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Molecularly imprinted solid-phase extraction for the screening of antihyperglycemic biguanides

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Abstract

A new molecularly imprinted polymer (MIP) was specifically synthesized as a smart material for the recognition of metformin hydrochloride in solid-phase extraction. Particles of this MIP were packed into a stainless-steel tubing (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, 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 with a 49 ± 2% binding. A systematic investigation of binding selectivity was conducted with respect to sample composition (including the solvent, matrix, pH, buffer and surfactant effects). 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.

Keywords: Molecular imprinting; Solid-phase extraction; Biguanides; Guanides; Metformin; Phenformin

1. Introduction

Metformin is a biguanide that is prescribed as an oral antihyperglycemic agent in the management of non-insulin-dependent diabetes mellitus (NIDDM) [1]. 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 [2]. 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 [3–5]. A rapid, simple and sensitive ion-pair HPLC method was recently developed for the determination of metformin in human plasma [6]. At a mobile phase flow rate of 1.5 ml/min, the retention time of metformin was 3.4 min. This translated to an analysis time of 4 min and a solvent consumption of 6 ml. Over a 24-h analysis of 360 plasma samples, the total solvent consumption would be 2.2 l approximately. Moreover, there exists a potential risk of supplementary errors due to exogenous diet ingredients (e.g. caffeine) that might be absorbed into the blood stream to give interference peaks.

Molecularly imprinted polymers (MIPs) are a class of smart materials with pre-determined selectivity for analytical separation. They hold promise in the development of highly selective solid-phase extraction (SPE) methods for the determination of trace analytes, particularly in complex pharmaceutical and biomedical samples [7,8]. Karlsson et al. highlighted the breadth of binding conditions that might be employed, such as polarity, pH and ionic strength [9]. In the present work, a method was developed for the determination of metformin based on molecularly imprinted solid-phase extraction–pulsed elution (MISPE-PE) [10,11]. This method was demonstrated to afford rapid matrix clean-up and analyte pre-concentration. A differential pulsed elution (DPE) step was required to eliminate potential interference due to the binding of structural analogues. Phenformin was a challenging interferent that could only

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be eliminated quantitatively by DPE with 5% picric acid in acetonitrile.

2. Experimental

2.1. Reagents and materials

Metformin·HCl and phenformin·HCl were obtained from Sigma (St. Louis, MO, USA). Stock solutions of 1 mg/ml each were prepared in distilled deionized water. They were stable when stored at 4 °C for several months. Working solutions of 60 μg/ml were prepared afresh for daily use.

Picric acid, didodecyldimethylammonium bromide (DDAB), dodecyltrimethyl-ammonium bromide (DTAB), hexamethonium bromide (HMB) hydrate, and myristyltrimethylammonium bromide (TTAB) were obtained from Aldrich (Milwaukee, WI, USA). All 50-mM aqueous buffers (phosphate for pH 2.5 and 7.0) were obtained from Beckman Coulter (Fullerton, CA, USA).

2.2. Metformin MIP preparation

The MIP polymer was synthesized in a 10-ml Pyrex vial containing 41 mg of metformin·HCl (0.25 mmol, print molecule), 140 mg of trifluoromethacrylic acid (TFMAA, 1.0 mmol, functional monomer) and 3.0 ml of acetonitrile (solvent). 1.2 ml of trimethylolpropane trimethacrylate (TRIM, 3.8 mmol, cross-linker) and 12 mg of 2,2′-azobis(2-methylproprionitrile) (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 colourless translucent bulk of solid MIP was obtained. The MIP was crushed and ground in a mortar with a pestle. The ground MIP particles were sieved to a size range of 38–63 μm in diameter. These particles were slurry-packed into a stainless-steel tubing (50 mm × 0.8 mm i.d.) equipped with an exit frit [11].

A control polymer was also synthesized in the same conditions as described above, but without the addition of metformin·HCl.

2.3. MISPE-PE instrumentation

The MISPE-PE instrumentation was set up with an Eldex Duro Series CC-30s micrometer pump (San Carlos, CA, USA), a Valco Cheminert VIGI C2XL injector valve (Houston, TX, USA) equipped with a 20-μl sample loop, a Bischoff Lambda 1010 UV detector (Leonberg, Germany), and a Dionex 4270 integrator (Sunnyvale, CA, USA). Acetonitrile was driven as the mobile phase through the MIP micro-column at a flow rate of 0.5 ml/min.

