. This procedure is not as rapid or inexpensive as other methods, but it is beneficial for the analysis of CBZ and its metabolites which are more polar, and thus can provide more information from a single measurement.

3.2. Method validation

The developed method was validated for CBZ and five metabolites (EP-CBZ, DiOH-CBZ, 2-OH-CBZ, 3-OH-CBZ and 10-OH-CBZ). Method-performance parameters are listed in Table 1. Good linearity (R^2 ranged from 0.9974 to 0.9999) was observed for the calibration curve in the range of 0.05–200 μg L^{-1}. LOQs ranging from 3.0 to 7.2 ng L^{-1} were set, enabling trace analysis of the target analytes in the urine samples. Chromatograms for the target compounds in the calibration standard (1 μg L^{-1}) and in a blank urine sample fortified to 10 ng L^{-1} are presented in Figs. S1 and S2 (Supplementary material), respectively. Recovery (assessed at 5, 10, 25, 100 and 2000 ng L^{-1}) values were in the range of 80–120% (Table 1). Only 3-OH-CBZ exhibited recovery values lower than 60%. Good performance of the method (relative standard deviation [RSD] < 20%) was observed at all tested levels. Thus we developed a selective, sensitive and accurate technique for the detection of CBZ and its main metabolites in urine at trace concentrations.

3.3. De-conjugation test

A de-conjugation test was carried out to assess the presence of CBZ in the urine samples in glucuronide-conjugated form. Studies of the metabolism of patients treated with this drug report high proportions of conjugated CBZ in the urine (Bahlmann et al., 2014). In our study, the aim was to determine whether conjugated CBZ is also present in the urine of people who are exposed to very low doses of CBZ via their diet. We denoted these urine samples as “real samples”. The test consisted of two phases: first, the efficiency of the de-conjugation procedure was determined using fortified samples; then, three real samples that tested positive for CBZ were analyzed before and after de-conjugation. The efficiency was assessed by spiking blank urine samples (five replicates) with CBZ-O-glucuronide (500 ng L^{-1}), and then incubating them with β-glucuronidase. Then samples were analyzed for the de-conjugation product 10-OH-CBZ. Average efficiency of de-conjugation was 96%, highlighting the high productivity of the enzymatic procedure in de-conjugating CBZ-O-glucuronide. De-conjugation of real samples used in this study revealed insignificant levels of glucuronidated CBZ. The differences between incubated and non-incubated samples were below 10%, with no statistical differences. This finding

