Solid Bar Microextraction and HPLC/DAD Determination of Diuretic Drugs and its Application to Spiked Human Urine

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ABSTRACT

In this study, solid bar microextraction combined with liquid chromatography-diode array detector (HPLC-DAD) was used for the simultaneous determination of furosemide and spironolactone in spiked human urine samples. The target drugs were extracted from aqueous sample solutions into a solid extracting sorbent located inside the lumen of a polypropylene hollow fiber with two ends sealed. After ending the extraction, analytes were desorbed by immersing the solid bar microextraction device in a micro-volume of suitable organic solvent. The effects of different variables on the extraction efficiency were investigated in order to obtain high extraction efficiency. Under the optimized experimental conditions, the calibration curves were linear in the concentration range of 10 to 100 µg L-1, with very good precision (RSD ≤ 1.53%) and low limits of detection/quantitation (LODs/LOQs were 1.45/4.85 and 2.09/6.92 µg L-1 for furosemide and spironolactone, respectively). Ultimately, the applicability of the current method was assessed by the extraction and simultaneous determination of both analytes in spiked human urine samples. The solid bar microextraction method proved to be simple, sensitive and environmentally friendly. Thus, it is a good choice for the determination of trace levels of furosemide and spironolactone in human urine using the common HPLC instrument without further cleanup procedures.

Keywords: Diuretics, Furosemide, Spironolactone, Solid bar microextraction, Urine, HPLC/DAD.

1. INTRODUCTION

Diuretic medications comprise various classes of drugs which are used for the treatment of many diseases like heart, lung and kidney diseases.[1-4] These medications have been abused by athletes in order to meet weight categories of some sports by rapid weight reduction, or to reduce the concentration of other doping drugs in urine and prevent their detection by increasing the volume of urine, resulting in a dilutional effect.[5,6] Thus, all classes of diuretics were banned by the World Anti-Doping Agency (WADA) and the International Olympic Committee (IOC).[7]

Furosemide (structure shown in Figure 1) is a potassium-wasting diuretic, which acts by reducing the reabsorption of salts in the blood, leading to their excretion in urine.[8], while spironolactone (Figure 1) is an aldosterone antagonist that acts as a potassium-sparing diuretic by increasing the excretion of sodium and the reabsorption of potassium.[9] The combination of these two drugs in one formulation to avoid hypokalemia is approved and available in many marketed formulations.[10-14]

Furosemide and spironolactone are potent drugs that are mainly excreted unchanged in urine in trace levels.[15,16] Consequently, analytical techniques for the quantification of these drugs in urine play an important role for evaluation and interpretation of pharmacokinetic, bioavailability and therapeutic bioequivalence data.

The widely used analytical methods for the simultaneous extraction and determination of furosemide and spironolactone in urine are based on chromatographic separation and mass spectrometry detection such as liquid

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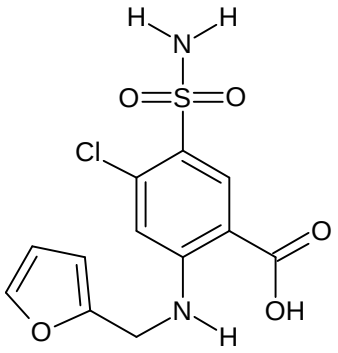
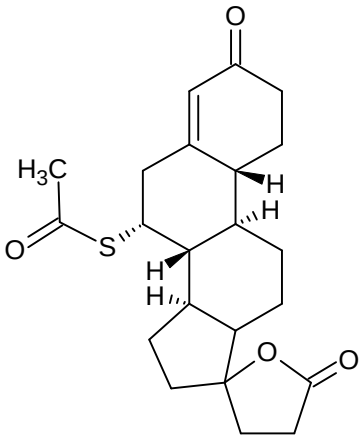
chromatography-mass spectrometry (LC-MS)[17-20] and gas chromatography-mass spectrometry (GC–MS).[21-23] However, although mass spectrometry (MS) detection may provide the required selectivity, the cost of the instrument is high, and the sensitivity might be insufficient for some applications.[24-28]



Furosemide



Spironolactone

Figure 1: Chemical structure of furosemide and spironolactone

Therefore, to determine both compounds at low levels in urine, sample preparation procedures comprising suitable cleanup and high enrichment factor are needed. Liquid-liquid extraction (LLE) and solid-phase extraction (SPE) are commonly referred methods for concentrating these analytes in aqueous samples, thus enhancing the analytical sensitivity.[2,3,17,24-29]. Nevertheless, these methods suffer from the consumption of large amounts of time as well as potentially harmful organic solvents.[30]

Non-conventional solid phase microextraction (SPME) has been considered a brilliant invention for ***sample pretreatment*** and the determination of trace ***drugs*** in ***biological*** samples.[31-33] The SPME technique merges extraction and preconcentration of the selected drugs simultaneously and thus, results in lowering the amount of solvent used and time spent, while increasing sensitivity as compared to other extraction techniques. However, the technique is costly, and the SPME fibers suffer from sample carryover and a short lifespan.[34]

The solid bar microextraction (SBME) technique is an interesting alternative to the multistep SPE method for extraction and preconcentration of analytes in complex samples.[34,35] SBME inherits the advantages of SPME, but with comparatively lower cost. The technique as reported previously[34,35] was based mainly on using a few milligrams of a sorbent covered with a hollow fiber microtube. The porous structure of the hollow fiber membrane works as a filter to exclude particles from the sample matrix while allowing the molecules of the analytes to diffuse through and adsorb onto the sorbent. Thereafter, the analytes are desorbed by submerging the SBME device in a microscale (< 1 mL) volume of suitable organic solvent.

The present work describes the development and validation of an analytical method based on SBME followed by high-performance liquid chromatography with diode array detection (HPLC-DAD) for the simultaneous determination of two diuretics: furosemide (FUR) and spironolactone (SPI) in spiked human urine specimens. Besides the optimization of SBME parameters, a comparison with previously reported methods, highlighting the main advantages and disadvantages is also discussed.