2.4. Human plasma analysis

Human plasma samples were obtained from the Ottawa General Hospital (Ottawa, Canada). Several 0.2-ml aliquots of a plasma sample was spiked with 20-μg/ml metformin and 300-μg/ml phenformin to provide a series of working standard solutions over the concentration range from 0.1 to 10 μg/ml metformin and 60-μg/ml phenformin. 1 ml of acetonitrile–50 mM phosphate buffer (pH 7) (9:1) was added to precipitate the proteins [12]. The mixture was thoroughly vortexed and then centrifuged at 5000 rpm for 20 min. The supernatant was filtered through Supor Acrodisc syringe filter (GelmanSciences, pore size 0.45 μm). A 20-μl volume of filtered supernatant was injected onto the MIP micro-column for MISPE-DPE-final pulsed elution (FPE) analysis. A standard calibration curve for MISPE-DPE-FPE was constructed by performing MISPE with CH_3CN as the mobile phase at a flow rate of 0.5 ml/min, followed by DPE with 5% picric acid in CH_3CN and FPE with 3% trifluoroacetic acid (TFA) in CH_3OH. The recovery of metformin was determined by analyzing plasma sample (0.2 ml) that contained 3 μg/ml metformin and 60 μg/ml phenformin.

3. Results and discussion

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-buffered 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 Fig. 1. When a FPE of metformin for direct UV detection was performed using 3% TFA in

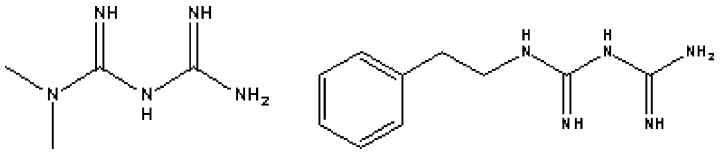

Fig. 1. Molecular structures of metformin and phenformin.

methanol, the phenformin would also be detected to result in a positive interference.

3.2. 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 [13]. 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 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 [14–17]. DPE was an intermediate wash step that would remove phenformin and other structural analogues from the MIP micro-column. Two nonionic (Triton X-100, Tween 20) surfactants were tested for their DPE effectiveness. Unfortunately, phenformin could not be removed using 1% Triton X-100 in 0.1 M 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 [18]. Table 1 shows the DPE effectiveness of 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.

Table 1
Effectiveness of using surfactant solutions for DPE after loading 60 μg/ml phenformin in CH_3CN–50 mM phosphate buffer (pH 7) (9:1)

DPE solvent	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. The % phenformin remaining can be compared with 100% without DPE.

Table 2
Effectiveness of adding surfactants into sample solution of 60 μg/ml phenformin in CH_3CN–50 mM 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 can be compared with 49% without adding surfactant.

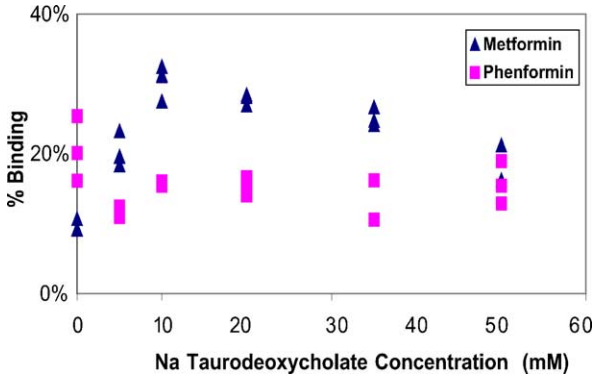

Fig. 2. Effect of sodium taurodeoxycholate concentration in sample solution on % binding of metformin and phenformin.

Second, surfactants were added as a matrix in the sample solution to prevent non-specific binding of phenformin on the MIP micro-column. As shown in Table 2, 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 Fig. 2, the surfactant could not prevent the non-specific binding of phenformin on the MIP micro-column.

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 [13], 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 3 to show no significant effects overall.

3.3. DPE with organic acids

Several organic acids were evaluated for their DPE effectiveness. As summarized in Table 4, 10% benzoic acid

Table 3
Effect of HMB, DTAB, TTAB and DDAB on % binding of metformin and phenformin

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

Table 4
Evaluation of organic acids for DPE effectiveness

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_3CN	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

($pK_a = 4.19$) in CH_3OH was poor for the DPE removal of phenformin. Eighteen percent pentafluorobenzoic acid ($pK_a = 1.99$) in CH_3CN left behind 20% of phenformin on the micro-column. 30% acetic acid ($pK_a = 4.74$) in CH_3OH was fairly good for the removal of phenformin, but only 10% of metformin remained on the micro-column. Two percent 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 ($pK_a = 0.30$–0.53) was strategically selected from among the strongest organic acids that were commercially available [19–21]. Different concentrations of picric acid in acetonitrile were evaluated for the best DPE result. After DPE with each picric acid, TFA ($pK_a = -0.23$–0.0) was used in FPE to elute all of the remaining phenformin. As shown in Fig. 3 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 [22,23], picric acid could form a stronger charge-transfer complex with phenformin than with metformin. Note that the functional monomer, TFMAA ($pK_a = 1.9$–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 $37 \pm 3\%$ 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. Five percent 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) and phenformin (60 μg/ml). The results verified that, over the concentration range studied, a reasonably constant $37 \pm 2\%$ of the bound metformin remained on the MIP micro-column while phenformin was quantitatively removed by the DPE.

