Research Article

Inverted Dispersive Liquid–Liquid Micro Extraction of Nicotinic Acid from Human Plasma and its Determination by High-Performance Liquid Chromatography

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Abstract

Extraction and determination of nicotinic acid from human plasma was performed using inverted dispersive liquid-liquid microextraction and HPLC. The parameters affecting extraction recovery such as type and volume of extracting and disperser solvents, pH of sample solution, salt addition and extraction time were optimized. Optimal extraction conditions were: 150µL tributyl phosphate as extraction solvent, 400µL methanol as disperser solvent, and pH of sample = 4.5, concentration of NaCl = 3M, without effect of extraction time. Under the optimal conditions a linear range of 30-1000 ng mL^{-1} (R^2 = 0.9994) was obtained. Limit of detection, the extraction recovery and preconcentration factor were 10 ng mL^{-1}, 68% and 53 respectively. The method was successfully applied for the extraction and determination of nicotinic acid in human plasma sample.

Keywords: Inverted dispersive liquid liquid micro extraction; Nicotinic acid; HPLC; Plasma

Introduction

Nicotinic acid (niacin or pyridine-3-carboxylic acid, Figure 1) is a water soluble B-complex vitamin present in many foods including fish, milk and green vegetables [1,2]. The deficiency of nicotinic acid results in pellagra, affecting the skin and central nervous system [3]. High-dose of nicotinic acid may cause thickening of the retina and increase the level of uric acid in the blood [4]. Thus, determination of nicotinic acid in human plasma is very important for health.

The analytical techniques, such as flow injection analysis [4], thin-layer chromatography [5], liquid chromatography-mass spectrometry [6], capillary chromatography [7], and high-performance liquid chromatography [8] were used for determination of nicotinic acid in human plasma. However, many of analytical techniques may need to preconcentrate target compounds before analysis. Preconcentration methods such as drop-to-drop solvent microextraction [9], solid phase extraction [10] and Reactive Extraction [11] are difficult and time consuming. Hence, simple and rapid preconcentration method is required to extract nicotinic acid from human plasma. Dispersive Liquid-Liquid Microextraction (DLLME) is a mode of Liquid-Liquid Extraction (LLE) in smaller level, which in comparison with the LLE method, its consumption of organic extracting solvent and environmental contamination significantly lower, and the obtained preconcentration factor is much higher. DLLME employs a mixture of a high-density extracting solvent and water miscible polar disperser solvent. In DLLME After a rapid injection of an appropriate mixture containing extracting and disperser solvents into the aqueous sample a cloudy state is formed. The contact area between the extracting solvent and the sample solution is very large. Thus the extraction equilibrium is achieved rapidly. After centrifugation, the extracted

phase is settled at the bottom of the conical test tube [12]. In 2009, Farajzadeh et al. [13] designed inverted dispersive Liquid-Liquid Microextraction (IDLLME). IDLLME is invert of DLLME because in IDLLME the extracting solvent is lighter than water; therefore the separated phase was collected at the top of the sample solution. Thus in the present work, IDLLME-HPLC is used for the extraction and determination of nicotinic acid in plasma sample. This method is based on the extraction of nicotinic acid from plasma sample into an organic solvent. Parameters affecting extraction recovery of nicotinic acid, such as selection types and volume of extracting and disperser solvents, pH of sample solution, salt addition and extraction time were optimized.

Experimental

Chemicals and stock solutions

Nicotinic acid was purchased from Sigma–Aldrich (Steinheim, Germany). HPLC grade (Methanol, acetonitrile, acetone), sodium hydroxide, hydrochloric acid, and sodium chlorid were obtained from Merck (Darmstadt, Germany). Xylene, n-hexan, toloen, dodecane, tributyl phosphate and 1-octanol were obtained from
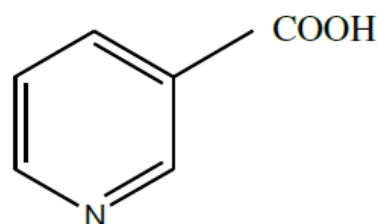

Figure 1: Chemical structure of nicotinic acid.