2. Experimental

2.1. Chemicals and materials

Furosemide, spironolactone, sodium dihydrogen phosphate (all with purity grades > 98%) and hydrochloric acid (37%) were purchased from Sigma-Aldrich (Steinheim, Germany). The sorbent materials Chromabond C4 (C4), Chromabond C8 (C8) and Chromabond C18 (C18) (all with particle size 45 µm) were obtained from Macherey-Nagel (Düren, Germany). HPLC-grade organic solvents were purchased from Merck (Darmstadt, Germany). The Q3/2 Accurel polypropylene hollow fiber membrane (600 µm i.d., 200 µm wall thickness, and 0.2 µm pore size) was purchased from Membrana (Wuppertal, Germany).

2.2. Preparation of standard and spiked urine solutions

Standard stock solutions of FUR and SPI (1 mg mL^{-1}) were prepared in methanol in volumetric glassware. Stock solutions were stored in a refrigerator at 4°C when not in use.

The blank human urine from healthy male volunteers was stored at -20°C and kept at 4°C before use. Aliquots of 15 mL of spiked human urine samples with different concentrations of FUR and SPI were centrifuged for 10 minutes at 3500 rpm, after which the supernatants were transferred to a clean vial and stored at 4°C until use. The spiked urine samples were diluted 1:1 with distilled water to minimize the matrix effects as reported elsewhere.[36] Finally, 30 mL of spiked and diluted urine samples were adjusted to a pH of 2 by using 4.0 M HCl before transferring to the SBME apparatus.

2.3. Preparation and extraction procedure of the SBME

Preparation of the SBME device was based on previously reported procedure.[34,35] The SBME device consisted of 2 mg of solid sorbent packed inside the lumen of a polypropylene microtube 2.5 cm in length with two ends sealed. Each device was cleaned by ultrasonication in methanol for 3 minutes and stored in methanol until use.

A 30-mL aliquot of the sample solution was added to a beaker (50 mL) as shown in Figure 2. A magnetic stirring bar (15 mm × 5 mm) was placed in the solution. Next, the SBME device was added to the sample solution that was stirred at 500 rpm. After the extraction (50 minutes), the device was removed, rinsed, dried and placed in a 300-µL desorption microvial. Then, 250 µL of methanol were added, and ultrasonication was used to desorb the analytes for 10 minutes. After desorption, the SBME device was removed, and the vials were placed directly in the autosampler of the HPLC-DAD system for analysis.

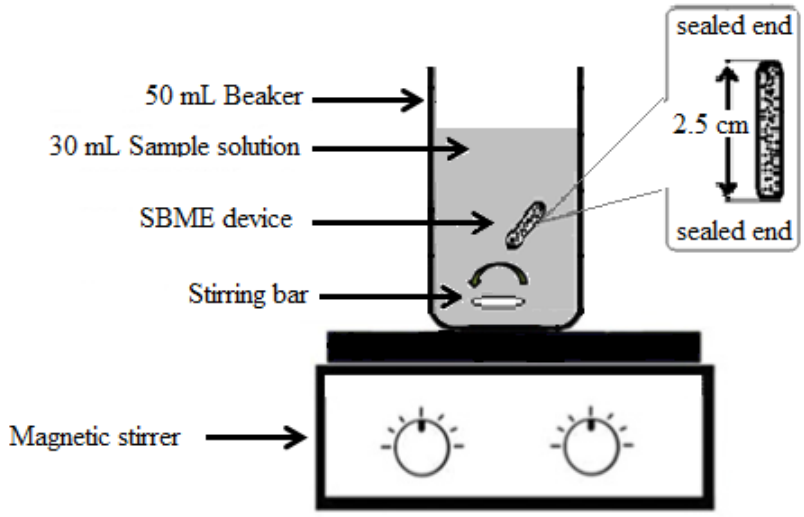


Figure 2: SBME graphic diagram[35]

2.4. HPLC-DAD instrumentation and conditions

All experiments were performed on an HPLC Ultimate 3000 system (Dionex, Germering, Germany) consisting of the following: a quaternary pump, an autosampler, a column heater and a UV/DAD detector. Chromatographic analytical method was developed on an Inertsil ODS-3, C18 (150 mm × 4.6 mm × 5 µm) column (Merck, Germany). The mobile phase consisted of a binary gradient of 20% acetonitrile as solvent A and 80% acetonitrile as solvent B, containing 0.25% acetic acid for both solvents. The gradient program of HPLC began at 91% A for 1 minute, and then reduced to 36% A within 2.4 minutes, followed by 100% B for 1.3 minutes, and eventually the HPLC program was returned to the starting conditions within 0.5 minute. The total run time was set to 8 minutes with a flow rate at 1.5 mL min^{-1}. Detection was performed at 230 nm, and injection volume was 10 µL, with column temperature set to 32°C. Retention times (t_r) of FUR and SPI were 3.73 and 4.97 minutes, respectively.

2.5 Calculation

The extraction efficiency or recovery percentage for the selected diuretics using the SBME system was calculated as the ratio of the detected amount of analyte obtained by SBME (N_d) to their initial amount in sample solution (N_s):[37,38]

Extraction efficiency or recovery =

$$\frac{N_d}{N_s} \times 100\% = \frac{C_d \times V_d}{C_s \times V_s} \times 100\%.$$

C_d and C_s are the analyte concentrations obtained by SBME (after desorption with organic solvent) and concentrations in the original sample solution, respectively. V_d is the volume of desorbed solvent, and V_s is the volume of the sample solution.

3. Results and discussion

3.1. Optimization of SBME

Different factors affecting extraction efficiency and analytical sensitivity were optimized. Optimization of these factors included controlling adsorption (i.e. the type of sorbent materials, the pH of sample solution, stirring speed and extraction time) and controlling desorption (i.e. the solvent, volume and time).