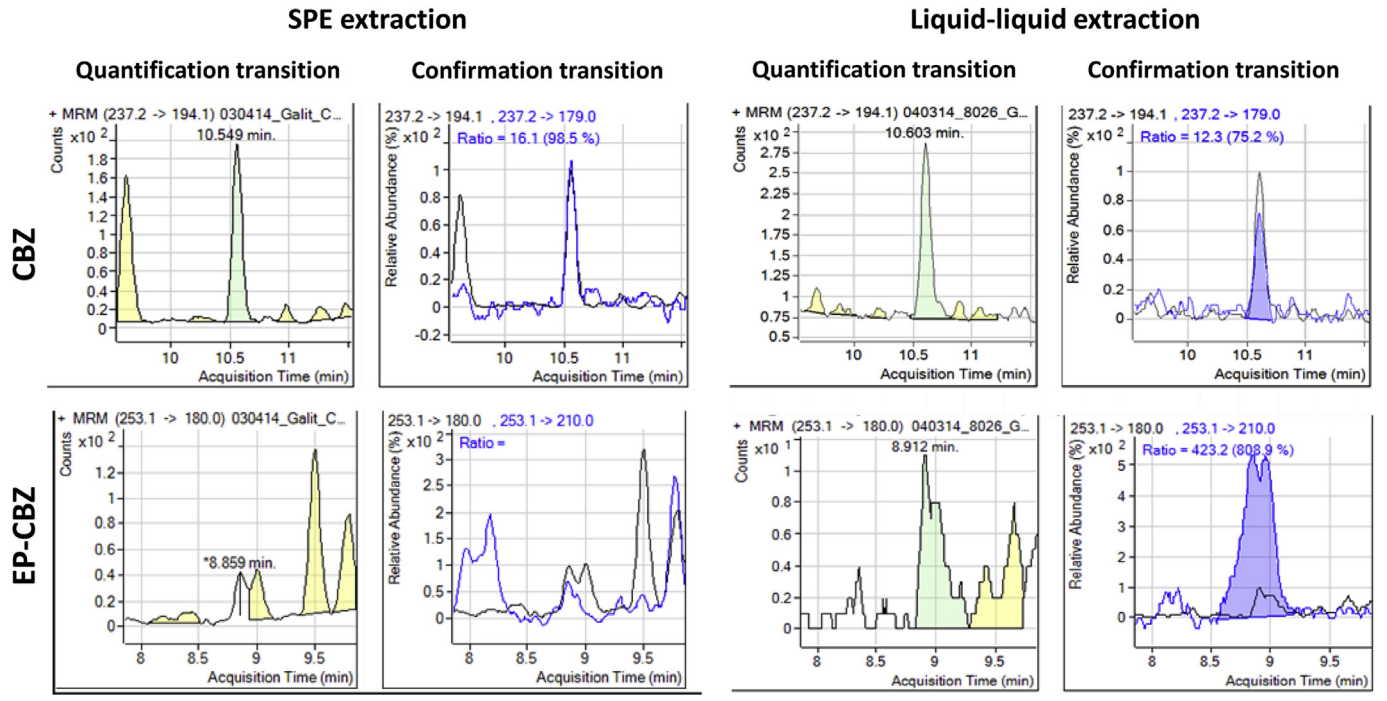

Fig. 1. Chromatograms for carbamazepine (CBZ; top) and epoxy-carbamazepine (EP-CBZ; bottom) in a blank urine sample fortified to 10 ng L^{-1} obtained from commonly used extraction methods: solid-phase extraction (left) and liquid–liquid extraction (right). Target analytes (CBZ and EP-CBZ) are marked in green for quantification transition and in blue for confirmation transition. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