Aldrich (Milwaukee, WI, USA). Water used was double distilled deionized which purchased from Power Plant in sari city. Stock solution of nicotinic acid (1.0 mg/L) was prepared in methanol and stored in the dark at 4°C. Noted to the LC chromatogram this solution was stable for two months. Working standard solutions were diluted with deionizer double distilled water at concentration of 100.0 ngmL⁻¹ when ever needed.

Instrumentation and operating condition

Chromatographic measurements were carried out using a HPLC system equipped with a series 10-LC pump, UV detector model LC-95 set at 293 nm and model 7725i manual injector with a 20 μL sample loop (Perkin-Elmer, Norwalk, CT, USA). Column used was C_{18} (250 × 4.6 mm, 10 μm particle size) from Dr. MaischGmbH (Ammerbuch-Entringen, Germany). To select the composition of mobile phase, several mobile phases (based on peak shape, retention time, resolution) consist of water-methanol (80:20, 70:30, 60:40, v/v), were tested and finally the selected mobile phase was water-methanol (70:30, v/v) at a flow rate of 1.0 mL/min at room temperature. The pHs of solutions were measured by a 3030 Jenway pH meter (Leeds, UK).

Inverted dispersive liquid–liquid microextraction procedure

A 10 mL of sample solution containing 100 ng mL⁻¹ of nicotinic acid was placed in a handmade centrifuge tube with narrow neck (~4 mm i.d.) which was specially designed for ease of taking supernatant phase. A mixture of 400μL methanol (as disperser solvent) and 150μL tributyl phosphate (as extracting solvent) was rapidly injected into the sample solution using 1.0 mL syringe, and mixed by vortex mixer at 500 g stirring rate, so that a cloudy solution was formed. The cloudy solution was centrifuged for 5 min at 3500 g, and extraction product (supernatant phase) were collected in the neck of the tube (about 130 ± 2 μL). Finally, this supernatant phase was injected in to the HPLC. All the experiments were carried out in triplicates and the average of the result was reported.

Calculation of Recovery and Preconcentration Factors

Extraction recovery (ER %) and preconcentration factor (PF) for nicotinic acid were calculated according to the following equations [14]:

Where and are the number of moles of analytes present in the initial sample and analytes finally collected in the supernatant phase, respectively and are the volume of sample solution, volume of the supernatant phase, initial analytes concentration within the sample and final concentration of analytes in the supernatant phase, respectively.

Results and Discussion

To obtain good sensitivity, precision and the highest extraction recovery of nicotinic acid various experimental parameters which influence the extraction recovery of IDLLME procedure including extracting and disperser solvents as well as their volume, pH of sample solution, salt addition and extraction time were optimized using one variable-at-a-time optimization method.
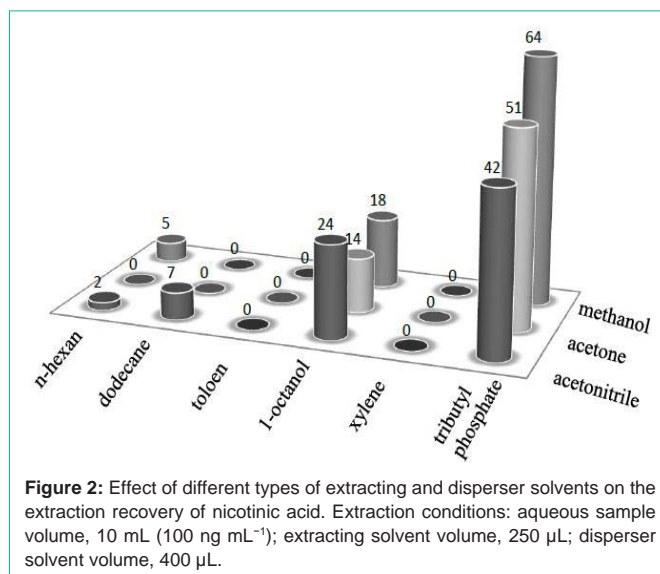

