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Research Article

Molecularly imprinted SPE and MEKC with in-capillary sample preconcentration for the determination of digoxin in human urine

Molecularly imprinted solid-phase extraction (MISPE) combined with MEKC was used for clean-up, preconcentration and determination of digoxin in the presence of its aglycon digoxin (digoxigenin) in human urine samples. In addition, the use of an in-capillary sample concentration electrophoretic technique by sweeping was investigated to enhance the concentration sensitivity in MEKC. The highly selective, fast and effective sample pretreatment by MISPE along with the preconcentration by sweeping could overcome the low sensitivity of the highly efficient capillary electrophoresis separation with UV detection. The optimization of the variables affecting the separation as well as MISPE conditions procedure was carried out to select the best conditions of selectivity and sensitivity to determine digoxin at low concentration levels in urine. To demonstrate the suitability of the developed method several analytical characteristics (selectivity, linearity, accuracy, precision, and LOD) were evaluated. Satisfactory results were obtained in terms of linearity ($r > 0.99$), recovery (95.4–96.5% with RSD from 1.3% to 2.6%), precision (RSD from 0.3% to 1.7% for migration times and from 2.1% to 7.3% for corrected peak areas), and sensitivity (LODs of 6 μg/L with 5 mL of sample or 1.2 μg/L with 25 mL). The proposed MISPE-MEKC method was satisfactorily applied to the analysis of spiked human urine samples achieving a concentration factor up to 7500-fold.

Keywords:
Digoxin / In-capillary sample preconcentration / Micellar electrokinetic chromatography / Molecularly imprinted solid-phase extraction / Sweeping

1 Introduction

The cardiac glycoside digoxin has been cornerstone of the treatment of congestive heart failure and certain cardiac arrhythmias for more than two centuries [1, 2]. Digoxin, a short-acting glycoside, is mainly excreted unchanged in urine and has a serum half-time between 36 and 48 h, being its bioavailability easily affected by kidney or liver failure and the dosing of other medicaments. Therefore, since its therapeutic range is very narrow (0.8–2.0 ng/mL in serum) [2], it is important to accurately measure digoxin from digitalized patients due to the possibility of severe toxic effects [1, 2].

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Abbreviations: DLIS, digoxin-like immunoreactive substances; **EDMA**, ethylene dimethacrylate; **MAA**, metacrylic acid; **MeOH**, methanol; **MIP**, molecularly imprinted polymer; **MISPE**, molecularly imprinted solid-phase extraction; **NIP**, nonimprinted polymer; **SC**, sodium cholate; **THF**, tetra hydrofuran

In that regard, rapid as well as sensitive and selective analytical procedures for digoxin analysis are required. Immunological and chromatographic methods have been widely used to determine digitalis glycosides levels in biological fluids. Immunoassays including radioimmunoassay [3], enzyme immunoassay [4–6], or fluorescence polarization immunoassay [7] are routinely used for digoxin determination. In addition, the combination of immunoassay with liquid chromatography (HPLC) [8] or CE [9, 10] is an alternative approach in which the antidigoxin antibodies are detected by HPLC or CE. However, in all immunological methods the commercially available antibodies show a high cross-reactivity with digoxin metabolites (i.e., digoxigenin), as well as with endogenous digoxin-like immunoreactive substances (DLIS) [1]. DLIS cross-react with antidigoxin antibodies and falsely elevate serum and urine digoxin concentrations interefering in the interpretation of results for therapeutic digoxin monitoring.

With respect to chromatographic methods, they seem to be suitable due to their high selectivity without interferences from DLIS. In addition, the enhanced sensitivity of fluorescence detection has attracted more recent attention to solve the sensitivity problems. Thus, the formation of fluorescent derivatives enabled the determination of digoxin and its metabolites in urine or feces [11–13]. Recently, the use of

LC/MS [14, 15] or LC-MS/MS [16] allowed the sensitive and accurate quantitation of digoxin in small volumes of biological samples. However, this instrumentation is expensive and difficult to setup, so it is not accessible to all routine analysis laboratories.

As an alternative, CE has proven to be a highly efficient and rapid analytical technique for various applications. To the best of our knowledge, only one CE method has been published on the analysis of cardiac glycosides [17] in drug products. The major advantages of CE over HPLC are high efficiency, high resolution, short analysis time, low consumption of samples and electrolytes, and the need for little or no organic solvent [18]. However, CE has poor concentration sensitivity, especially with the most widely used UV detection method, due to minute sample volume and limited optical path-length for on-capillary photometric detection.

