Molecularly Imprinted Solid Phase Extraction – Pulsed Elution and Capillary Electrophoresis for Rapid Screening of Metformin in Human Plasma

by

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ABSTRACT

A new molecularly imprinted polymer (MIP) was specifically synthesized as a smart material for the recognition of metformin in solid-phase extraction. Particles of this MIP were packed into a stainless-steel tube (50 mm×0.8 mm i.d.) equipped with an exit frit. This micro-column was employed in the development of a molecularly imprinted solid-phase extraction (MISPE) method for metformin determination. The MISPE instrumentation consisted of a micrometer pump, an injector valve equipped with a 20-μl sample loop, a UV detector, and an integrator. With CH_3CN as the mobile phase flowing at 0.5 ml/min, a total binding capacity of 1600 ng metformin was determined for the 20 mg of MIP particles. A 95±2% binding could be achieved for 1200 ng of metformin from one injection of a phosphate-buffered sample solution (pH 2.5). Methanol+3% trifluoroacetic acid was good for quantitative pulsed elution (PE) of the bound metformin. The MISPE-PE method, with UV detection at 240 nm, afforded a detection limit of 16 ng (or 0.8 μg/ml) for metformin. However, the micro-column interacted indiscriminately with phenformin, a structural analogue, to attain 49±2% binding. A systematic investigation of binding selectivity was conducted with respect to sample solvent, matrix, pH, surfactant effects and buffer compositions. An intermediate step of differential pulsed elution used acetonitrile with 5% picric acid to remove phenformin and other structural analogues. A final pulsed elution of metformin for direct UV detection was achieved using 3% trifluoroacetic acid in methanol. Each MISPE–DPE–FPE analysis required less than 5 min to complete. Application of this MISPE-DPE-FPE method, is demonstrated for accurate determination

of metformin in human plasma over a linear range of 0.1 μg/mL to 10 μg/mL, which represented the typical therapeutic range of metformin in patients.

In addition, a SPE-CE method was developed for the determination of metformin and phenformin in human plasma. Coupled at line to capillary electrophoresis (CE), solid phase extraction (SPE) using a C18 cartridge was employed to remove most of the water and proteins from the plasma sample. Analyte detectability was increased due to trace enrichment during the SPE process. Elution of metformin and phenformin was achieved with methanol + 3% acetic acid. CE analysis was performed using a non-aqueous buffer, acetonitrile + 20 mM ammonium acetate + 5% acetic acid, which afforded rapid separation of metformin from phenformin within 3 min. The present SPE-CE method, with an electrokinetic injection time of 6 s and UV detection at 240 nm, was useful for monitoring down to 1 μg/mL of metformin and phenformin in human plasma. When the electrokinetic injection time was increased to 36 s, the detection limits were improved to 12 ng/mL for metformin and 6 ng/mL for phenformin.

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GLOSSARY

AIBN	2,2'-Azobisisobutyronitrile
CE	Capillary Electrophoresis
DDAB	Didodecyldimethylammoniym bromide
DPE	Differential pulsed elution
DTAB	Dodecyltrimethylammonium bromide
FIA	Flow injection analysis
FPE	Final pulsed elution
HMB	Hexamethonium bromide
LOD	Limit of detection
LOQ	Limit of quantification
MAA	Methylacrylic acid
MIP	Molecularly imprinting polymer
MISPE	Molecularly imprinted solid Phase extraction
NIDDM	Non-Insulin Dependent Diabetes Mellitus
PE	Pulsed elution
RSD	Relative standard deviation
SPE	Solid phase extraction
TFA	Trifluoroacetic acid
TFMAA	2-(Trifluoromethyl)acrylic acid
TRIM	trimethylolpropane trimethacrylate
TTAB	Myristyltrimethylammonium bromide
UV	Ultra-violet

CHAPTER I

INTRODUCTION

1.1 Molecular Imprinting

1.1.1 Historical development of molecular imprinting

Molecularly imprinted polymers (MIPs) represent a new class of materials that have artificially created receptor structures[i,ii,iii]. Molecular imprinting has gained increasing research interest during the past few years. However, although interest in the technique is new, the concept itself has a long history. The lock-and-key principle formulated by Emil Fischer as early as the end of the 19th century has still not lost any of its significance for the life sciences. The idea of molecular specificity that could be tailored in sorbents has been around for a long time. In 1931, Polyakov[iv] demonstrated that molecular specificity could be imprinted into silica gel by pre-treating silicic acid with organic adsorbates before polycondensation. Later, Dickey[v, vi] performed more extensive investigations. Modern molecular imprinting technology appeared in the 1970s, when Klotz[vii] and Wulff[i] reported that chemical memories could be prepared in synthetic polymers. The latter report showed enantiomer recognition. The group of Wulff then presented a series of papers dealing with what is called the covalent approach to molecular imprinting, since reversible covalent interactions are the basis for molecular recognition in these systems. In the early 1980s the Mosbach group developed the non-covalent approach to molecular imprinting[viii]. This approach, in which the molecular recognition is based on non-covalent interaction (such as hydrogen bonding, ionic interactions, hydrophobic interactions, etc.) has been used as a more general approach to molecular imprinting, since the number of compound classes that could be imprinted was dramatically increased. A third approach, which is based on metal-ion chelation between the template and the polymer, has also been reported [ix]. From that time, an increasing

interest has been seen in the potential application of highly selective molecularly imprinted polymers. MIPs are adeptable to several analytical techniques, including liquid chromatography [x, xi], solid phase extraction [xii], immunoassay [xiii], capillary electrophoresis and capillary electrochromatography [xiv].

1.1.2 The concept of molecular imprinting

Molecularly imprinted polymers may be prepared according to a number of approaches that are different in the way the template is linked to the functional monomer and subsequently to the polymeric binding sites. Currently, the most widely applied technique to generate molecularly imprinted binding sites is represented by the non-covalent approach which is more common and flexible concerning the choice of functional monomers, possible template and application of the imprinted materials. This is based on non-covalent self-assembly of the template with functional monomers prior to polymerization, free radical polymerization with a cross-linking monomer, and then template extraction followed by rebinding *via* non-covalent interactions. Although the preparation of a MIP by this method is technically straightforward, it relies on the successful stabilization of the individually weak non-covalent interactions between the template and the functional monomers. This stabilization will in turn result in the incorporation of the functional binding groups to the templated cavities.

A typical imprinting system consists of a template molecule, at least one type of functional monomer, a cross-linking monomer, and a porogenic solvent. To induce radical polymerization, an appropriate initiator is added to the mixture. Formation of the initial template-functional monomer complex (imprinting), as well as of the template-

MIP complex (re-binding) may be driven by non-covalent interactions or reversible covalent bonds. In the process of molecular imprinting, a molecular template (print molecule) is used to direct the arrangement of the functional monomers around the template, which are then chemically fixed by co-polymerization with a cross-linking monomer. This results in a rigid polymer matrix embedding the template. The resulting bulk polymer is ground and sieved to prepare particles of appropriate size. Afterwards, the print molecule can be removed from the polymer by simple solvent extraction. This creates recognition sites specific to the template based on the position of the functional groups and the shape of the cavity (Figure 1). The MIP particles can be evaluated for the presence of selective recognition sites by using them either in batch experiments or as the stationary phase in a chromatographic column.

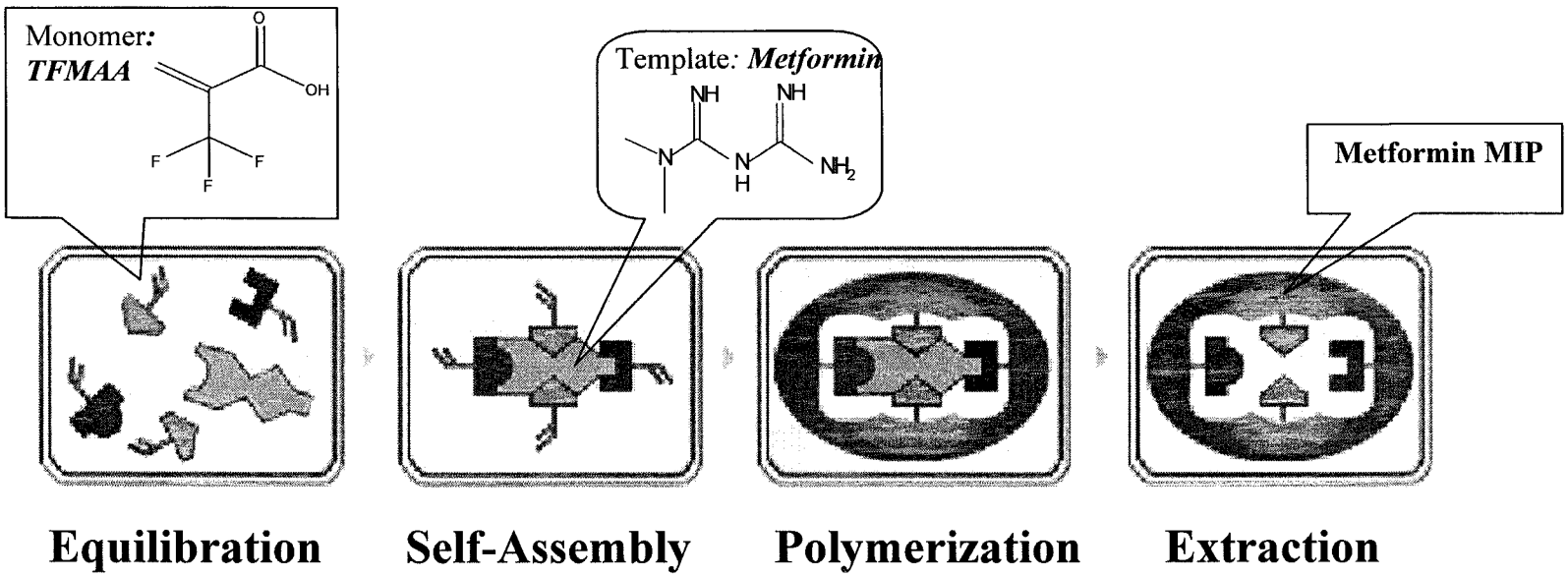


Figure 1. **Scheme of the molecular imprinting process**

1. The selected template molecule is mixed with functional monomers in a solvent. 2. The template molecule assembles with the functional monomers to form a complex. 3. Polymerization of the resulting complex with an excess of cross-linking monomer to form a rigid MIP. 4. Extraction of the template molecule to create binding cavities.

1.1.3 Applications of MIPs

As antibody and receptor binding mimics, MIPs have displayed very attractive features that have made MIPs the goal of intensive investigation: (1) their high affinity and selectivity for many target molecules, which are similar to those of natural receptors; (2) their unique stability which is superior to that demonstrated by natural biomolecules; and (3) the simplicity of their preparation and the ease of adaptation to different practical applications. The favorable physical and chemical robustness of MIPs allow these artificial antibodies to be used under harsher conditions such as organic solvents, pH extremes, high pressures and elevated temperatures, where biomolecules are often denatured.

The technique of molecular imprinting has been applied with success to the preparation of materials with high affinity for separations[15,16], binding assays[17], sensor science[18,19,20], and catalysis[21], wherever a highly specific interaction of the target molecule (analyte) with the MIP is demanded. A wide variety of MIPs have been screened to meet the present demand of higher selectivity in various fields, such as environmental control, drug development, health protection, forensics, and biotechnology [22,23,24,25].

Non-covalent molecular imprinting relies on multiple non-covalent interactions between the print molecule and the monomers. The association/dissociation kinetics of non-covalent MIPs is in general faster than that observed on MIPs prepared by the covalent approach. For this reason, the former polymers are more attractive for use as the stationary phase in chromatography.

1.1.4 Molecularly imprinted solid phase extraction (MISPE)

The first reported study on molecularly imprinted polymer based solid phase extraction (MISPE) by Sellergren[26] employed a pentamidine-selective MIP for demonstration of on-line sample enrichment of a spiked urine sample. Since then, molecular imprinting has been brought to the forefront of selective chromatographic sorbents with potential applications in widely different fields such as selective sorbents in SPE for trace analysis, sorbents for scavenging purposes, and polymeric selectors for preparative chiral separations. The selectivity of a MIP can be pre-determined by the choice of template and functional monomers employed for its preparation. One major benefit of MISPE relates to the high efficiency of sample clean up. Different modes of MISPE have been tried, including various modes of on-line SPE[26,27], conventional SPE where the MIP is packed into columns or cartridges[28,29,30], and batch mode SPE where the MIP is incubated with the sample[31].

MISPE with pulsed elution (PE) was first developed by Mullett and Lai[27] for the analysis of theophylline in serum sample. The method made use of a MIP column for the on-line enrichment of theophylline, followed by injection of a small volume of methanol to produce a rapid pulsed desorption of the analyte. The sample was applied in chloroform since in this solvent a complete retention of the analyte was observed. The matrix constituents, and other potential interferences that were not recognized by the binding sites, were rapidly eluted with chloroform. Then a rapid and quantitative recovery of theophylline was accomplished in a pulsed format through injection of 20 μL of methanol. In this way, the eluted analyte could be detected directly by UV for quantification. Further improvement to the MISPE-PE method was achieved by a novel

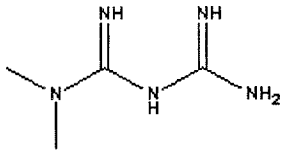
differential pulsed elution (DPE) to eliminate the nonspecific adsorption of interfering drugs, without sacrificing the selective binding of theophylline[32].

1.2 Metformin

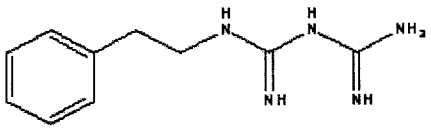
1.2.1 Current clinical applications

Compounds containing the guanidine group play an important role in biochemistry. Metformin (1,1-dimethylbiguanide) and phenformin (phenylethylbiguanide) are biguanide agents used as oral antihyperglycemic agents in the treatment of type 2 (non-insulin-dependent) diabetes mellitus (Figure 2). However, phenformin was withdrawn in 1977 because of its association with a high incidence of lactic acidosis. In 2002, metformin ranked 38th of the top 200 drugs in prescriptions worldwide. It is fairly widely marketed and used in the treatment of NIDDM [Non-Insulin Dependent Diabetes Mellitus][33]. Metformin has clinically been used, in combination with a high protein-low carbohydrate diet, to restore normal menstrual cycles in teenage females with polycystic ovary syndrome (PCOS) [34]. Metformin is also included in HIV clinical trials to assess its efficacy in the treatment of the metabolic disturbances (fat redistribution, insulin resistance, and hyperinsulinemia) associated with HIV lipodystrophy syndrome [35].

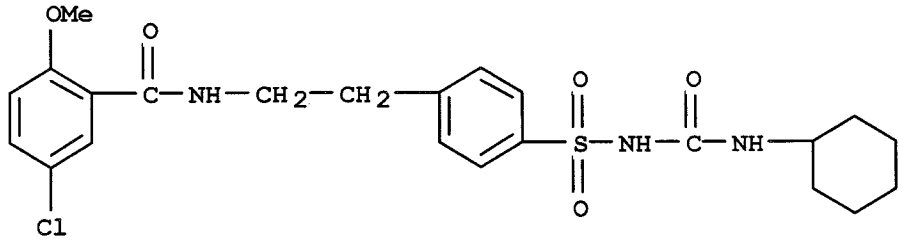
Glyburide, which is an oral blood-glucose-lowering drug of the sulfonylurea class, is usually combined with metformin hydrochloride. Under the brand name of Glucovance, these two antihyperglycemic agents (with complementary mechanisms of action) could improve glycemic control in patients with type II diabetes [36].



Metformin



Phenformin



Glyburide

Figure 2. Molecular structures of metformin, phenformin and glyburide.

1.2.2 Clinical pharmacology of metformin

Metformin HCl (N,N-dimethylimidodicarbonimidic diamide hydrochloride) has been the only clinically available drug that can significantly improve insulin sensitivity[37]. This drug improves glucose tolerance in patients with type II diabetes, lowering both

basal and postprandial plasma glucose. It decreases hepatic glucose production, decreases intestinal absorption of glucose, and improves insulin sensitivity by increasing peripheral glucose uptake and utilization [36]. Metformin acts principally by improving the sensitivity of peripheral tissue (chiefly skeletal muscle) and the liver, to insulin, thus opposing the insulin resistance. Metformin does not increase pancreatic insulin secretion and does not induce hypoglycemia.

Metformin has an absolute oral bioavailability of 40% to 60%, and gastrointestinal absorption is apparently complete within 6 hours of ingestion. Metformin is rapidly distributed following absorption and negligibly bound to plasma proteins. Following administration at usual doses, peak plasma drug concentrations are usually attained within 2 to 4 hours and steady-state plasma concentrations (generally < 1 μg/mL) are reached within 24 to 48 hours. Therapeutic level may be 0.5 to 1.0 μg/mL in the fasting state and 1-2 μg/mL after a meal. During controlled clinical trials, maximum metformin plasma levels did not exceed 5 μg/mL. Metformin is excreted unchanged in the urine and does not undergo hepatic metabolism or biliary excretion.

1.2.3 Adverse effects and toxicity of metformin

Lactic acidosis is a rare, but serious, metabolic complication that can occur as a result of metformin accumulation. In general, lactic acidosis is fatal in approximately 50 percent of cases. While the reported incidence of this condition in patients taking metformin is very low, the risk of metformin accumulation and lactic acidosis increases with the degree of renal function impairment and the patient's age. Concerns about lactic

acidosis are particularly relevant in patients with HIV infection, given the potential for increased lactate levels[38] .

Gastrointestinal adverse effects (including diarrhea, abnormal stools, nausea, vomiting, abdominal bloating or cramping, flatulence, and taste disorder) were severe enough to require drug discontinuation in about 4 percent of study participants [38] . Additionally, the following adverse reactions were reported by study patients: hypoglycemia, myalgia, lightheadedness, headache, dyspnea, nail disorder, rash, increased sweating, chest discomfort, chills, flu syndrome, flushing, and palpitation[39] .