Figure 2: Effect of different types of extracting and disperser solvents on the extraction recovery of nicotinic acid. Extraction conditions: aqueous sample volume, 10 mL (100 ng mL⁻¹); extracting solvent volume, 250 μL; disperser solvent volume, 400 μL.

Selection of extracting and disperser solvents

To obtain a good extraction recovery for IDLLME of nicotinic acid, selection of an appropriate solvent is a major factor for IDLLME process. The extracting solvent has to meet two properties: having less density than water and having low solubility in water. Hence, xylene (density, 0.86 g mL⁻¹), n-hexan (density, 0.65 g mL⁻¹), dodecane (density, 0.75 g mL⁻¹), toloen (density, 0.87 g mL⁻¹), 1-octanol (density, 0.82 g mL⁻¹) and tributyl phosphate (density, 0.97 g mL⁻¹) were tested for this purpose. In order to choose disperser solvent in IDLLME, the miscibility of it in organic phase (extracting solvent) and aqueous phase (sample solution) is a key factor. Acetonitrile, acetone and methanol were examined as the disperser solvent in the extraction of nicotinic acid. To obtain a high recovery factor, all combinations using xylene, n-hexan, toloen, tributyl phosphate, dodecane and 1-octanol (250 μL) as extracting solvent with acetone, acetonitrile, methanol (400μL) as dispersive solvent were examined. Results in (Figure 2) indicate that tributyl phosphate as the extracting solvent and methanol as the disperser solvent had the maximum extraction recovery of about 64%. Therefore, tributyl phosphate-methanol combination was selected for subsequent experiments.

Effect of extracting solvent volume on the extraction recovery: To examine effect of the extraction solvent volume, experiments involving different volumes of tributyl phosphate were used for extraction of nicotinic acid from standard sample solution with 400 μL methanols as disperser solvent. At the extraction solvent volumes of lower than 50μL, no supernatant organic phase was collected on the top of aqueous phase. (Table 1) shows the supernatant phase volume, extraction recovery and preconcentration factor with the extraction

Table 1: Effect of extracting solvent (tributyl phosphate) volume (μL) on supernatant phase volume (μL), ER (%) and PF.

Extracting solvent volume	Supernatant phase volume	ER (%)	PF
100	82	55	45.1
150	130	65	50
200	192	62	32.3
250	225	63	28
300	279	63	21.2
350	335	61	18.2

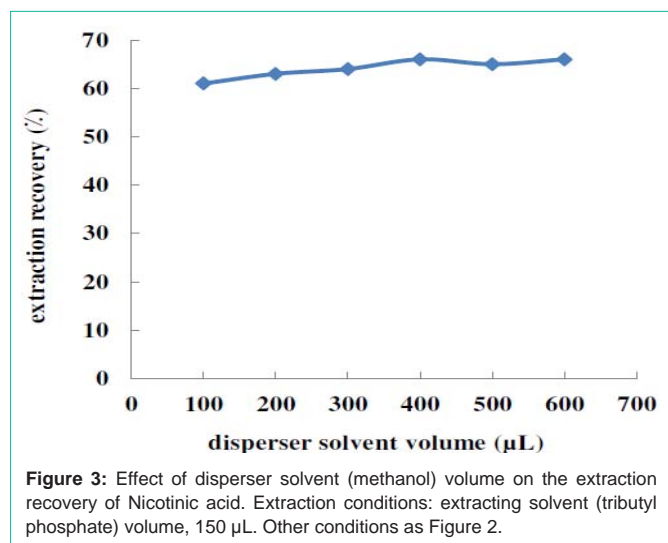Extraction conditions: disperser solvent (methanol) volume, 400 μL.