SPE is a routine sample preparation technique for extracting analytes from a complex matrix, being one of the most effective approaches for sample clean-up because the sample matrix can be removed further improving concentration sensitivity. Thus, SPE has widely been proposed for sample cleanup and preconcentration at trace levels prior to CE separation, due to its simplicity, high preconcentration and clean-up efficiencies, versatile use and time effectiveness [18]. Nevertheless, the nonselective sorbents used in SPE often result in the coextraction of many matrix components and decrease the enrichment efficiency.

In that context, the need for efficient methods for sample preconcentration as well as clean up in clinical analyses is constantly increasing. Thus, other materials with higher selectivity, such as molecularly imprinted polymers (MIPs), have been recognized as useful materials for SPE in the analysis of biological samples [19]. MIPs are synthetic materials possessing specific cavities designed for a target molecule (template). These cavities artificially generated have recognition sites within the polymer matrix that are adapted to the 3-D shape and functionalities of an analyte of interest or a group of structurally related species [19–22]. Thus, MIPs can recognize and bind the target analyte selectively as the antibody does. Furthermore, MIPs have advantages such as physical robustness, high mechanical strength, resistance to elevated temperatures and pressures, inertness towards organic solvents, acids or bases, and reusability [20–22]. The use of MIPs as selective sorbent materials, named molecularly imprinted solid-phase extraction (MISPE) [26], may enable higher enrichment and clean-up efficiencies than traditional SPE cartridges, due to the coupling of the high specificity, selectivity and sensitivity of the molecular recognition mechanism with the high resolving power of the separation methods [21, 23]. Finally, coupling MISPE with CE combines highly selective cleanup and enrichment technique with a highly efficient separation method and may be a powerful analytical method for trace analyses in complex samples [24, 25].

The aim of this work was the development of a new and reliable analytical methodology for the determination of digoxin by MEKC using an in-capillary concentration strategy based on electrophoretic principles. In addition, previous to the determination, a simple extraction procedure based on MISPE was carried out for clean up and preconcentration of the samples. The method was applied to the analysis of digoxin in human urine samples.

2 Materials and methods

2.1 Reagents

All reagents used for the preparation of the separation buffers were of analytical grade. Sodium hydroxide, ortophosphoric acid, boric acid, SDS and sodium cholate (SC) were supplied by Merck (Darmstadt, Germany). Sodium chloride was from Panreac (Barcelona, Spain). ACN, methanol (MeOH), hydrochloric acid and THF were obtained from Scharlab (Barcelona, Spain). Digoxin and digoxigenin were from Sigma-Aldrich (Madrid, Spain). Reagents for the MIP polymerization: methacrylic acid (MAA), ethylene dimethacrylate (EDMA), and AIBN were purchased from Sigma-Aldrich (Madrid, Spain).

Distilled water purified through a Milli-Q System from Millipore (Bedford, MA, USA) was used for the preparation of buffers and working solutions. Buffers solutions were prepared diluting phosphoric acid or dissolving boric acid with Milli-Q water, adjusting the pH to the desired value with NaOH 1 M. The BGE was elaborated dissolving SDS or SC in the corresponding separation buffer. BGEs and all prepared solutions were degassed in an ultrasonic bath. All solutions were stored at 4°C.

2.2 Standard solutions and samples

Stock solutions of digoxin and digoxigenin were prepared at 500 mg/L in methanol and suitably diluted as reference solutions in MilliQ water. The conductivity of samples was adjusted with 1M NaCl to be the same as the BGE.

Human urine samples were collected from a healthy volunteer. These samples were spiked with digoxin reference solution at 8 or 40 μg/L to demonstrate the applicability of the MISPE procedure to the extraction of digoxin from real samples.

2.3 Synthesis of molecularly imprinted polymer

MIP was prepared by bulk polymerization using digoxin as the template molecule, according to the noncovalent approach and based on described earlier works [26, 27]. For the synthesis 2×10^{-3} mmol of digoxin, 2 mmol MAA, 10 mmol EDMA, and 0.24 mmol AIBN as radical initiator were dissolved in 10 mL of ACN in a 25 mL glass tube. This mixture was degassed in an ultrasonic bath for 5 min then sparged with oxygen-free nitrogen for 10 min. Later an ultraviolet lamp Vilber Lourmat CN-6T (J. P. Selecta, Barcelona, Spain) was used to induce the polymerization process with

UV source (365 nm) at 10°C for 24 h. The monolithic polymer obtained was crushed in a mechanical mortar and sieved into the desired particle size range (200–355 μm). Then the template was removed by Soxhlet extraction with MeOH/ACN (50/50, v/v) over a period of 20 h.

