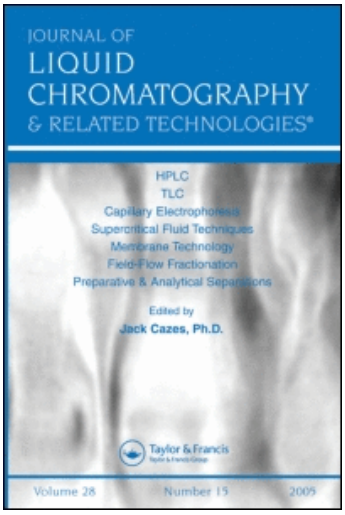

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Determination of Clinically Very Basic Compounds by Nanoscale Liquid Chromatography with Tandem Mass Spectrometry

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Abstract: A simple nanoscale liquid chromatographic method with tandem mass spectrometry (LC-MS-MS) was developed for the determination of very basic compounds, biguanides, such as metformin and phenformin. Plasma samples were deproteinated and acidified with heptafluorobutyric acid (HFBA) and the resulting supernatant injected into the nanoscale separation system. Chromatography was carried out using a short reversed-phase C_{18} column under isocratic elution with methanol-40 mM aqueous HFBA (75:25, v/v) at a flow rate of 800 nL/min. Therapeutic monitoring on metformin and phenformin to minimize adverse side effects is important. The main advantage of the nanoscale method is the lower consumption of organic solvent. This nanoscale method is suitable for the investigation and monitoring of the concentration of oral antihyperglycemic drugs in clinical patients.

Keywords: Nanoscale, Basic compounds, Biguanide, Tandem mass spectrometry

INTRODUCTION

Metformin and phenformin are classified as biguanides that are used as oral antihyperglycemic drugs in the treatment of non-insulin dependent diabetes mellitus

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(NIDDM). Biguanides are currently used to overcome insulin resistance, especially in obese NIDDM patients who fail to diet and do exercise therapy. All biguanides can cause lactic acidosis, but the incidence is highest in patients using phenformin.[1,2] For metformin, maximal plasma concentrations after an oral dose of 500 to 1000 mg are 1 to 2 μg/mL.[3] However, in patients with lactic acidosis and renal impairment, a significant accumulation may occur and high plasma concentrations have been reported.[4,5] Metformin and phenformin are guanidine skeleton with different side chains, dimethyl and phenyl, respectively. They are highly polar and strongly basic compounds having a pK_a over 11,[6] it is difficult to direct determination of biguanides by chromatographic methods. It also has a very short retention time when separated using reversed-phase chromatography (and so may coincide with the rapidly eluting endogenous substances from the plasma) and cannot be eluted using normal phase chromatography due to its very strong retention.[7]

Recently, the growing public concern over protecting the natural environment has led us to limit pollution. Green chemistry standards have become alternatives to the traditional pollute and cleanup practice.[8] The uncontrolled disposal of chemical waste in analytical laboratories, such as organic solvents, may also exert an adverse influence on the environment. Therefore, development of green chemistry should also include the development of green analytical chemistry. The goal of green analytical chemistry is eliminating or minimizing the use of chemical reagents, particularly organic solvents, from analytical methods.[9,10]

In order to develop a green analytical method, we describe a nano-analytical technique for the determination of metformin and phenformin from human plasma. The nanoscale method is based on ion pair liquid chromatography with tandem mass spectrometry (LC-MS-MS). Metformin and phenformin exhibited retention behavior with the reversed-phase mode in the C_{18} LC column by using HFBA as an ion pair agent. This was a useful characteristic for direct injection into the LC system after elution of analytes without evaporation or reconstitution steps. The nanoscale system exhibited excellent performance in terms of the low cost of organic solvents, short run time, and simplicity of sample preparation. Our goal was to develop a nanoscale LC-MS-MS method for the analysis of biguanides in human plasma, providing a green analytical technique and employing simple and fast sample preparation.

EXPERIMENTAL

Reagents and Materials

Metformin hydrochloride and phenformin hydrochloride were purchased from Sigma (St. Louis, MO, USA). HPLC grade methanol was supplied by E. Merck (Darmstadt, Germany). Heptafluorobutyric acid (HFBA) was obtained from Aldrich (Milwaukee, WI, USA). Deionized water from a

Milli-Q system (Millipore, Bedford, MA) was used at all times. Reference solutions of metformin, phenformin, and HFBA solution were prepared by dissolving the appropriate amounts of the respective compounds in deionized water. The protein precipitating solution was prepared by adding HFBA to water to give a concentration of 100 g/L. Fused silica capillaries (75, 100, and 150 μm inner diameter, 375 μm outer diameter) were purchased from Polymicro Technologies (Phoenix, AZ, USA). The 3 μm reversed-phase C_{18} particle was obtained from Vydac (Hesperia, CA, USA). Drug free human plasma samples were obtained from normal volunteers.