1.2.4 Current analytical methods for metformin

Monitoring of metformin plasma concentration usually has little clinical value, except when lactic acidosis is suspected or diagnosed. Metformin accumulation can induce serious metabolic complications such as lactic acidosis when the metformin concentration in plasma becomes too high[35]. For this reason, the determination of plasma metformin should always be done during treatment. Indeed, when lactic acidosis occurs in metformin-treated patients, early determination of the metformin plasma concentration appears to be the best criterion for assessing this acute condition. Determination of metformin has previously been reported in human plasma, urine, breast milk and pharmaceutical preparations by high-performance liquid chromatography (HPLC) with spectrophotometric detection [39,40,41,42,43,44], capillary electrophoresis (CE) using field-amplified sample stacking technique [45], voltammetry on a glassy carbon electrode [46], potentiometry, and spectrofluorimetry[47]. One challenge with measuring very low metformin concentrations in human plasma (in pharmacokinetic studies) is due to the high polarity of this compound. It is difficult to extract the drug directly from biological

fluids by solvent extraction. A method based on the extraction of metformin with bromothymol blue (as an ion-pair) into chloroform had previously been described, using phenformin as internal standard [45].

Its direct determination by chromatographic analytical techniques is rather difficult because of its strong base properties ($pK_a = 13.1 \pm 0.5$) and high polarity [48]. Gas chromatographic methods require a pre-derivatization of the analyte with acetic anhydride [49]. In the analysis of biological fluids such as human plasma and urine, liquid chromatographic methods require a pre-extraction with solid phase sorbents to clean up the matrix complexity [50],[51],[52]. Recently, an ion-pair HPLC method was developed for the determination of metformin in human plasma. The sample preparation involved protein precipitation and no evaporation step was required [53]. These sample pretreatment procedures are potentially sources of supplementary errors to the analysis.

1.3 MISPE-DPE-FPE for determination of metformin

Generally, analytical methods comprise a sample-pretreatment step followed by either separation and detection steps that can discriminate to a variable degree between the analyte and the matrix components, or quantification in a competitive binding assay. The general idea is that all elements of a method should contribute to its required sensitivity and selectivity. In many instances, however, bioanalytical sample pretreatment is required as a step to get rid of water, salts and bulk amounts of proteins present in the samples. In trace analysis, many other compounds, some of which may have very similar structures to the analyte of interest, are also present as interferences. These compounds can also interfere with analyses. Highly selective phases that can discriminate and isolate the target analyte from the multitude of other compounds in the

sample are desirable. Therefore, MISPE has become widely evaluated for analyte pre-concentration and sample matrix cleanup. To date MISPE methods are available for the rapid screening of many drugs (including aminopyridine, bupivacaine, cephalexin, darifenacin, 7-hydroxycoumarin, nicotine, pentamidin, propranolol, sameridine, tamoxifen, and theophylline)[32]. Metformin is more basic than all of these drugs previously reported, and was chosen as a challenging molecule (with a high pKa of 13.1 ± 0.5 and a large number of functional groups for binding interactions) to re-establish generic rules that would enable speedy development of MISPE-DPE-FPE methods.

In the present study, a new MIP was synthesized by using metformin as the print molecule and trifluoromethacrylic acid (TFMAA, pKa = 2.1± 0.2) as the functional monomer. A micro-column packed with these MIP particles was evaluated for its ability to bind metformin selectively in acetonitrile. Molecularly imprinted solid phase extraction followed by pulsed elution (MISPE-PE) was demonstrated to be a useful procedure for matrix clean up and analyte pre-concentration. A differential pulsed elution (DPE) step was added to eliminate potential interferences due to the binding of structural analogues. A schematic diagram of MISPE-DPE-FPE-UV system for on-line determination of metformin was shown in Figure 3.

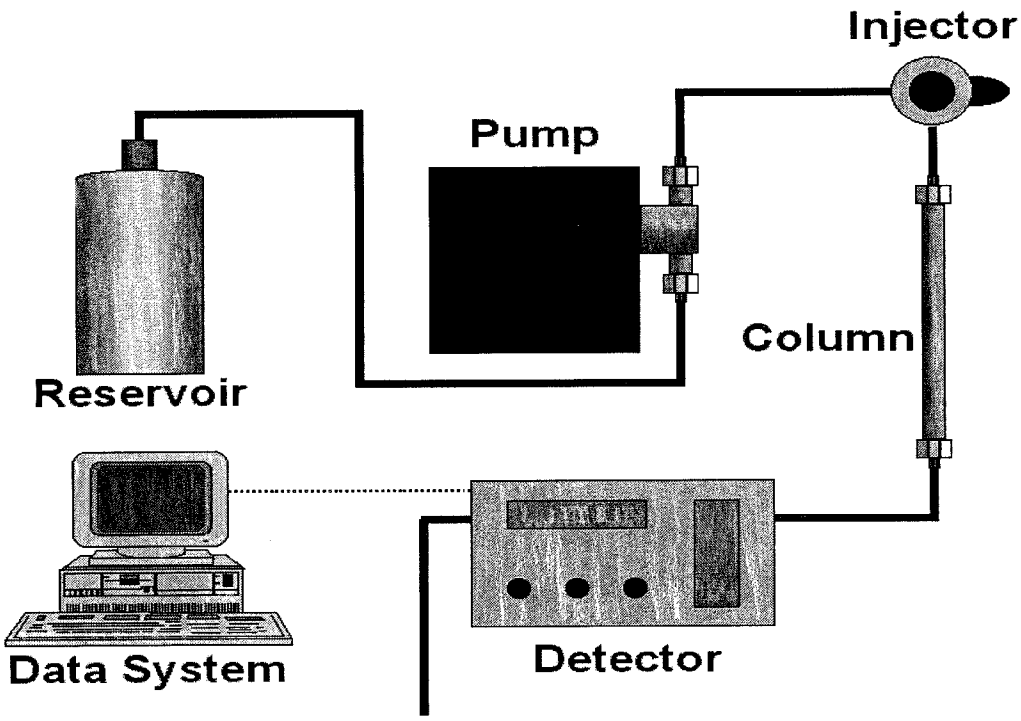


Figure 3. Schematic diagram of MISPE-DPE-FPE-UV system for on-line determination of metformin

1.4. Capillary Electrophoresis (CE)

1.4.1 General introduction of CE

Capillary electrophoresis (CE) is a relatively new separation technology that combines both aspects of gel electrophoresis and HPLC. The main advantages of CE over HPLC are the much higher separation efficiency, lower solvent (or buffer) consumption, and shorter analysis time. Like gel electrophoresis, the separation depends upon differential migration in an electrical field. Since its first description in the late 1960s[54], several capillary electrophoretic techniques have been demonstrated: zone electrophoresis, displacement electrophoresis, isoelectric focusing, and sieving separations. CE is characterized by high resolving power, and commercial CE instruments became available since 1980s. Narrow-bore capillaries with excellent heat dissipation properties enable the use of very high field strengths, which decrease analysis time and minimize band diffusion. When separations are performed in the presence of electroosmotic flow (EOF), the plug-flow characteristics of EOF also contribute to high efficiency.

Because of its many advantages, CE shows great promise in the analysis of hydrophilic molecules and highly charged solutes. These techniques have led to the rapid separation and quantitation of: basic pharmaceuticals[55], proteins[56], glycoproteins, peptides[57], carbohydrates, chiral isomers[58], inorganic ions, and organic ions [59,60]. Especially, CE has been applied to the analysis of various pharmaceuticals and their mixtures[61].

1.4.2 Theory of capillary electrophoresis

As the name implies, capillary electrophoresis (CE) is performed by a narrow-bore fused-silica capillary filled with a buffer electrolyte. A schematic of CE system is presented in Figure 4.

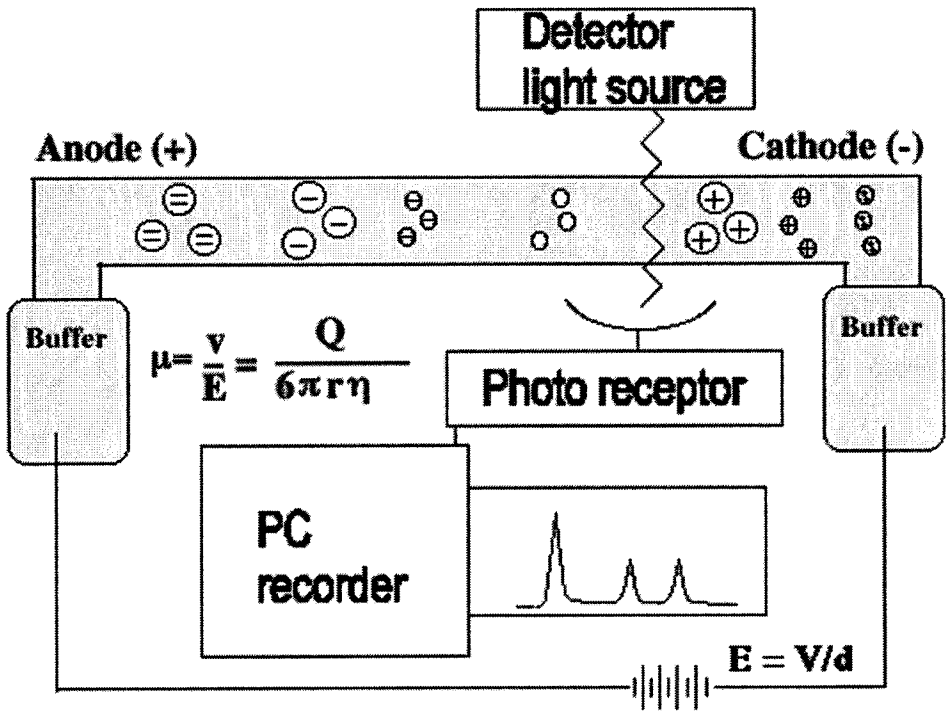


Figure 4. Schematic diagram of capillary electrophoresis (CE) system.

The magnified view depicts the capillary interior with molecules of different size and charge separating. As illustrated, in capillary electrophoresis, the positive molecules migrate toward the negative electrode while the electroosmotic flow carries the neutral and negatively charged molecules in the same direction.

Capillary zone electrophoresis (CZE) is the simplest form of CE. High voltages are used to separate molecules based on the charge-to-size ratio of the analytes. The choice of the running buffer pH plays a crucial role because it influences the charge of the analytes. Injection into the capillary is accomplished by immersing the end of the capillary into a sample vial and applying pressure, vacuum or voltage. As soon as the high voltage power supply of the instrument is switched on, an electric field E along the capillary with length L will be generated:

$$V = E \times L \tag{1}$$

where V is the total applied voltage. Without consideration of the electroendosmotic flow, the sample ions are moving with the speed ν_{EP}, the velocity of migration, which is given by:

$$\nu_{EP} = \mu_{EP} \times V / L \tag{2}$$

where μ_{EP} is the electrophoretic mobility, V is the applied voltage, and L is the capillary length. Generally, the capillary surface is charge carrier (negative in the case of fused silica capillaries because of the dissociated silanol groups). An electric double layer at the solid-liquid interface is formed, due to preferential adsorption of ions. Cations or partial positive charges in the medium are electrostatically attracted to the capillary wall, thus forming the electric double layer that is responsible for the electroosmotic flow. The velocity of electroosmotic flow, ν_{EO}, is described by:

$$\nu_{EO} = \mu_{EO} \times V / L \tag{3}$$

where μ_{EO} is the coefficient of electroosmotic flow. The total migration time, t, is thus calculated as follows:

$$t = L / (\nu_{EP} + \nu_{EO}) = VL^2 / (\mu_{EP} + \mu_{EO}) \tag{4}$$

During the migration time, diffusion continues to occur. If an initially infinitely thin zone is allowed to diffuse for a time t, the spatial variance σ^2_L of the zone will be:

$$\sigma^2_L = 2D = 2DL^2V / (\mu_{EP} + \mu_{EO}) \quad (5)$$

where D is the diffusion coefficient of the analyte. The number of plates, N, can be calculated according to N = L / H, where H is the height per theoretical plate:

$$N = (\mu_{EP} + \mu_{EO}) \times V / 2D \quad (6)$$

From the last equation it follows that, in CE separations, high voltages result in high separation efficiencies. This can be achieved by narrow bore capillaries with efficient heat dissipation.

1.4.3 SPE-CE for determination of metformin

The availability of fast, precise and reliable analytical methods for the determination of drug levels is essential in clinical chemistry. Over the past two decades, different techniques have been developed for drug monitoring in body fluids and biological tissue specimens, with clinical relevance such as dosage adjustments, compliance control, and determination of efficient drug levels or overdoses[62]. With its distinct advantages, modern CE is an attractive tool for fast and quantitative monitoring of drugs.

One challenge with measuring very low metformin concentrations in human plasma is due to the high polarity of this compound. It is difficult to extract the drug directly from biological fluids by solvent extraction. In the present work, a new solid phase extraction (SPE) method was developed for the pre-treatment of human plasma

samples. Then, non-aqueous CE was employed for the rapid separation of metformin from phenformin, glyburide and other drugs.

Several advantages should be mentioned: (1) minute quantities of buffer (milliliter) are required for replicate analyses; (2) only microliters of a patient sample are needed for analysis; (3) the capillary can be rinsed and regenerated for subsequent runs in minutes; and (4) the potential to implement fully automatic procedures. As a net result, the SPE-CE method provides rapid, highly efficient and reproducible separation for metformin in low-volume plasma samples.

1.5 Objectives

The main objective of this research was to choose metformin as a challenging molecule for advancing our understanding of molecular imprinting, and re-establishing generic rules that would enable speedy development of MISPE-DPE-FPE methods. In the present work, a new MIP was specifically synthesized (by non-covalent imprinting) as a smart material for the recognition of metformin. The molecular recognition properties of the MIP were investigated and optimized. In order to eliminate potential interference due to the binding of structural analogues and sample matrix components, a differential pulsed elution (DPE) technique was investigated and developed.

The second objective involved the method development of solid phase extraction (SPE) and capillary electrophoresis (CE) for the determination of metformin and other antidiabetic drugs in human plasma samples. Separations performed in a non-aqueous buffer system were explored and studied. Furthermore, the MISPE-DPE-FPE method and SPE-CE method for metformin determination were validated for pharmaceutical and biomedical analyses.

CHAPTER II

EXPERIMENTAL

2.1 MISPE-DPE-FPE

2.1.1 Preparation of metformin-MIP

2.1.1.1 Reagents and materials

Metformin, phenformin, glyburide and caffeine were obtained from Sigma (St. Louis, MO). Stock solutions of 1 mg/mL each was prepared in distilled deionized water. They were stable when stored at 4°C for several months. Standard solutions of 60 μg/mL each were prepared afresh by dilution with acetonitrile for daily use.

Acetonitrile (CH_3CN) and methanol (CH_3OH) were HPLC-grade solvents obtained from Caledon (Georgetown, ON). Acetic acid (HAc) was purchased from Anachemia (Toronto, ON). Trifluoroacetic acid (TFA), 2-trifluoromethacrylic acid (TFMAA), picric acid, benzoic acid and pentafluorobenzoic acid were obtained from Aldrich (Milwaukee, WI). Trimethylolpropane trimethacrylate (TRIM) and 2,2'-azobis-2-methylproprionitrile (AIBN) were purchased from Sigma (St. Louis, MO) and Caledon (Georgetown, ON).

All 50-mM aqueous buffers (glycine for pH 1.7, phosphate for pH 2.5, acetate for pH 4.5, MES for pH 6.0, phosphate for pH 7.0 and Tris for pH 8.0) were obtained from Beckman Coulter (Fullerton, CA). Ammonium acetate (NH_4OAc) and glacial acetic acid (HAc) were obtained from Anachemia (Toronto, ON).

Didodecyldimethylammonium bromide (DDAB), dodecyltrimethyl-ammonium bromide (DTAB), hexamethonium bromide (HMB) hydrate, and myristyl-tri-methylammonium bromide (TTAB) were obtained from Aldrich (Milwaukee, WI, USA).

Human plasma and serum samples were supplied by the Biochemistry Laboratory, Ottawa General Hospital.

2.1.1.2 Metformin MIP synthesis

The MIP was synthesized in a 10-mL pyrex vial containing 16 mg of metformin HCl (0.10 mmol, print molecule), 101 mg of TFMAA (0.72 mmol, functional monomer) and 3.0 mL of acetonitrile (porogenic solvent). 0.23 mL of TRIM (0.72 mmol, cross-linker) and 12 mg of AIBN (0.07 mmol, initiator) were added. The mixture was purged with nitrogen and sonicated in a water bath for 5 min. Polymerization was then initiated by UV light and left overnight at 60°C. A colorless translucent bulk of solid MIP was obtained. The bulk MIP was broken down and ground in a mortar with a pestle. The ground MIP particles were sieved to a size range of 38-63 μm in diameter. A control polymer was also synthesized in the same conditions as described above, without the addition of metformin HCl.

2.1.1.3 MIP micro-column packing

An adequate amount of the sieved MIP particles was suspended in methanol. After sonication in a water bath for 10 min, the slurry of suspended MIP particles was poured into a stainless-steel HPLC tubing (35 mm length, 0.8 mm i.d.) equipped with an exit frit. A zero-volume union was put on each end of the newly packed column before acetonitrile was pumped through for 2-3 hours to achieve uniformly tight packing. Approximately 20 mg of MIP particles were contained in the micro-column.

2.1.1.4 Removal of template molecule from metformin MIP

The packed MIP micro-column was installed by connecting with an Eldex Duro Series CC-30s micrometer pump (San Carlos, CA). A Valco Cheminert VIGI C2XL injector valve (Houston, TX) equipped with a 20-μL sample loop was used for sample injection. A Bischoff Lambda 1010 UV detector (Leonberg, Germany) was used to monitor the removal of metformin template. The retention times and peak areas were recorded by a Dionex 4270 integrator (Sunnyvale, CA).

Removal of the metformin template was achieved by performing an on-line flushing using methanol + 3% TFA at a flow rate of 0.5 mL/min, until a stable baseline was attained for UV absorbance at 240 nm. This was followed by flushing with 100% CH_3CN for 2 hours.

2.1.2 Investigation of molecular recognition

2.1.2.1 MISPE-PE instrumentation

The MISPE-PE instrumentation was set up with an Eldex Duro Series CC-30s micrometer pump (San Carlos, CA), a Valco Cheminert VIGI C2XL injector valve (Houston, TX) equipped with a 20-μL sample loop, a Bischoff Lambda 1010 UV detector (Leonberg, Germany), and a Dionex 4270 integrator (Sunnyvale, CA). Acetonitrile was driven as the mobile phase through the MIP micro-column at a flow rate of 0.5 mL/min. A previous work had studied the effects of flow rate on the recovery and PE efficiency for a cephalexin MIP micro-column with an identical i.d.[63]. A slower flow rate than 0.5 mL/min was not adopted mainly for the sake of a rapid analysis time (under 5 min).