2.4 CE conditions

All analyses were performed on an Agilent HP^{3D} CE instrument (Agilent Technologies, Waldbron, Germany) equipped with a DAD. The instrument was controlled by a PC running the 3D-CE ChemStation from Agilent Technologies. MEKC was performed on uncoated fused-silica capillaries of 48.5 cm (40 cm effective length) × 50 μm ID purchased from Composite Metal Services Ltd. (Worcester, England). Samples were injected by pressure (50 mbar) for different times. The electrophoretic separation was achieved with a voltage of –15 kV (reverse polarity mode) at about 20°C in 50 mM phosphate buffer (pH 2.5) with 100 mM SDS plus 5% of ACN. Detection was carried out at 223 nm ± 4 nm (anode at the detection side) using a reference wavelength of 330 ± 40 nm. Before its first use, the new capillary was rinsed with 1 M NaOH for 30 min, 5 min of 0.1 M HCl, 5 min of Milli-Q water and finally 30 min with the separation buffer. Between injections of samples, the capillary was conditioned with Milli-Q water (3 min), 5 min 0.1 M phosphate buffer (pH 2.5) and BGE (4 min). A pressure of 1 bar was applied in all steps.

2.5 MISPE conditions

A 100-mg amount of dry MIP was packed into empty SPE cartridges of 3 mL between two frits. Before samples were processed, MISPE cartridges were conditioned with 5 mL of water. The optimum MISPE protocol was loading with water 5 or 25 mL of standard solutions or urine samples, washing with water:ACN (85:15, v/v) (5 mL for standard solutions and 20 mL for urine sample), and eluting with 3 mL methanol. MISPE steps were carried out at about 1.0 mL/min on a vacuum manifold from Supelco. Prior to the washing step, a drying-step flowing air for 3 min was passed through the sorbent to remove amounts of solvent that could affect the subsequent washing step. All the applied fractions were collected and evaporated to dryness (with an evaporation system Labconco, MO, USA) at 70°C. The residues were dissolved in 100 μL of MilliQ water and they were analyzed by MEKC using the above-described method.

3 Results and discussion

3.1 Development of an in-capillary sample concentration methodology by MEKC

An in-capillary sample concentration technique in MEKC was used to select the best conditions for digoxin determination.

Table 1. Comparison of the response factor (peak height/concentration) and its repeatability ($n = 3$) for digoxin using acid and basic BGEs at different injection times

Injection time	Basic BGE[a)]		Acid BGE[b)]	
	Response factor	RSD (%)	Response factor	RSD (%)
5 s	0.089	2.5	0.12	1.8
10 s	0.16	3.0	0.25	1.9
50 s	0.90	3.5	1.3	2.1
100 s	1.8	4.2	2.5	2.3
150 s	2.9	8.0	4.1	4.8
200 s	–	–	4.5	16.7

Experimental conditions: capillary, 50 μm × 40 cm to the detector; temperature, 20°C; hydrodynamic injection, different times at 50 mbar; UV detection, 223 nm; 10 mg/L digoxin.
a) 50 mM borate buffer at pH 9.0 with 100 mM SDS and an applied voltage of 30 kV.
b) 50 mM phosphate buffer at pH 2.5 with 100 mM SDS and an applied voltage of –30 kV.

First, a preliminary study to evaluate the sweeping effect of different surfactants was carried out with digoxin standard prepared in water and adjusting the conductivity with NaCl to that of the BGE to promote sweeping effect [28, 29]. In this study, two anionic surfactants, SDS and SC at different concentrations (50, 100, and 150 mM) in a 50 mM borate buffer at pH 9.0 were examined (standard operating conditions in MEKC). The optimum surfactant was SDS at 100 mM concentration. SC showed lower affinity for digoxin, which produced broadened peak and did not improve the sweeping effect.

The pH is also a factor that affects the analyte–micelle interactions, so several works described that acidic condition can promote these interactions [28, 29]. Thus, given the high affinity digoxin–SDS micelle, reverse migration mode with acid buffer (almost no EOF) was tested. Table 1 shows the results obtained using a hydrodynamic injection for digoxin at different times (from 5 to 200 s) comparing an acid phosphate buffer at 50 mM (pH 2.5) with basic borate buffer at 50 mM (pH 9.0) both with 100 mM SDS. It can be observed that in acid medium it was possible to increase about 1.4 times the response factors (peak height/concentration), possibly due to increased digoxin–micelle interaction in this medium. The acidic conditions favor the analyte–micelle interaction because the higher ionic strength of acid phosphate buffer with respect to that of basic borate buffer decreases the CMC of SDS providing the formation of micelles and increasing the analyte–micelle retention factor [30]. The results showed that it was possible to inject up to 150 s increasing linearity (with a correlation coefficient >0.999) up to 30 times the response factor. However, longer injection times produced a significant loss in linearity between response factor and injection time due to unwanted band broadening (i.e., there is an insufficient sweeping), and a significant increase in the RSD value.

