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Detection of digoxin in urine samples by surface-assisted laser desorption/ionization mass spectrometry with dispersive liquid–liquid microextraction

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ABSTRACT

A novel method for the detection of digoxin using dispersive liquid–liquid microextraction (DLLME) coupled to the surface-assisted laser desorption/ionization mass spectrometric detection (SALDI/MS) was developed. Acetone and chloroform were used as the disperser solvent and extraction solvents, respectively. After the extraction, digoxin was detected using SALDI/MS with colloidal palladium as the matrix. Under optimal extraction and detection conditions, the calibration curve, which ranged from 0.01 to 0.50 μM, was observed to be linear. The limit of detection (LOD) at a signal-to-noise ratio of 3 was 2 nM for digoxin. With a sample-to-extract volume ratio of 400, the enrichment factor for digoxin was calculated to be 252. This novel method was successfully applied for the determination of digoxin in human urine samples.

1. Introduction

Digoxin is a cardiac glycoside that has become the most widely prescribed medication for treating congestive heart failure and arrhythmias [1]. Unfortunately, digoxin has a small therapeutic range; when the concentration of digoxin in plasma exceeds 2 ng mL^{-1} (2 nM), intoxication may occur [2]. Therefore, a simple and sensitive analytical procedure for monitoring the concentration of digoxin in biological fluids is required to ensure optimum efficacy while minimizing the risk of toxicity and other adverse effects.

Traditional digoxin detection has been performed using microbiological assays [3], but these methods are often time-consuming and lack the specificity and reproducibility needed for analytical purposes. High performance liquid chromatography (HPLC) has been proposed for the analysis of digoxin. However, because digoxin does not contain a chromophore or fluorophore in its structure (Fig. 1), the compound is difficult to detect with a UV-based detector, with the exception of low UV wavelengths [4] for which the detection limits are not favorable. Fluorescence detection [5] and mass spectrometric (MS) detection [6–7] have also been reported for HPLC analysis of digoxin. Among these methods, LC/MS is the most popular technique due to its sensitivity and specificity, as well as its ability to identify unknown compounds. However, LC/MS instruments require complicated sample preparation and are expensive. Recently, an optical sensor using a molecularly imprinted polymer (MIP) membrane was developed for the determination of digoxin in serum samples [8].

Despite the high sensitivity achieved by many analytical methods, an extraction/preconcentration step is generally required for the determination of digoxin in biological samples. Liquid–liquid extraction (LLE) [9,10] and solid-phase extraction (SPE) [4,11] using commercial SPE cartridges are the most common procedures for extraction/preconcentration of digoxin from urine and plasma samples. However, these sample pre-treatment procedures are tedious and time-consuming. Recently, dispersive liquid–liquid microextraction (DLLME) has become an important sample preparation technique due to its rapidity, ease of operation, and low cost [12,13]. DLLME is based on a ternary solvent system in which the extraction and disperser solvents are rapidly injected into the aqueous sample to form a cloudy solution. Extraction equilibrium is quickly achieved due to the large amount of surface contact between the droplets of the extraction solvent and the aqueous sample. Therefore, the extraction time is very short. After the centrifugation of the cloudy solution, the extraction solvent generally settles at the bottom of the tube and is aspirated with a microsyringe for instrumental analysis. The DLLME technique, coupled with GC and LC, has been widely applied to the analysis of PAH and pesticides [14–15]. Despite

Abbreviations: surface-assisted laser desorption/ionization mass spectrometry, SALDI/MS; dispersive liquid-liquid microextraction, DLLME; nanoparticles, NPs