2.1.2.2 % Binding measurements

With acetonitrile as the mobile phase at a flow rate of 0.5 mL/min, 20 μL of a 60-μg/mL metformin solution was tested for binding on the MIP micro-column. The breakthrough peak of metformin was recorded and the remaining analyte bound to the MIP micro-column was removed with a 20 μL pulsed elution (PE) with 3% TFA in methanol. The breakthrough and PE peaks were detected at an optimal wavelength of 240 nm. These peak areas were blank-subtracted before their ratio was calculated to determine the % binding for metformin. A similar binding test and calculation was done for phenformin.

2.1.2.3 Evaluation of binding capacity

Binding capacity of metformin MIP micro-column was investigated by multiple injections of a 7.5-μg/mL metformin solution in 9:1 CH_3CN / 50 mM phosphate buffer (pH 2.5) onto the metformin MIP micro-column, with acetonitrile as the mobile phase at a flow rate of 0.5 mL/min. Binding saturation was finally observed when the breakthrough peak area of each injection became identical to the flow injection analysis (FIA) peak area. A similar binding capacity evaluation was done for phenformin.

2.1.2.4 Effect of sample solvent on % binding

The hydrodynamic ability of the metformin MIP micro-column to bind and retain metformin was evaluated for various aqueous and acetonitrile standard solutions. The effect of sample solvent in the molecular recognition process of MISPE-PE was

investigated by injection of 60-μg/mL metformin solutions with various ratios of acetonitrile to water. Acetonitrile was employed as the mobile phase in all subsequent investigations for the benefit of minimal non-specific binding during MISPE-PE. Breakthrough and PE peak areas were measured in separate 20-μL loading injections at 240 nm by UV absorbance detection.

2.1.2.5 Effect of buffer pH in sample solution on % binding

Binding of metformin onto the MIP micro-column was examined over a pH range from 1.5 to 8.0. Different types of aqueous buffers were added to the 60-μg/mL metformin solutions in acetonitrile, in a constant v/v ratio of 1:9. The breakthrough and PE peaks were detected at an optimal wavelength of 240 nm. These peak areas were blank-subtracted before their ratio was calculated to determine the % binding for metformin.

2.1.2.6 Effect of phosphate buffer on % binding

To test the effect of phosphate buffer on % binding for metformin, the sample solutions were phosphate-buffered (at pH 2.5 or 7.0). The % binding of 60-μg/mL metformin in CH_3CN, in 9:1 CH_3CN / 50 mM phosphate buffer (pH 2.5), and in 9:1 CH_3CN / 50 mM phosphate buffer (pH 7.0) were compared by single loading injections.

2.1.2.7 Effect of metformin concentration on % binding

For further investigation of the relation between metformin concentration and % binding, metformin standard solutions of different concentrations over the range of 7.5-

60 μg/mL in 9:1 CH_3CN / 50 mM phosphate buffer (pH 2.5) were tested. CH_3CN was used as the mobile phase at a flow rate 0.5 mL/min. MISPE was performed by single injection of individual metformin standard solution, followed by PE with 3 % TFA + CH_3OH. The breakthrough peak area after injection of each individual metformin concentration was measured to determine the % binding achieved.

2.1.2.8 Binding selectivity of metformin MIP

To investigate the binding selectivity of metformin MIP micro-column, the % binding of metformin (imprinting template), phenformin (structural analogue), caffeine (most common interference in human plasma samples) and glyburide (sulfonylurea antidiabetic drug) were measured separately under the same MISPE condition. 20-μL aliquots of metformin, phenformin, caffeine and glyburide solutions (60 μg/mL in 9:1 CH_3CN / 50 mM pH 7 phosphate buffer) were loaded separately on the metformin MIP micro-column, followed by PE with 3% TFA in CH_3OH. Acetonitrile was employed as the mobile phase at a flow rate of 0.5 mL/min. The breakthrough and PE peaks were detected at optimal wavelengths of 230-260 nm, and their ratio was calculated to determine the % binding.

2.1.3 MISPE-DPE-FPE

2.1.3.1 Final pulsed elution (FPE) of metformin

Acetonitrile was used as the mobile phase at a flow rate of 0.5 mL/min. TFA is a strong organic acid with a pKa value ranging from -0.23 to 0.0 as reported in the literature. In the present MISPE method development, various concentrations of TFA

over the range from 0.025% to 3% in methanol were tested to remove bound metformin from the MIP micro-column for UV detection at 240 nm. Finally, 3% TFA in methanol was chosen as the optimal FPE solvent.

2.1.3.2 Differential pulsed elution (DPE) of phenformin

Acetonitrile was used as the mobile phase at a flow rate of 0.5 mL/min. An intermediate wash step, differential pulsed elution (DPE), was added to eliminate potential interference due to the binding of structural analogues. Phenformin is a guanidine derivative that is structurally analogous to metformin. As a strong base with a pKa value of 12.7±0.5, phenformin was used as a model interferent molecule to challenge the selectivity of this MISPE method. DPE was studied as a means to eliminate phenformin quantitatively while leaving behind an adequate amount of metformin for UV detection. A final pulsed elution (FPE) of metformin for direct UV detection was achieved using 3% TFA in methanol.

2.1.3.3 Evaluation of organic acids for DPE effectiveness

Acetonitrile was used as the mobile phase at a flow rate of 0.5 mL/min. Several organic acids: benzoic acid, pentafluorobenzoic acid, acetic acid, trifluoroacetic acid (TFA), trifluoromethacrylic acid (TFMAA) and picric acid in acetonitrile or methanol were evaluated for their DPE effectiveness. After DPE with each organic acid, 3% TFA + CH_3OH was used in FPE to elute all of the remaining phenformin.

2.1.3.4 DPE with trifluoroacetic acid (TFA)

Acetonitrile was used as the mobile phase at a flow rate of 0.5 mL/min. After single injection of 20-μL of phenformin solution (60-μg/mL in 9:1 CH_3CN / 50 mM pH 7 phosphate buffer) onto the metformin MIP micro-column, different concentrations of TFA ranging from 0.025% to 2% in methanol were tested for DPE individually. After each DPE, 3% TFA + CH_3OH was used in FPE to elute all of the remaining phenformin.

2.1.3.5 DPE with picric acid

Acetonitrile was used as the mobile phase at a flow rate of 0.5 mL/min. Picric acid (pKa = 0.3 to 0.53 as reported in the literature) was strategically selected from among the strongest organic acids that were commercially available. After a single 20-μL injection of phenformin solution (60 μg/mL in 9:1 CH_3CN / 50 mM pH 7 phosphate buffer) onto the metformin MIP micro-column, different concentrations of picric acid over the range from 1.7% to 22% in acetonitrile were evaluated for the best DPE result. After DPE with each picric acid solution, 3% TFA (pKa = -0.23 to 0.0) in methanol was used in FPE to elute all of the remaining phenformin. The ΔFPE peak area was determined and plotted versus the concentration of picric acid. Metformin standard solution, with concentrations ranging from 7.5 to 50 μg/mL in 9:1 CH_3CN / 50 mM phosphate buffer (pH 7.0), were tested.

2.1.3.6 Standard calibration curve for MISPE-PE determination of metformin

Under isocratic mobile phase conditions of 100% acetonitrile, a standard calibration curve was performed for MISPE-PE determination of metformin in

acetonitrile (phosphate buffered at pH 7.0), using 3% TFA in methanol as PE solvent. The concentration of metformin standards was over the range from 3.75 to 60 μg/mL in 9:1 CH_3CN / 50 mM phosphate buffer (pH 7.0). The MISPE-PE peak area was plotted vs. the mass of metformin loaded on the MIP micro-column.

The detection limit was determined for metformin, based on 3 standard deviations of the blank.

2.1.4 Human plasma analysis

Human plasma samples were obtained from the Ottawa General Hospital (Ottawa, Ontario, Canada). Several 0.2-mL aliquots of a plasma sample were spiked with 20–μg/mL metformin and 300-μg/mL phenformin to provide a series of working solutions over the concentration range from 0.1 to 10 μg/mL metformin and 60 μg/mL phenformin. 1 mL of 9:1 acetonitrile / 50 mM phosphate buffer (pH 7.0) was added to precipitate the proteins [64]. The mixture was thoroughly vortexed and then centrifuged at 5000 rpm for 20 min. The supernatant was filtered through SuporAcrodisc® syringe filter (Gelman Sciences, pore size 0.45 μm). A 20-μL of filtered supernatant was injected onto the MIP micro-column for MISPE-DPE-FPE analysis.

CH_3CN was used as the mobile phase at a flow rate of 0.5 mL/min. A standard calibration curve for MISPE-DPE-FPE was constructed by triplicate 20-μL injections of the above standard solutions onto the metformin MIP micro-column. This was followed by DPE with 5% picric acid in CH_3CN and FPE with 3% TFA in CH_3OH. The % recovery of metformin was determined by analyzing a plasma sample (0.2 mL) that had been spiked with 3-μg/mL metformin and 60-μg/mL phenformin.

2.2 Capillary zone electrophoresis

2.2.1 Reagents and materials

Ammonium acetate (NH_4OAc) was obtained from Anachemia (Toronto, ON).

2.2.2 Conditioning of the capillary

CE separation was performed on a fused silica capillary (65 μm i.d., 360 μm o.d., 30 cm total length, 22.5 cm effective length to detector) obtained from Polymicro Technologies (Phoenix, AZ). The new fused silica capillary was flushed with 1 M NaOH, rinsed with deionized distilled water and cleaned with the running buffer. Before use, the capillary was conditioned with the running buffer at an applied voltage of 15 kV for 30 min. One blank run (no sample injection) was allowed before the first analysis.

2.2.3 Capillary zone electrophoresis

CE analyses were performed on a laboratory-built system that included a Spellman CZE1000R high voltage power supply. The electrolyte was composed of acetonitrile, and different concentrations of buffer, ammonium acetate (NH_4OAc) and acetic acid (HOAc). The optimal non-aqueous buffer (pH 5.1) for CE was composed of 20 mM NH_4OAc and 5% HOAc in CH_3CN. When the buffer was run under an applied voltage of 15 kV, the capillary was equilibrated with the ambient temperature of 22-25°C. The samples were degassed by sonication, and electrokinetic injections were made by applying 15 kV for 6 s typically.

A Bischoff Lambda 1010 (Leonberg, Germany) UV detector was employed to monitor the elution of analytes at a wavelength of 240-280 nm. The detector output

signal was acquired by both a Dionex 4270 integrator (Sunnyvale, CA) and a personal computer running the Peak Simple Chromatography Data System (SRI model 203, Torrance, CA). After each run, the capillary was conditioned by running the buffer at 15 kV for 1 min before the next analysis.

2.2.4 Effect on buffer composition

A non-aqueous CE buffer was previously reported by Siren *et al* [65] to provide separation of polar drugs. In the present work, a non-aqueous separation medium, which was acetonitrile modified by NH_4OAc and glacial HOAc, was employed. In the CE technique, after laboratory optimization, the final buffer solution was chosen to be 20 mM NH_4OAc and 5% HOAc in acetonitrile (pH 5.1).

To investigate the effect of the concentration of electrolytes, buffer solutions were prepared by varying the NH_4OAc from 1 to 20 mM and the HOAc from 1% to 5% in acetonitrile. The prepared buffers were degassed by ultrasonication before use. The sample solutions, which contained 60 μg/mL of metformin and phenformin in acetonitrile, were electrokinetically injected at 15 kV for 6 s. The analyte peaks were detected by UV detector at 240 nm. Finally, 20 mM NH_4OAc and 5% HOAc in acetonitrile was chosen as the non-aqueous media that gave the best separation for the analytes.

2.2.5 Sample Matrix Effect on CE-UV Peak Area

Various standard mixtures which contained 60-μg/mL of metformin and 60-μg/mL of phenformin were prepared in H_2O, 20 mM NH_4OAc + 5% HOAc, CH_3OH + 3% HOAc, and CH_3OH + 3% TFA, respectively. These sample solutions were analyzed by CE to investigate the sample matrix effects on CE-UV peak areas.

2.2.6 Human plasma analysis

Each 0.5-mL human plasma sample was spiked with metformin, phenformin (as an internal standard [66]) and glyburide (as potential interference) from aqueous standard solutions. After dilution to 2 mL with deionized water, extraction treatment was carried out on a C18 SPE cartridge (T. Baker, 7020-03). The cartridge was dried by helium gas, before the extracted metformin, phenformin and glyburide were eluted with 2 mL of methanol + 3% acetic acid. The eluate was collected and electrokinetically injected (at 15 kV for 6-36 s) to the capillary for CE analysis using a running buffer of 20 mM NH_4OAc + 5% HOAc in acetonitrile. A standard calibration curve was constructed by performing SPE-CE-UV analysis of human plasma samples that were spiked with metformin and phenformin in the concentration ranges from 1 μg/mL to 30μg/mL.

CHAPTER III

RESULTS AND DISCUSSION

3.1 Synthesis of metformin-MIP

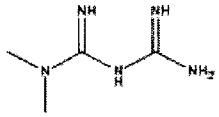
3.1.1 Preparation of metformin-MIP

In non-covalent molecular imprinting, functional monomers are chosen to allow hydrogen bonds, ionic interactions, π-π interactions and/or hydrophobic interactions with the imprint molecules. The strength and positioning of the monomer-template interactions are of importance to obtain a MIP with good molecular recognition properties. The broad applicability of MAA as a functional monomer is related to the fact that the carboxylic acid group serves as a hydrogen bond and proton donor, as well as a hydrogen bond acceptor [67]. In aprotic solvents such as acetonitrile, carboxylic acids and amine bases form hydrogen-bonded assemblies where the association strength for a given functional monomer acid increases with the basicity of the print molecule [68]. Often, a large excess of MAA in the synthesis step is required for recognition to be observed and then only in solvents of low to medium polarity and hydrogen bond capacity [69]. Moreover, the excess of functional monomer results in a portion not containing imprinted cavities. These cavities interact non-selectively with solutes with affinity for carboxylic acids (from the functional monomer) and limit the degree of separation that can be achieved. Hence, MAA, although broadly applicable, is not a universal monomer for the generation of high affinity sites. Instead, trifluoromethyl acrylic acid (TFMAA) can enhance selectivity in a terpolymer with MAA [70].

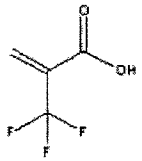
Therefore, in this study, 2-(trifluoromethyl)acrylic acid (TFMAA) was selected as the functional monomer for its ability to interact by non-covalent bond with metformin, the print molecule. Compared with methacrylic acid (MAA), TFMAA has a higher acidity and would afford stronger ionic interaction with metformin. Before

polymerization, the monomers and the print molecules were self-assembled elaborately by dissolving them in an appropriate solvent. Cross-linker (TRIM) is added and the polymerization was initiated. The print molecules were subsequently extracted from the highly cross-linked polymeric network obtained.

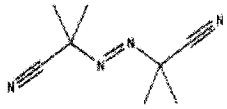
The molecular structure of metformin has characteristics that make it suitable for successful molecular imprinting (Figure 5). It was not difficult to foresee a priori how the formation of hydrogen bonding interactions with multiple TFMAA in the polymerization mixture would be possible because of the primary, secondary and ternary amine groups on metformin. Multiple N-H···O= interactions between metformin and oxygen lone pairs had previously been reported by Kataky *et al.* [71]. Only one kind of functional monomer was deemed necessary for the present MIP preparation. The molar ratio of TFMAA to the print molecule was varied by adjusting the amount of print molecule added to an otherwise constant pre-polymerization mixture. A molar ratio of 1:7 (metformin : TFMAA) was found to be optimal in ensuring specific binding and reducing non-specific binding.



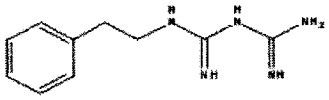
Metformin



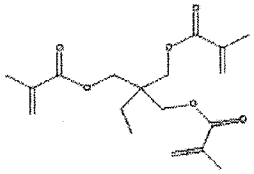
TFMAA



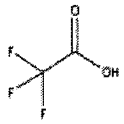
AIBN



Phenformin



TRIM



TFA

Figure 5. Molecular structures of metformin, phenformin, TFMAA, TRIM, AIBN and TFA.

3.1.2 Selection of solvent

MIPs often display good recognition in organic solvents, whereas significant non-specific binding is seen in aqueous solutions [72]. Generally, in most successful non-covalent imprinting systems, an aprotic solvent of low to medium polarity (*e.g.* CH_2Cl_2, toluene, CH_3CN) was used as diluent or porogen. Similarly, the rebinding to MIPs is strongly dependent on the medium. The properties of MIPs are often correlated with the solvent conditions employed during polymerization and thus optimum recognition is frequently observed using this same solvent [73,74,75]. Previously, when the same solvent was used in the rebinding as in the polymerization step, the strength and selectivity of the template rebinding increased with decreasing polarity and hydrogen bond capacity of the solvent. For instance, a MIP using chloroform as the diluent exhibited higher affinity for the template in a chloroform medium than that of a polymer prepared using acetonitrile as diluent and evaluated in an acetonitrile medium [76]. Interestingly when evaluating the latter polymer in the less polar chloroform medium, a much lower affinity was observed. Likewise, the polymer prepared in chloroform showed a lower rebinding affinity in acetonitrile. Similar solvent dependence has been observed in a number of other systems. This indicates that the binding sites are complementary to the template, including part of the solvation shell, in the solvent used as diluent, and/or that the polymer chains need to be solvated by the same solvent used in the synthesis in order to adopt a conformation for optimum rebinding of the template.

In a study of the influence of the diluent properties in the recognition properties of MIPs, a series of L-PA (L-phenylalanine anilide) MIPs were prepared in the presence of various solvents (diluents) with different polarity and hydrogen bond capacities [77]. Acetonitrile or benzene resulted in mesoporous low-swelling polymers while non-porous swellable polymers were obtained by using chloroform or dichloromethane as diluents. In present study, acetonitrile was eventually chosen as a suitable solvent since it is classified as poor in hydrogen bonding and thus has little ability to compete for the hydrogen bonding sites of the template or the MIP binding sites [77]. Furthermore, it solvates the methacrylate polymer backbone well [78] and is polar enough to dissolve a large number of compounds, including the polar metformin and phenformin.