Figure 1 shows the electropherograms corresponding to the separation of digoxin and digoxigenin under different experimental conditions. First, when the conductivity of the

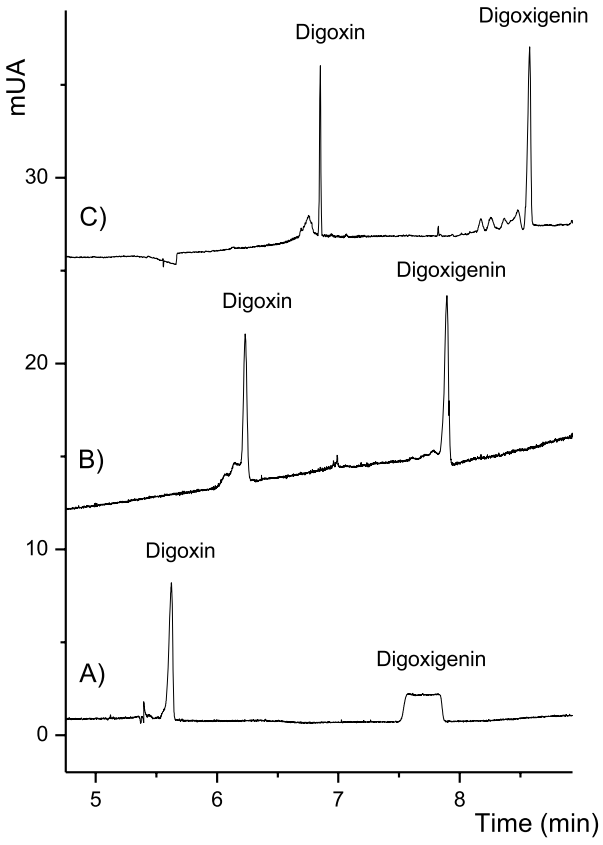

Figure 1. Electropherograms corresponding to the separation of digoxin and digoxigenin by Sweep-MEKC using: (A) a sample with conductivity adjusted to the BGE, 50 mM phosphate buffer at pH 2.5 with 100 mM SDS; (B) a sample without adjusting its conductivity, BGE is the same as A; and (C), a sample without adjusting its conductivity using as BGE 50 mM phosphate buffer at pH 2.5 with 100 mM SDS and 5% (v/v) of ACN. Other experimental conditions: injection, 50 mbar × 150 s; applied voltage, –30 kV; temperature, 20°C; capillary, 50 μm × 40 cm to the detector; detection, 223 nm; sample, 4 mg/L of each compound.

sample was adjusted to that of the BGE with NaCl, the peak of digoxigenin did not suffer the sweeping effect desired (see Fig. 1A) possibly due to the lower affinity of digoxigenin for the SDS micelle. Then, the possibility of using samples without adjusting their conductivity (aqueous sample matrix with lower conductivity than the BGE) was investigated [31–33]. Figure 1B shows that the peak of digoxigenin suffered a significant narrowing effect while the peak of digoxin maintained an almost equal peak width. In conclusion, the effects of sweeping can allow achieving an adequate preconcentration of digoxin in samples with adjusted or unadjusted conductivity to the BGE (Fig. 1A and 1B), a fact that has already been reported in the literature [28, 31–33]. On the other hand, in the case of digoxigenin, the situation is that where a mixture of an analyte in the aqueous and micellar phases is in dynamic equilibrium and moves like a single substance with a certain effective electrophoretic mobility. Regular stacking of such a substance proceeds by the same mechanisms as described for nonmicellar CZE. Therefore, in the MEKC system described there are two principal stacking modes to be distinguished: regular MEKC stacking and sweeping [34]. Finally, due to the large volume of sample injected, small bands appeared partially overlapping the peak of digoxin and digoxigenin. To improve the resolution, the addition to the BGE of different organic solvents (ACN and MeOH) as modifiers of the separation was considered. Percentages of 2%, 5%, 10%, and 15% were tested. The addition of 5% ACN to the BGE allowed to achieve an optimum resolution and provided an analysis time lower than 9 min (Fig. 1C). Above this percentage, the migration time of digoxigenin greatly increased. This result demonstrated the focusing of the digoxin band by sweeping with a concentration factor up to 30-fold allowing its selective determination in the presence of its major metabolite (digoxigenin).