3.1.1 Type of sorbent material

The properties of sorbent material packed inside the lumen of the porous hollow fiber microtube play a crucial role in the SBME procedure; an appropriate sorbent may facilitate extraction efficiency and speed. The silica-based sorbents C18, C8 and C4 were applied in this work for the extraction and cleanup of diuretics from urine samples, since both drugs are highly hydrophobic.[39,40] A fresh SBME device was used in each experiment to avoid any possible memory effect.

Comparison of SBME efficiency for FUR and SPI using different sorbent materials is listed in Table 1. The extraction conditions were as follows: a 30-mL sample volume containing 7 µg L^{-1} drugs in water, 5 pieces of SBME devices, 60 minutes of extraction time, a 500-rpm stirring speed, 250 µL of methanol as desorption solvent and 20 minutes as desorption time.

Table 1. The peak area of extracted analytes using SBME with different sorbent materials

Sorbent material	Peak area (RSD%, n=3)	
	FUR	SPI
C18	6.14 (0.61)	18.49 (0.53)
C8	2.31 (1.07)	13.66 (1.33)
C4	0 (0)	3.74 (2.45)

As the sample solution was water, the non-ionizable SPI molecules[41] are more favorably adsorbed onto the long alkyl chain sorbent (C18) than the shorter chains C8 and C4. Moreover, in comparison with the shorter alkyl chains of C4 and C8, the longer alkyl chain of C18 exhibits less dipole-dipole interactions between the acidic analyte FUR[4] and the silanol group of silica. Thus, there is easier desorption of FUR from the C18 surface sorbent. Therefore, the C18 sorbents showed the highest peak areas for both diuretics and was selected for the remaining studies.

3.1.2 Optimizing the pH of sample solution

The adjustment of pH for sample solution is an important step to achieve the optimum extraction efficiency in SBME after choosing the C18 as the sorbent material. SPI is a non-ionizable diuretic,[41] whereas FUR is an acidic diuretic with pKa of 3.8.[42] Therefore, the polar acidic analyte (FUR) should be kept in undissociated form in order to increase the affinity of FUR molecules toward nonpolar C18 sorbent. For this purpose, different pH values (1 to 6) were tested (see Figure 3).

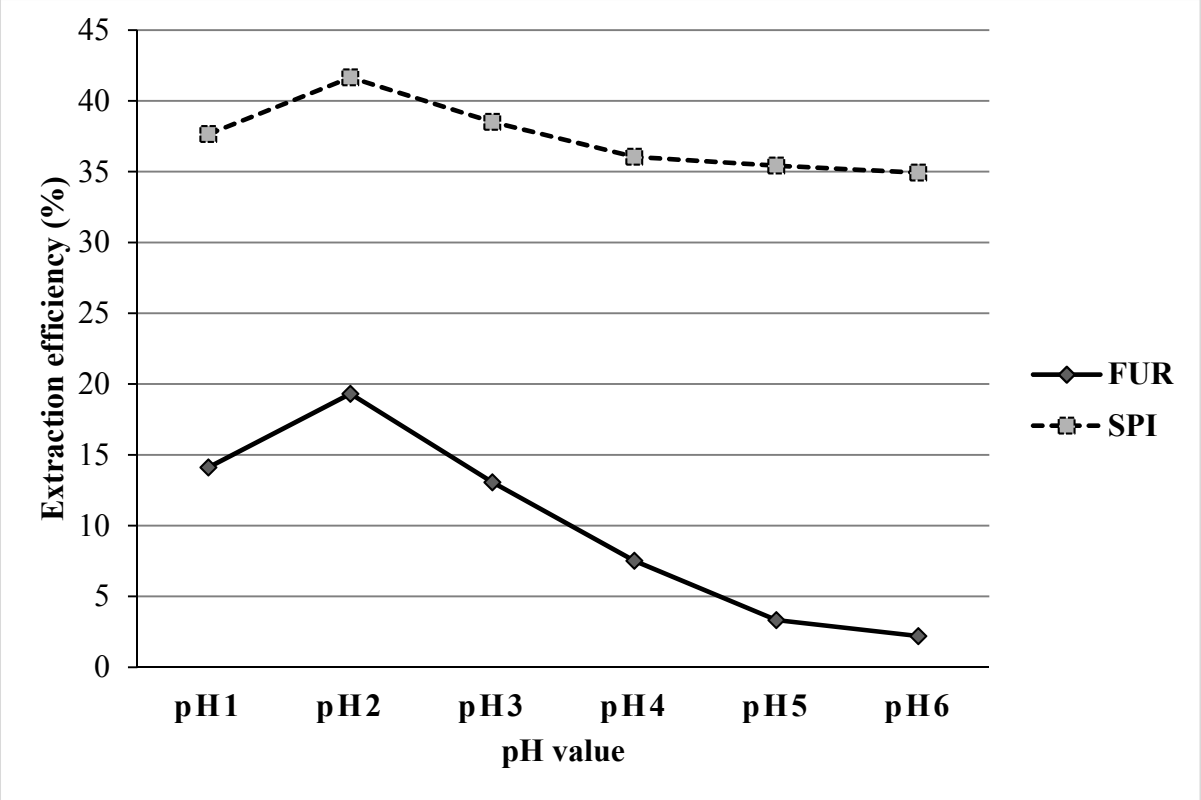


Figure 3: Optimization of the pH of the sample solution (using 30 mL of sample solution, 5 pieces of SBME devices, 60 minutes of extraction time, 500 rpm stirring speed, 250 µL of methanol as desorption solvent and 10 minutes as desorption time)

When the pH of the solutions is lower than the pK_a value of FUR, the drug will predominantly exist in a nonionic form. Thus, a pH of 2 gave the highest sorption efficiency due to the hydrophobic interaction between nonionic forms of both drugs (FUR and SPI) and the nonpolar, long alkyl chain sorbent (C18) as shown in Figure 3. However, decreasing the pH of the sample solution to 1 resulted in lowering the affinity of analytes

toward the nonpolar C18 sorbent probably due to the ionization of the weakly basic amine group in FUR and protonation of carbonyl groups in SPI.