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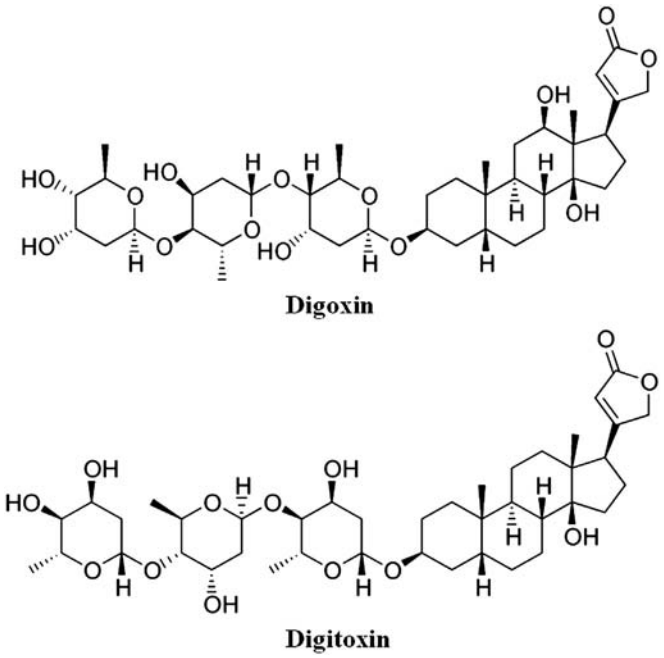

Fig. 1. Structural formulas for digoxin and digitoxin.

the wide applicability of DLLME coupled with GC and LC, there are a few reports on the use of DLLME coupled with the surface-assisted laser desorption/ionization mass spectrometry (SALDI/MS) in the literature [16]. Additionally, DLLME has not yet been applied to the extraction of digoxin.

SALDI/MS has become a popular tool for the analysis of drugs due to its excellent capability for high-throughput, reduced background noise in the low-mass region, and better shot-to-shot reproducibility. Nanomaterials, such as gold nanoparticles (NPs) [17,18], silver NPs [19,20], titanium dioxide NPs [21], colloidal graphite [22], nanostructured silicon [23], and diamond nanowire [24] have been recognized as effective SALDI matrices. Although a variety of NPs have been successfully applied as matrices in SALDI/MS, to date, no application of palladium NPs in SALDI/MS has been reported. The maximum absorption wavelength of Pd NPs is 320 nm [25,26], which is close to the emission wavelength of the most commonly used nitrogen laser (337 nm) in SALDI/MS. Pd NPs may efficiently absorb laser energy, rapidly transfer laser energy to the analytes, and then induce the desorption and ionization of the analytes. Our goal in this study is to examine whether the colloidal Pd can be used as a SALDI matrix for digoxin. In addition, we combined the DLLME technique with SALDI/MS for the analysis of digoxin. The factors that influence the extraction efficiencies and analyte detection were investigated. The applicability of the method for the determination of digoxin in human urine was also demonstrated. To our knowledge, this is the first report that demonstrates the use of SALDI/MS for the analysis of digoxin.

2. Experimental methods

2.1. Chemicals and solutions

Digoxin, digitoxin and carbon tetrachloride were purchased from Sigma (St. Louis, MO, USA). Dichloromethane was obtained from J.T. Baker (Phillipsburg, NJ, USA). Chloroform was obtained from Showa (Tokyo, Japan). Colloidal suspensions of palladium (10 ppm), gold (20 ppm), and silver (10 ppm) were purchased from Purest Colloids (Westhampton, NJ, USA). All chemicals were used as received without further purification. Water that was purified with a Millipore Synergy water purification system (Billerica, MA, USA) was used for all solutions.

Stock standard solutions (1 mM) of digoxin and digitoxin were prepared in methanol and were diluted to the desired concentrations with 5 mM phosphate buffer (pH 7). The digoxin and digitoxin solutions were stored at 4 °C for a month.

