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Matrix-assisted laser desorption/ionization mass spectrometry for quantitative determination of β-blocker drugs in one-drop of human serum sample

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ABSTRACT

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) has been applied for the quantitative determination of β-blocker drugs in one-drop of human serum samples using drop-to-drop solvent microextraction (DDSME) as a preconcentrating probe. The optimum experimental conditions for β-blocker drugs were investigated and 1.8 μL volume of toluene for 10 min extraction time with the 5% addition of NaCl under pH 11.0 was found to be the best conditions for the separation and preconcentration of drugs from 30 μL of serum sample from a patient with high blood pressure. The optimized methodologies for DDSME/MALDI-MS analyses exhibited a good linearity with intra- and inter day precision value of 8.5–10.5% and 9.4–12.6%, respectively. The proposed DDSME/MALDI-MS offers a very simple, rapid and low-cost technique for the determination of β-blocker drugs in one drop of serum sample. The reported method has been successfully applied for the determination of propranolol and nadolol in small volume of serum sample from patient suffering from high blood pressure. In future, this technique could be applied for pharmacokinetic and clinical studies.

1. Introduction

Propranolol and nadolol are non-selective β-(beta)-blocker drugs used in the treatment of high blood pressure, migraine headaches, and chest pain. In addition, β-blockers are used to prevent symptoms of hyperthyroidism and some forms of tremor. The side effect of β-blocker drugs is congestive heart failure, bradycardia, fatique and brochospasms [1–3]. Several analytical methods based on, spectrophotometry, phosphorescence, gas chromatography, high performance liquid chromatography (HPLC), electrospray ionization-mass spectrometry (ESI-MS) and HPLC–MS have been described in the literatures for the determination of the β-blocker drugs from various samples [4–10]. HPLC, HPLC–MS and ESI-MS are found to offer better sensitivities for the determination of β-blocker drugs. These techniques require more amount of organic solvents, extensive sample preparation and long analysis time. Therefore, a simple, rapid and efficient method for the determination of β-blocker drugs in serum sample is required in order to assess the side effect of the drug on human health.

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) is a rapid and powerful technique for the analysis of biomolecules, including peptides, proteins and nucleic acids [11–16]. Recently, MALDI-MS has been applied for the monitoring of drugs and environmental toxicants due to its high sample throughput and easy operations [17–20]. The proposed method is rapid and cost effective for analysis of β-blocker drugs in serum samples. Sample preparation in MALDI-MS is the most important step for obtaining a good quality spectrum, which could be helpful for easy identification of particular target compounds in complex samples. Several sample preparation methods are regularly practiced for the MALDI-MS analysis of drugs, proteins, and pollutants from complex matrices samples [13–20]. The most widely used techniques for the separation and preconcentration of trace amount of analytes include, liquid–liquid extraction (LLE) [21] and solid-phase extraction (SPE) [22]. But the disadvantages of such techniques are lengthy time consuming extraction process and requirement of large amount of organic solvents for the extraction of analytes. Solid-phase microextraction (SPME) is an excellent alternative technique. The drawbacks of SPME are that fibers used are expensive with a limited lifetime and sample carry-over can be a problem [23]. In recent years, liquid-phase microextraction (LPME) such as single drop microextraction (SDME) [24] and hollow fiber liquid-phase microextraction [25] has been developed as solvent-minimized sample pretreatment procedures since they are inexpensive and have a minimal exposure to toxic organic solvents. Recently, drop-to-drop solvent microextractions (DDSME) have been reported for separation and preconcentration of various molecules from different types of biological samples prior to instrumental analysis. The main disadvantage of the DDSME is lower sensitivity with a possibility of increasing the sensitivity by increas-

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ing the volume of the drug sample (>30 μL) taken for the extraction. DDSME is a simple and rapid sample preparation technique where the micro amount (15.0–30.0 μL) of biological sample is used for the analysis of target compound [18,26,27].

In the present study, DDSME technique was applied for the separation and enrichment of β-blocker drugs from 30 μL of serum sample followed by MALDI-MS analysis with the addition of CHCA as a matrix to ionize the drugs in the MS. The parameters that affected the separation and preconcentration of β-blocker drugs were optimized to carryout the determination of drugs from serum samples. Internal standard (IS) compound was used to improve shot-to-shot and sample-to-sample to reproducibility in order to determine the concentration of drugs in serum samples.