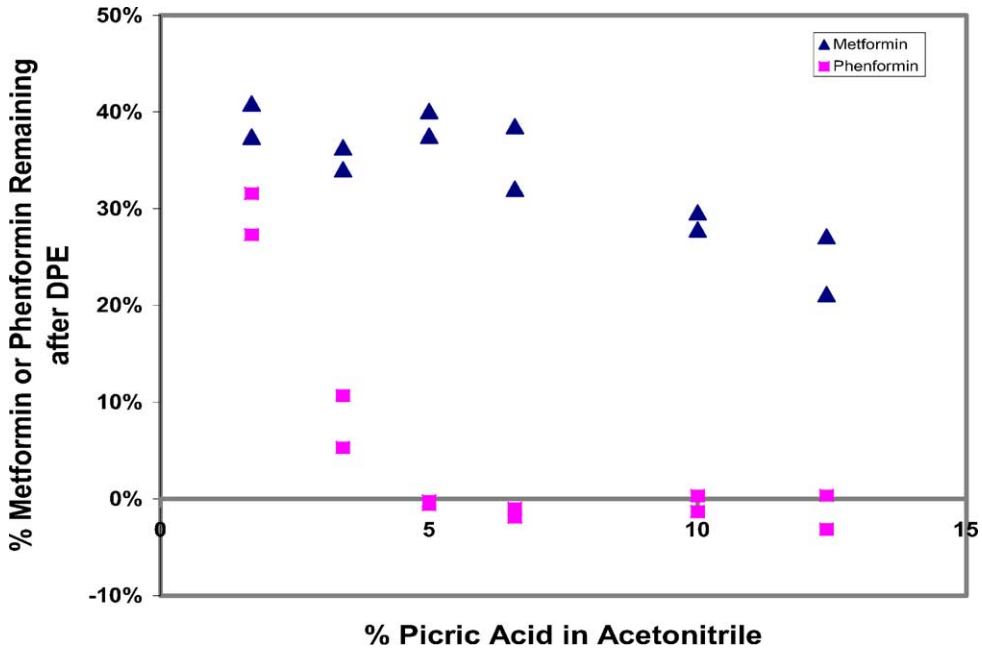

Fig. 3. % Metformin or phenformin remaining on MIP micro-column after DPE with varying % of picric acid in acetonitrile.

Table 5
Summary of DPE solvents for various drug compounds bound onto five different MIP micro-columns

Analyte	pK_a of analyte	Interferents	pK_a of interferents	Solvent for MISPE of analytes and interferents	Solvent for elution of interferents by DPE	Solvent for elution of analyte
Theophylline [14,15]	8.68	Dyphylline Nicotinic acid	5.36 4.80	$CHCl_3$	CH_3CN	CH_3OH
Nicotine [16]	8.02	Myosin		CH_3CN	CH_3OH	Water + 1% TFA
4-Aminopyridine [17]	9.26	2-Aminopyridine	6.67	$CHCl_3$	DMSO	CH_3OH + 1% TFA
Cephalexin [10]	5.3 and 7.3	Cefradine Cefadroxil	2.6 and 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

Table 5 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 solvent 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.

3.4. Analytical figures of merit

At a mobile phase flow rate of 0.5 ml/min, the MISPE-PE-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 [11], 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 absorbed into the blood stream to give interference peaks.

3.5. 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 [3,24]. Good linearity (R^2 = 0.9919) was observed in the metformin concentration range from 0.1 to 10 μg/ml, 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.

4. 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) [14]. In the present study, a robust MISPE-DPE-FPE method was developed for metformin determination by UV detection. A special reagent, 5% picric acid in acetonitrile, has demonstrated great success in DPE to eliminate phenformin. 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 [25], the reproductive system in patients with polycystic ovary syndrome [26], human ovarian steroidogenesis [27], as well as body mass index, menstrual cyclicity, and ovulation induction in women with polycystic ovary syndrome [28].

Acknowledgements

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