2.2. DLLME procedure

An aliquot (1 mL) of buffered solution containing the digoxin was placed in a 1.5 mL sample vial. The disperser solvent acetone (140 μL) and the extraction solvent chloroform (60 μL) were rapidly injected into the sample solution using a 1.0 mL syringe (Hamilton, Reno, NV, USA), and a cloudy solution was formed. The mixture was gently shaken for 5 min. The mixture was then centrifuged at 3500*g* for 5 min, and the dispersed fine droplets of the extraction solvent settled at the bottom of the sample vial. Fifty-five microliters of the sediment phase was transferred to a separate sample vial using a 100 μL HPLC syringe (Hamilton, Reno, NV, USA). The extract was evaporated to dryness. The extract residue was re-dissolved with 2.5 μL of 5 mM phosphate buffer (pH 7) containing I.S. (5 μM).

2.3. SALDI/MS measurements

One microliter of the extract solution was mixed with 1 μL of colloidal Pd solution. Then, 1 μL of the mixture was deposited onto a stainless steel target and was allowed to dry at room temperature.

Mass spectrometry experiments were performed in positive-ion mode on a reflectron-type time-of-flight mass spectrometer (Microflex, Bruker Daltonics) with a flight length of 1.96 m. The samples were irradiated with a 337 nm nitrogen laser at 20 Hz. The generated ions were accelerated at a voltage of 19 kV. To obtain good signal-to-noise ratios, the laser energy settings were adjusted to slightly exceed the threshold, and each spectrum was acquired from an average of 100 laser pulses.

2.4. Preparation of urine samples

Human urine samples were collected from healthy volunteers who were not receiving any pharmaceutical treatment at the time of sampling. The urine samples were stored at −20 °C until analysis. An aliquot (990 μL) of the urine sample was spiked with 10 μL of the digoxin standard. Urine samples of various digoxin concentrations were prepared by spiking the urine with the desired amounts of digoxin. A blank urine sample was prepared by spiking 10 μL of DI water into 990 μL of urine. The digoxin-spiked urine (1 mL) was treated with DLLME following the procedure described above.

3. Results and discussion

3.1. Determination of digoxin by SALDI/MS

Previously, Au and Ag NPs were the most commonly used SALDI matrices. We investigated the use of colloidal Au and Ag as SALDI matrices for the detection of digoxin. Fig. 2A and B shows the mass spectra of digoxin using colloidal Au and Ag as SALDI matrices, respectively. The ion signal at $m/z=803.42$ corresponded to the sodium adduct ion of digoxin. Although colloidal Au and Ag contributed some background ions, the results indicated that colloidal Au and Ag successfully desorbed and ionized digoxin. Because the maximum absorption wavelength of Pd NPs is 320 nm, colloidal Pd may be a more efficient SALDI matrix when