2. Experimental

2.1. Reagents and solution preparations

All the chemicals used were of analytical reagent grade. The structures of propranolol (PN) and nadolol (ND) used are given in Fig. 1. Both the drugs, α-cyano-4-hydroxycinnamic acid (CHCA) and trifluoroacetic acid (TFA) were obtained from Sigma (St. Louis, MO, USA). Toluene, chloroform, n-hexane, n-butane, chloromethane and sodium chloride were purchased from Merck (Darmstadt, Germany). Stock solution of propranolol nadolol and quinine (QN, used as IS) was prepared separately by dissolving 1 mg of substance in 1 mL of methanol. Working standard solutions of concentrations (50, 100, 200, 500, and 1000 ng mL^{-1}) were prepared by diluting the stock solution with deionized water. A solution of CHCA (26.4×10^{-3} M) was prepared by dissolving an appropriate amount of substance in 50% ACN containing a 0.1% TFA. The patients were given a dose of 160 mg of propranolol after every 24 h. Blood samples were collected before giving another dose to patient. Blood samples were collected in cleaned polyethylene tubes from healthy persons and patients of high blood pressure with the help of medical staff. The blood samples were centrifuged for 5 min at 12,000 rpm to separate red blood cells from serum. The serum sample obtained from blood sample was stored in refrigerator at −15 °C until the DDSME/MALDI-MS analysis.

2.2. Procedure for separation and preconcentration of β-blocker drugs using DDSME and followed by MALDI-MS analysis

For DDSME, 30 μL of spiked serum (500 ng mL^{-1} of propranolol, nadolol and quinine) was placed in a 100 μL glass vial. The micro syringe (10F, SGE Australia) containing 1.8 μL volume of organic solvent was clamped above the vial and needle was immersed into the sample. Before extraction, the micro syringe was washed at least 10 times with extracting solvent in order to eliminate the bubbles from the barrel and the needle. The micro syringe fixed with a stand and clamps was then inserted through the septum of the sample vial and the plunger was pushed down to expose the micro drop in to the sample solution to extract β-blocker drugs for prescribed extraction time. After, the completion of extraction time, the solvent was retracted back to syringe and directly spotted on to the MALDI target plate for the determination of drugs with same amount of CHCA matrix containing an IS.

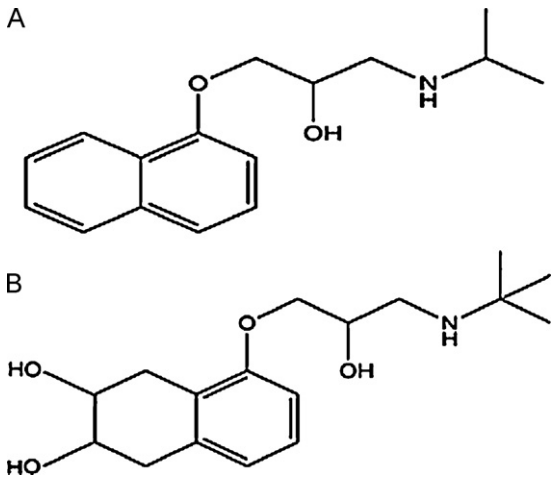

Fig. 1. Structure of propranolol and nadolol β-blocker drugs.

2.3. MALDI-MS analysis of β-blocker drugs

β-Blocker drugs were analyzed using a Biflex III (Bruker Daltonics, Billericia, MA) MALDI-MS with a nitrogen laser of 337 nm operated in positive ion mode for the desorption and ionization of analytes from the sample source. The acceleration voltage and pulse voltage were maintained at 20 kV and 1300 V, respectively, and the extraction delay time was 225 ns. Spectra were obtained with the summation of all individual shots for 1 min. The spectra were the average of 200 laser shots.