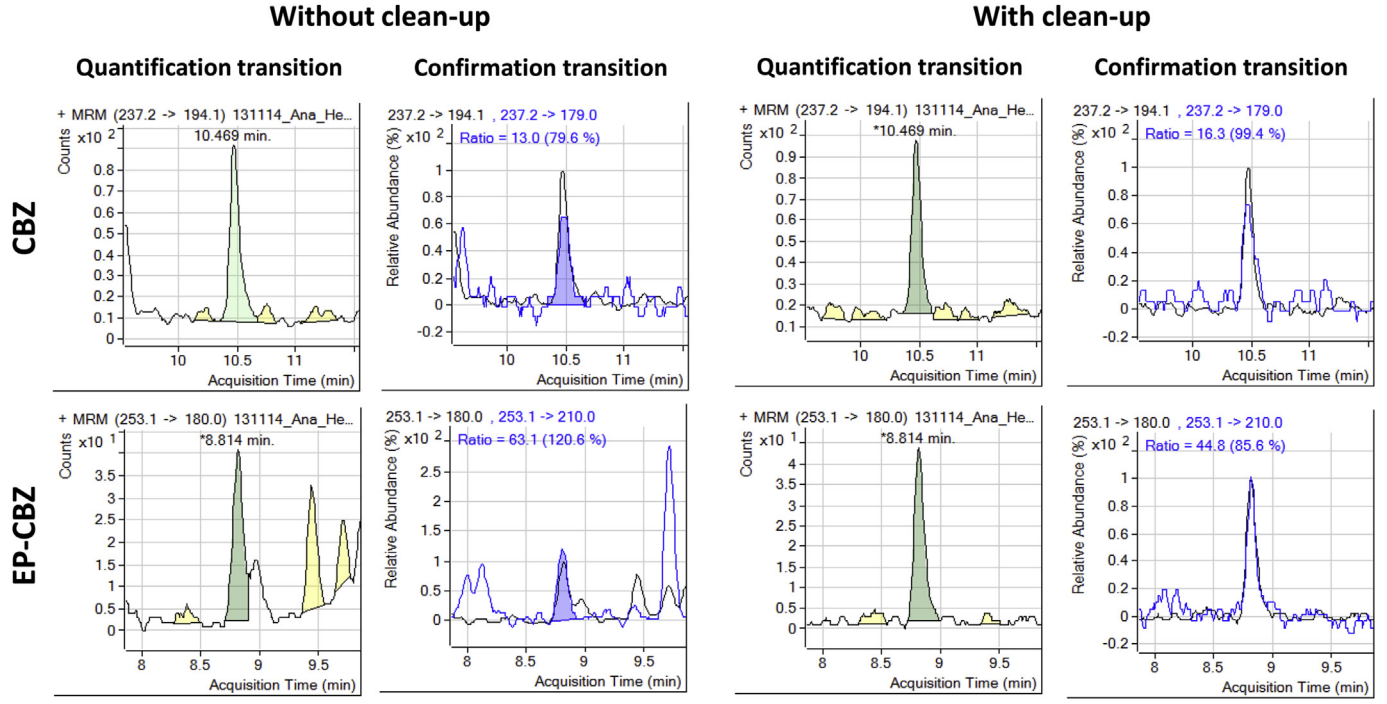

Fig. 2. Chromatograms for carbamazepine (CBZ; top) and epoxy-carbamazepine (EP-CBZ; bottom) in blank urine samples fortified to 10 ng L^{-1} and obtained by freeze–drying and acetonitrile extraction with and without clean-up (right side and left side, respectively). Target analytes are shown in green for quantification transition and in blue for confirmation transition. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Characteristics of method performance. Relative standard deviations of five replicates are shown in brackets.

Recovery (%)								
Compound	Internal standard	R^2[a]	LOQ (ng L^{-1})	LOD (ng L^{-1})	5 ng L^{-1}	10 ng L^{-1}	25 ng L^{-1}	100 ng L^{-1}
CBZ	CBZ D2,13C	0.9996	3.6	1.2	141 (5)	129 (9)	117 (3)	93 (6)
EP-CBZ	EP-CBZ D8	0.9982	3.1	1.0	76 (20)	68 (9)	61 (9)	80 (15)
DiOH-CBZ	10-OH-CBZ D3	0.9974	7.2	2.4	95 (17)	85 (8)	112 (8)	124 (9)
2-OH-CBZ	CBZ D2,13C	0.9975	3.0	1.0	98 (10)	76 (16)	79 (4)	68 (12)
3-OH-CBZ	CBZ D2,13C	0.9992	3.4	1.1	63 (13)	55 (6)	55 (9)	65 (13)
10-OH-CBZ	10-OH-CBZ D3	0.9999	3.6	1.2	93 (20)	90 (14)	82 (4)	100 (6)

[a] *R*-squared coefficient of linear regression for the calibration curve.

suggests that the metabolism of CBZ at low doses is different from that at high therapeutic doses.

3.4. Application of the developed techniques to analysis of real samples

The concentrations of CBZ and its metabolites in the real samples are presented in Table 2. The concentrations of only three compounds were above the LOQ: CBZ, EP-CBZ and DiOH-CBZ; all other metabolites (2-OH-CBZ, 3-OH-CBZ, 10-OH-CBZ) were below the limit of detection. Urine samples collected from 22 individuals contained at least one target compound. DiOH-CBZ was the most frequently detected metabolite in real samples, and its concentration was higher than those of CBZ or EP-CBZ. The concentration of DiOH-CBZ ranged from <LOQ to a maximum 497 ng L^{-1}. For the subjects with EP-CBZ > LOQ, the average EP-CBZ concentration was 51 ng L^{-1}. EP-CBZ was detected in only 10 samples, at lower concentrations (average 4.6 ng L^{-1}). EP-CBZ is known to quickly metabolize to DiOH-CBZ in the human liver (Kitteringham et al., 1996). CBZ was found in only 9 out of 34 samples at an average concentration of 2.8 ng L^{-1} (for the subjects with CBZ concentration > LOQ). These findings correspond to reports showing that DiOH-CBZ is the dominant metabolite in patients treated with CBZ (Bernus et al., 1994).