Under optimal conditions, analytical parameters of the Sweep-MEKC method were established (see Table 2). These parameters included linearity, precision, LOD (signal-to-noise ratio of 3), and LOQ (signal-to-noise ratio of 10). Good linearity was obtained for digoxin in the range from 1 to 20 mg/L (correlation coefficient of 0.998 and intercept not different from zero for a confidence interval of 95%). Satisfactory precision was achieved in terms of instrumental repeatability and intermediate precision for migration times (RDS $\leq$ 1.7%) and corrected peak areas (RSD $\leq$ 7.3%). The LOD and LOQ values obtained were 0.3 and 1.0 mg/L, respectively, excellent values for CE with molecules with a weak carbonyl chromophore such as digoxin. However, these values were not sufficient to determine digoxin traces at the μg/L level existing in complex biological samples. Highly selective and efficient MISPE as clean-up and enrichment strategy was deemed to be necessary prior to Sweep-MEKC.

Table 2. Analytical characteristics of the optimized Sweep-MEKC method for the determination of digoxin

LOD (mg/L)	0.30 mg/L			
LOQ (mg/L)	1 mg/L			
Linearity				
Range	1–20 mg/L			
Linear equation	$y = 0.1357\,x + 0.0095$			
Correlation coefficient	0.998			
	1 mg/L		20 mg/L	
Precision	Area	Time	Area	Time
Instrumental repeatability [a] ($n = 6$)	2.4%	0.3%	2.1%	0.3%
Intermediate precision [b] ($n = 3$)	7.3%	1.4%	5.0%	1.7%

Experimental conditions: BGE, 50 mM phosphate buffer at pH 2.5 with 100 mM SDS and 5% (v/v) of ACN; injection, 150 s at 50 mbar; applied voltage, –30 kV; temperature, 20°C; capillary, 50 μm × 40 cm to the detector; detection, 223 nm.
a) Instrumental repeatability obtained from six consecutive injections on the same day.
b) Intermediate precision assessed from three standard solutions freshly prepared on the three different days.

3.2 Development of MISPE procedure for the determination of digoxin

In this work, the optimized conditions for the synthesis of a digoxin MIP used as a sensor by Paniagua et al. [27] were taken as starting conditions. The morphological and imprinting characters of this monolith polymer were: specific surface area, 31.8 m^2/g; micropores volume, 0.011 cm^3/g; mesopores volume, 0.086 cm^3/g; partition coefficient, 2.9; and imprinted factor, 2.74.

In order to establish the selectivity of MISPE, digoxigenin was probed as analog (compound structurally related to the template) due to the small difference with digoxin (an additional OH group of secondary alcohol on the aglycone residue at 12-position). In addition, digoxigenin is the most possible interfering compound that can significantly affect clean-up efficiency. Therefore, removal of this analog was used as an index to evaluate the clean-up efficiency in this study [35, 36].

The operational use of SPE consumables containing MIPs is very similar to the use of the other SPE sorbents for preconditioning, sample loading, washing and elution. First, the cartridges were conditioned with 5 mL of water, and then different solvents (THF, ACN and water) to load the sample (5 mL of 0.2 mg/L of digoxin and digoxigenin) were tested. Results showed that digoxin or its analog were not retained in the MIP when ACN or THF were used as loading solvent, being detected in the loading step. However, when water was used as loading solvent digoxin and its analog were fully retained on the MIP and NIP. This indicated that the binding between MIP and digoxin was strong but nonspecific (based on hydrophobicity), being water suitable to be used as the loading solvent (i.e., urine itself).