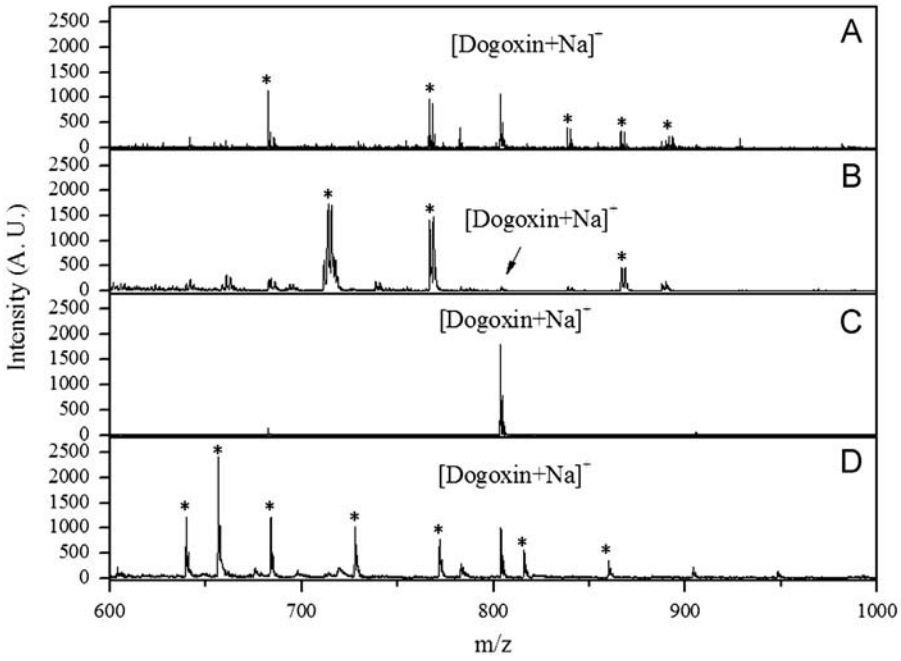

Fig. 2. Mass spectra of digoxin with (A) colloidal Au, (B) colloidal Ag, (C) colloidal Pd and (D) α-CHCA matrices. The ions at m/z=803.42 corresponded to the sodium adduct ions of digoxin. The sample solutions were prepared in 5 mM phosphate buffer, pH 7. The concentration of the digoxin was 10 μM. A total of 50 pulsed laser shots were applied using a laser that was adjusted to energy levels that slightly exceed the threshold. Those peaks marked with asterisks were generated from matrices.

using a nitrogen laser. Fig. 2C shows the mass spectrum of digoxin using colloidal Pd as the SALDI matrix. The sodium adduct ions of digoxin were obtained. The signal intensity of digoxin was 1.3 and 20.1 times greater than that obtained using colloidal Au and Ag as the SALDI matrices, respectively. The reproducibility of the SALDI sample preparation was examined. The signal intensities of the sodium adduct ions of digoxin varied 10.7% over 21 sample spots (3 samples) in the presence of internal standard. In comparison, we used α-cyano-4-hydroxycinnamic acid (α-CHCA), a common organic matrix for MALDI/MS, to facilitate the ionization of digoxin. As shown in Fig. 2D, digoxin was ionized as [digoxin +Na]$^+$. The signal intensity of [digoxin+Na]$^+$ when using α-CHCA was lower than the signal intensity of [digoxin+Na]$^+$ obtained when colloidal Pd was used as the matrix. Compared with α-CHCA, using colloidal Pd as the SALDI matrix resulted in higher sensitivity, less complex mass spectra, and fewer problems associated with nonhomogeneous samples.

Using Pd as the SALDI matrix, typical mass spectra obtained from colloidal Pd and colloidal Pd with digoxin (1μM) are shown in Fig. 3A and B. The ions at m/z=803.42 and 787.42 corresponded to the sodium adduct ions of digoxin and the I.S., respectively. A calibration curve for digoxin was constructed over the concentration range of 1–50 μM using digitoxin (5 μM) as an I.S. A plot of the ratio of the digoxin/I.S. signal intensity versus concentration exhibited good linearity (y=0.1169x−0.0020) with a correlation coefficient (r) of 0.9984 (n=7). The limit of detection (LOD) for digoxin, calculated as three times the signal-to-noise (S/N) ratio, was 390 nM. To further lower the LOD, DLLME was employed to enrich the analytes prior to the SALDI/MS analysis.

3.2. Optimization of DLLME

To optimize the extraction efficiency by DLLME, the amount of extracted digoxin was monitored based on the ratio of the digoxin/I.S. ion intensities of sodium adduct ions in the three samples. The selection of the disperser solvent and the extraction solvent is a critical factor in DLLME. All combinations of dichloromethane, carbon tetrachloride, and chloroform as extraction solvents with acetonitrile, tetrahydrofuran, and acetone as disperser solvents were investigated. Sixty microliters of extraction solvent was dissolved in 140 μL of the disperser solvent. The combination of chloroform and acetone provided the highest extraction efficiency. The effect of the disperser solvent volume on the signal intensity was investigated. The results showed that the signal intensities increased when the disperser solvent volume increased from 110 to 140 μL. At lower volumes of acetone, the cloudy solution did not form well, resulting in decreased signal intensities. At higher volumes of acetone, the solubility of the digoxin in water increased and the signal intensities decreased. To investigate the effect of the extraction solvent volume, several volumes of chloroform were subjected to the same DLLME procedures. From those samples, 25, 35, 45, 55, and 65 μL of the sediment phases was collected for analysis from chloroform solutions of 30, 40, 50, 60, and 70 μL, respectively. Fig. 4 shows that the signal intensities increased as the extraction solvent volume increased from 30 to 60 μL. Hence, 60 μL of extraction solvent volume was used for all subsequent experiments.