3.1.3 Effect of the number of SBME devices

The effect of the number of SBME devices on the extraction efficiency was also tested by using 30 mL of pH 2 sample solutions spiked at 7 μg L^{-1} of selected analytes. As predicted, by increasing the number of SBME devices (from 1 to 5), the extraction efficiency was increased (Figure 4). Nevertheless, no additional enhancement was detected when more than five SBME devices were used. Consequently, five SBME devices were used as an optimum number for further experiments.

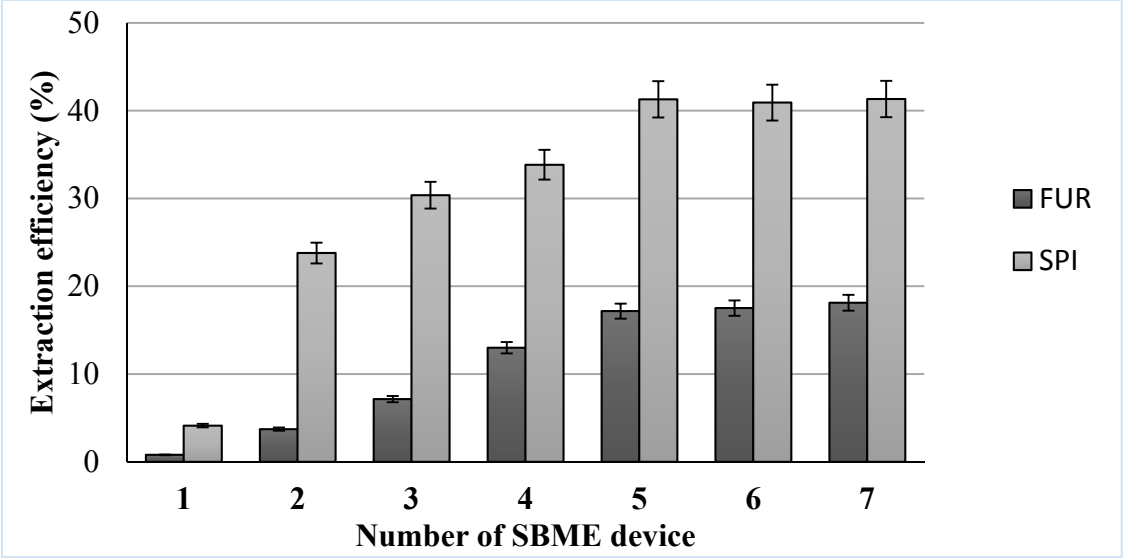


Figure 4: Effect of the number of SBME devices on the extraction efficiency of selected analytes (using sample solution with a pH of 2 and other extraction conditions as in Figure 3); error bars correspond to standard deviation

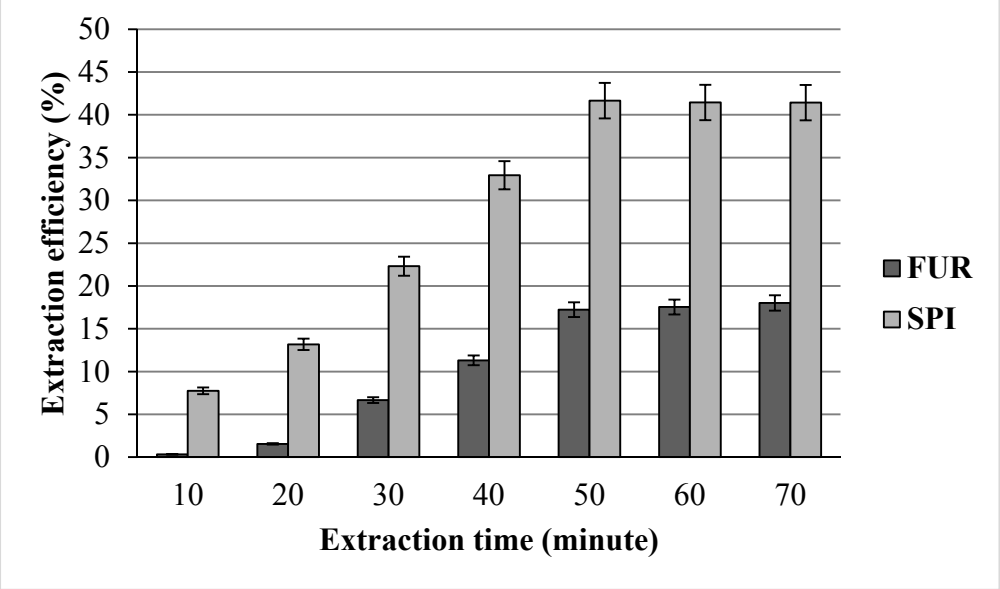


Figure 5: SBME time profile of studied drugs (with other extraction conditions as in Figure 4)