3. Results and discussion

The determination of organic compounds in MALDI-MS is a difficult task due to the poor shot-to-shot and sample-to-sample reproducibility guided by the heterogeneous crystal formation of matrix with analyte molecules. Thus, in the present study, quinine was added as IS in order to overcome the variability in signal response when laser light moves across the sample surface. Fig. 2(A) and (B) shows the MALDI-MS spectra of spiked β-blocker drugs in serum sample without (directly analysis) and with the use of DDSME for the separation and preconcentration of drugs with the addition of CHCA as a matrix. The peaks were observed at m/z 260.2 $[PN+H]^+$, m/z 310.2 $[ND+H]^+$, and m/z 325.3 $[QN+H]^+$ in the mass spectrum. The prominent signals observed were at m/z 190.5 $[CHCA+H]^+$ and m/z 379.8 [2 CHCA+H]$^+$ additionally small peaks were observed possibly from serum when DDSME was not used prior to MALDI-MS analysis, shown in Fig. 2(A). A 12–15-fold enhancement in the signal intensity of drugs was obtained when DDSME was used as separating and preconcentrating probes for the determination of drugs from serum samples (Fig. 2(B)). Thus, DDSME/MALDI-MS is important in the determination of β-blocker drugs from serum samples.

3.1. Optimization of DDSME for the extraction of β-drugs in MALDI-MS

The extraction efficiency of β-drugs in MALDI-MS was monitored as the relative abundance of β-blocker drugs with respect to IS at m/z 325.4 $[QN+H]^+$ for three successive DDSME extractions. Several parameters that controlled the DDSME extractions, such as a nature of extracting solvent, exposure volume of solvent, extraction time, pH and ionic strength of solution were separately evaluated to optimize the extraction for the determination of β-blocker drugs.

3.1.1. Effect of organic solvents on the extraction of β-blocker drugs using DDSME/MALDI-MS

The type of organic solvent used in LPME is an essential consideration for an efficient extraction of target analyte from aqueous solution into the organic solvent. Generally, there are several requirements for the choice of organic solvent. Firstly, it should be able to provide a high distribution coefficient for the target analytes. Secondly, it should have a low solubility in water and