The optimal extraction time was investigated by plotting the ratio of the digoxin/I.S. signal intensity versus the extraction time within the range of 1–15 min, while holding all other parameters constant. As shown in Fig. 5, the observed signal intensities of digoxin increased as the extraction times increased from 1 to 5 min, and reaching a plateau after 5 min. Thus, the extraction time was set to 5 min. Due to the large amount of surface contact between the solvent droplets and the aqueous sample, the mass transfer from the sample solution to the extraction solvent is very rapid. This rapid mass transfer is the most important advantage conferred by the DLLME technique.

3.3. Analytical characteristics

A calibration curve for digoxin in 5 mM phosphate buffer (pH 7) was constructed over the concentration range of 0.01–0.50 μM using digitoxin (5 μM) as an I.S. A plot of the ratio of the digoxin/I.S. signal intensity versus concentration presented good linearity (y=29.4114x+0.1446) with a correlation coefficient (r) of 0.9974 (n=7). The LOD and limit of quantitation (LOQ, S/N=10) values for

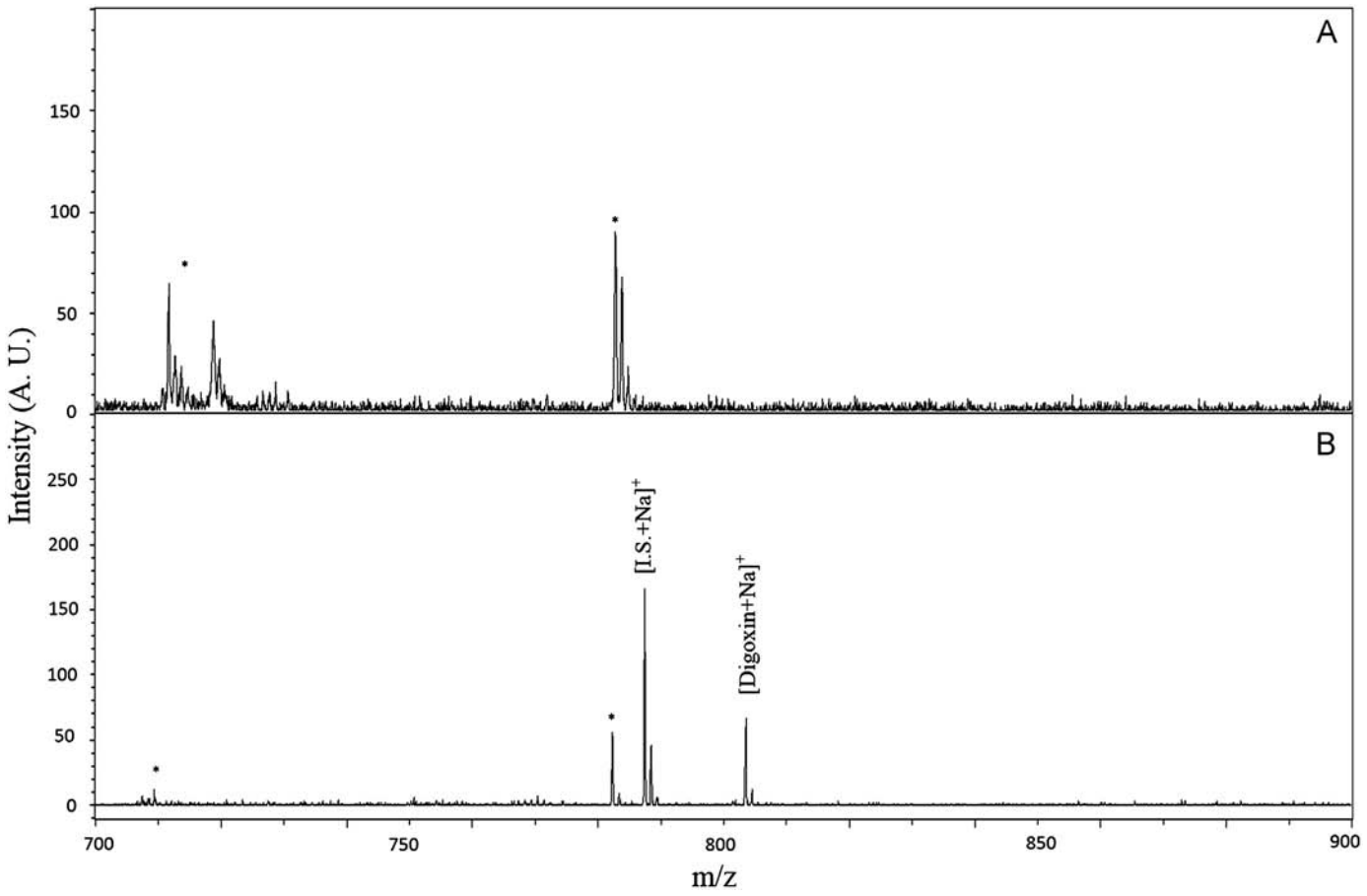

Fig. 3. Mass spectra of (A) colloidal Pd and (B) colloidal Pd with digoxin. The concentrations of digoxin and the I.S. were 1 μM and 5 μM, respectively. Those peaks marked with asterisks were generated from Pd matrix. The other conditions were the same as those provided in Fig. 2.

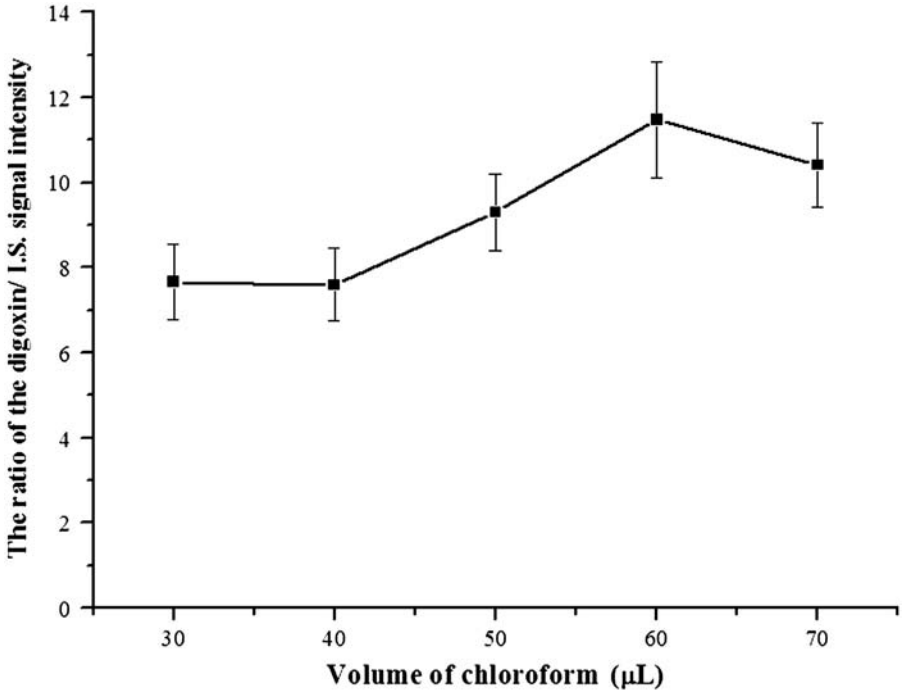

Fig. 4. Effect of the chloroform volume on the signal intensity ratio of digoxin to digitoxin obtained from DLLME. Experimental conditions: sample volume, 1 mL; extraction time, 5 min; dispersive solvent, acetone (140 μL); and digoxin concentration, 10 μM.