Figure 3: Effect of disperser solvent (methanol) volume on the extraction recovery of Nicotinic acid. Extraction conditions: extracting solvent (tributyl phosphate) volume, 150 µL. Other conditions as Figure 2.

solvent volume. Result in this table show that, 150µL of the extraction solvent, produced the highest recovery and preconcentration factor. Thus this volume was selected as an optimum volume for the extraction solvent.

Effect of disperser solvent volume on the extraction recovery: To obtain optimized volume of disperser solvent, extractions were carried out by changing the volume of methanol in the range of 100-600 µL. Results in (Figure 3), show that with increasing the volume of methanol up to 400 µL, the extraction recovery increased and had no change after this volume. The lower extraction recovery at volume of methanol less than 400 µL can be attributed to the fact that, cloudy state was not well formed and the extracting solvent (tributyl phosphate) could not be well dispersed among aqueous solution in the form of very little droplet. So for the following experiments, 400 µL methanols were used as optimal disperser solvent volume.

Effect of sample pH on the extraction recovery: In IDLLME pH of the sample solution is a key factor for extraction of acidic and basic compounds. To obtain high extraction recovery for acidic compounds, the sample solution should be acidified to deionizer the analytes and consequently increase their transfer from the sample
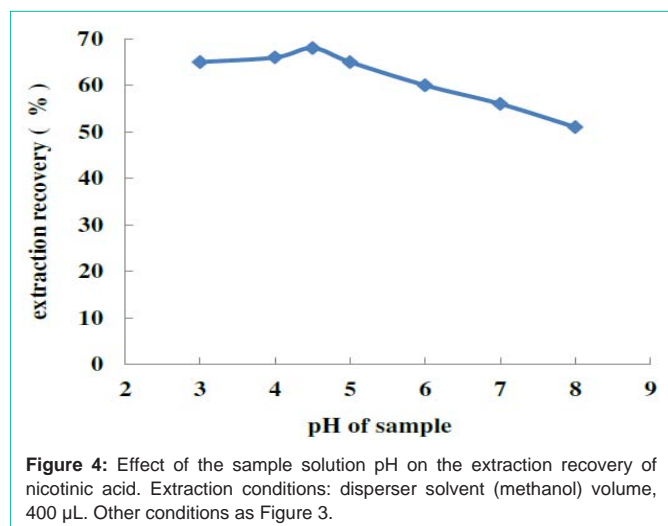

Figure 4: Effect of the sample solution pH on the extraction recovery of nicotinic acid. Extraction conditions: disperser solvent (methanol) volume, 400 µL. Other conditions as Figure 3.

solution into the organic phase. Thus, pH of sample solution should be adjusted to make neutral molecular forms of the analytes prior the microextraction step. For this purpose, effect of pH of the sample solution on the extraction recovery of nicotinic acid was investigated in the range of 3-8. Values of pH higher than 8.0 were not examined, because nicotinic acid is a weak acid (pK =2.2), and can be hydrolyzed at basic pH. (Figure 4) shows that, the highest extraction recovery is obtained at pH = 4.5 and subsequent experiments were performed at this pH.

Effect of extraction time on the extraction recovery: In IDLLME, extraction time is defined as gap time between injection the mixture of disperser and extraction solvent into the aqueous sample and start of centrifugation. In this work, effect of the extraction time was examined from 0 to 30 min. The obtained results showed that the variations of extraction recovery with the extraction time are not significant. This observation can be explained by the fact that after injection cloudy solution was formed rapidly, and the contact area between extraction solvent and aqueous phase is very large. Thereby, migration of the analyte from aqueous phase to the extraction solvent is very fast, so the equilibration is fast and the extraction time is short. This is an advantage of IDLLME technique, with respect to the others.