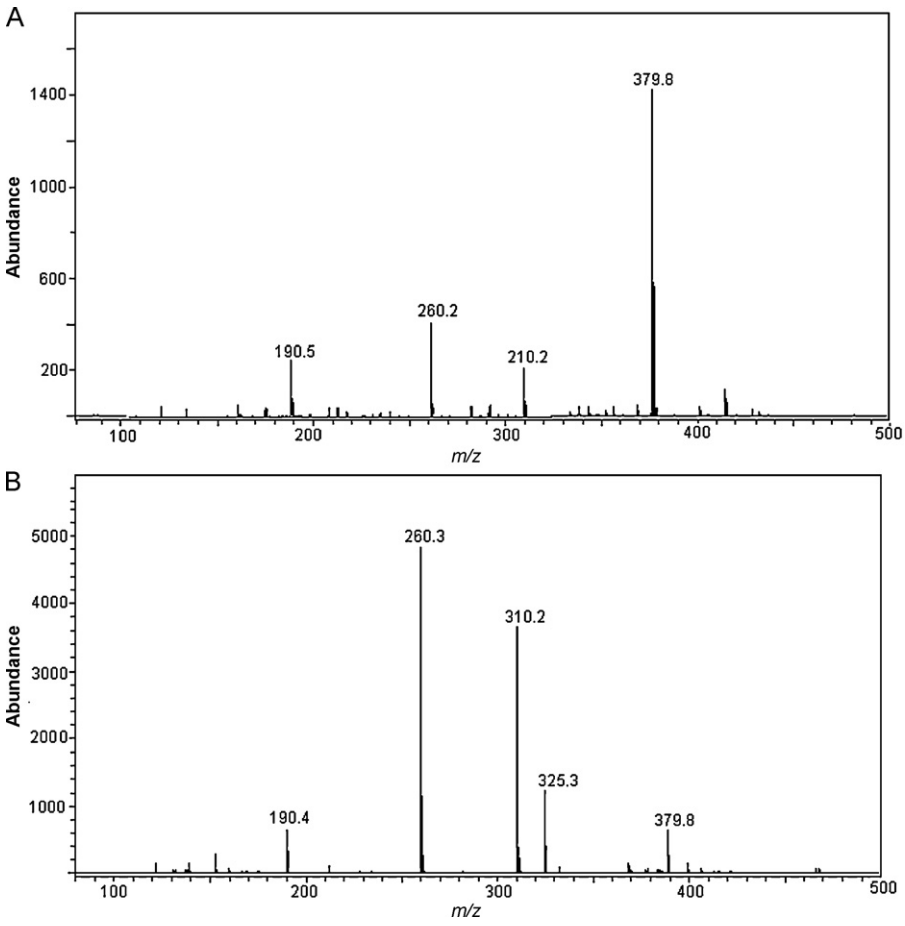

Fig. 2. MALDI-MS spectra of β-blocker drugs which obtained from spiked serum (500 ng mL^{-1} of propranolol and nadolol) sample by direct analysis (A) and with DDSME using 1.8 μL exposure volume of toluene as an extracting solvent for 10 min extraction time at pH 11.0 without the addition of salt.

non-volatile to prevent solvent loss during extraction, especially when longer extraction times are applied. Additionally, the organic solvent should be a comparative in the formation of crystal with MALDI matrix on the target plate for ionization of analyte in MS. Thus, n-hexane, iso-octane, cyclohexane, chloroform and toluene were tested to extract the β-blocker drugs from serum sample of a human. The evaluations were performed with the extraction of spiked drugs (500 ng mL^{-1} of propranolol and nadolol) from 30 μL of the sample solution. As can be seen (Fig. 3), toluene was the most suitable extraction solvent since it yielded the highest signal response for drugs. Therefore, toluene was selected for the separation and preconcentration of β-blocker drugs from serum sample solution.

3.1.2. *Effect of extraction time on the extraction of β-blocker drugs using DDSME/MALDI-MS*

The effect of extraction time was examined in the range of 2–15 min under constant experimental conditions, shown in Fig. 4. The extraction efficiency of β-blocker drugs was monitored as a relative abundance of analytes (ratio of β-blocker drugs to IS) used in MALDI-MS analyses. The results showed that extraction efficiency of the drugs was increased with the extraction time up to 10 min, and then started decreasing, possibly due to the depletion of solvent on the tip of a syringe. The similar effect was also observed in our previous DDSME extractions of drugs from a sample solution [18,26]. Hence in the present investigations, 10 min of extraction time was adopted for further studies to get maximum sample throughput and good reproducibility of the results.

3.1.3. *Effect of exposure volume of solvent on the extraction of β-blocker drugs using DDSME/MALDI-MS*

The effect of volume of organic solvent on the extraction efficiency was also examined in the range of 0.5–2.0 μL for 10 min of extraction time. The effect of exposure volume from 0.5 to 2.0 μL

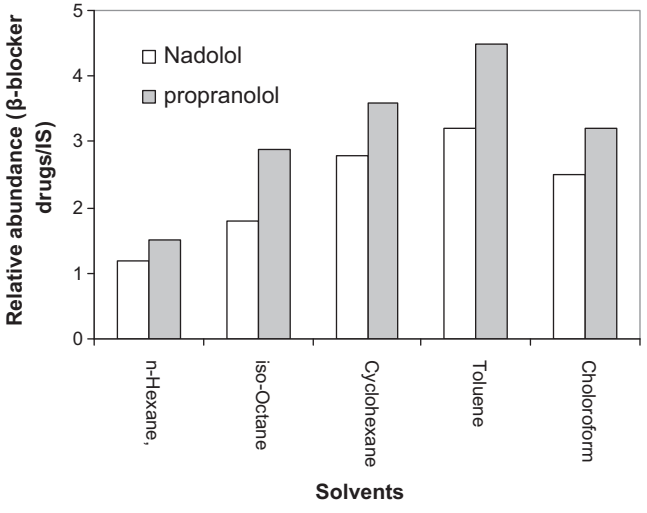

Fig. 3. Effect of solvents on the relative abundance of β-blocker drugs from spiked serum sample (500 ng mL^{-1} of propranolol and nadolol) obtained from DDSME using 1.8 μL exposure volume of solvent for 10 min extraction time at pH 11.0 without the addition of salt.