3.1.3 Choice of cross-linker

EDMA is the most commonly used cross-linker for the methacrylate-based systems, primarily because it yields polymers with mechanical and thermal stability, good wetability in most rebinding media and rapid mass transfer with good recognition properties [79]. Comparing with EDMA, as a trimethacrylate monomer, TRIM could provides similar recognition properties for a large variety of target molecules. MIPs that were prepared by TRIM possessed a higher sample load capacity and better performance than similar MIPs prepared using EDMA as cross-linker [80]. Therefore, TRIM was used as cross-linker in this present study.

3.1.4 Removal of template molecule from metformin MIP

Before the MIP can be used in any application, the template molecules have to be removed to leave behind cavities (or recognition sites) with affinity for the target analyte. In addition, the aim is to reduce template bleeding to levels that would allow the use of the MIP as affinity phases for extraction of the target analyte from samples at low concentrations. Otherwise, the remaining template can constitute a problem as it might bleed from the polymer during the elution step of solid-phase extraction (SPE), giving erroneous results and/or an increased limit of quantification (LOQ). Therefore, it was of prime concern to search for methods capable of removing the metformin template from the MIP.

Careful and effective wash procedure was needed to remove the metformin template from the MIP. Continuous extraction using a Soxhlet apparatus typically results in the removal of up to 99% of the template. Several studies, however, have shown that a small portion of the template remains unextracted even after extensive washing using various organic solvents containing acid or base additives[81,82,83,84].

To remove the metformin template, methanol was combined with trifluoroacetic acid (TFA) in order to disrupt the binding interactions between metformin and the MIP. TFA is by far the strongest organic acid, which has a pKa value of -0.25. In the present experiment, the metformin MIP particles were packed into a stainless steel HPLC tubing equipped with an exit frit, and treated by extensive on-line washing with methanol containing 3% TFA at 0.5 mL/min. The baseline was monitored by UV detection at 240 nm. Bleeding was gradually reduced until a flat baseline was observed. Comparing with Soxhlet extraction, this on-line washing procedure was very simple and straightforward

in terms of operation and efficiency. After optimization, 3% TFA in methanol was proven to be effective in eluting the template metformin molecules entrapped in the MIP particles. No further eluted metformin was detected when the MIP micro-column was flushed with acetonitrile for 2 hours. This indicated that, within the time interval, bleeding of the template molecules was significantly reduced to levels acceptable for trace level analysis.

3.2 Investigation of molecular recognition

3.2.1 % Binding measurements

% Binding is a criterion for evaluating the molecular recognition ability of the MIP particles for metformin and its structural analogues. It was calculated by subtracting the breakthrough peak area from the flow injection analysis (FIA) peak area (with the sample solution bypassing the metformin MIP micro-column) and dividing by the latter:

$$\% \text{ Binding} = \frac{\text{FIA peak area} - \text{Breakthrough peak area}}{\text{FIA peak area}} \times 100\% \qquad (3\text{-}1)$$

When CH_3CN was used as the mobile phase for MISPE, 53% binding of metformin could be achieved on this metformin MIP micro-column at the 60-μg/ml concentration level.

3.2.2 Binding selectivity

Phenformin is a guanidine derivative that is structurally analogous to metformin. As a strong base with a pKa value of 12.7±0.5 [92], phenformin was used as a model interferent molecule to challenge the selectivity of this MISPE method. Fig. 6 compares the % bindings obtained for metformin and phenformin at the 60-μg/ml concentration level when CH_3CN was used as the mobile phase for MISPE. Clearly, the MIP exhibited much stronger binding with metformin than phenformin in all of the three sample solvents. The MISPE selectivity (= % binding for metformin / % binding for phenformin) was calculated to be 2.3 in acetonitrile, 1.9 in 9:1 acetonitrile/phosphate buffer (pH 2.5, 50 mM), and 1.8 in 9:1 acetonitrile/phosphate buffer (pH 7.0, 50 mM), respectively.

To further test the binding selectivity, phenformin (structural analogue), caffeine (common diet ingredient) and glyburide (sulfonylurea anti-diabetic drug) were chosen as potential interferences. % Binding was tested by using 60 μg/mL of metformin, phenformin, caffeine and glyburide in 9:1 CH_3CN/ 50 mM phosphate buffer (pH 2.5), separately. Caffeine is a common organic molecule found in many beverages such as coffee, tea, and cola. Glyburide is a sulfonylurea anti-diabetic agent which is usually combined with metformin, to improve glycemic control in patients with type 2 diabetes. As expected, this metformin MIP micro-column has very good binding selectivity for metformin (Figure 7).

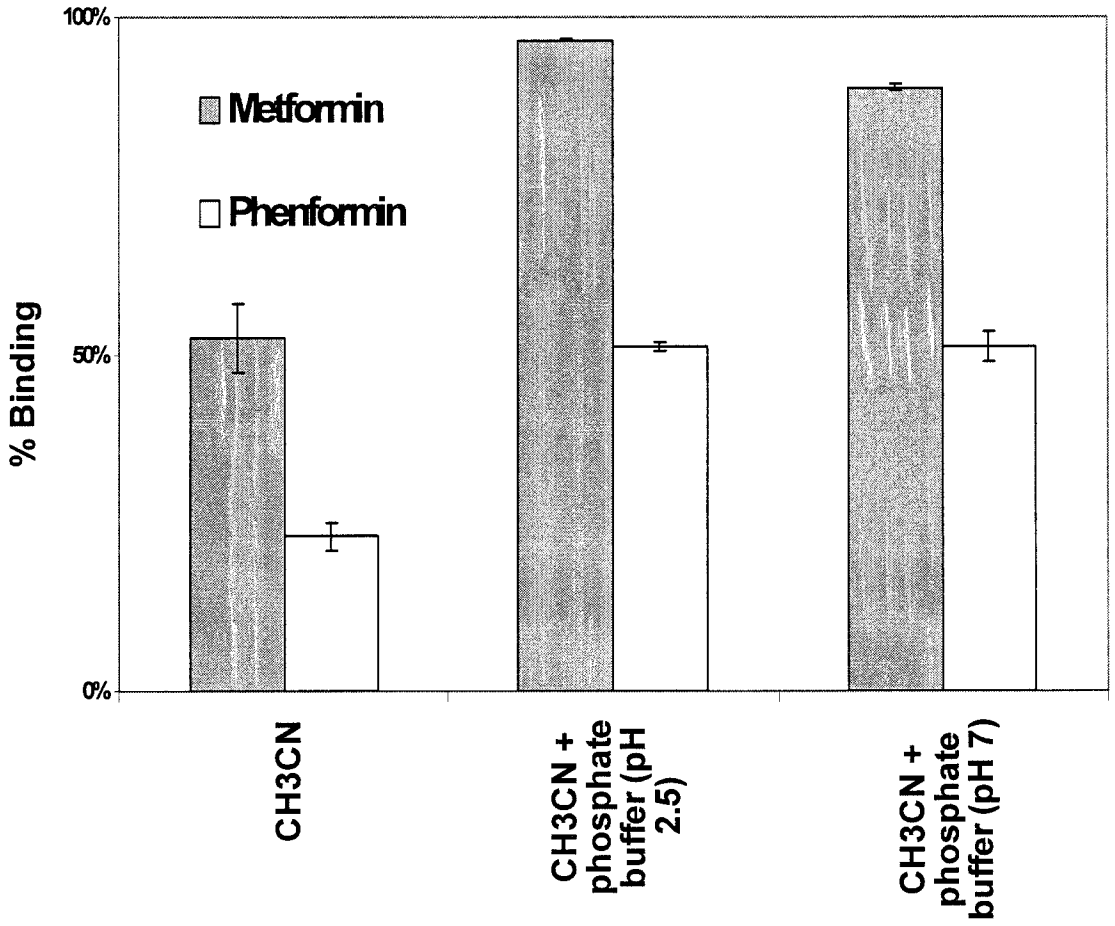


Figure 6 **Comparison of % bindings for 60 μg/mL metformin and 60 μg/mL phenformin in acetonitrile, 9:1 acetonitrile/phosphate buffer (pH 2.5, 50 mM), and 9:1 acetonitrile/ phosphate buffer (pH 7.0, 50 mM).**

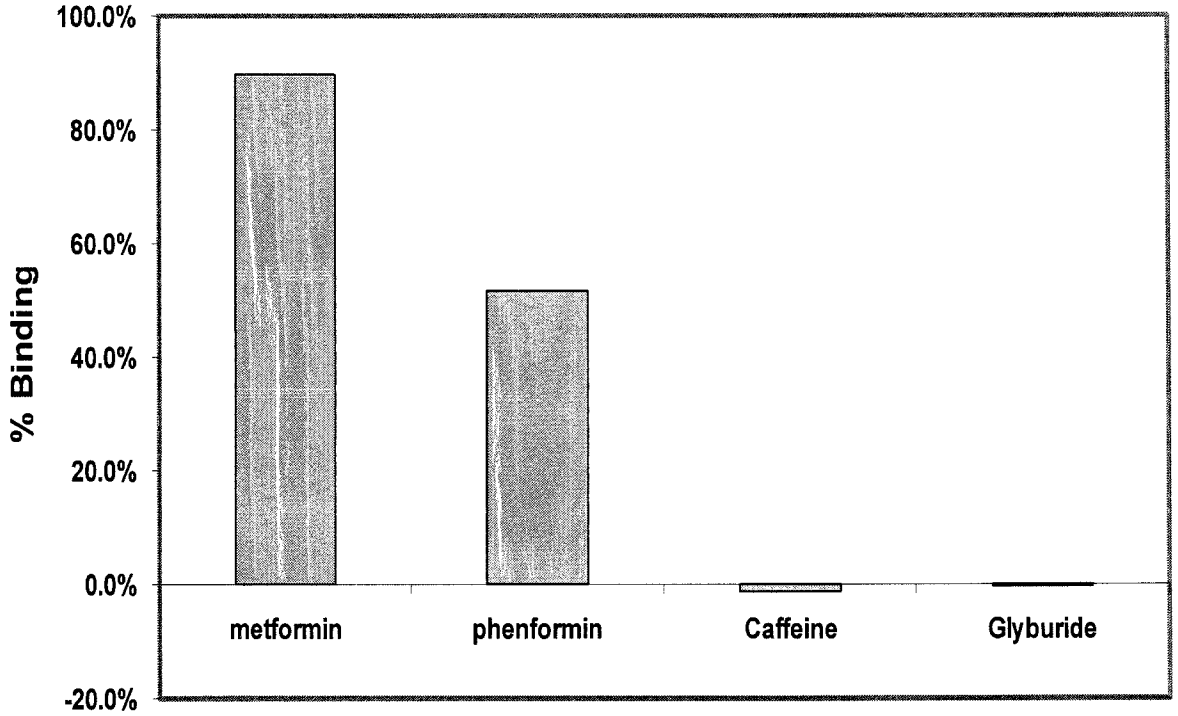


Figure 7. **Comparison of % bindings for 60 μg/mL metformin, phenformin, caffeine and glyburide in 9:1 acetonitrile/phosphate buffer (pH 2.5, 50 mM), separately.**

3.2.3 Evaluation of binding capacity

Binding capacity and binding constant, as determined by the method of multiple loading injections [85], are important indices for evaluating the molecular recognition

ability of a MIP. Metformin binding onto the MIP micro-column was evaluated by multiple 20-μL loading injections of a 7.5-μg/mL metformin standard in 9:1 CH_3CN/50 mM phosphate buffer (pH 2.5), with acetonitrile as mobile phase at a flow rate of 0.5 mL/min (Figure 8). As showed in Table 1, this metformin MIP micro-column showed a much higher binding recognition for metformin than for phenformin. The binding capacity and binding constant were 2 and 3 times higher for metformin than phenformin.

Table 1. Comparison of binding capacity and binding constant values, as determined for 20 mg of MIP particles in micro-column.

Micro-column	Metformin	Phenformin
Binding capacity	1630 ng	780 ng
Binding constant (*K*eq)	13.6	4.4

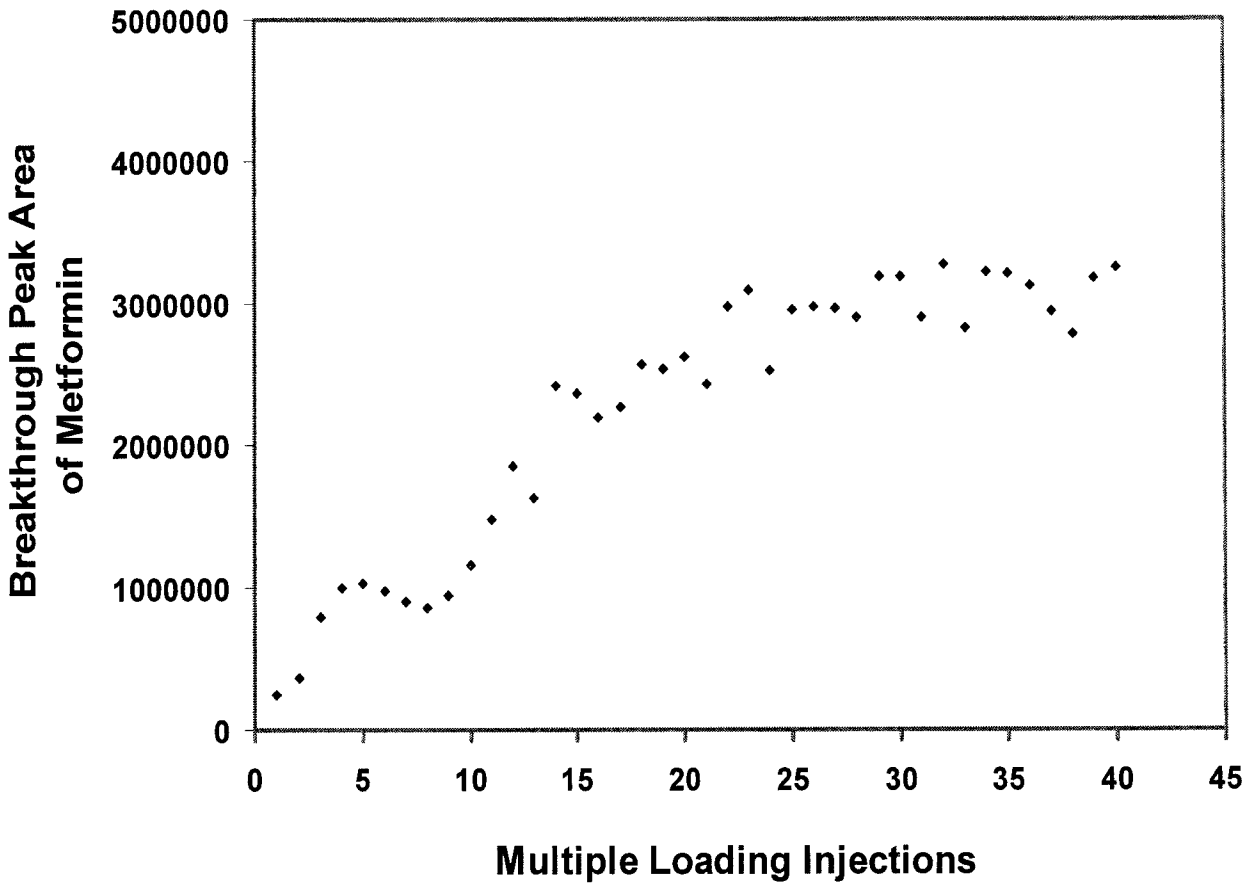


Figure 8. Metformin binding saturation test

3.2.4 Effect of metformin concentration on % binding

The effect of metformin concentration on % binding was investigated by testing metformin standard solutions over the range of 7.5-60 μg/mL (150 -1200 ng) in 9:1 CH_3CN/50 mM phosphate buffer (pH 2.5). As shown in Figure 9, the mass of metformin loaded has no significant effect on % binding, and 86.0-96.6 % binding could be achieved over the tested concentration range. These results show that the metformin MIP behaves well to recognize metformin molecules at various concentration levels.

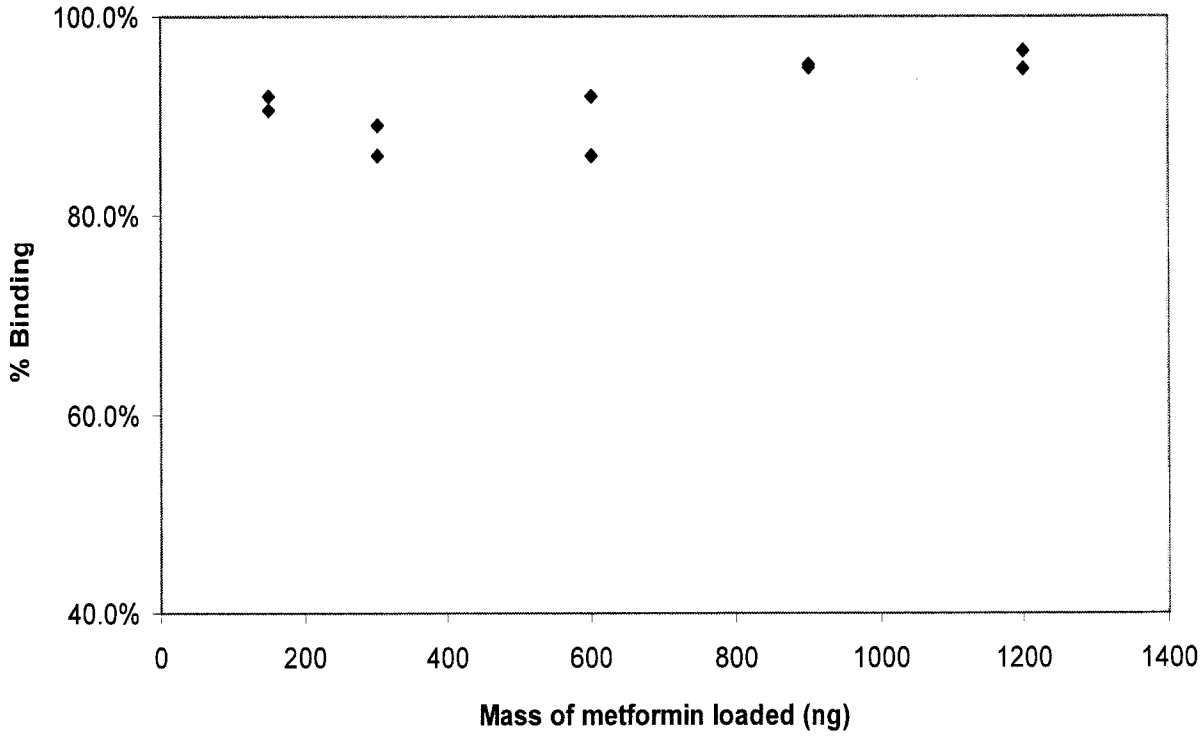


Figure 9. Relationship of % binding and mass of metformin loaded

3.2.5 Effect of sample solvent on % binding

MIPs can be used to extract the target analytes from either organic or aqueous solutions. Swelling and shrinkage is an inherent property of even highly cross-linked MIPs [86]. Shrinkage can reduce the analyte binding because of collapsing cavities and diffusion hindrance in the MIP. Swelling can often be disregarded in aqueous solutions, whereas organic solutions will affect the polymer properties. Hence, it is important to investigate the performance of a new MIP in different sample media for the potential application of MISPE in various assay formats and application areas.