An optimum washing step is the key to obtain the best selectivity and recovery [35, 36]. Generally, MIP exhibits better molecular recognition in the solvent used as a porogen during polymerization [21, 35, 36]. To obtain the most appropriate washing solvent up to 5 mL (one to one) mixtures of different ratios of ACN and water were tested. The results showed that digoxigenin together with digoxin was completely retained by the polymer when the washing solvent was water. An increase in clean-up efficiency and selectivity was observed when a 85:15 (v/v) water–ACN mixture was used as washing solvent, being digoxin fully retained in the MIP and the washing recovery of digoxin increasing to 53.7% in NIP, while the washing recovery of digoxigenin increased to 45.6% in MIP and 76.4% in NIP. However, 80:20 (v/v) water–ACN increased the washing recovery of digoxin up to 47.2% in MIP. Therefore, water–ACN 85:15 (v/v) was used as optimum washing solvent. These results showed that the MIP exhibited highly selective binding affinity for digoxin, and demonstrated that the adsorption of this compound was due to imprinted binding sites and not to nonspecific binding. Finally, the optimization of the elution step was performed using MeOH, and testing different volumes of this eluting solution. 3 mL of MeOH was sufficient to completely elute digoxin from the MISPE cartridge. Then the eluent was taken to dryness and reconstituted in only 100 μL, which leads a preconcentration factor that depended on the volume of sample loaded (50 times for 5 mL, and so on).

Table 3. Recovery and repeatability for the determination of digoxin (at different spiked concentration) using different loading volumes by the optimized MISPE-Sweep-MEKC methodology

Loading volume (mL)	Spiked concentration (μg/L)	Recovery[a)] (%)	RSD[a)] (%)
5	20	95.4	2.4
5	40	96.5	1.5
5	80	95.7	1.3
10	20	94.3	2.4
25	8	92.9	2.1

MISPE protocol: washing step, 5 mL of water:ACN (85:15, v/v); and eluting step, 3 mL of MeOH.
a) Methodological repeatability obtained from three standard solutions freshly prepared on the same day.

Validation of MISPE was performed evaluating the accuracy and repeatability of the MISPE protocol. The accuracy was evaluated by calculating the recovery obtained for digoxin using three replicates of 5 mL at three concentration levels and each one injected by Sweep-MEKC in triplicate. Recoveries ranging from 95.4% to 96.5% were obtained for digoxin concentrations ranging from 20 to 80 μg/L (Table 3). The intraday RSD was less than 2.4%. The preconcentration achieved with the MISPE protocol enabled the detection of only 6 μg/L and the quantitation of 20 μg/L when 5 mL of samples were analyzed (a concentration factor up to 50-fold). To further evaluate the enrichment efficiency of MISPE, 1 mL of standard solution (0.2 mg/L) was diluted to different volumes (5, 10, and 25 mL) and was loaded onto the MISPE cartridge. Table 3 shows that recoveries of digoxin were constant in the loading volume range. The use of a MISPE with a large sample volume may increase the enrichment factor (up to 250-fold) and to provide a good strategy for overcoming the low sensitivity of the CE–UV method, making it possible to reduce the detection limit of MISPE-Sweep-MEKC (LOD of 1.2 μg/L and LOQ of 4 μg/L when 25 mL of samples were analyzed) and to determine trace amounts of digoxin in complex samples.

3.3 Analysis of human urine samples

Taking into account that the levels of digoxin in urine are higher than in blood (about 40 μg/L, between 15 and 30 times higher than in blood [37, 38]), the reliability of the MISPE-Sweep-MEKC methodology developed was evaluated with a human urine sample spiked with digoxin.

Although it was necessary to increase the volume of the washing step to 20 mL of water:ACN (85:15, v/v) to clean up the urine sample, the recoveries obtained for digoxin were 96.5% for urine sample and 95.7% for standard solution. These results demonstrated the possibility of increasing the volume of washing solvent until 20 mL without significant