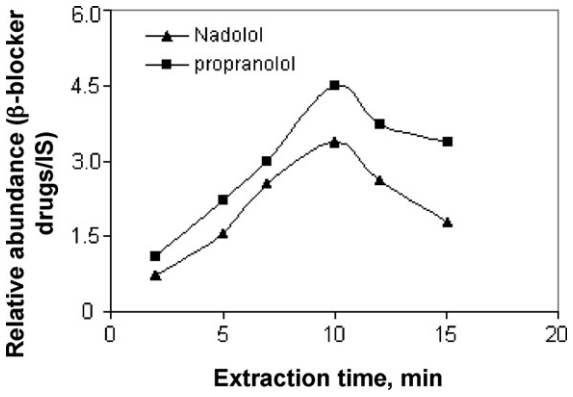

Fig. 4. Effect of extraction time on the relative abundance of β-blocker drugs from spiked serum sample (500 ng mL^{-1} of propranolol and nadolol) obtained from DDSME using 1.8 μL exposure volume of toluene as an extracting solvent for 10 min extraction time at pH 11.0 without the addition of salt.

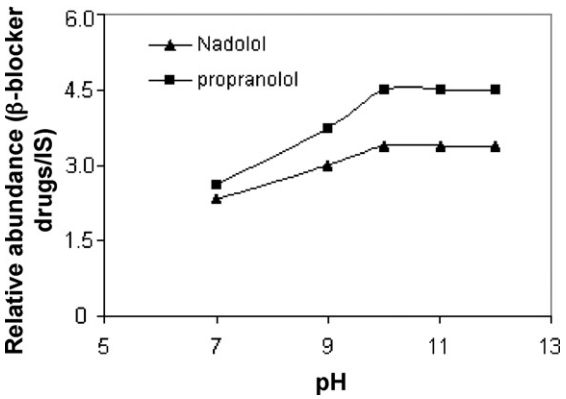

Fig. 6. Effect of pH of sample solution on the relative abundance of β-blocker drugs from spiked serum sample (500 ng mL^{-1} of propranolol and nadolol) obtained from DDSME using toluene as an extracting solvent for 10 min extraction time without the addition of salt.

is shown in Fig. 5. The results indicated that the extraction efficiency increased linearly up to 1.8 μL of exposure volume of the acceptor phase and thereafter a decrease in the signal intensity was observed possibly due to the depletion of solvent in the aqueous solvent. Therefore, 1.8 μL exposure volume of the acceptor phase was adopted in the following study; as it could be stably persist on the needle of the micro syringe.

3.1.4. Effect of pH on the extraction of β-blocker drugs using DDSME/MALDI-MS

The pH of the sample solution is expected to influences the extraction efficiency of analytes in LPME. The pH of the sample solution should be higher than the pK_a of drugs (propranonol = 9.4 and nadolol = 9.7), which could cause the transfer of analytes into the organic solvent. In this work, the pH of sample was varied from 7.0 to 12.0 using 0.1 N HCl or NaOH solutions. As can be seen from Fig. 6, the extraction efficiency of drugs was increased as the pH of sample solution increased from 7.0 to 10.0 and remained constant. Thus, pH 11.0 was selected for further experiments.

3.1.5. Effect of salt on the extraction of β-blocker drugs using DDSME/MALDI-MS

In conventional LLE, salting out effect is often used to increase the partition coefficient of polar analytes to the organic solvent. This effect can be enhanced by adding salt to the sample solution. To investigate the influence of the ionic strength on the performance of DDSME, various experiments were performed by adding different amounts of NaCl in the range of 0–15%. The extraction efficiency of drugs was increased with increase in the salt concentration from 0 to 5.0% and decreased thereafter. The results are shown in Fig. 7. Hence, 5% of NaCl was added throughout the experiments in order to obtain better extraction efficiency of β-blocker drugs from sample solution.