Effect of Salt concentration on the extraction recovery: Some researchers have reported that the addition of salt to the sample solution has been beneficial for the extraction recovery in microextraction procedures [15,16]. It is well known that the addition of salt to the aqueous sample is usually made to improve the extraction of analytes when LLE is used, because the increase in ionic strength brings a reduction on the solubility of the analytes in the water solution and causes the transfer of molecules into organic phase [9]. Thus, in this study, effect of salt addition on the extraction recovery was examined by adding different amounts of NaCl into the sample solution of nicotinic acid in the range of 0.0- 5.0*M*. (Figure 5) shows that the highest extraction recovery of nicotinic acid was obtained at 3*M* concentration of NaCl.

Analytical performance of the IDLLME-HPLC for determination of nicotinic acid

To evaluate the analytical performance of the IDLLME technique, figures of merit of this method including Limit of Detection (LOD), linear range, extraction recovery and preconcentration factor were investigated for extraction of nicotinic acid from the standard aqueous
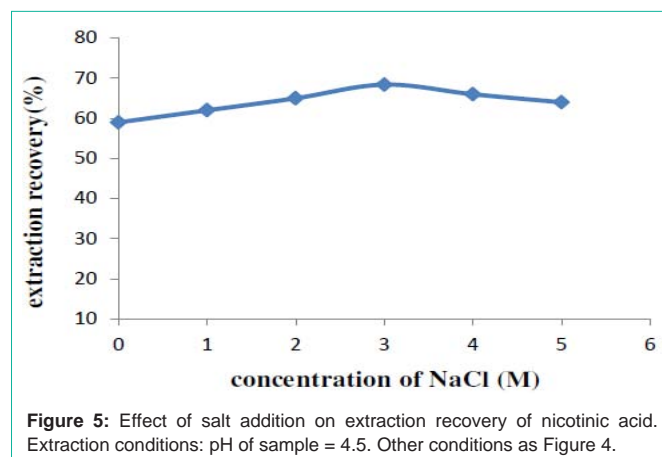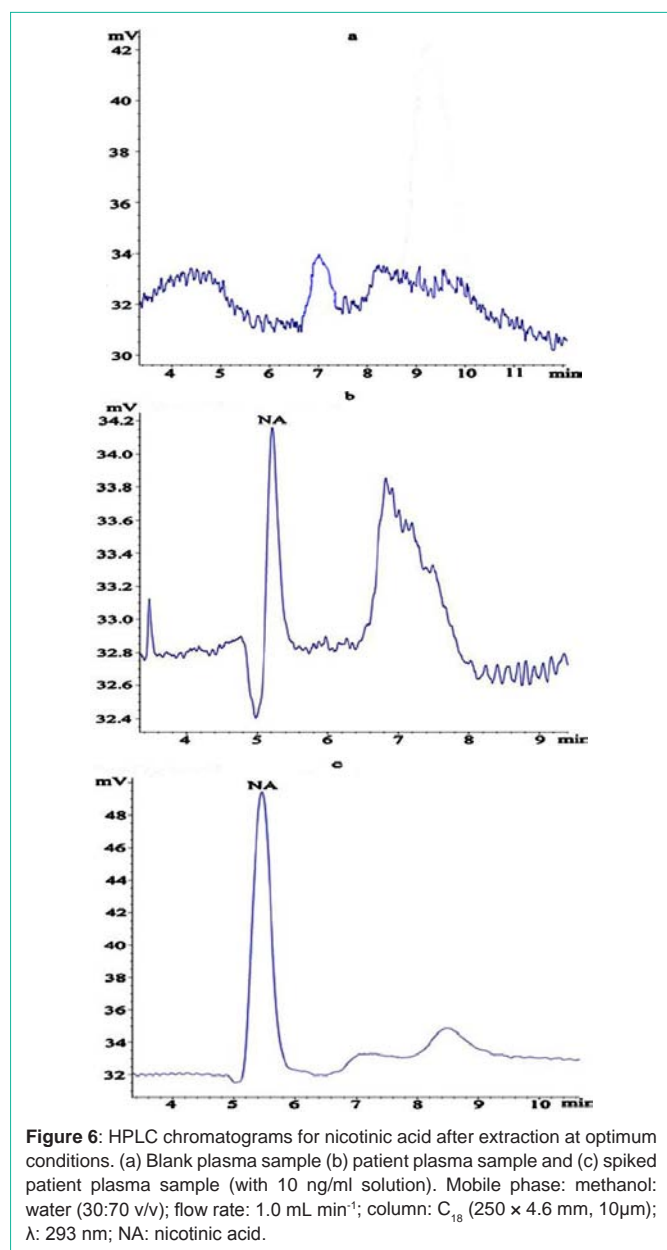