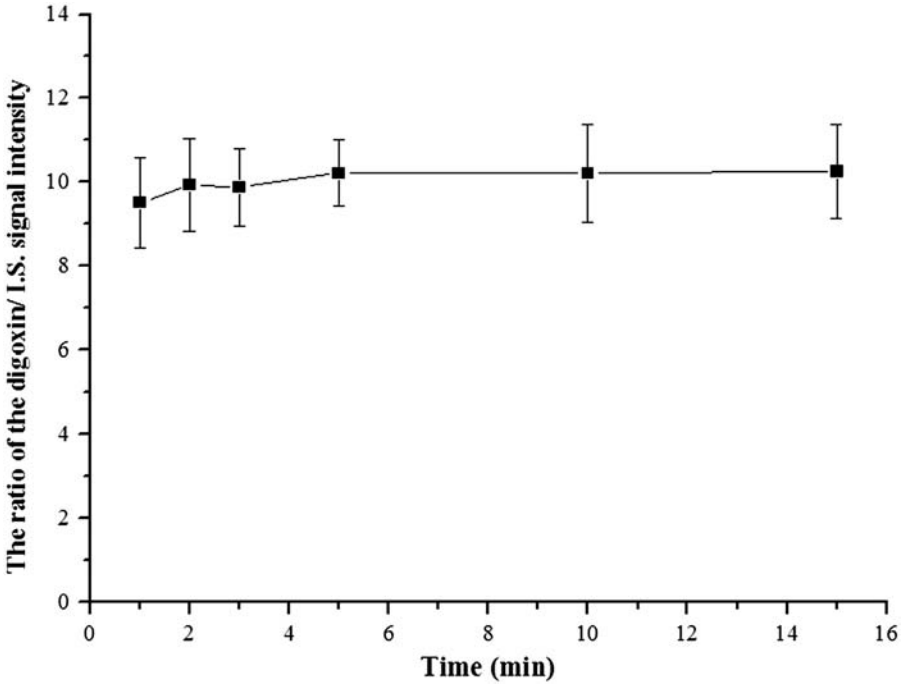

Fig. 5. Effect of extraction time on the signal intensity ratio of digoxin to digitoxin obtained from DLLME. The other conditions were the same as those provided in Fig. 4.