3.2. Analytical figures of merit of the method for the determination β-blocker drugs using DDSME/MALDI-MS

The analytical figures of merit, of the proposed method, such as calibration curve, limit of detection (LOD), limit of quantitation (LOQ), intra- and inter-assay precision and accuracy at different concentration levels on independently prepared quality control samples, stability and matrix effects using different serum samples have been tested. The linearity of the method was evaluated using spiked serum sample with the selected drugs at five different concentration levels. The calibration curves for β-blocker drugs were plotted using the ratio of the absolute abundance of analyte to the IS. Quinine was used as IS for the determination of β-blocker drugs in serum samples. The results of linearity and slope obtained on different days are in good agreement and thus it can be used for the quantification of β-blocker drugs. A good linearity was obtained for β-blocker drugs in the range of 50–1000 ng mL^{-1} for propranolol and 25–700 ng mL^{-1} for nadolol with correlation coefficient (R^2) in the range of 0.995–0.998. The slope of the calibration curve at different days was calculated and values obtained were 0.0086 (1st Day), 0.0084 (2nd Day), 0.0087 (3rd Day), 0.0086 (4th Day) and

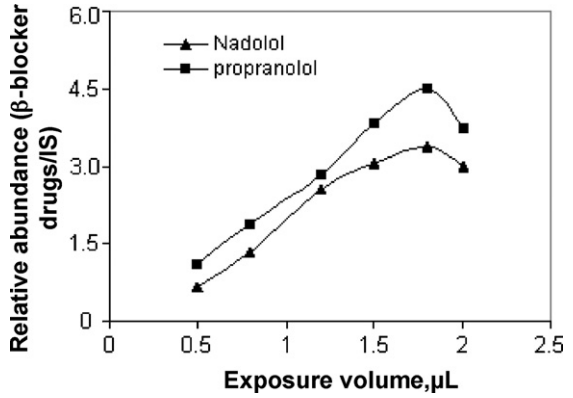

Fig. 5. Effect of exposure volume of solvent on the relative abundance of β-blocker drugs from spiked serum sample (500 ng mL^{-1} of propranolol and nadolol) obtained from DDSME using toluene as an extracting solvent for 10 min extraction time at pH 11.0 without the addition of salt.

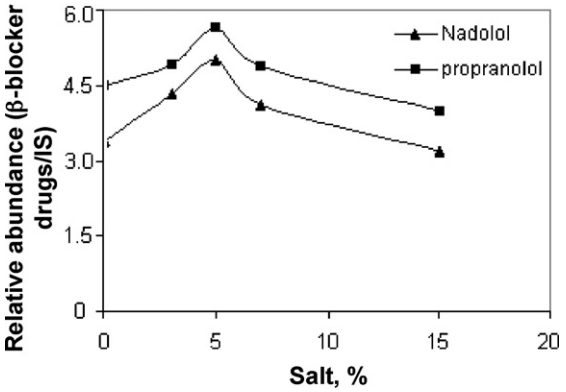

Fig. 7. Effect of the addition of salt on the relative abundance of β-blocker drugs from spiked serum sample (500 ng mL^{-1} of propranolol and nadolol) obtained from DDSME using toluene as an extracting solvent for 10 min extraction time at pH 11.0.