Figure 5: Effect of salt addition on extraction recovery of nicotinic acid. Extraction conditions: pH of sample = 4.5. Other conditions as Figure 4.

Figure 6: HPLC chromatograms for nicotinic acid after extraction at optimum conditions. (a) Blank plasma sample (b) patient plasma sample and (c) spiked patient plasma sample (with 10 ng/ml solution). Mobile phase: methanol: water (30:70 v/v); flow rate: 1.0 mL min^{-1}; column: C$_{18}$ (250 × 4.6 mm, 10µm); λ: 293 nm; NA: nicotinic acid.

solutions under the optimal conditions (150µL tributyl phosphate as extraction solvent, 400µL methanol as disperser solvent, pH of sample = 4.5, concentration of NaCl = 3M, without effect of extraction time). The LOD (10 ng mL^{-1}) was calculated based on $3S_b/m$ where S_b is the standard deviation of blank and is equal to Peak-Peak noise when only mobile phase was passing through the column for 45 min and m is the slope of calibration curve. Linear range was 30 to 1000 ng mL^{-1} with determination coefficient of $R^2 = 0.9992$ using peak area. The extraction recovery and preconcentration factor were 68% and 53 respectively.

Plasma sample preparation

Blank plasma sample was provided by a healthy student volunteer. Real plasma sample was from a patient after administration of an oral dose (5 mg) of nicotinic acid after 2 hours (life span of drug). In order to eliminate the protein binding of the drug in plasma (greater than 99%) [17], the pretreatment was performed as outlined in the work of Tahmasebi et al. [18]. In order to eliminate protein binding of nicotinic acid, 3 mL of methanol was added to 2 mL of the plasma and the resulting mixture was strongly vortexes for 10 min. The mixture was placed in an ice bath for 10 min, and centrifuged at 3500 g for 10 min. The supernatant was transferred into a 10 mL volumetric flask and diluted to the mark with deionizer double distilled water after adjusting pH=4.5 using 2 M formic acid solution and the extraction procedure was done under the optimized conditions.

Application of IDLLME for analysis of nicotinic acid in plasma sample

Due to the importance analysis of nicotinic acid in plasma sample, the optimized method (150 µL of tributylphosphat as extracting solvent, 400 µL of methanol as disperser solvent, pH of sample = 4.5, with 3M of salt) was applied to determine the concentration of this drug in the plasma sample from a patient under nicotinic acid treatment. The chromatograms of IDLLME extracts from blank plasma sample, patient plasma sample and spiked patient plasma sample are shown in (Figure 6) (a, b and c). The concentration of nicotinic acid in patient plasma sample was 134 ng/mL.

Conclusion

The extraction and determination of nicotinic acid in aqueous sample solution and human plasma were performed using IDLLME and HPLC. Result show that IDLLME is an effective method for preconcentration of nicotinic acid from the biological sample. This method presented a high preconcentration and good sensitivity, while enabled efficient sample clean-up. IDLLME provides a simple, inexpensive, easy to use and benign to the environment method for extraction of nicotinic acid from plasma samples.

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