Apparatus

The nanoscale liquid chromatographic system consisted of an Agilent (Palo Alto, CA) 1100 Series CapLC pump, a binary pump, an autosampler, and a switching valve (Vici, Schenkon, Switzerland). The MS-MS system consisted of a Micromass Q-TOF mass spectrometer (model Q-Tof-2, Massachusetts, USA) with a nanospray ESI source.

LC Conditions

A 1 cm short reversed-phase C_{18} capillary column was prepared in our laboratory and this method was established in our previous study.[11] Briefly, the fused silica capillary was cut and sealed at one end using bare silica material and then sintered by a pocket microtorch. The needle of the syringe and the fused silica capillary was connected by a piece of PTFE tubing. The C_{18} stationary phase suspension (in methanol) was then transferred into the capillary by sedimentation with gravity and a hand push. After a slurry packing procedure, the fused silica capillary was finally filled with 1 cm reversed-phase C_{18} stationary phase. The short capillary column was used and provided the stationary phase to retain analytes. The nanoscale analytical system applied in this work was shown in Figure 1. The mobile phase of the binary pump for sample loading was 40 mM HFBA and the CapLC pump for sample analysis was methanol: 40 mM HFBA = 75:25 (v/v) at a flow rate of 1 μL/min and 800 nL/min, respectively. The sample loading time was 2 min. After sample loading, the valve was switched and mobile phase from pump 2 eluted the analyte of interest to the nanospray source.

ESI-MS-MS Conditions

The mass was operated in positive ion mode with a source temperature of 80°C and a cone voltage of 45 V. A voltage of 3.2 kV was applied to the

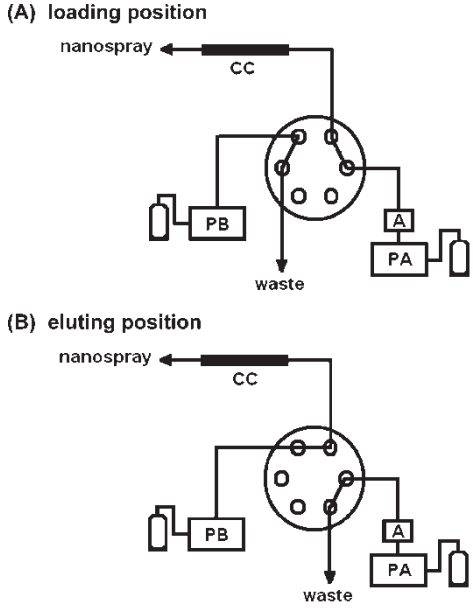

Figure 1. The nanoscale analytical system with a switching valve: A = autosampler; CC = 1 cm capillary column; PA = binary pump and PB = CapLC pump. (A) and (B) represented the loading and eluting positions of the switching valve.

source capillary. The desired molecular weights of metformin and phenformin selected for collision induced dissociation (CID) are 130 and 206, respectively. MS-MS spectra were collected for each of these precursor ions by MS-MS scan mode of the scan function. The collision gas was argon and the collision energy was set at 20 V during the MS-MS scans. The eluent was directed to the nanospray source with a 20 μm i.d. × 90 μm o.d. fused silica capillary. MS-MS spectra were collected and processed by using MassLynx 4.0 software (Micromass).

Sample Preparation

Drug free human plasma samples spiked with 1 μg/mL metformin and phenformin were used. Human plasma samples (10 μL) were added in Eppendorf vials (500 μL) then the protein precipitating solution (5 μL) was added. The sample vials were vortexed (30 s) and centrifuged at 10,000 rpm (2 min). The supernatant was obtained and 1 μL was subjected to nanoscale LC-MS-MS system.

RESULTS AND DISCUSSION

Chromatography and Column Efficiency

The chemical structures of these two biguanides are similar and phenformin is more lipophilic than metformin. Figure 2 shows the structures of metformin and phenformin. Since they are very hydrophilic and not retained by the reversed-phase column, separation was achieved by ion pair chromatography. A heptafluorinated ion pair agent, HFBA, was used in this method due to its compatibility with the mass detector. Because of the high polarity of metformin and phenformin, they are rapidly eluted from reversed-phase columns, even if the mobile phase has a very low organic content.[7] In this study, we used ion pair chromatography to elute metformin and phenformin. LC separation was performed in an isocratic mode with a 1 cm capillary column at a nanoscale flow rate. First, analytes were loaded by binary pump and trapped on the C_{18} stationary phase. Secondly, metformin and phenformin were eluted by the CapLC pump to the nanospray source.