Acetonitrile was the mobile phase of choice in terms of minimal non-specific binding during MISPE. The hydrodynamic ability of the MIP micro-column to bind and retain metformin was evaluated for various aqueous and acetonitrile standard solutions (60 μg/mL). Breakthrough peak area measurements were made in separate 20-μL loading injections. Acetonitrile afforded a 35±2% binding for metformin while distilled deionized water attained a 55±3%. It was apparent that hydrophobic (or non-ionic) polar interactions between the MIP and metformin molecules played a determining role in the strength of binding when measured for water as a polar, protic sample solvent [87]. In comparison, water/acetonitrile (1:9 v/v) afforded a 35 ±3% binding for metformin. These results agree with the previous findings of Karlsson et al's that the entropy-driven hydrophobic effect would be significant in aqueous buffers and polar solvents, whereas enthalpy driven binding interactions would dominate in non-polar solvents [88]. Since they had observed a maximum level of MIP binding selectivity in pure acetonitrile, this organic solvent was employed as the mobile phase in all subsequent investigations.

3.2.6 Effect of buffer pH in sample solution on % binding

Binding of metformin onto the MIP micro-column was examined over a pH range from 1.5 to 8.0. Different types of aqueous buffers were added to the 60-μg/mL standard solutions of metformin in acetonitrile, in a constant ratio of 1:9 v/v. Given the basic nature of metformin (pKa = 13.1±0.5), the positively-charged protonated metformin was expected to interact strongly with the negatively-charged carboxylic groups in the MIP at pH ≤ 7.0. The results in Figure 10 show a marked effect of buffer pH on the MIP micro-column binding ability. Relatively high % bindings were achieved at pH 2.5, 4.5 and 7.0. No general trend in pH effect could be established because a low % binding was observed at the intermediate pH of 6.0. It is interesting to note that the two phosphate buffers (pH 2.5 and 7.0) afforded significantly higher % bindings of metformin than the other buffers. One plausible explanation is that some buffer acids might compete with metformin for the MIP carboxylic acid functional group. Consider acetic acid for instance:

$$\text{Metformin} + CH_3COOH + \text{HOOC-MIP}$$

$$\rightleftharpoons \quad \text{Metformin} + CH_3CO\cdots HO \quad OH\cdots OC\text{-MIP}$$

where two hydrogen bonds (=CO···HO-) could exist to form a dimeric interaction between the acetic acid molecule and the MIP carboxylic acid functional group. According to this hypothetical model, a critical parameter would be the distance between the =CO oxygen and the –OH hydrogen in the buffer acid molecule. This distance should

ideally match the distance between the =CO oxygen and the –OH hydrogen in the MIP carboxylic acid functional group. Calculations were performed at the semi-empirical molecular orbital level (AM1) using PC Spartan Plus for the buffer acids and base studied, with their molecular structures shown in Figure 11. As summarized in Table 2, the distances calculated for glycine, acetic acid, 2-(4-morpholino)ethanesulfonic acid and tris(hydroxymethyl)-methylamine are comparable to that for the MIP. Interestingly, the distance for phosphoric acid is significantly larger. This mismatch explains why the phosphate buffers (both pH 2.5 and pH 7.0) did not compete favorably for the MIP carboxylic acid functional group. As a result, the % bindings for metformin onto the MIP micro-column were significantly higher in these two phosphate buffers.

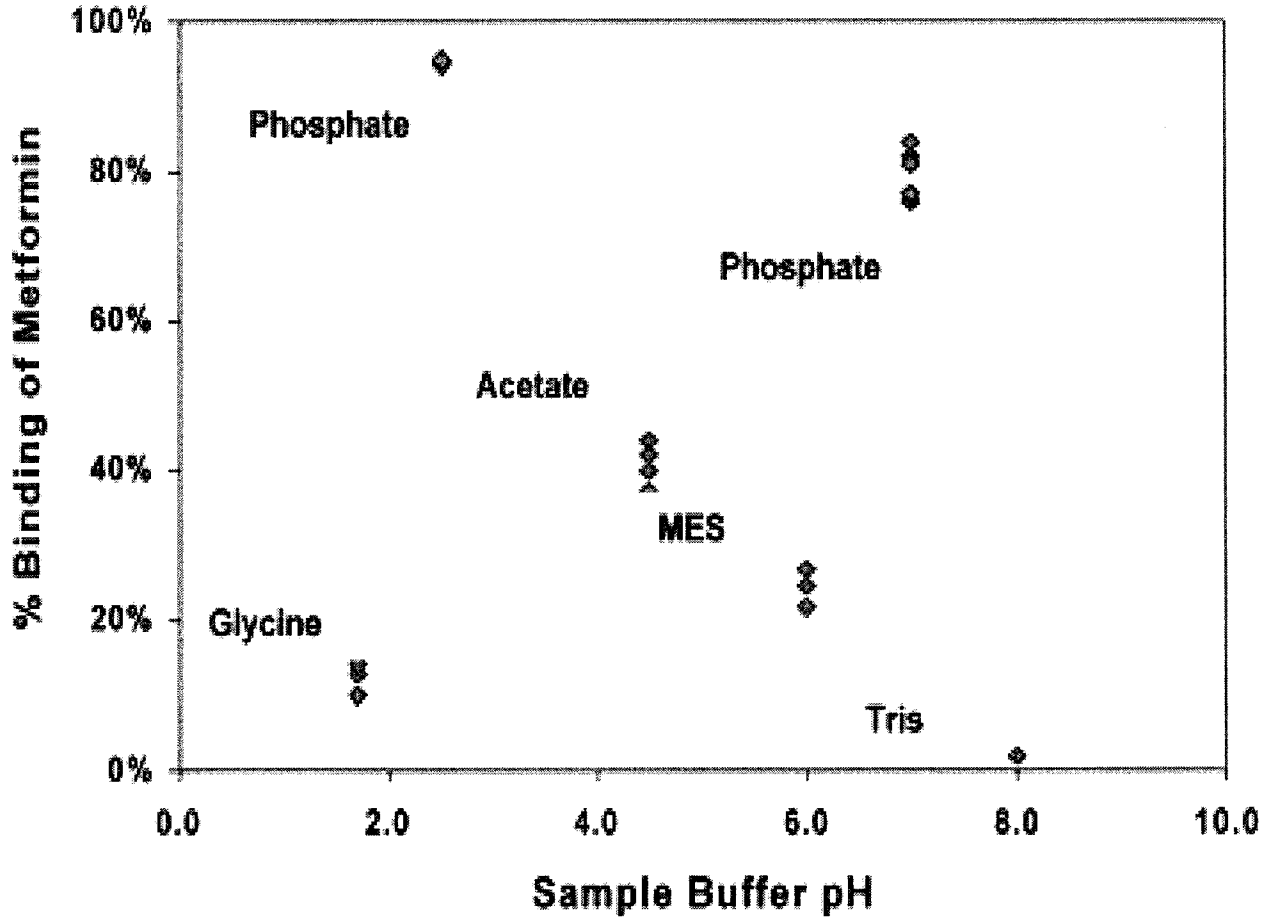


Figure 10. Effect of buffer pH (at constant buffer concentration of 50 mM) in sample solution on % binding for 60 μg/ml metformin.

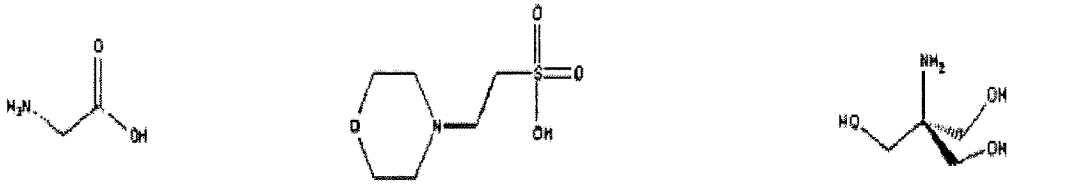


Figure 11. Molecular structures of buffer acids and base studied.

Table 2. Distances between =CO oxygen (or $-NH_2$ nitrogen) and –OH hydrogen calculated for the MIP and different buffer acids and base. TFA is included as a useful reagent for pulsed elution of metformin from the MIP micro-column.

Molecular structure	pH	Distance between O (or N) and H (Å)
2-Trifluoromethylpropranoic acid (MIP)		2.192
Glycine	1.7	2.194
Phosphoric acid (pKa = 2.2, 7.2 and 12.2)	2.5	2.832
Acetic acid	4.5	2.214
2-(4-Morpholino)ethanesulfonic acid	6.0	2.369
Phosphate	7.0	
Tris(hydroxymethyl)methylamine	8.0	2.136
Trifluoroacetic acid		2.195

3.3 Molecularly imprinted solid phase extraction

Sorbents for SPE need to work only in "on" or "off" modes, strongly adsorbing and easily releasing an analyte, while moderate strength of retention with good resolution is required for chromatographic stationary phases. Furthermore, the column efficiency, negatively affected by peak broadening and tailing, is much less significant in SPE applications. SPE is thus regarded as an application suitable for MIPs. SPE was previously reported using a dispersion polymer for enrichment of a specific sample[89]. Some other examples of MIP-based SPE have also been reported [90, 91].

3.3.1. Molecularly imprinted solid-phase extraction

The new MIP was specifically synthesized as a smart material for the recognition of metformin hydrochloride. Particles of this MIP were packed into a micro-column for the development of a molecularly imprinted solid-phase extraction (MISPE) method. With CH_3CN as the mobile phase flowing at 0.5 ml/min, 95±2% binding could be achieved for up to 1200 ng of metformin from one 20-µl loading injection of a 60-µg/ml metformin solution (phosphate buffer at pH 2.5 or 7.0). However, the micro-column interacted indiscriminately with phenformin with a 49±2% binding. The molecular structures of metformin and phenformin are very similar as shown in Figure 5. When a FPE of metformin for direct UV detection was performed using 3% TFA in methanol, the phenformin would also be detected to cause a positive interference.

3.3.2 Pulsed elution of metformin

TFA is a strong organic acid with a pKa value of 0.7±0.2 [92]. It had previously been reported that elution with 2% TFA in acetonitrile yielded approximately 80% recovery of the analytes (ropivacaine and bupivacaine) that was bound on the MIP, and that the extracts were less pure than elution with 2–10% triethylamine (TEA) [93]. We expected that 2% TEA in acetonitrile would transfer to the present MISPE method development because this elution solvent contained a strong base (TEA) that could effectively displace metformin from the MIP binding sites. However, TEA could not be employed for the PE of metformin because it is a strong base itself that would bind on the MIP micro-column to hamper MISPE analysis of the next sample. Fortunately, improvement to 98±1% recovery was achieved when 3% TFA in methanol (not acetonitrile) was evaluated for the PE of metformin. Increasing the TFA content up to 10% in methanol, however, only marginally improved the recovery of metformin but significantly increased the blank signal to jeopardize low-concentration metformin analysis.

3.3.3 Standard calibration curve for MISPE-PE determination of metformin

A standard calibration curve for MISPE–PE determination of metformin in acetonitrile (phosphate buffered at pH 2.5), using 3% TFA in methanol for PE, is shown in Figure 12. The MISPE–PE peak area was linearly related (R^2=0.9895) to the mass of metformin loaded on the MIP micro-column up to 900 ng. Deviation from linearity

began near 1200 ng, which was approaching the micro-column binding capacity of 1600 ng for metformin. The detection limit was found to be 0.8 μg/ml (or 16 ng in 20 μl of sample injected) for metformin, based on 3 standard deviations of the blank.

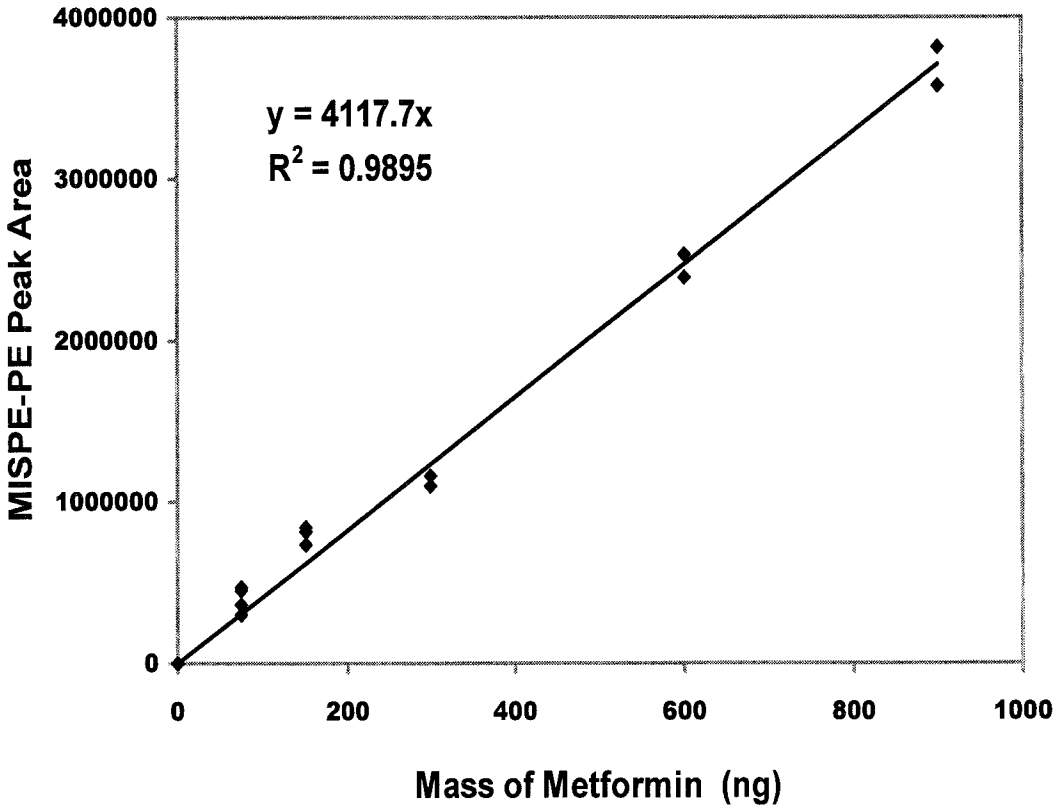


Figure 12. Standard calibration curve for MISPE-PE determination of metformin in acetonitrile (phosphate buffer at pH 2.5), using CH_3OH+3% TFA for PE.

3.3.4 Elimination of non-specific binding for phenformin

3.3.4.1 Effectiveness of surfactants

Previously, Andersson *et al.* had investigated the influence of the type and concentration of detergent in the buffer on specific binding and non-specific binding [94]. They found that Triton X-100, Tween 20 and Brij 35 (0.1–0.5%) were able to eliminate non-specific adsorption while analyte specific binding was left essentially unaffected. A systematic investigation of enhancing binding selectivity with detergents (or surfactants) was conducted for the purpose of eliminating phenformin interference. The three approaches were Differential pulsed elution (DPE), sample matrix addition and mobile phase composition. They all involved a variation of surfactants, differing mostly in their consumption of chemicals and simplicity of operation.

First, MISPE-DPE approach was adopted [95,96,97,98]. DPE was an intermediate wash step used to remove phenformin and other structural analogues from the MIP micro-column. Two nonionic (Triton X-100 and Tween 20) surfactants were tested for their DPE effectiveness. Unfortunately, phenformin could not be removed using 1% Triton X-100 in 0.1M aqueous NaOH as the DPE solution. One reason might be that Triton X-100 could not form a charge-transfer complex with phenformin because they are both electron donors [99]. Table 3 shows the DPE effectiveness observed for Tween 20 at three different % in CH_3OH over multiple injections. On the average, 62±4% of the phenformin remained on the MIP micro-column. With acetonitrile as the mobile phase, it seemed hard to quantitatively eliminate the non-specific binding of phenformin on the micro-column by using non-ionic surfactants in DPE.

Second, surfactants were added as a matrix component in the sample solution to prevent non-specific binding of phenformin on the MIP micro-column. As shown in Table 4, as much as 11% of the injected phenformin could bind with the micro-column even though the Tween 20 content was increased up to 50%. A plausible explanation is that the surfactant failed to competitively occupy the binding sites because hydrophobic interactions in the non-aqueous solvent were too weak for the surfactant molecules to adsorb readily on the MIP particles. One anionic surfactant, Na taurodeoxycholate, was then added in varying concentrations to different sample solutions. As illustrated in Figure 13, the surfactant could not prevent the non-specific binding of phenformin on the MIP micro-column.