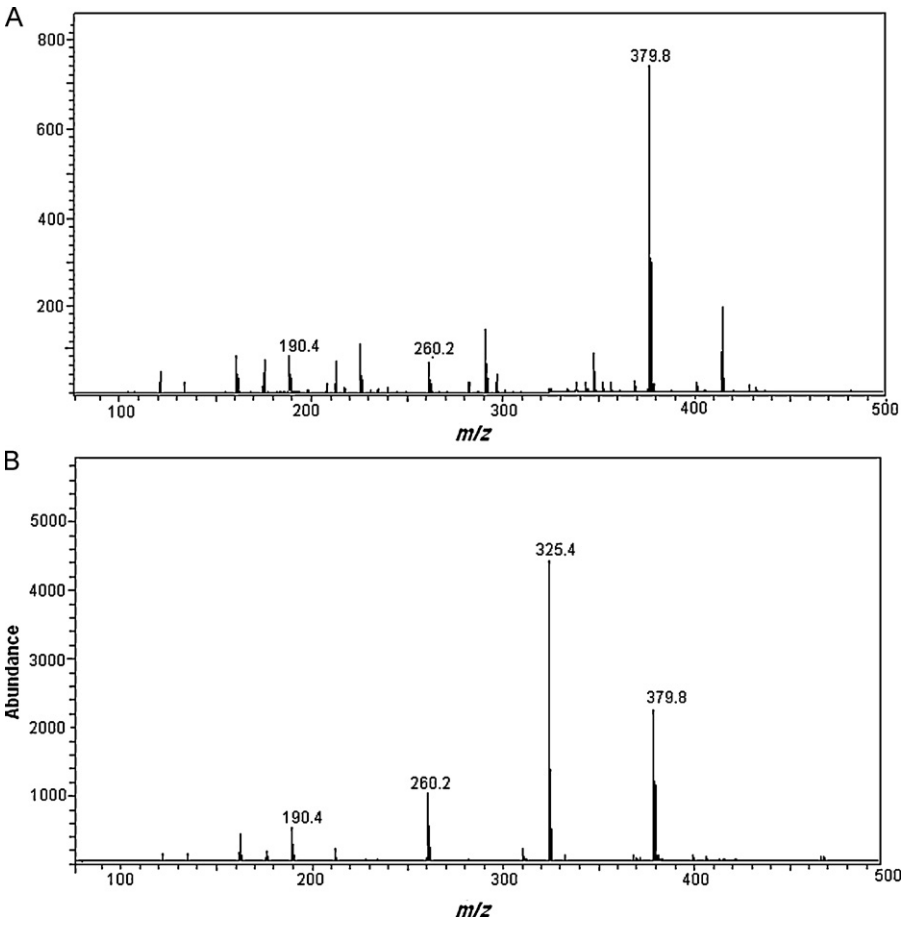

Fig. 8. MALDI-MS spectra of β-blocker drugs which obtained from serum sample of patient of high blood by direct analysis (A) and with DDSME for 10 min of extraction time at pH 11.0 with the addition of salt.

0.0084 (5th Day) for propranolol at five different days. These results suggested that quinine can be used as an IS for the quantification of β-blocker drugs in serum samples.

Precision was expressed as relative standard deviation (RSD, %) and was determined by performing a consecutive extractions of β-blocker drugs from sample solutions. Intra-day precision was calculated based on the analysis of three concentrations (50, 200, and 500 ng mL^{-1}) in triplicate analyses ($n = 3$) in same day and found to be 8.5–10.5%. However, inter-day precision was determined on three consecutive days for analyzing three concentrations (50, 200, and 500 ng mL^{-1}) and found to be 9.4–12.6%. Accuracy is defined as the relative deviation in the calculated value (E) of a standard from that of its true value (T) expressed as percentage ($RE\%$) = $[(E - T)/T] \times 100$. The value of accuracy obtained for the determination of propranolol in serum sample was 7.5–13.5%. LOD was calculated based on the signal-to-noise ratio (S/N) of 3 at the lowest concentration with precision value <20%. The LOD obtained for propranolol and nadolol was found to be 15 and 6 ng mL^{-1}, respectively. The LOQ calculated based on (S/N) of 10, was found to be 40 ng mL^{-1} for propranolol and 16 ng mL^{-1} for nadolol. The enrichment factor, defined as the ratio between the final analyte concentration in the organic solvent and the initial aqueous sample concentration, was found to be in the range 10–14 at the optimized conditions of DDSME/MALDI-MS analysis. Stability of the sample solution was studied as a function of time and storage with three different concentrations and kept at room temperature for maximum time and two concentrations were submitted to freezing/thawing in triplicate cycles. Stability after three freeze thaw cycles was in the variation range of 8.5–10.5%.

3.3. Effect of urea on the determination of β-blocker drugs using DDSME prior to MALDI-MS

The presence of urea in biological samples often causes the suppression of signal intensity in MALDI-MS analysis and thus the interpretation of interested molecules in the mass spectrum is difficult. Therefore, different concentrations of urea from 1.0 to 10.0 M were spiked into the sample solution containing β-blocker drugs with the intention to know whether the presence of urea in sample solution could interfere on the determination of targeted drugs from sample solution. The results showed that the presence of urea up to a concentration of 8 M has no effect on the determination of the drugs and a decrease in the signal intensity of drugs was observed when the concentration of urea was >8 M. Thus, we concluded that the use of 8.0 M of urea in the sample solution did not affect the determination of β-blocker drugs in MALDI-MS analysis.