In order to demonstrate the separation efficiency of these 1 cm capillary columns in this nanoscale system, capillary columns with different inner diameters (75, 100, and 150 μm) were tested. The retention time of the method was tested on the separation of metformin and phenformin, each, at 1 μg/mL. Figure 3 shows the influence of methanol concentration (from 40 to 80% in the mobile phase) on the retention time of metformin and phenformin. For capillary columns with 75 and 100 μm inner diameters, the retention time of metformin was almost the same (Figure 3A). For the 150 μm inner diameter capillary column the retention of metformin is increased, as the aqueous content of the mobile phase is up to 50% (Figure 3B). For capillary columns with 75 and 100 μm inner diameters, the retention time of phenformin is increased as the aqueous content of the mobile phase is up to 50% (Figure 3A). For capillary columns with 150 μm inner diameter, the greatest retention was observed below 30% methanol for phenformin (Figure 3B). Therefore, the reasonable explanation is that the 150 μm capillary column contains more abundant stationary phase than the 75 and 100 μm inner diameter columns to retain the desired analytes. Besides, metformin is more hydrophilic than phenformin, it is more difficult to retain in the hydrophobic column. The results

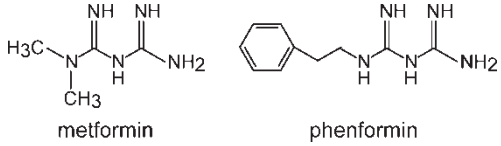

Figure 2. Structures of metformin and phenformin.

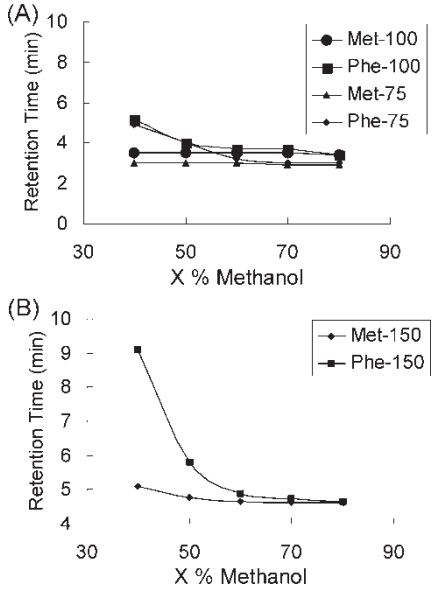

Figure 3. Influence of the methanol concentration in mobile phase on the retention time of biguanides. Mobile phase: X% methanol: (100 − X)% 40 mM HFBA = (v/v) where methanol varied from 40 to 80%. Met = metformin; Phe = phenformin; 75, 100, and 150 represented the inner diameters of the capillary columns.

indicated that the nanoscale analytical system coupled with ion pair chromatography has the ability to separate very basic and hydrophilic compounds in a suitable running time. For the analysis of biguanides in biological samples, the capillary column with a 150 μm inner diameter is more suitable than 75 and 100 μm inner diameter columns.

Application

The application of the nanoscale system was tested on the separation of very hydrophilic compounds, biguanides, in biosamples. Clinically basic drugs, metformin and phenformin, each at 1 μg/mL, were spiked in human plasma. Because of their high polarity and strong base properties, it is difficult to directly determine these drugs from biological fluids by solvent–solvent extraction. For minimum sample preparation, direct injection of samples to mass spectrometry after protein precipitation by HFBA was performed. The plasma samples prior to LC-MS-MS analysis were prepared by using HFBA as protein precipitation. For data acquisition, the set masses were m/z 130 and 206 for $[M + H]^+$ of metformin and

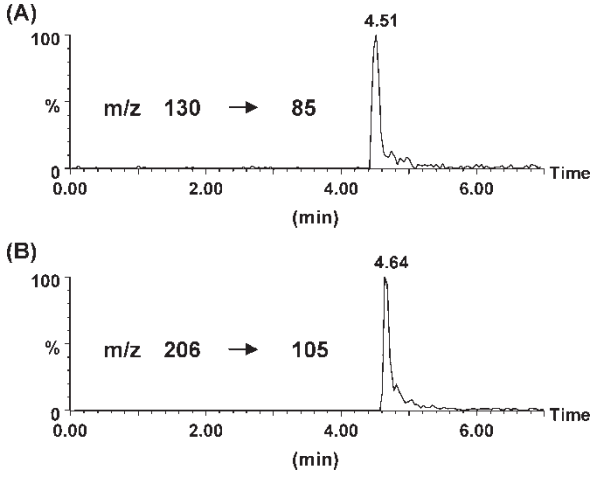

Figure 4. Extracted LC-MS-MS chromatograms of the product ions of metformin (m/z 85) and phenformin (m/z 105) from human plasma: (A) blank plasma spiked with 1 μg/mL metformin; (B) blank plasma spiked with 1 μg/mL phenformin. Column inner diameter = 150 μm; LC conditions: methanol: 40 mM HFBA = 75:25 (v/v); flow rate = 800 nL/min.