Table 3. Effectiveness of using surfactant solutions for DPE after loading 60 μg/ml phenformin in CH_3CN–50mM phosphate buffer (pH 7.0) (9:1 v/v)

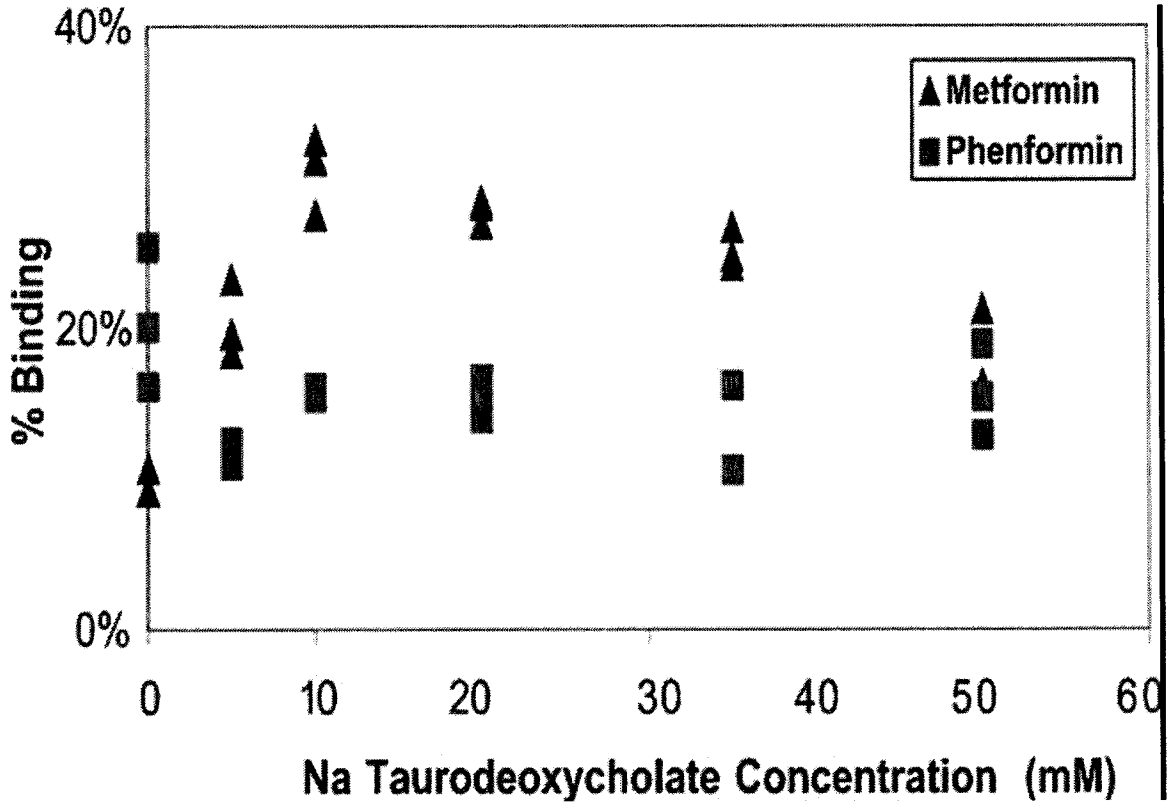
Concentration of surfactant solution for DPE	Phenformin remaining on micro-column after DPE (%)
1% Tween 20 in CH_3OH (4 × 20 μl)	58
10% Tween 20 in CH_3OH (6 ×20 μl)	58
20% Tween 20 in CH_3OH (3 ×20 μl)	67

Mobile phase: acetonitrile at 0.5 ml/min; FPE solvent: 3% TFA in methanol. These % phenformin remaining results can be compared with 100% without DPE.

Table 4. Effectiveness of adding surfactants into sample solution of 60 μg/ml phenformin in CH_3CN–50mM phosphate buffer (pH 7) (9:1)

Surfactant added	Binding of phenformin (%)
20% Tween 20	19
50% Tween 20	11

Mobile phase: acetonitrile at 0.5 ml/min; FPE solvent: 3% TFA in methanol. The % phenformin binding results can be compared with 49% without adding surfactant.



Figuree 13. Effect of sodium taurodeoxycholate concentration in sample solution on % binding of metformin and phenformin.

Third, surfactants were added in the mobile phase to pre-occupy the binding sites on the MIP micro-column. When 0.5% Tween 20 + 5% ethanol in acetonitrile was employed as the mobile phase [94], the 52±2% binding result for phenformin was statistically the same as before when using pure acetonitrile as the mobile phase. Several cationic surfactants (HMB, DTAB, TTAB, DDAB) were last evaluated as modifiers.

After injection of 20-μl aliquots of these solutions to pre-condition the MIP, phenformin and metformin sample solutions were loaded on the micro-column. Their % binding results are presented in Table 5 to show no significant effects overall.

Table 5. Effect of HMB, DTAB, TTAB and DDAB on % binding of metformin and phenformin.

Surfactant solution	Binding of metformin (%)	Binding of phenformin (%)
0.1M HMB in CH_3CN–water (8:2)	75	43
0.1M DTAB in CH_3CN–water (8:2)	76	48
0.1M TTAB in CH_3CN–water (8:2)	75	47
0.1M DDAB in CH_3CN–water (8:2)	77	46

3.3.4.2 Differential pulsed elution (DPE) with organic acids

DPE method was chosen for elimination of phenformin from the MIP micro-column, while metformin was selectively retained. Several organic acids were evaluated for their DPE effectiveness. As summarized in Table 6, 10% benzoic acid (pKa = 4.19) in CH_3OH was poor for the DPE removal of phenformin. 18% pentafluorobenzoic acid (pKa = 1.99) in CH_3CN left behind 20% of phenformin on the micro-column. 30% acetic acid (pKa = 4.74) in CH_3OH was fairly good for the removal of phenformin, but only 10% of metformin remained on the micro-column. 2% TFA in CH_3OH was very good at

removing phenformin, but only 5% of metformin remained. Ideally, DPE must eliminate phenformin quantitatively while leaving behind an adequate amount of metformin for UV detection. Picric acid (pKa = 0.30–0.53), as shown in Figure 14, was strategically selected from among the strongest organic acids that were commercially available [100,101,102]. Different concentrations of picric acid in acetonitrile were evaluated for the best DPE result. After DPE with each picric acid, TFA (pKa = −0.23–0.0) was used in FPE to elute all of the remaining phenformin. As shown in Figure 15 for 60 μg/ml phenformin, a picric acid concentration of 5% or higher proved to be effective in the quantitative elution of phenformin. As a π-electron acceptor [103,104], picric acid could form a stronger charge-transfer complex with phenformin than with metformin. Note that the functional monomer, TFMAA (pKa = 1.9–2.3), must be able to hold a significant amount of metformin on the micro-column during DPE. When the evaluation was repeated for 60 μg/ml metformin, the result indicated that 37±3% of metformin remained on the micro-column after DPE with 5% picric acid. This suggested that picric acid would be an effective reagent for the quantitative DPE of phenformin in the presence of bound metformin. 5% picric acid was evaluated as the DPE solvent for mixtures of metformin (5–50 μg/ml in acetonitrile–50 mM pH 7 phosphate buffer, 9:1 v/v) and phenformin (60 μg/ml). The results verified that, over the concentration range studied, a reasonably constant 37±2% of the bound metformin remained on the MIP micro-column while phenformin was quantitatively removed by the DPE.

Table 6. Evaluation of organic acids for DPE effectiveness

(% metformin remaining was not determined unless % phenformin remaining on micro-column was under 3%.)

DPE solution	Phenformin remaining on micro-column after DPE (%)	Metformin remaining on micro-column after DPE (%)
10% Benzoic acid in CH_3OH	87	
20% Acetic acid in CH_3OH	10	
20% Acetic acid in CH_3OH	5	
30% Acetic acid in CH_3OH	3	10
18% Pentafluorobenzoic acid in CH_3CN	20	
0.025% TFA in CH_3OH	68	
0.05% TFA in CH_3OH	50	
0.1% TFA in CH_3OH	2	20
0.5% TFA in CH_3OH	1	5
2% TFA in CH_3OH	1	5

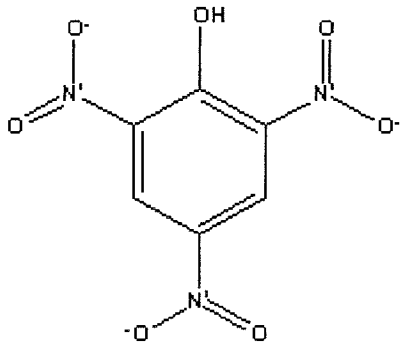


Figure 14. Structure of picric acid.

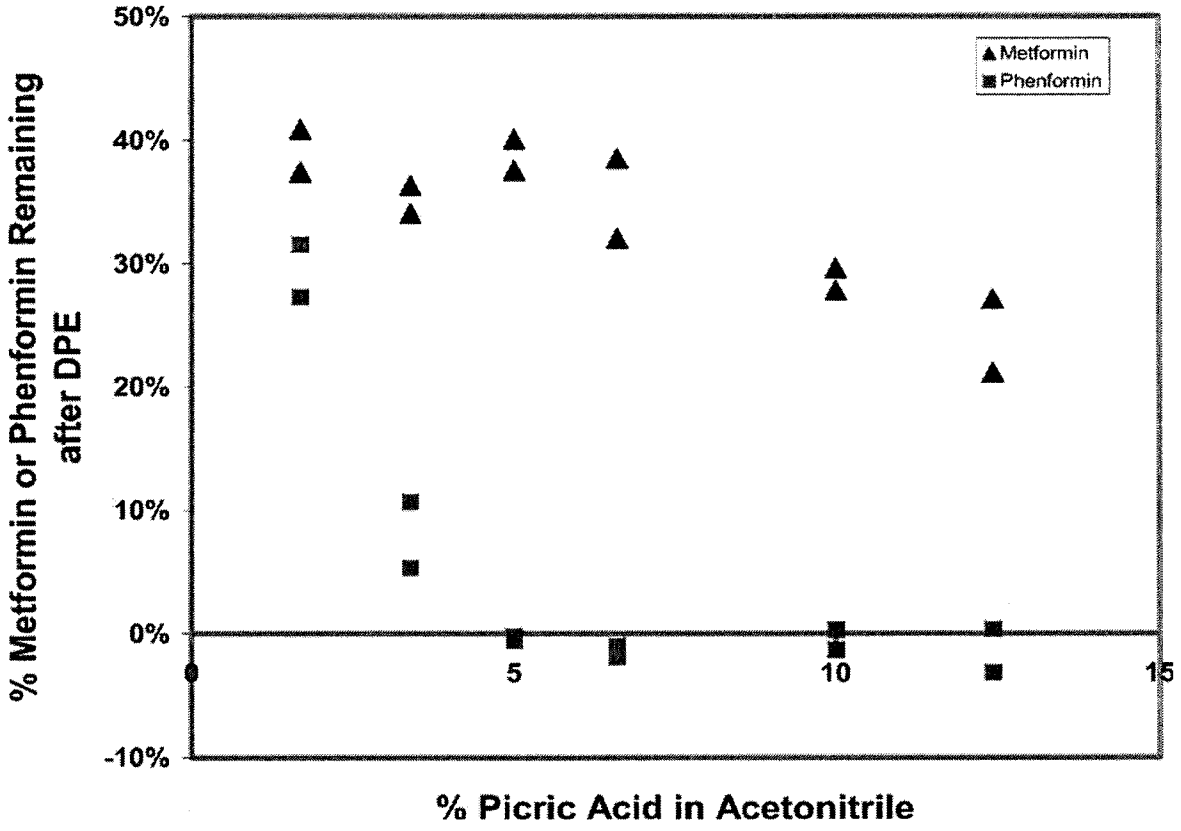


Figure 15. % Metformin or phenformin remaining on MIP micro-column after DPE with varying % of picric acid in acetonitrile.

Table 7 is a summary of the DPE solvents that have been reported in the literature as required for various drug compounds bound on five different MIP micro-columns. In the first three cases, different organic solvents could be effective for optimal DPE. In the fourth case, the DPE solvent was optimized by systematically increasing the % acetic acid in acetonitrile. It cannot be overemphasized how critical it was in the present work

to find picric acid as an effective reagent for the DPE of phenformin in the presence of metformin. The final increment from 3.4 to 5% picric acid made a big difference between incurring a risk of 8±2% interference by phenformin and enjoying an interference-free determination of metformin in the FPE step.

Table 7. Summary of DPE solvents for various drug compounds bound onto five different MIP micro-columns

Analyte	pKa of Analyte	Interferents	pKa of interferents	Solvent for MISPE of analytes and interferents	Solvent for elution of interferents by DPE	Solvent for elution of analyte
Theophylline[95, 96]	8.68	Dyphylline Nicotinic acid	5.36 4.80	$CHCl_3$	CH_3CN	CH_3OH
Nicotine[97]	8.02	Myosin		CH_3CN	CH_3OH	H_2O + 1% TFA
4-Aminopyridine[98]	9.26	2-Aminopyridine	6.67	$CHCl_3$	DMSO	CH_3OH + 1% TFA
Cephalexin[63]	5.3&7.3	Cefradine Cefadroxil	2.6&7.3	$CHCl_3$	CH_3CN + 10% CH_3COOH CH_3CN + 12% CH_3COOH	CH_3OH + 1% TFA
Metformin	13.1	Phenformin	12.7	CH_3CN	CH_3CN + 5% picric acid	CH_3OH + 3% TFA

3.3.5. Analytical figures of merit

At a mobile phase flow rate of 0.5 ml/min, the MISPE-DPE method required an analysis time of 6 min and a solvent consumption of 3 ml. Over a 24-h analysis of 360 samples, the total solvent consumption would be 1.1 L approximately. This affords a substantial reduction in the costs of solvent purchase and waste disposal. With UV detection at 240 nm, a standard calibration curve of ΔFPE peak area versus metformin concentration showed good linearity in the range from 5 to 50 μg/ml. The limit of detection was 20 ng metformin, or 1 μg/ml with a 20-μl loading injection of sample. Since the total binding capacity was determined to be 1600 ng metformin for 20 mg of MIP particles in the micro-column [105], loading injection of a larger-volume sample could easily be adopted to improve the detection limit. Moreover, there is no potential risk of supplementary errors due to exogenous diet ingredients (e.g. caffeine) that might be observed in human blood to give interference peaks.

3.3.6 Human plasma analysis

The usefulness and applicability of the MISPE-PE-DPE method was tested on human plasma samples. Human plasma samples spiked with known quantities of metformin and phenformin were analyzed to confirm the % recovery of the method. A mean recovery of 94(±4)% for metformin showed good concordance with two previous reports [106,107]. Good linearity ($R^2 = 0.9919$) was observed in the metformin concentration range from 0.1 to 10 μg/mL (Figure 16), which represented the typical therapeutic range of metformin in patients. The limit of detection (LOD) is 57 ng/ml (expressed as

3×standard deviation of the plasma blank) was adequate for human plasma analysis. This method would be useful for pharmacological and biomedical applications.

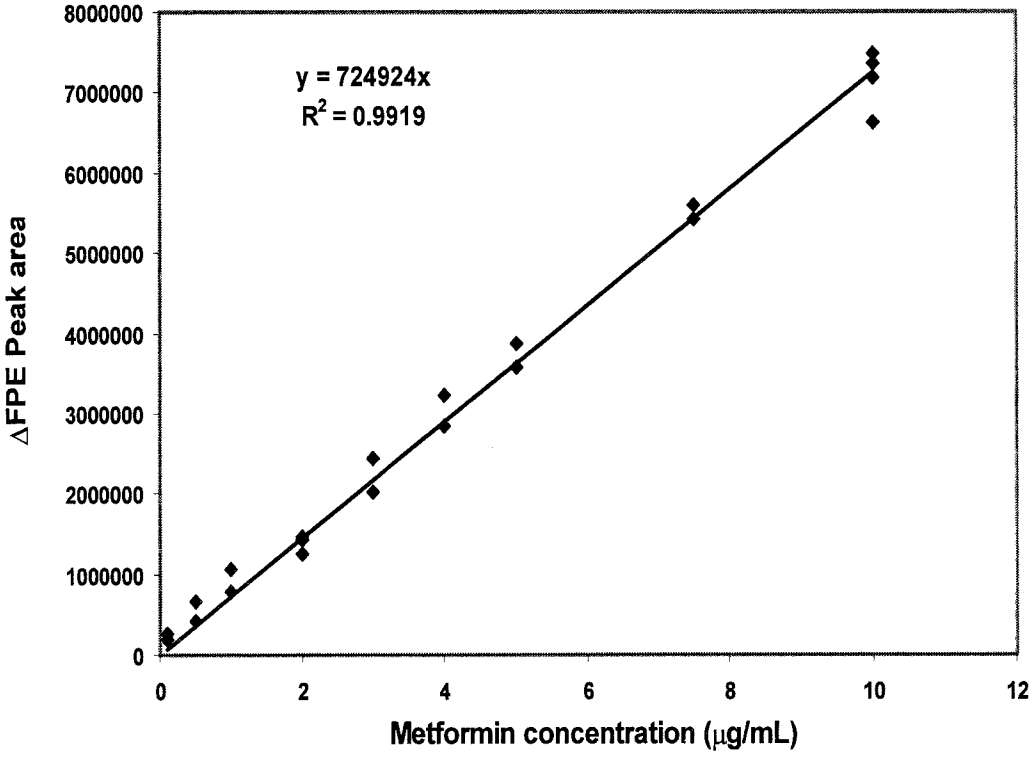


Figure 16. Standard calibration curve of MISPE-FPE-DPE for plasma analysis of metformin.

3.4 Capillary zone electrophoresis

3.4.1 Capillary zone electrophoresis

A capillary electrophoresis (CE) method had previously been described for the determination of metformin in human plasma based on the extraction of an ion-pair with bromothymol blue into chloroform. Phenformin was used as an internal standard, and field-amplified sample stacking was employed[108]. When the method was adopted in our laboratory, the separation results turned out to be rather different. As shown in Figure 17. using 50 mM phosphate buffer (pH 2.5) in water as the medium, separation of metformin from phenformin was attained at 4.33 min and 6.40 min, respectively. These migration times were deemed to be relatively long.

Song *et al* had previously demonstrated a general CE method development process of starting at low pH for basic compounds[108]. Low pH (<3.0) would be ideal for basic compounds with cathodic CE. At a low pH, peak tailing interactions with the capillary would be minimized, providing the best peak shape. At mid pH (5.0-8.0), basic compounds might still have a positive charge but different selectivity would result. Changing the pH could be used to optimize selectivity in most CE method development.

In the present study, the successful CE method employed 20 mM NH_4OAc and 5% HOAc in acetonitrile as the running buffer (pH 5.1), which was modified from the non-aqueous separation medium of 20 mM NH_4OAc and 1 M HOAc in acetonitrile-alcohol (50:49 v/v) reported by Siren *et al* [109]. The CE analysis results are illustrated in Figure 18, showing good resolution, high separation efficiency and short migration times for metformin (2.48 min) and phenformin (3.47 min). When the CE analysis was repeated by employing 20 mM NH_4OAc and 5% HOAc in water as the running buffer

(pH 3.0), the results exhibited peak tailing, low separation efficiency and increased migration times as shown in Figure 19. This comparison demonstrates that acetonitrile, a dipolar-aprotic solvent, was really contributing to the good separation of metformin and phenformin shown in Figure 18.