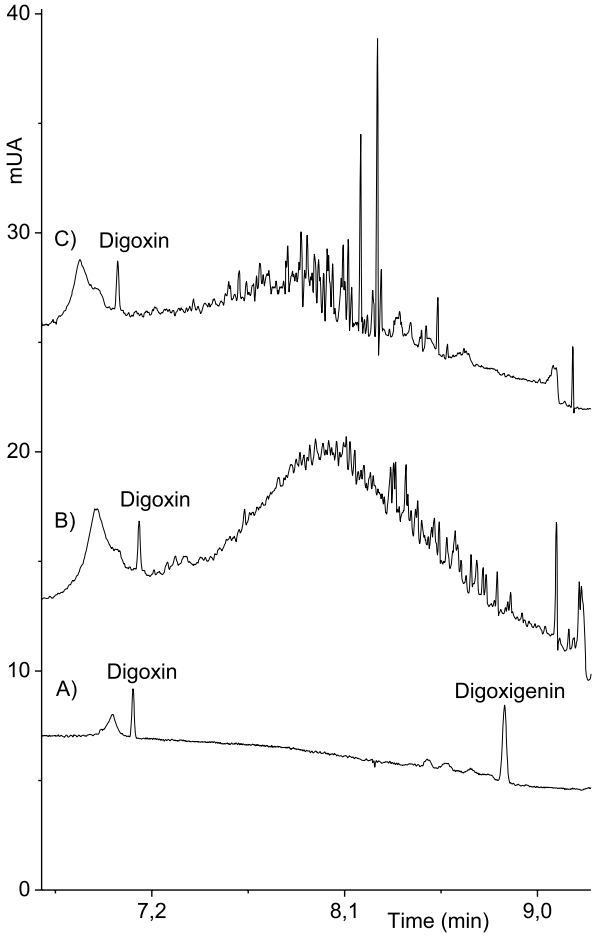

Figure 2. Electropherograms corresponding to the determination of digoxin by MISPE-Sweep-MEKC in (A) 25 mL of aqueous standard solution spiked with 8 μg/L of digoxin and digoxigenin after MISPE protocol washing with 5 mL of water:ACN (85:15, v/v) and eluting with 3 mL methanol; (B) 5 mL of human urine sample spiked with 40 μg/L of digoxin and digoxigenin after MISPE protocol washing with 20 mL of water:ACN (85:15, v/v) and eluting with 3 mL methanol; and (C) 25 mL of human urine sample spiked with 8 μg/L of digoxin and digoxigenin after MISPE protocol washing with 20 mL of water:ACN (85:15, v/v) and eluting with 3 mL methanol. Other experimental conditions as in Fig. 1C.

loss of digoxin. Figure 2 shows the electropherograms corresponding to 5 and 25 mL of urine samples spiked with 40 and 8 μg/L of digoxin and digoxigenin, respectively, and a standard sample of 8 μg/L of digoxin and digoxigenin after MISPE protocol. The results showed the applicability of this method for the detection and quantitation of this analyte in urine samples. Thus, it is possible to determine the digoxin levels in patients from the digoxin doses administered and the digoxin excreted in urine allowing the routine digoxin determination laboratory work in a rapid and easy way.

The developed CE method with UV detection enables to achieve a digoxin LOQ in urine of only 4 μg/L while an HPLC method with fluorescence detection and precolumn derivatization has a LOQ of 5 μg/L with RSD over 10% [12]. The use of expensive instrumentation like SPE–HPLC–MS/MS provides a slight decrease of LOQ to 1.2 μg/L in only 3 min with a RSD of 9% [16]. Other methods like RIA can be used in the same digoxin range in urine, 1–100 μg/L [3] but there is a possibility of high cross-reactivity with digoxin metabolites [1].

4 Concluding remarks

In this work, a reliable analytical methodology has been developed for the determination of digoxin by MEKC using a sweeping injection mode to improve the detection sensitivity about 30 times against a standard injection, also allowing its selective determination in the presence of its major metabolite (digoxigenin). In addition, due to the use of SPE, using a MIP as selective sorbent, the cleanup and preconcentration of the samples up to 250 times were possible. Thus, a concentration factor up to 7500-fold can be obtained with the combination of the MISPE procedure and Sweep-MEKC, reducing the LOD of MISPE-Sweep-MEKC up to only 1.2 μg/L. In conclusion, the high sensitivity and selectivity for digoxin achieved by this combination allowed its determination at the level required in human urine samples. The analytical characteristics demonstrated the suitability of the developed methodology showing that this method has considerable potential use in clinical analyses for the determination of digoxin in urine.

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5 References

[1] Cook, J. D., Koch, T. R., Cook, M. S., Knoblock, E. C., *Clin. Biochem.* 1988, *21*, 353–357.

[2] Smith, T. W., *N. Engl. J. Med.* 1993, *329*, 51–53.

[3] Christenson, R. H., Hammond, J. E., Hull, J. H., Bustrack, J. A., *Clin. Chem. Acta.* 1982, *120*, 13–19.

[4] Kaneki, N., Xu, Y., Kumari, A., Halsall, B. H., Heineman, R. W., Kissinger, T. P., *Anal. Chim. Acta.* 1994, *287*, 253–258.

[5] Self, H. C., Dessi, L. J., Winger, A. L., *Clin. Chem.* 1994, *40*, 2035–2041.

[6] Borges, N. C., Guermani, A., Mazucheli, J. A., Mendes, G. D., Moreno, R. A., *Intern. J. Clin. Pharm. Therap.* 2007, *45*, 366–372.

[7] Porter, W. H., Haver, V. M., Bush, B. A., *Clin. Chem.* 1984, *30*, 1826–1829.

[8] Ikeda, Y., Fujii, Y., Yamazaki, M., *J. Nat. Prod.*, 1992, *55*, 748–752.