Three samples (#18, 24 and 26; Table 2), which exhibited concentrations above LOQ for all three analytes, were re-analyzed in triplicate to evaluate reproducibility and RSD. For sample #18, average concentrations (RSDs are given in brackets) were 10 (14%), 6.7 (8%) and 620 (8%) ng L^{-1} for CBZ, EP-CBZ and DiOH-CBZ, respectively. For sample #24, they were 17 (9%), 3.5 (20%) and 56 (7%) ng L^{-1} for CBZ, EP-CBZ and DiOH-CBZ, respectively; for sample #26, they were 4.4 (7%), 4.5 (14%) and 104 (12%) ng L^{-1} for CBZ, EP-CBZ and DiOH-CBZ, respectively. When compared with the results obtained from the first analyses, the difference in concentrations for the second analysis was less than 30% for CBZ and DiOH-CBZ, whereas significantly lower concentrations of EP-CBZ were observed. This might be due to loss of EP-CBZ by twice freeze–thaw cycles (during the extraction for the first measurement, and after 1 month for the second extraction for the method control). EP-CBZ was also found to be unstable in the analytical standard solution when stored at 4°C for more than 1 week. Therefore, it is not surprising that the EP-CBZ concentration changed during storage. Instability of EP-CBZ should be considered when analyses of CBZ and its metabolites in urine samples are conducted.

Table 2
Concentrations of carbamazepine (CBZ) and its metabolites epoxy-carbamazepine (EP-CBZ) and dihydroxy-carbamazepine (DiOH-CBZ) in the "real urine samples" of healthy volunteers. For each analyte, the data are shown in ng L^{-1} and as creatinine-normalized values (ng μg^{-1} creatinine [CR]).

Volunteer	CBZ		EP-CBZ		DiOH-CBZ	
	ng L^{-1}	ng μg CR^{-1}	ng L^{-1}	ng μg CR^{-1}	ng L^{-1}	ng μg CR^{-1}
1	<LOQ	<LOQ	3.1	2.3	50	37
2	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
3	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
4	<LOQ	<LOQ	<LOQ	<LOQ	75	75
5	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
6	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
7	7.1	11	<LOQ	<LOQ	33	50
8	<LOQ	<LOQ	<LOQ	<LOQ	99	70
9	<LOQ	<LOQ	<LOQ	<LOQ	31	16
10	<LOQ	<LOQ	<LOQ	<LOQ	34	17
11	<LOQ	<LOQ	<LOQ	<LOQ	53	13
12	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
13	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
14	<LOQ	<LOQ	<LOQ	<LOQ	21	30
15	<LOQ	<LOQ	<LOQ	<LOQ	16	13
16	<LOQ	<LOQ	<LOQ	<LOQ	17	18
17	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
18	13	9.8	10	7.6	497	376
19	<LOQ	<LOQ	<LOQ	<LOQ	21	44
20	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
21	<LOQ	<LOQ	<LOQ	<LOQ	47	81
22	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
23	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
24	23	83	11	40	44	159
25	<LOQ	<LOQ	11	6.9	15	9.5
26	5.7	7.2	10	13	85	107
27	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
28	4.5	4.1	8.1	7.4	26	24
29	25	9.6	76	29	388	148
30	3.7	6.2	3.9	6.5	32	53
31	<LOQ	<LOQ	<LOQ	<LOQ	28	75
32	6.6	6.8	12	12	81	83
33	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
34	5.8	15	11	29	26	69

4. Conclusions

Urine contains components that interfere with analytes and hence this matrix can make analysis very difficult. The technique developed here proved to be selective and sensitive for quantification of CBZ as well as its main metabolites in urine at environmentally relevant concentrations (i.e., nanograms per liter). Quantification of low levels of pharmaceutically active compounds in the urine of healthy people can provide important information about their exposure to pharmaceuticals from their diet. This analytical protocol can be used to provide the scientific community and stakeholders with real data which can be used for risk assessments and to design policies that will ensure safe use of wastewater for crop irrigation.

Acknowledgments

The study was financially supported by the Environment and Health Fund; the Lady Davis Fellowship Trust; the Ministry of Education, Youth and Sports of the Czech Republic - projects "CENAKVA" (No. CZ.1.05/2.1.00/01.0024), "CENAKVA II" (No. LO1205 under the NPU I program).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2016.02.027.

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