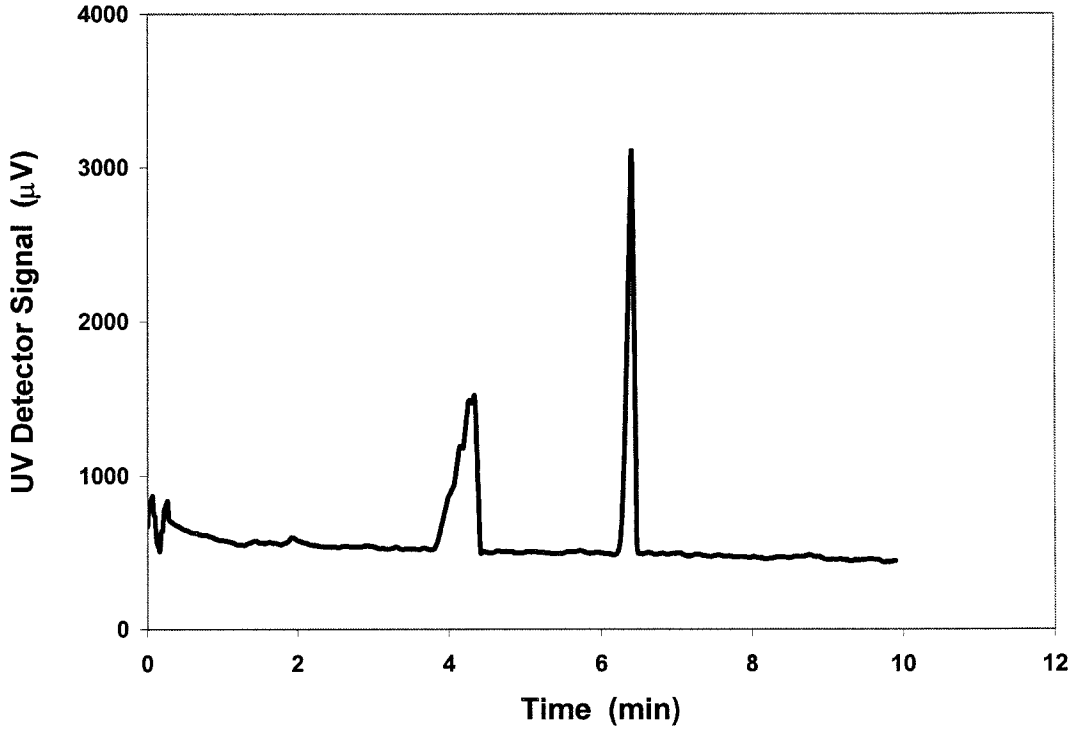


Figure 17 **Separation of metformin (at 4.33 min) from phenformin (at 6.40 min), 60 μg/mL each in aqueous sample. 50 mM phosphate buffer (pH 2.5) in water as the running buffer; CE under 15 kV; room temperature = 22°C; electrokinetic injection at 15 kV for 6 s; UV detection at 240 nm.**

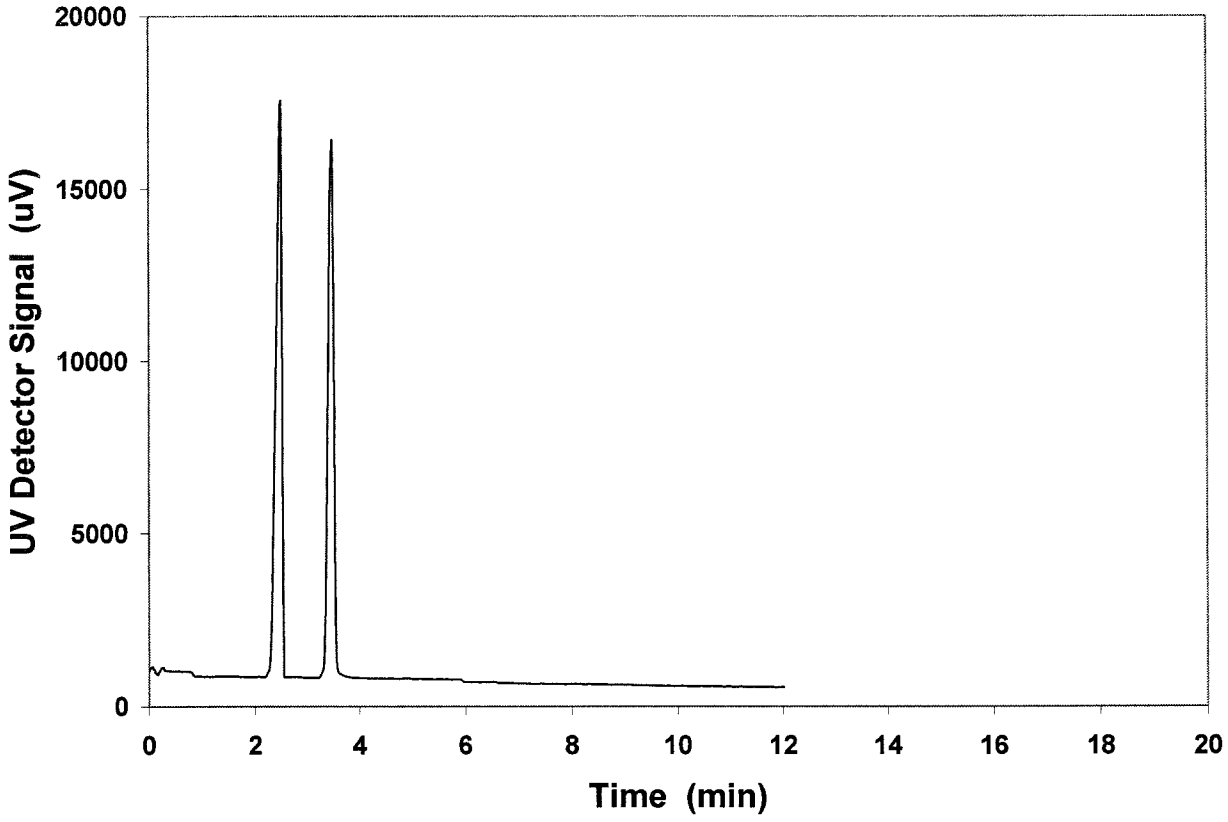


Figure 18 **Separation of metformin (at 2.48 min) from phenformin (at 3.47 min), 60 μg/mL each in aqueous sample. 20 mM NH_4OAc + 5% HOAc in acetonitrile as the running buffer; CE under 15 kV; room temperature = 22°C; electrokinetic injection at 15 kV for 6 s; UV detection at 240 nm.**

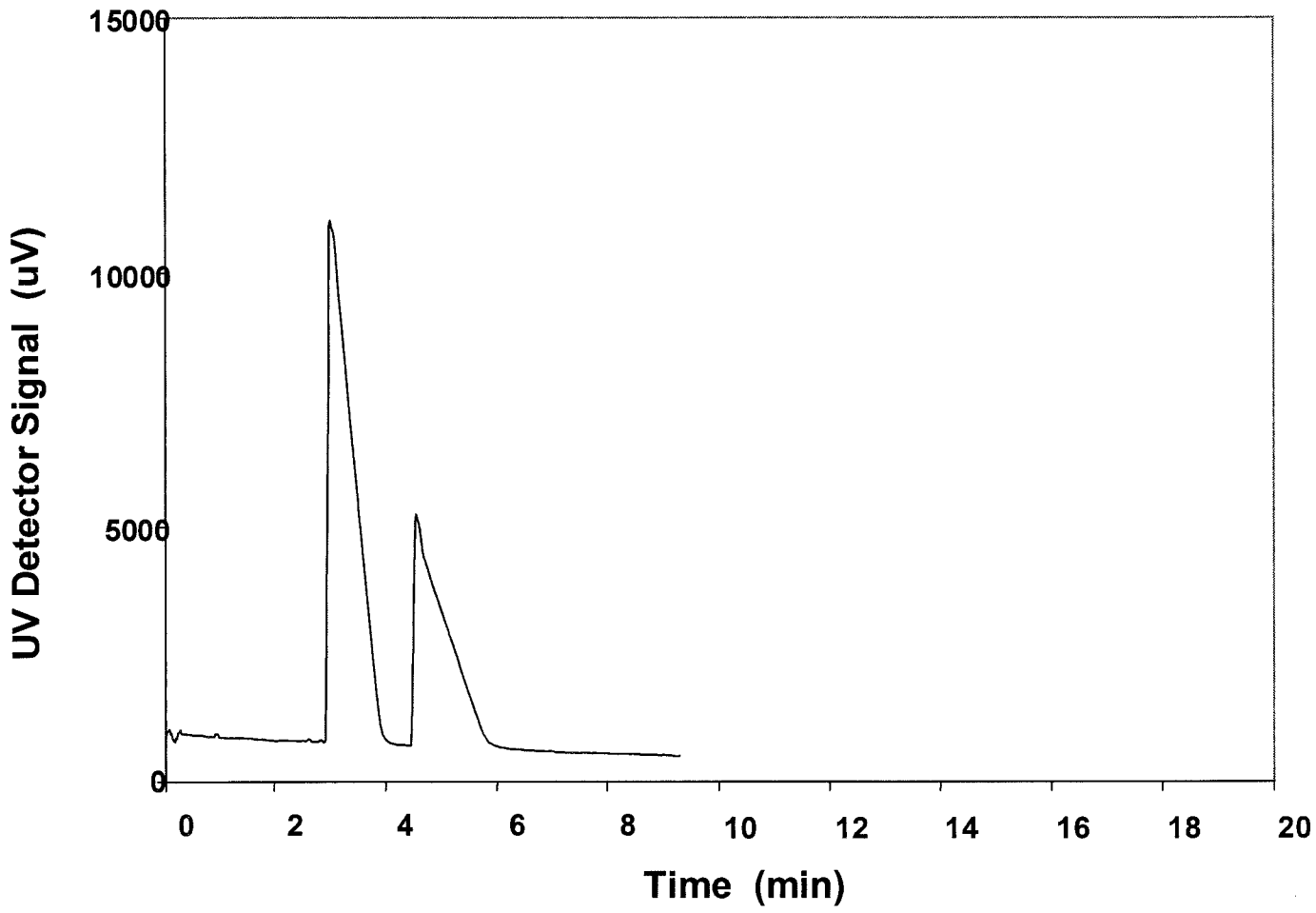


Figure 19 **Separation of metformin (at 3.00 min) from phenformin (at 4.55 min), 60 μg/mL each in aqueous sample. 20 mM NH_4OAc + 5% HOAc in water as the running buffer; CE under 15 kV; room temperature = 22°C; electrokinetic injection at 15 kV for 6 s; UV detection at 240 nm.**

3.4.2 Effect on buffer composition

Non-aqueous CE involves the separation of analytes in a medium composed of organic solvents. The viscosity and dielectric constants of organic solvents affect both sample ion mobility and the level of electroosmotic flow. The use of non-aqueous medium allows additional selectivity options in method development, and it is also valuable for the separation of water-insoluble compounds. Acetonitrile is one of the most commonly used solvents for background electrolytes in non-aqueous CE. It is inexpensive, is easily available at high purity, and has a low absorption at commonly used UV detection wavelengths. Furthermore, combined with other organic solvents or buffer electrolytes, the mobility and separation selectivity could be altered in different ways to greatly expand the range of CE applications. The lower currents present in non-aqueous solvents allow the use of high electric field strengths and wide bore capillaries, the latter in turn allowing larger sample load. In this way, the detection sensitivity can be further enhanced.

To investigate the effects of buffer solutions, the content of ammonium acetate was varied from 1 to 20 mM in acetonitrile. In these solutions, the acetic acid concentration was maintained between 1 and 5%. The sample solutions, which contained 60 μg/mL of metformin and phenformin in acetonitrile, were electrokinetically injected at 15 kV for 6 s. Finally, 20 mM ammonium acetate and 5% acetic acid in acetonitrile were determined to be the non-aqueous medium giving the best separation for the two analytes in terms of resolution and total analysis time.

3.4.3 Sample Matrix Effects on CE-UV Peak Areas

Since metformin and phenformin became protonated in the acidic sample matrix to form cations, high stacking efficiencies were afforded by the modest matrix conductivity during electrokinetic injection. Figure 20 correlates the CE-UV peak areas and conductivity in different sample matrices. These results indicate that methanol + 3% HOAc was an optimal sample matrix. A more acidic matrix like methanol + 3% TFA actually made it worse due to its increased conductivity, as illustrated in Figure 21.

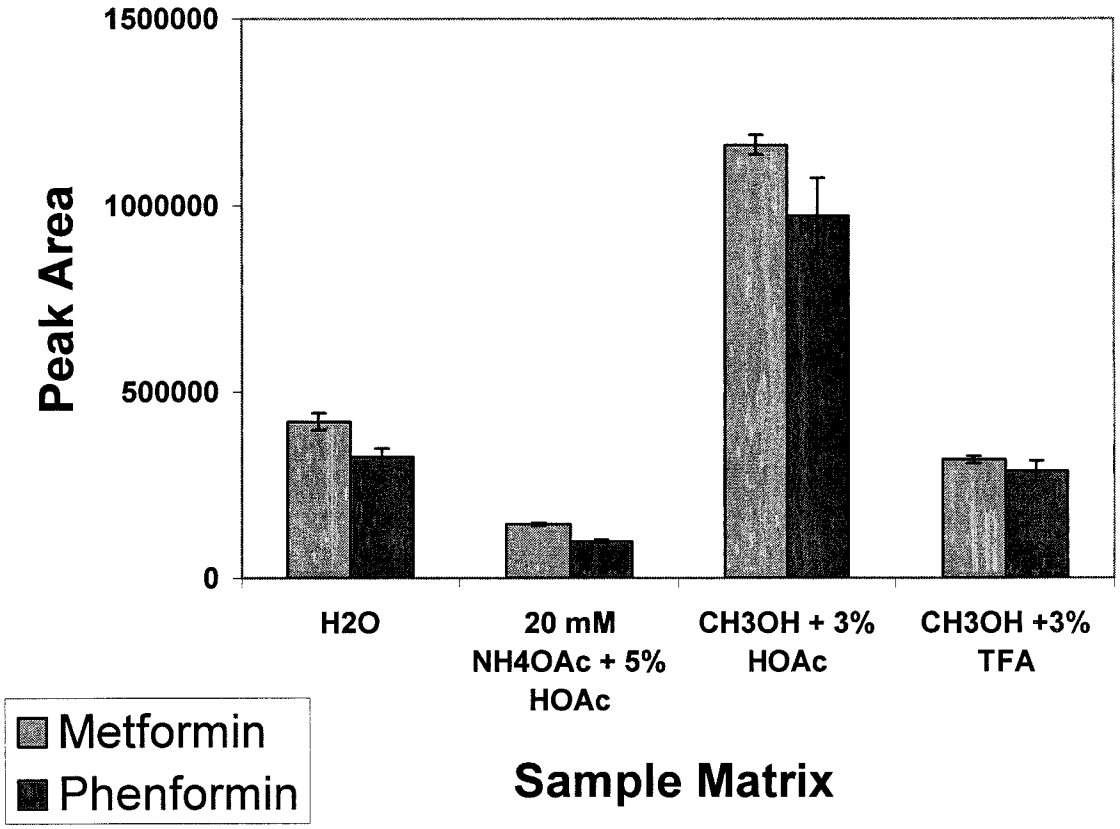


Figure 20. **Effect of sample matrix on CE-UV peak area for 60 μg/mL metformin and 60 μg/mL of phenformin. 20 mM ammonium acetate and 5% acetic acid in acetonitrile as running buffer; CE under 15 kV at 13 μA; room temperature = 22°C; electrokinetic injection at 15 kV for 6 s; UV detection at 240 nm.**

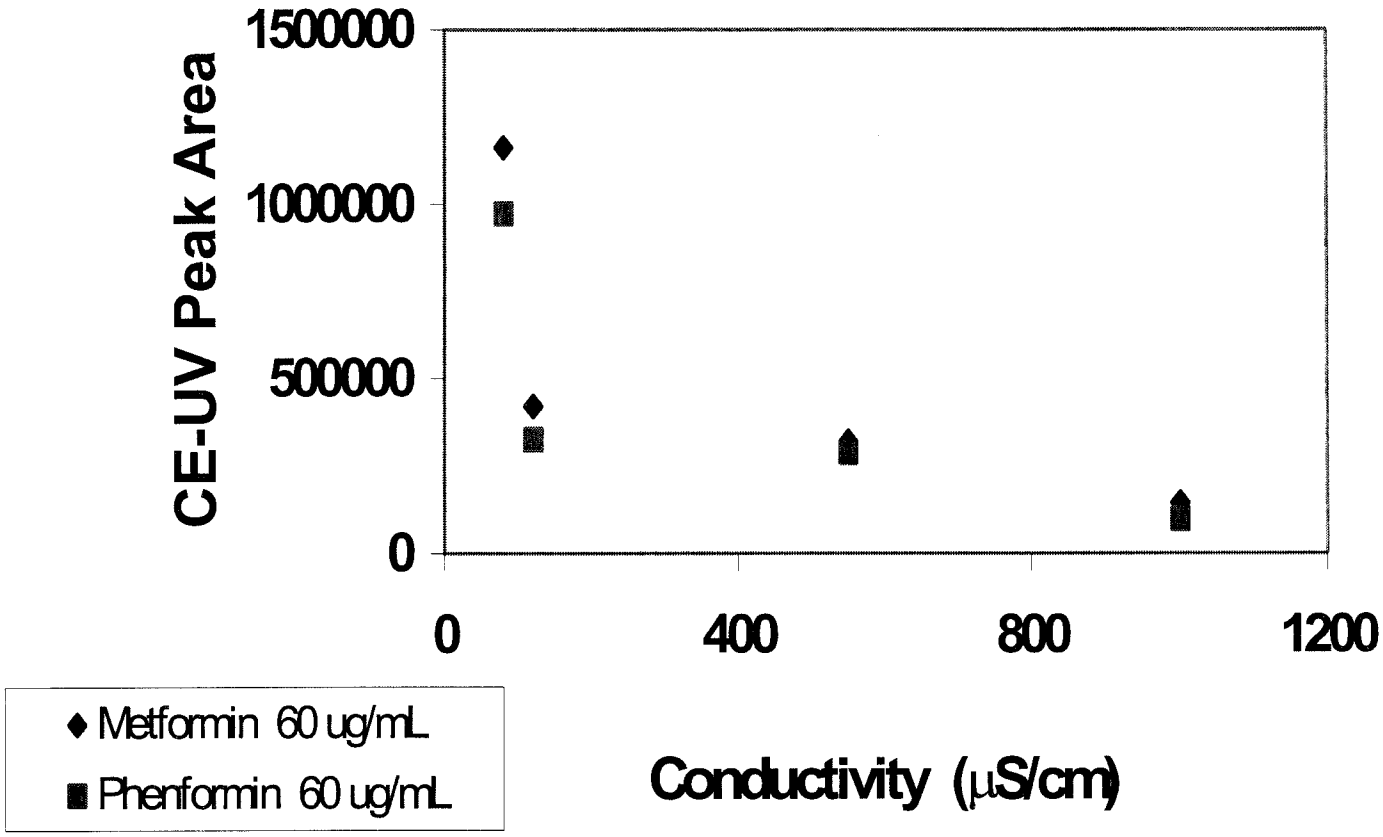


Figure 21. **Dependence of CE-UV peak areas for 60 μg/mL metformin and 60 μg/mL of phenformin on sample conductivity. 20 mM ammonium acetate and 5% acetic acid in acetonitrile as running buffer; CE under 15 kV at 13 μA; room temperature = 22°C; electrokinetic injection at 15 kV for 6 s; UV detection at 240 nm.**

3.4.4 Human plasma analysis

Metformin HCl tablets contain 500-850 mg of metformin HCl. In addition, each tablet contains the following inactive ingredients: povidone, magnesium stearate and hydroxypropyl methylcellulose (hypromellose) coating. There are a lot of ionic substances and proteins in human plasma. Ineffective stacking and unsatisfactory electropherograms would be obtained unless the plasma samples are deionized. For CE analysis of human plasma and serum, solid phase extraction (SPE) was considered to be a suitable sample preparation technique. The commercially-available C18 SPE cartridge was hence evaluated for binding metformin and phenformin in spiked plasma samples. When metformin and phenformin were next eluted from the cartridge with methanol + 3% HOAc, a high recovery and precision was achieved for both compounds.