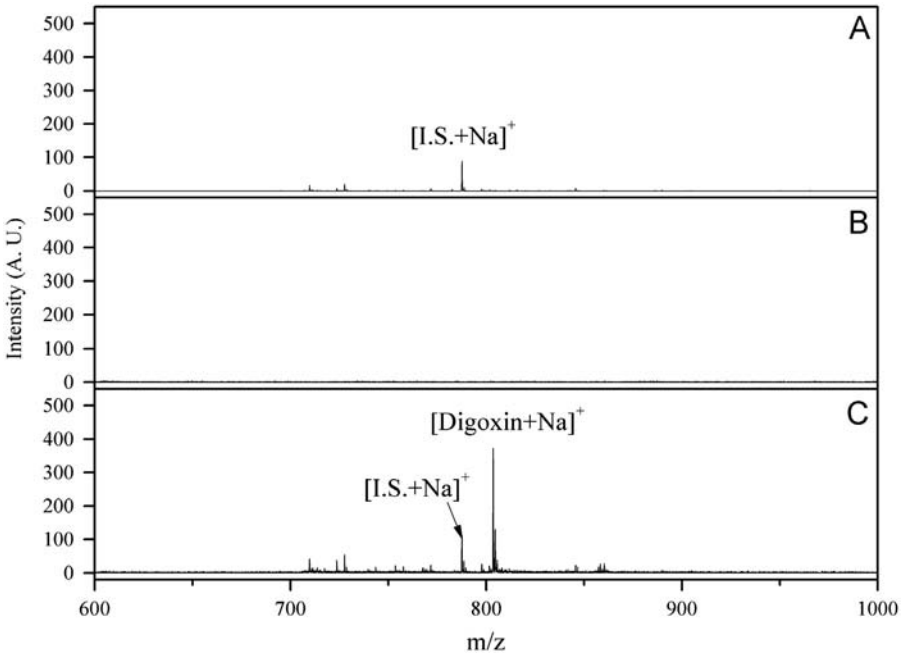

Fig. 6. Mass spectra of (A) human urine samples obtained without pretreatment, (B) blank urine sample after extraction by DLLME and (C) digoxin-spiked urine sample after extraction by DLLME. The ions at m/z=803.42 and 787.42 corresponded to the sodium adduct ions of digoxin and the I.S., respectively. Experimental conditions: urine sample volume, 1 mL; organic extractant, chloroform (60 μL); dispersive solvent, acetone (140 μL); extraction time, 5 min; digoxin concentration spiked, 0.3 μM; and I.S. concentration, 5 μM. The other conditions were the same as those provided in Fig. 2.

digoxin in aqueous solution were calculated to be 2 nM and 8 nM, respectively. An enrichment factor (EF) was estimated following a previously reported method [27]. With a sample-to-extract volume ratio of 400, the EF for digoxin was calculated to be 252.

3.4. Applications

To evaluate the applicability of this method for biological and clinical analyses, human urine spiked with digoxin was used as a test sample. The digoxin-spiked urine (1 mL) was treated with the DLLME technique using the procedure described above. Fig. 6A presents the result from the direct analysis (without DLLME) of the digoxin-spiked urine sample. No digoxin ion was present because the concentration was below the LOD of digoxin with direct SALDI/MS analysis. After treatment using the DLLME method, typical mass spectra obtained from blank and digoxin-spiked urine samples are shown in Fig. 6B and C. The ions at m/z=803.42 and 787.42 corresponded to the sodium adduct ions of digoxin and the I.S., respectively. Although the

urine sample matrix was complex, it did not interfere with the signals of I.S. and digoxin. A calibration curve was constructed using the digoxin-spiked urine in the concentration range of 0.05–0.50 μM. The plot of the ratio of the digoxin/I.S. peak area versus concentration exhibited good linearity ($y=20.3893x+0.1870$) with a correlation coefficient (r) of 0.9901 ($n=6$). The LOD and LOQ values for digoxin in urine were calculated to be 12 nM and 41 nM, respectively. Although LC/MS coupled with SPE [2,28] provides a lower LOD value for digoxin in urine, more complicated sample preparation and instrumentation are required.

Analyzing blank urine samples obtained from six healthy adults tested the selectivity of this method. For the six samples that were tested, there were no endogenous compounds that interfered with digoxin or the I.S. The intra-day and inter-day accuracy and precision of this method were evaluated using urine samples spiked with 0.3 μM of digoxin. The intra-day and inter-day accuracy values for digoxin in urine, which are represented as relative error (RE), for the digoxin in urine were −5.5% and −8.1%, respectively. The intra-day and inter-day precision values for digoxin in urine, which are represented as relative standard deviation (RSD), were 5.2% and 10.2%, respectively. These values meet the accuracy and precision criteria set forth in the guidelines for the bioanalytical method validation [29]. The recovery of digoxin in urine samples was determined by the standard addition method base on peak height. The mean recovery of digoxin was found to be 64.4%. The analysis of digoxin in urine samples can be readily performed in less than 10 min. This newly developed SALDI/MS method, with its speed and ease of operation, is suitable for high-throughput screening of digoxin in human urine samples.

4. Concluding remarks

We have developed a DLLME method for the extraction of digoxin from aqueous solutions. Coupled with the SALDI/MS detection, this method provides the advantages of simplicity, rapidity, and high sensitivity. The analysis of digoxin in an aqueous solution can be readily performed in less than 10 min. We also successfully applied this newly developed method for the determination of digoxin in human urine samples. This SALDI/MS method, with its speed and ease of operation, is suitable for high-throughput screening of digoxin levels in human urine samples.

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