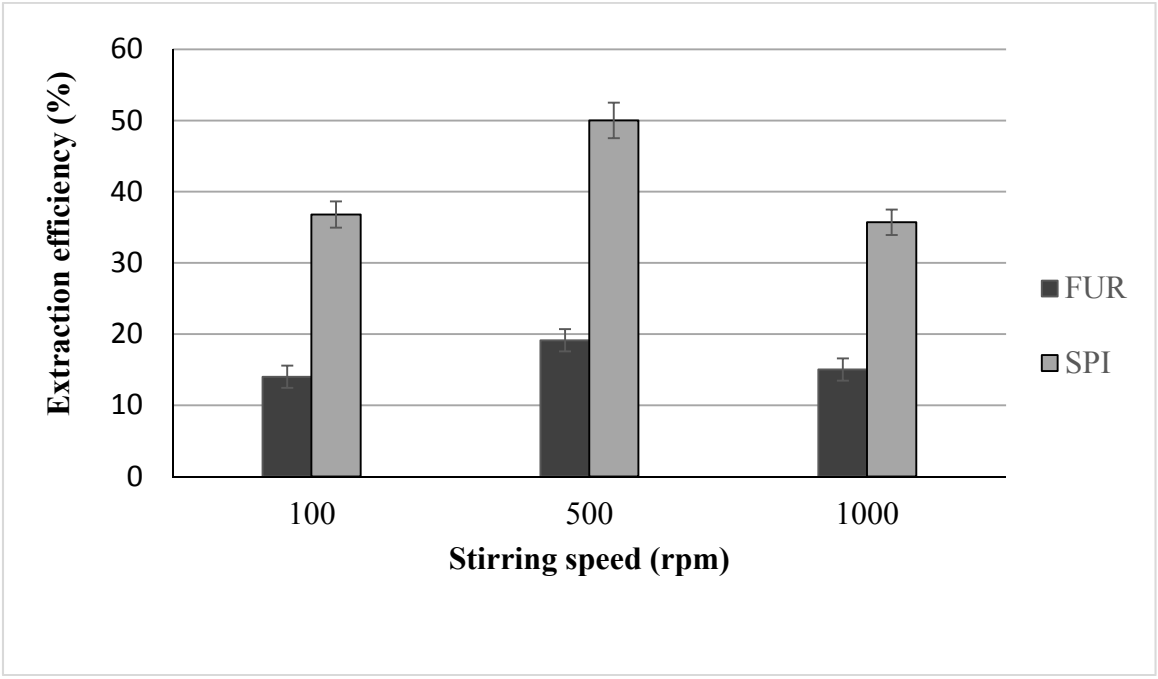


Figure 6: Influence of the stirring speed on the SBME efficiency (using an extraction time of 50 minutes and other extraction conditions as in Figure 4

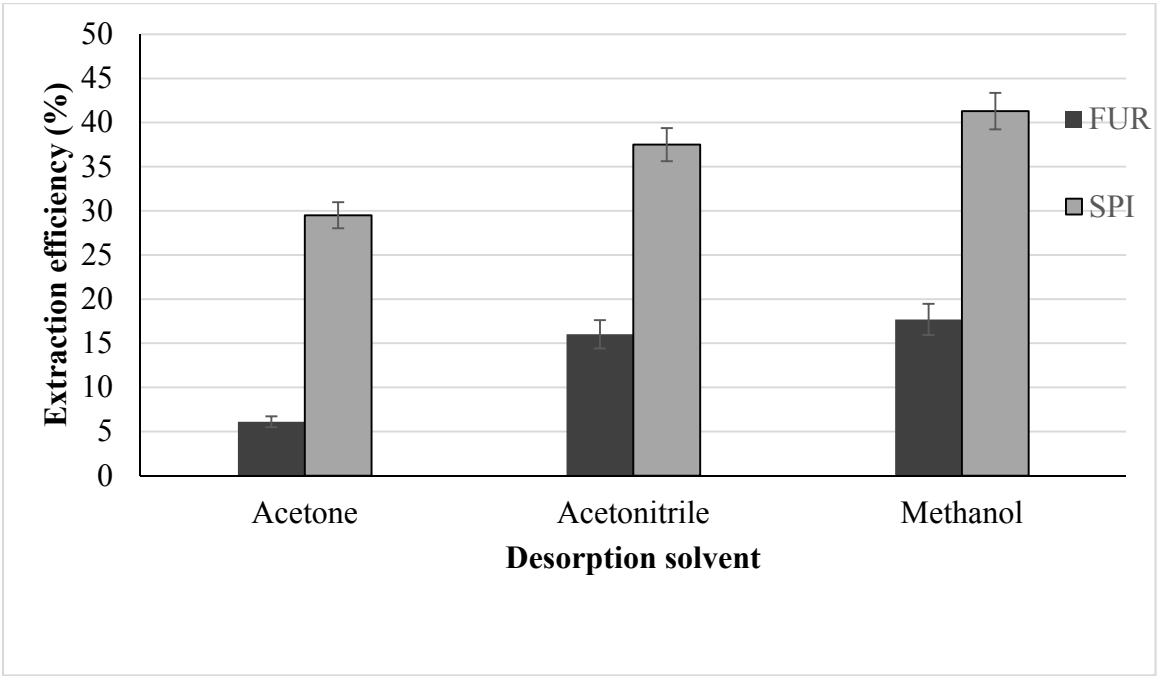


Figure 7: Effect of the type of desorption solvent on the SBME extraction efficiency (using other extraction conditions as in Figure 4)

Table 2. Validation parameters of the proposed method

Parameter	FUR	SPI
Calibration curve	y = 0.7671x + 3.7967	y = 0.3091x + 1.2207
Correlation coefficient (R)	0.9991	0.996
Precision (% RSD, n= 5)	1.53	0.75
LOD (µg L^{-1})	2.09	1.45
LOQ (µg L^{-1})	6.92	4.85
Linear range (µg L^{-1})	6.92-100	4.85-100
Recovery %, (% RSD, n= 3)	17.4% (1.8-3.1)	41.1% (1.3-2.7)

Table 3. Recovery of spiked urine samples by SBME (n=3)

Compound	Added (µg L^{-1})	Found (µg L^{-1})	Recovery (%)	RSD (%)
FUR	10	1.51	15.1	1.18
	50	7.40	14.8	1.13
	100	14.6	14.6	0.95
SPI	10	3.71	37.1	1.22
	50	18.70	37.4	0.77
	100	36.8	36.8	1.20

Table 4. Comparison of the developed SBME method with previous studies for the determination of FUR and SPI in urine samples

Instrument	Sample preparation	LOD (µg L^{-1})		Recovery (%)	RSD (%)	Ref.
		FUR	SPI			
HPLC-DAD	SBME	2.09	1.45	14.6-37.4	0.77-1.22	Current work
HPLC-UV	LLE	20	170	98.37-98.08	5.41-1.35	[25]
GC-MS	SPE	40	250	-	-	[21]
LC-MS/MS	SPE	50		104.1	3.06	[17]
UPLC-MS/MS	LLE	50	50	87.1-104.3	-	[45]

3.1.4 Optimization of the extraction time

The extraction processes of SBME are time-dependent,[35] thus the extraction time profile of the two diuretics under agitation conditions should be optimized. Figure 5 demonstrates the influence of extraction time (from 10 to 70 minutes) on the extraction efficiency of selected analytes.