[9] Liu, X. H., Xu, Y., *Anal. Chem.* 1995, *67*, 3211–3218.

[10] Wan, K. H., Le, X. C., *J. Chromatogr., B: Biomed Appl.*, 1999, *734*, 31–38.

[11] Knowng, E., McErlane, M. K., *J. Chromatogr. B: Biomed Appl.* 1986, *377*, 233–242.

[12] Shepard, T. A., Hui, J., Chandrasekaran, J., *J. Chromatogr. B: Biomed Appl.* 1986, *380*, 89–98.

[13] Cohen, A. F., Kroon, R., Schoemaker, H. C., Breimer, D. D., van Vliet_Verbeek, A., Brandenbuerg, H. C., *Br. J. Clin. Pharmacol.* 1993, *35*, 36–142.

[14] Kaiser, P., Akerboom, T., Wood, W. G., Reinauer, H., *Clin. Lab.* 2006, *52*, 37–42.

[15] Kirby, B. J., Kalhorn, T., Hebert, M., Easterling, T., Unadkat, J. D., *Biomed. Chromat.* 2008, *22*, 712–718.

[16] Salvador, A., Sagan, C., Denouel, J., *J. Liq. Chrom. Rel. Technol.* 2006, *29*, 1917–1932.

[17] Tseng, H. H., Wu, H. L., Lin, S. J., Chen, S. H., *J. Sep. Sci.* 2003, *26*, 1693–1697.

[18] Puig, P., Borrull, F., Calull, M., Aguilar, C., *Anal. Chim. Acta* 2008, *616*, 1–18.

[19] Andersson, L. I., *J. Chromatogr. B*, 2000, *739*, 163–173.

[20] Pichon, V., *J. Chromatogr. A*, 2007, *1152*, 41–53.

[21] Tamayo, F. G., Turiel, E., Martín-Esteban, A., *J. Chromatogr. A.* 2007, *1152*, 32–40.

[22] Pichon, V., Chapuis-Hugon, F., *Anal. Chim. Acta* 2008, *622*, 48–61.

[23] González, G. P., Hernando, P. F., Alegría, J. S. D., *Anal. Chim. Acta* 2006, *557*, 179–183.

[24] Meng, Z., Liu, Q., *Anal. Chim. Acta* 2001, *435*, 121–127.

[25] Lara, F. J., Lynen, F., Sandra, P., Garcia-Campana, A. M., Ales-Barrero, F., *Electrophoresis* 2008, *29*, 3834–384.

[26] González, G. P., Hernando, P. F., Alegría, J. S. D., *Anal. Chim. Acta* 2009, *394*, 963–970.

[27] González, G. P., Hernando, P. F., Alegría, J. S. D., *Anal. Chim. Acta* 2009, *638*, 209–212.

[28] Yang, S., Khaledi, M. G., *J. Chromatogr. A.* 1995, *692*, 301–310.

[29] Muijselaar, P. G., Otsuka, K., Terabe, S., *J. Chromatogr. A.* 1997, *780*, 41–61.

[30] Terabe, S., Otsuka, K., Ando, T., *Anal. Chem.* 1985, *57*, 834–841.

[31] Kim, J., Quirino, J. P., Otsuta, K., Terabe, S., *J. Chromatogr. A.* 2001, *916*, 123–130.

[32] Quirino, J. P., Kim, J., Terabe, S., *J. Chromatogr. A.* 2002, *965*, 357–373.

[33] Aranas, A. T., Guidote, A. M., Quirino, J. P., *Anal. Bioanal. Chem.* 2009, *394*, 175–185.

[34] Malá, Z., Slampová, A., Gebauer, P., Bocék, P., *Electrophoresis* 2009, *30*, 215–229.

[35] Zheng, Y. Q., Liu, Y. H., Guo, H. B., He, L. M., Fang, B. H., Zeng, Z. L., *Anal. Chim. Acta* 2011, *690*, 269–274.

[36] Shi, Y., Peng, D. D., Shi, C. H., Zhang, X., Xie, Y. T., Lu, B., *Food Chem.*, 2011, *126*, 1916–1925.

[37] Twittenhoff, W. S., Strauch, M., Koch, K., Shauman, W., *Int. J. Clin. Pharmacol. Ther. Toxicol.* 1981, *19*, 405–481.

[38] Shlipak, M. G., Smith, G. L., Rathore, S. S., Massie, B. R., Krumholz, H. M., *J. Am. Soc. Nephrol.* 2004, *15*, 2195–2203.