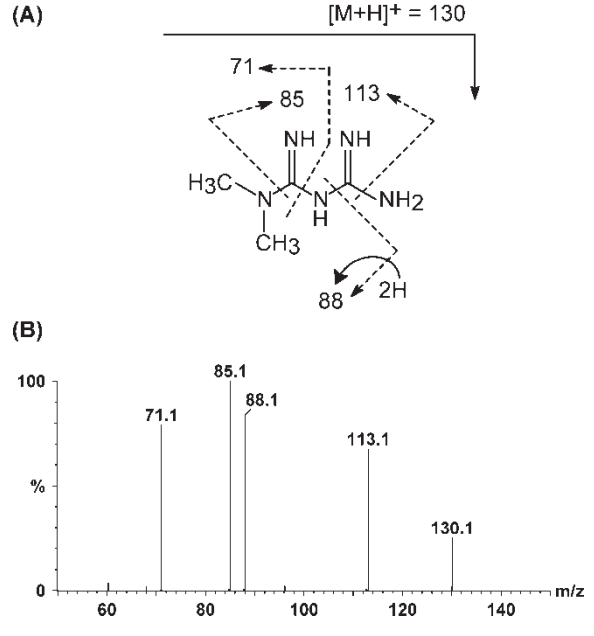

Figure 5. ESI-MS-MS fragment ions and spectrum of metformin: (A) fragment ions of metformin; (B) MS-MS spectrum of metformin.

phenformin, respectively. The LC-MS-MS chromatograms of metfot min and phenformin from human plasma are shown in Figure 4. The monitoring of compound specific fragments of the protonated molecule provides added confidence in the identification of the analyte. In this study, four fragment ions (m/z 71, 85, 88, and 113) of metformin and six fragment ions (m/z 60, 85, 105, 147, 164, and 189) of phenformin were detected for structural identification. The resulting MS-MS spectra of metformin and phenformin were presented in Figures 5 and 6. The nanoscale method only needed a simple protein precipitation procedure, which reduced the preparation time and lowered the consumption of organic solvents in the extraction steps.

For quantitative purposes, the major product ions at m/z 85 and 105 could be used for metformin and phenformin, respectively. It is possible to use one of the analytes as an internal standard for quantitation. In order to improve reproducibility and accuracy, using a structurally similar compound as the internal standard was necessary. A nanoscale LC-MS-MS method for determination of metformin and phenformin in human plasma has been successfully developed. To our knowledge, rarely has the nanoscale LC-MS-MS system to determine very basic compounds been reported. This method offers a suitable nanoscale system for pharmacokinetic studies of metformin and phenformin.

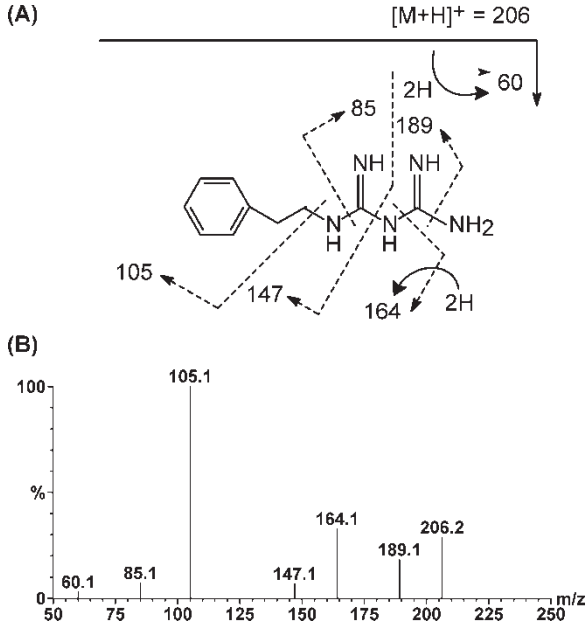

Figure 6. ESI-MS-MS fragment ions and spectrum of phenformin: (A) fragment ions of phenformin; (B) MS-MS spectrum of phenformin.

Also, this method could be useful for the analysis of metformin and phenformin in the study of the metabolic diseases in clinical patients.

CONCLUSION

A nanoscale method based on ion pair LC-MS-MS has been developed for the determination of metformin and phenformin in human plasma. The analytical method consists of a simple sample preparation without any further evaporation steps, and LC separation with a reversed-phase isocratic system followed by nanoscale LC-MS-MS in positive ion mode using nanospray ionization. This method reduces the time requirement for sample preparation, reduces the consumption of the organic solvents, and reduces the cost associated with the use of organic solvents. The method would be applied to the analysis of human plasma to study the relationship between the biguanides level and diabetic patients for routine clinical use.

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