The results suggest that the responses of the target compounds increased with extraction time, and, at 50 minutes, the extraction efficiency reached the highest level. Thus, 50 minutes was selected as the optimum extraction time.

3.1.5 Optimization of the stirring speed

Appropriate stirring of the sample solution may accelerate the mass transfer process of analytes and increase the extraction efficiency. The effects of stirring speed on the extraction efficiency of the target compounds were investigated in detail at stirring speeds of 100 to 1000 rpm. Overagitation (> 500 rpm) enhanced the formation of bubbles, which tended to adhere to the surface of the fiber and reduce the extraction efficiency. As shown in Figure

6, the speed of 500 rpm was therefore chosen as the best stirring speed for selected analytes.

3.1.6 Optimization of the desorption condition

Selection of a suitable desorption condition including the type of desorption solvent, volume of desorption solvent and desorption time were also evaluated. Methanol, acetonitrile and acetone were used with sonication to release selected diuretics from the C18 sorbent. In comparison with the other solvents used (see Figure 7), methanol showed the highest extraction efficiency, probably due to its higher capability to break the interactions between the C18 material and the reported drugs by forming intermolecular hydrogen bonds with the carbonyl groups of both drugs. Hence, it was adopted as the desorption solvent for the subsequent experiments.

The volume of desorption solvent (methanol) was investigated, ranging from 100 to 300 µL. A volume of 100 µL was not sufficient to immerse the SBME devices during the ultrasonication, and, as shown in Figure 8, the higher volume of methanol (> 250 µL) caused a decrease in the extraction efficiency resulting from dilution of the analyte. As a result, the optimum extraction efficiency for both analytes was reached by using 250 µL of methanol.

The effect of sonication time (5 to 15 minutes) with methanol on extraction efficiency was investigated. Higher extraction efficiency of both drugs was obtained after 10 minutes of sonication as shown in Figure 9. Increasing the time further resulted in lower extraction efficiency, which may be due to the chemical effect of ultrasound on methanol. Previous studies on the sonication of methanol have shown that sonication of this solvent generates free radicals such as superoxide ions, hydroxyl ions, solvated electrons, and atomic hydrogen that cause secondary oxidation-reduction reactions[44]. It is therefore possible that the reduction in the extraction efficiency of FUR and SPI with increased sonication time was due to the oxidation of these compounds by free radicals.

3.2. Validation of the optimized method

Based on the above described experiments, the optimal SBME conditions for the acidic FUR and neutral SPI diuretics were selected as follows: C18 as sorbent material, 5 SBME devices and a pH of 2 for the sample solution reached by adding 0.1 M HCl. In addition, SBME extraction time was performed for 50 minutes with 250 µL of methanol as desorption solvent with a stirring speed of 500 rpm.

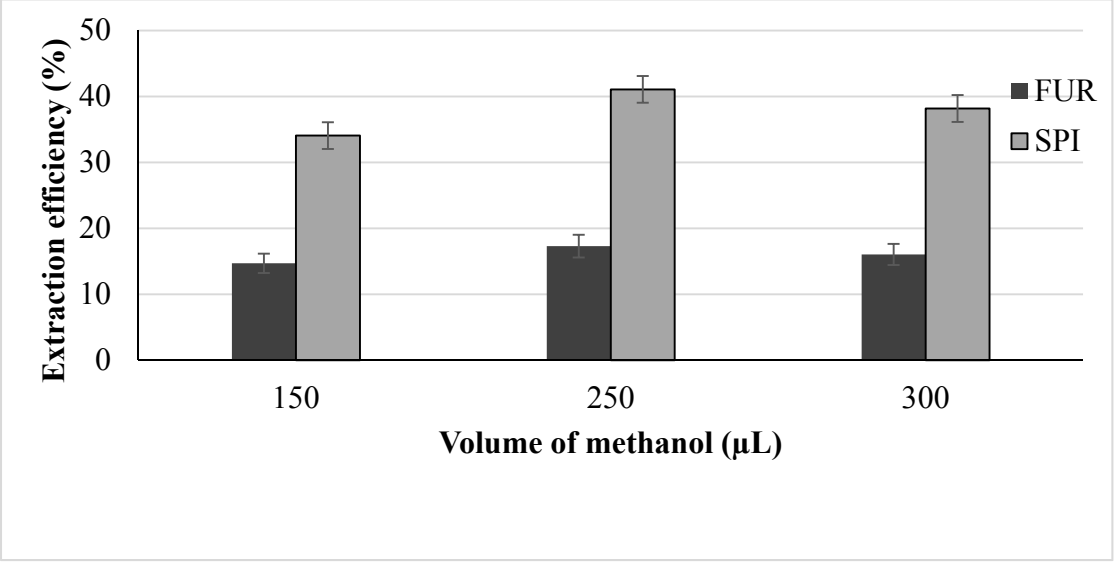


Figure 8: Effect of the volume of desorption solvent on the SBME efficiency (using other extraction conditions as presented in Figure 4)