A typical CE analysis of the methanol + 3% HOAc eluate from C18 SPE of human plasma is demonstrated in Figure 22. No endogenous peak was observed interfering with metformin (1.75 min) or phenformin (2.70 min). These results verified that metformin is negligibly bound to plasma proteins. No metabolites or conjugates of metformin could be identified. At the usual clinical doses and dosing schedules of metformin, steady state plasma concentrations of metformin are typically ~1 μg/mL. Peak metformin plasma levels would not exceed 5 μg/mL, even at maximum therapeutic doses of 850-1500 mg during controlled clinical trials[110].

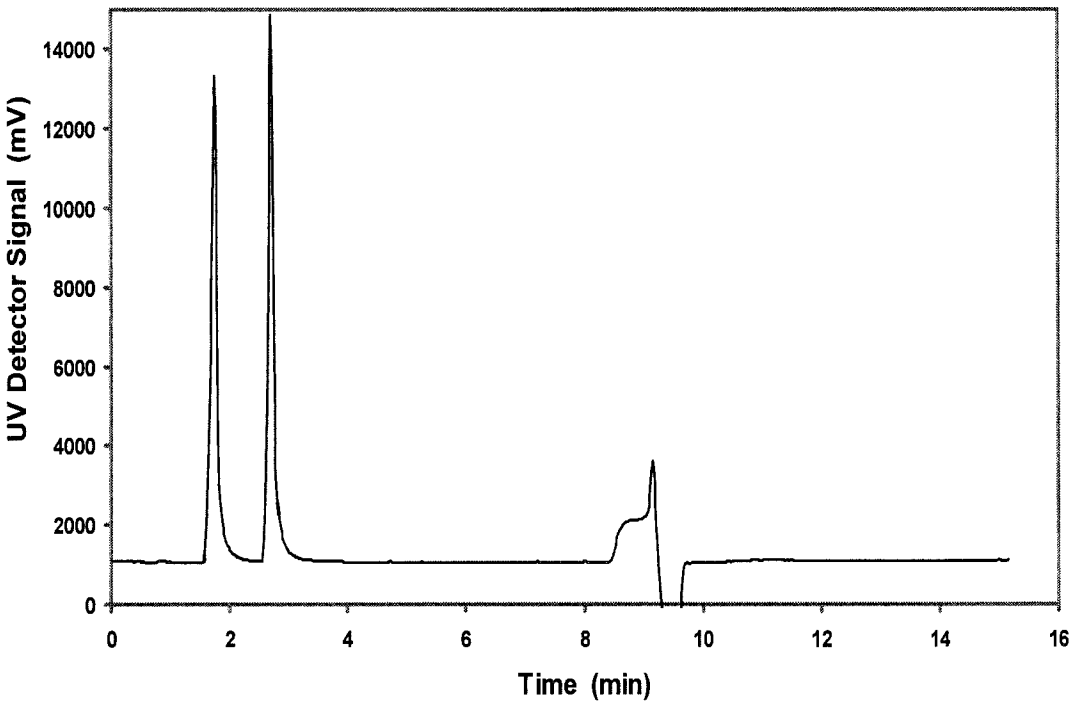


Figure 22. CE analysis of metformin and phenformin from the spiked human plasma

Furthermore, this SPE-CE-UV method was applied to analyze metformin, phenformin and glyburide simultaneously for human plasma. In clinical applications, glyburide is usually combined with metformin for improving glycemic control in patients with type 2 diabetes. As a potential interference, using 3% acetic acid in methanol as the sample matrix, glyburide (at 6.43 min) was totally separated from metformin (at 1.78 min) and phenformin (at 2.25 min) (Figure 23). These results demonstrated how easy it

was to separate the neutral glyburide molecules from the positively charged metformin and phenformin cations by CE.

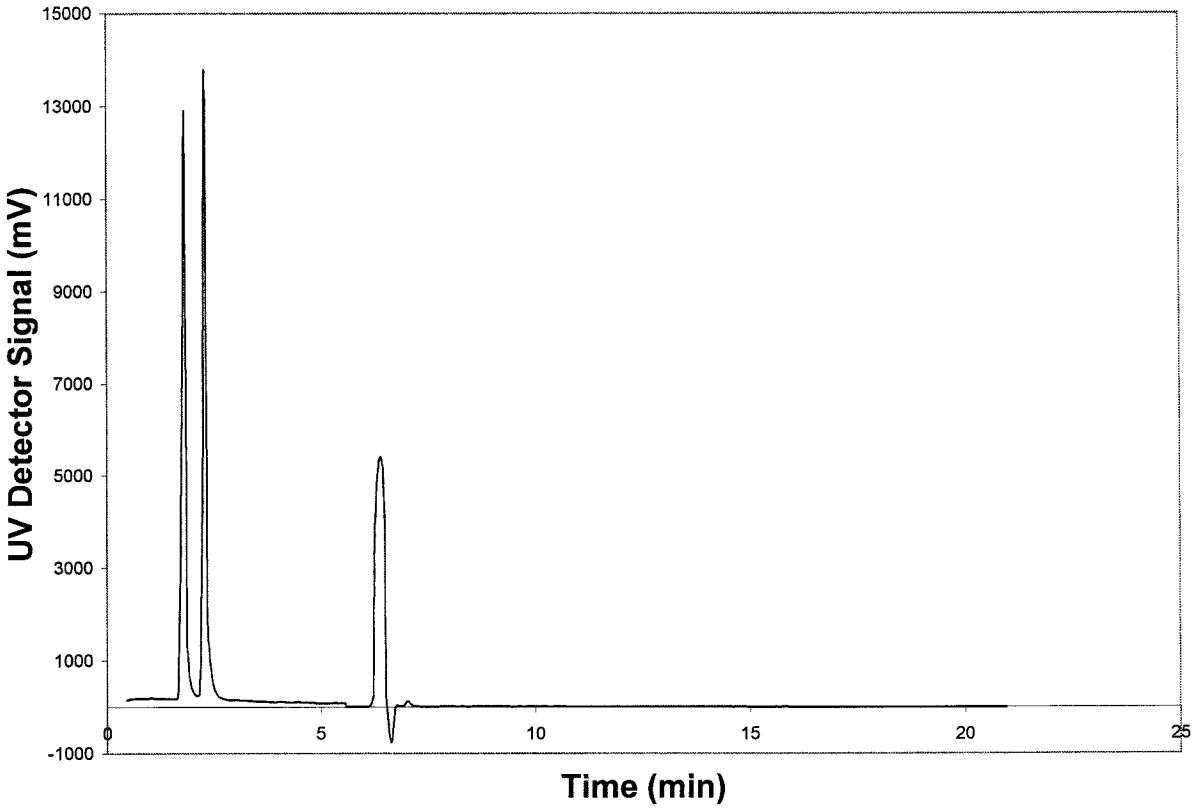
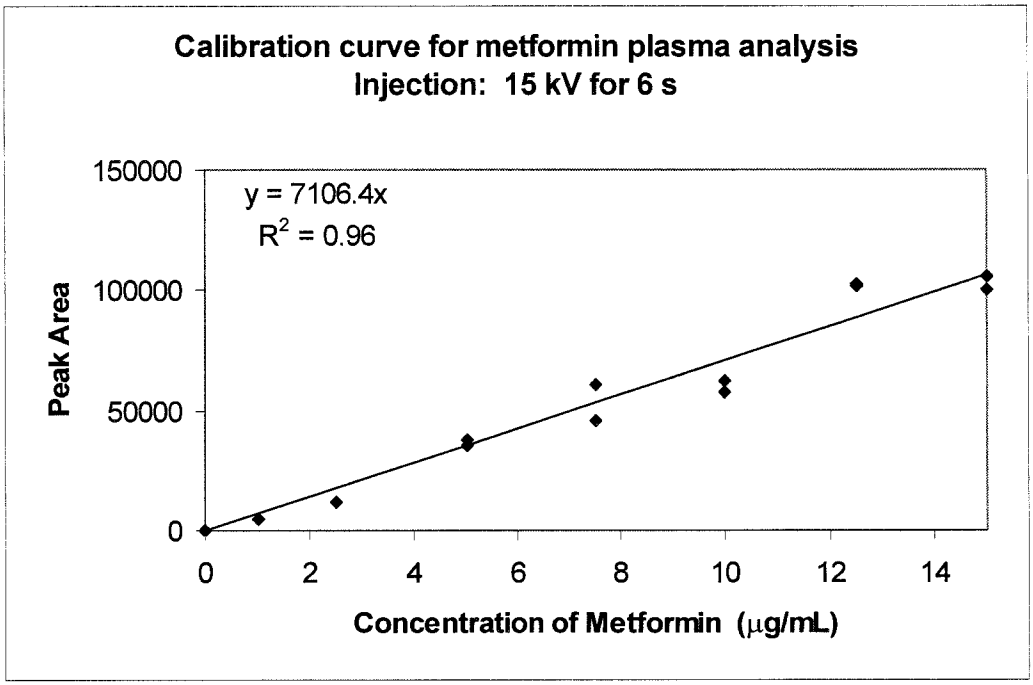


Figure 23. CE analysis of metformin, phenformin and glyburide from the spiked human plasma

3.4.5 Linear dynamic range and detection limit

When metformin hydrochloride capsules were administered in a previous study by Song *et al.* and blood samples were assayed at regular time intervals, the results showed that the concentration of metformin in plasma reached a maximum of C_{max} = 1.9 μg/mL at t_{max} = 3-4 hours[108]. A recent report by Tache *et al.* showed the variation of metformin concentration in plasma samples (from twelve healthy human volunteers) as a

function of time after a single 500-mg oral dose administration. The maximum metformin concentration was 1.2-1.3 μg/mL, as found at 2-3 hours after dose administration [111]. In the present SPE-CE method development, standard calibration curves were constructed over a concentration range from 1 μg/mL to 30 μg/mL metformin and phenformin in plasma. As shown in Figure 23, with an electrokinetic injection time of 6 s and UV detection at 240 nm, linearity was observed from 1 μg/mL to 15 μg/mL (R^2 = 0.9600 and 0.9506, and slopes = 7106 and 12300 arbitrary units per μg/mL, for metformin and phenformin respectively). Significant scattering of data points appeared between 15 μg/mL to 30 μg/mL. Hence the method would be best used for monitoring metformin and phenformin between 15 μg/mL to 1 μg/mL in human plasma. When the electrokinetic injection time was increased from 6 s to 36 s, the metformin and phenformin peak areas increased linearly (R^2 = 0.9804 and 0.9650, respectively). Further increase of injection time to 42 s began to cause splitting of the metformin peak. Using an optimal injection time of 36 s, progressive dilution of the 1 μg/mL plasma sample with methanol + 3% HOAc for CE-UV analysis demonstrated improvement in detection limits down to 12 ng/mL for metformin and 6 ng/mL for phenformin. However, both precision and linearity were compromised.



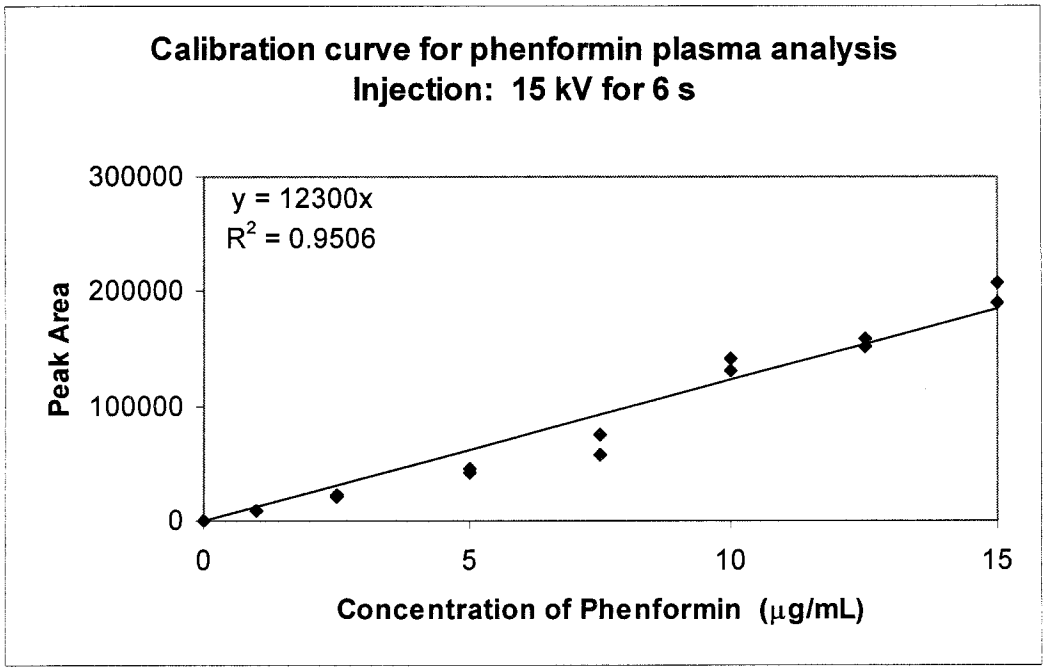


Figure 23. CE-SPE calibration curves for plasma analysis of metformin and phenformin

3.4.6 Internal standard method

Alternatively, the internal standard method was evaluated with an electrokinetic injection time of 6 s. By plotting the ratio of metformin and internal standard (phenformin) peak areas versus metformin concentration, a straight line was obtained in the concentration range of 0.2 to 3.5 μg/mL. The limit of detection was 0.1 μg/mL (S/N = 3). At the limit of quantification, an acceptable RSD of 20% (n = 5) was obtained.

CHAPTER IV

CONCLUSIONS

MISPE has become widely used for analyte preconcentration and sample matrix cleanup. To date MISPE methods are available for the rapid screening of many drugs (including aminopyridine, bupivacaine, cephalexin, darifenacin, 7-hydroxycoumarin, nicotine, pentamidin, propranolol, sameridine, tamoxifen, and theophylline)[112]. Metformin is more basic than all of these drugs previously reported, except for propranolol, which has a higher pKa of 13.8±0.2 but a smaller number of three functional groups for binding interactions[113]. In the present study, metformin was chosen as a challenging molecule (with a high pKa of 13.1±0.5 and a large number of five functional groups for binding interactions) to re-establish generic rules that would enable speedy development of MISPE–DPE–FPE methods. A new MIP with good binding and selectivity characteristics for metformin was synthesized. Specific recognition cavities allowed rebinding of metformin in CH_3CN based on molecular shape, hydrogen bonding, ionic attraction and hydrophobic interactions. A working range was mapped with respect to sample solvent and buffer compositions. These results highlighted the importance of optimization, in the breadth of potential assay conditions that could be employed for MISPE, without any modification of the mobile phase. A robust MISPE—DPE-FPE method was successfully developed for the determination of metformin by UV detection. A special reagent, 5% picric acid in acetonitrile, has demonstrated great success in DPE to eliminate phenformin. This MISPE–DPE–FPE method merits consideration for applications in pharmaceutical and biomedical analysis. Currently, the MISPE–DPE–FPE method is being applied in our research laboratory for the direct screening of metformin in human plasma samples. Modern applications in pharmaceutical and biomedical analysis could study the effects of metformin on fatal and nonfatal lactic acidosis in type

2 diabetes mellitus[114], the reproductive system in patients with polycystic ovary syndrome[115], human ovarian steroidogenesis[116], as well as body mass index, menstrual cyclicity, and ovulation induction in women with polycystic ovary syndrome[117].

Furthermore, the simultaneous determination of metformin, phenformin and other drug compounds in human plasma has been demonstrated by capillary electrophoresis with solid phase extraction. The present SPE-CE method is unique in that rapid separation of metformin and phenformin from other drug compounds is the merit of judicious optimization based on the basic and acidic functionalities of various SPE and CE steps. CE analysis was best performed using a non-aqueous buffer, acetonitrile + 25 mM ammonium acetate + 5% acetic acid. This buffer afforded rapid separation of metformin from phenformin within 3 min, depending on the sample matrix composition. Potential applications of this newly developed SPE-CE methodology may fall into two pharmaceutical and biomedical analysis areas. First, the absolute bioavailability of a 500-mg metformin hydrochloride tablet given under fasting conditions is approximately 50-60%. Studies using single oral doses of metformin tablets, from 500 mg to 2550 mg, have indicated that there is a lack of dose proportionality with increasing doses, which is due to decreased absorption rather than an alteration in elimination. Food decreases the extent of and slightly delays the absorption of metformin, compared to the same tablet strength administered fasting. Second, intravenous single-dose studies in normal subjects demonstrate that metformin is excreted unchanged in the urine and does not undergo hepatic metabolism (i.e., no metabolites have been identified in humans) nor biliary excretion. The clinical relevance of these two related areas is important, and further investigation would be greatly facilitated by the present SPE-CE methodology.

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