3.4. Application of DDSME/MALDI-MS for determination the β-blocker drugs in serum sample

The validity of the proposed method was tested for the determination of β-blocker drugs in 30 μL of serum sample from a patient of high blood pressure using DDSME/MALDI-MS. Fig. 8 shows the MALDI-MS spectra of serum sample which obtained without (A), and with (B) use of DDSME as separating and preconcentrating probes. Many peaks due to interferences were obtained in the mass spectrum when DDSME was not used as separating probe. However, when DDSME was used for the separation and preconcentration of drugs from serum sample, good signal intensity of

Table 1
Comparison of DDSME/MALDI-MS with SPE/ESI-MS for the determination of propranolol from serum sample.

Parameters	DDSME/MALDI-MS	SPE/ESI-MS
Extraction time (min)	10	90
Volume of sample used (μL)	30	300
Samples throughout (h)	6	0.6
Calibration curve (ng mL^{-1})	50–1000	0.20–25.0
LOQ (ng mL^{-1})	40	0.20
Intra- and inter-day precision (%)	8.5–10.5 and 9.4–12.6	1.5–4.3 and 1.4–11.3
Recovery (%)	90.5–97.4	94.7–104.6

Table 2
Determination of propranolol in serum samples using DDSME/MALDI-MS and SPE/ESI-MS.

Samples	DDSME/MALDI-MS		SPE/ESI-MS	
	ng mL^{-1}	RSD (%)	ng mL^{-1}	RSD (%)
Serum-1	20.2	8.5	19.8	1.5
Serum-2	10.3	10.5	10.8	2.5
Serum-3	15.1	9.4	15.7	3.0

propranolol was obtained in the mass spectrum. This concluded that the use of DDSME prior to MALDI-MS analysis removed most of the interferences from the sample solution.

3.5. Relative recovery studies to determine β-blocker drugs in serum samples using DDSME/MALDI-MS

The relative recovery studies were performed in serum sample to investigate the effect of matrix (interference) on the determination of β-blocker drugs using DDSME/MALDI-MS. The serum sample spiked with 50 and 100 ng mL^{-1} of β-blocker drugs was extracted by DDSME and analyzed using MALDI-MS. Results of relative recoveries (defined as the ratios of abundances of the respective spiked serum sample extracts to spiked ultrapure water extracts) for spiked serum ranged from 90.5% to 97.4% with RSD value from 8.5 to 10.3%. This means that the matrix had little effect on the DDSME extractions of drugs and making these procedure feasible for real biological sample analysis.

3.6. Comparison of DDSME/MALDI-MS and SPE/ESI-MS for determination of β-blocker drugs in serum samples

The results obtained by DDSME/MALDI-MS were compared with SPE/ESI-MS in order to find the potentiality of the present method. The results are given in Table 1. The detailed procedure and experimental conditions for the determination of propranolol using SPE/ESI-MS have been described in the literature [28]. The sensitivity of the SPE/ESI-MS was better than DDSME/MALDI-MS, however, the sensitivity of the proposed method can be enhanced using high amount of sample volume (>30 μL) for the extraction of drugs in DDSME. The number of samples throughput was higher in DDSME/MALDI-MS than SPE/ESI-MS. The recovery % for the extraction of drugs was found to be comparable to each other. The concentration of propranolol was determined in three serum samples using DDSME/MALDI-MS and SPE/ESI-MS to validate the method, given in Table 2. The results obtained by both the methods are close to each other.

4. Conclusions

DDSME/MALDI-MS has been successfully applied for the determination of β-blocker drugs in one drop of serum from patient of high blood pressure, where the volume of sample availability is very small. The present methodology is an easy, rapid, low cost and requiring very minute volume of sample solution for analysis of β-blocker drugs in serum sample of patient of high blood pressure. Further, we believe that the application of this proposed method will be useful for the pharmacokinetic studies and clinical analysis. We also believe that the proposed method could be useful for the analysis other drugs from complex biological samples.

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