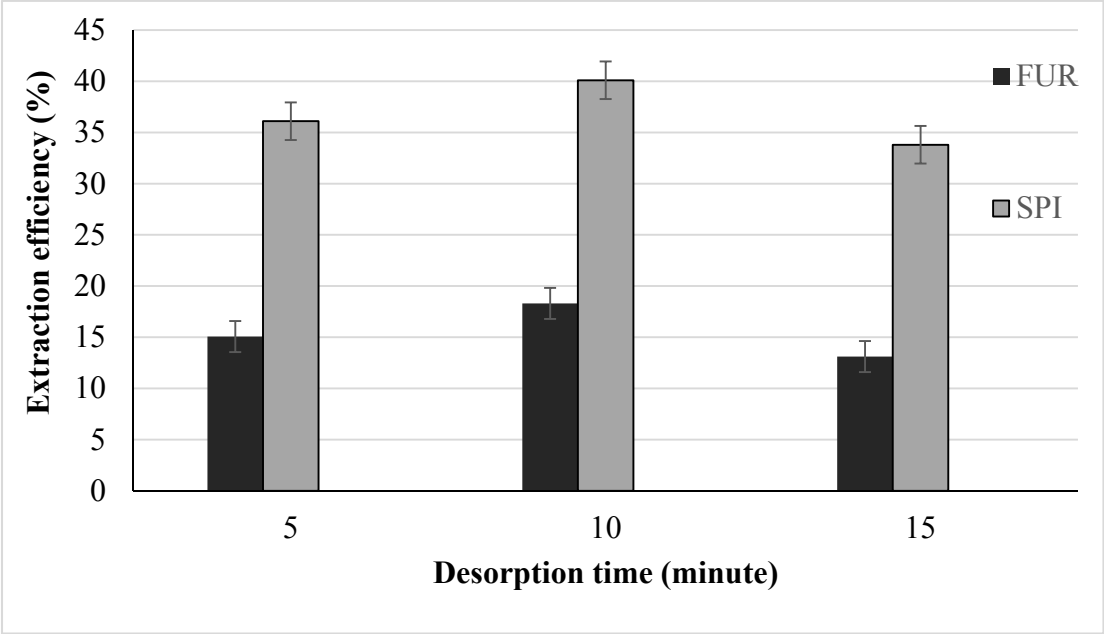


Figure 9: Influence of desorption time on the SBME efficiency (using other extraction conditions as presented in Figure 4)

The validation of this method was carried out by establishing linearity, reproducibility, accuracy, limit of detection (LOD) and limit of quantification (LOQ). Under the optimized conditions, calibration curves were constructed for SBME in the concentration range of 10 to 100 µg L^{-1}. The results shown in Table 2 indicate good linearity with correlation coefficients ≥ 0.996. Reproducibility was determined by five repeated extractions of 15 µg L^{-1} of FUR and SPI. The relative standard deviations (RSD, n=5) were 1.53 and 0.75% for FUR and SPI, respectively. The limits of detection (LOD) based on three standard deviations of ***the blank*** divided by the ***slope*** of the calibration curve (3 S_b/m) were 2.09 and 1.45 µg L^{-1} for FUR and SPI, respectively. Limits of quantification (LOQ) using (10 S_b/m) were 6.92 and 4.85 µg L^{-1} for FUR and SPI, respectively. The accuracy of the SBME method was verified by means of recovery studies following the approach indicated by Zhang et al.[2] More specifically, the recovery percentage was determined in water samples spiked at three concentration levels: low (10 µg L^{-1}), medium (50 µg L^{-1}) and high (100 µg L^{-1}), analyzing each sample three times. As listed in Table 2, the average recoveries of the developed method were 20.4% for FUR and 49.1% for SPI. The RSDs ranged from 1.8 to 3.1% for FUR, and from 1.3 to 2.7% for SPI.

3.3. Method application and comparison with previously reported methods

Due to the reliable quantitative results in the determination of the two analytes in water samples, the final step was to quantify them in the more complex urine matrix. The spiked urine samples (see section 2.2) with three different concentrations (10, 50 and 100 µg L^{-1}) of selected diuretics were analyzed using the developed method. The analytical results of SBME for these spiked urine samples are illustrated in Table 3. As shown in Figure 10, no interfering peaks due to endogenous substances were observed at the retention time of the compounds of interest. The relative standard deviation was less than 1.22% for both drugs, and the recovery was 17.6% and 47.8% for FUR and SPI, respectively.

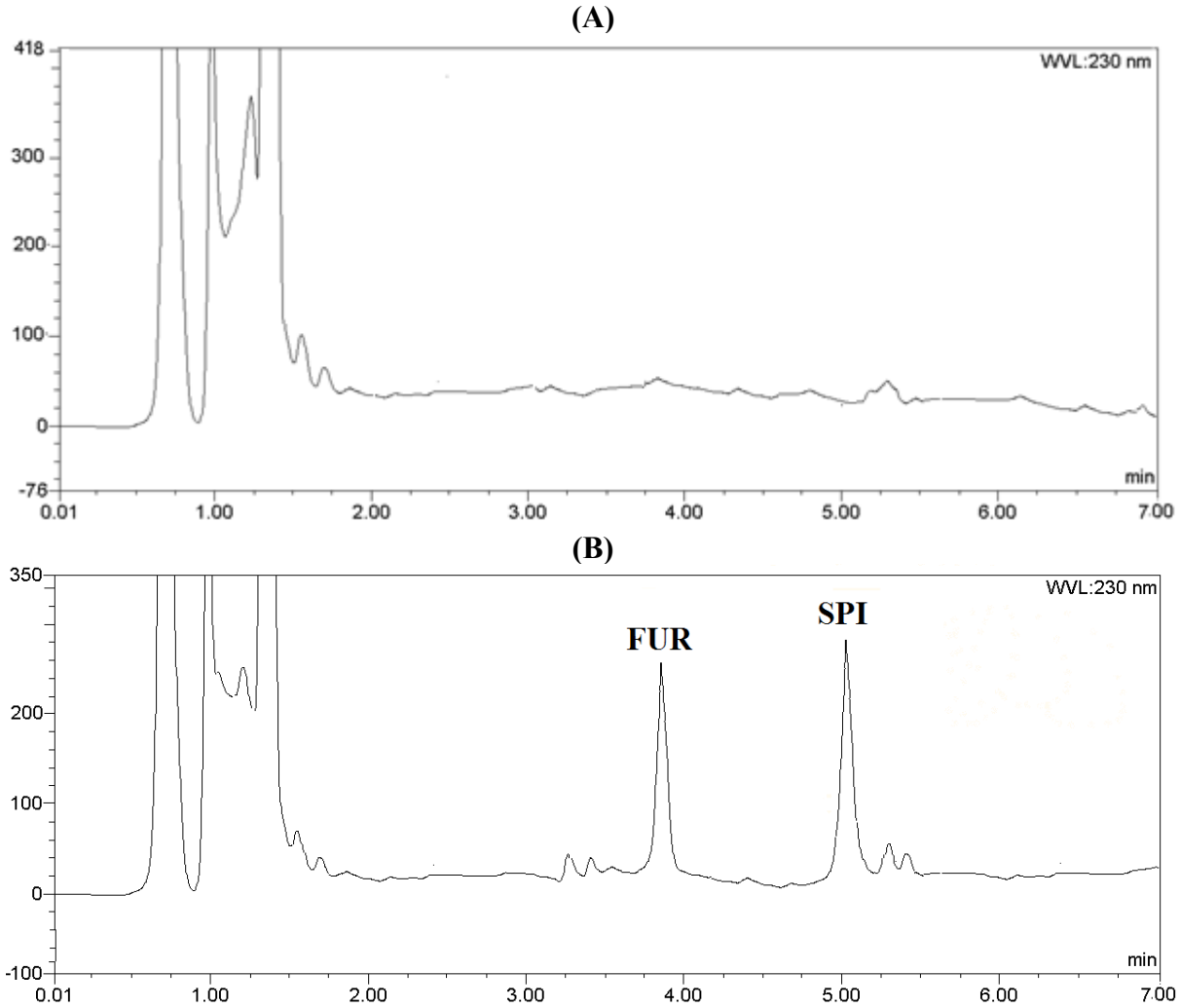


Figure 10: Chromatogram obtained from the SBME of 30 mL of (A) a blank urine sample and (B) a urine sample spiked with 10 µg L^{-1} of selected analytes

A comparison between the SBME and the previous reported methods in the extraction of FUR and SPI from urine samples is listed in Table 4. It is clear whether the developed method provided a better sensitivity (reflected by LOD) and repeatability than the more expensive HPLC-MS[17,45] and GC-MS[21] methods. However, the lower recovery percentage of SBME was expected, as the SBME is a non-exhaustive extraction technique[35].

4. Conclusion

In this work, the analytical method based on SBME and HPLC-DAD was set up for the determination of acidic (FUR) and neutral (SPI) diuretics in spiked human urine. The convenient detection limits have been successfully applied to the assay of both analytes in spiked human urine specimen. Analytical results indicate that this proposed low-cost method is environmentally friendly (using only 250 µL of organic solvent), highly sensitive and very suitable for the determination of trace diuretics from human urine. Furthermore, SBME could be used for extracting the selected diuretics directly from human urine samples without further cleanup procedures.

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استخدام تقنية قضبان الاستخلاص الدقيق الصلبة مع الكروماتوغرافيا السائلة عاليــة الكفاءة المرتبطة بكاشفة المجموعة الضوئية في تحديد الادوية المدرة للبول في عينات البول البشرية

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ملخص

في هذه الدراسة تم استخدام تقنية قضبان الاستخلاص الدقيق الصلبة مع الكروماتوغرافيا السائلة عالية الكفاءة المرتبطة بكاشفة المجموعة الضوئية في استخلاص وتقدير دوائي الفيوروسيمايد والسبيرونولاكتون في عينات البول البشرية. وتم أولاً فصل الأدوية المختارة من محاليل مائية (مضاف اليها الادوية المذكورة بتراكيز معروفة) إلى المادة الماصة المحشوة داخل تجويف ألياف البولي بروبلين والمغلقة من كلا الطرفين. بعد الانتهاء من مرحلة الاستخلاص، تم فصل الادوية المذكورة من تقنية قضبان الاستخلاص الدقيق الصلبة في حجم صغير من المذيبات العضوية المناسبة بمساعدة الأمواج فوق الصوتية. ومن أجل الحصول على اعلى كفاءة استخلاص فقد تم دراسة تأثير عدة عوامل من الممكن أن تؤثر في كفاءة الاستخلاص للتقنية المذكورة. تحت الظروف المثلى، كانت منحنيات المعايرة خطية في مدى تركيز من 10الى 100 ميكروغرام لكل لتر، مع دقة جيدة (الانحراف المعياري النسبي ≤ 1.53%) وحساسية ممتازة (حد الكشف/الحد الكمي كانت 2.09 / 6.92 و 4.85/1.45 ميكروغرام لكل من فيوروسيمايد والسبيرونولاكتون، على التوالي). في نهاية البحث، تم تطبيق الطريقة المطورة في استخلاص وتقدير كل من الفيوروسيمايد والسبيرانولاكتون في عينات البول البشري (المضاف اليها الادوية المذكورة بتراكيز معروفة) والمأخوذة من متطوعين

أثبتت النتائج بأن تقنية قضبان الاستخلاص الدقيق الصلبة مع الكروماتوغرافيا السائلة عالية الكفاءة المرتبطة بكاشفة المجموعة الضوئية غير مكلفة نسبيا وحساسة وصديقة للبيئة، بالإضافة إلى إمكانية تطبيقها بشكل مباشر لاستخلاص وتقدير دوائي الفيوروسيمايد والسبيرونولاكتون في عينات البول البشرية دون الحاجة لمزيد من إجراءات التنقية.

الكلمات الدالة: أدوية مدرة للبول، الفيوروسيمايد، السبيرونولاكتون، تقنية قضبان الاستخلاص الدقيق الصلبة، بول، الكروماتوغرافيا السائلة عالية الكفاءة.

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