รายงานวิจัยฉบับสมบูรณ์

โครงการ: การเลียนแบบเออร์กอตรีเซพเตอร์ด้วยการพิมพ์รอยโมเลกุลของโดปามีนและซีโรโตนิน

ผู้วิจัย

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Abstract

A temperature sensitive dopamine-imprinted polymer was prepared in 80% aqueous methanol solution by free-radical crosslinking copolymerisation of methacrylic acid and acrylamide at 60ºC in the presence of *N,N*-methylene-bis-acrylamide as the cross-linker and dopamine hydrochloride as template molecule. The resulting molecularly imprinted polymer (MIP) formed temperature responsive materials which could be used for the selective separation of appropriate dopamine and adrenergic compounds from a liquid matrix at ambient temperatures. The thermoresponsive MIP exhibited a swelling-deswelling transition in 80% aqueous methanol solution at about 35ºC. The capacity of the thermoresponsive MIP to recognise the template molecule when present in aqueous methanol solution changed with temperature, with the highest selectivity found at 35ºC. Additionally, binding parameters obtained from Scatchard analyses indicate that increasing temperature resulted in an increased affinity and binding capacity of specific binding sites, but had less effect on non-selective binding sites. Subsequently, the thermoresponsive MIP was tested for its application as a sorbent material, utilisable in the selective solid-phase extraction (SPE) of dopamine and other adrenergic compounds (epinephrine, isoproterenol, salbutamol and serotonin) from urine samples. It was shown that the compounds that were structurally related to dopamine could be removed by elution, while dopamine and serotonin, the analytes of interest, remained strongly adsorbed to the adsorbent during SPE applications. The thermoresponsive MIP displayed different efficiency in clean-up and enrichments using the SPE protocol at different temperatures.

A dopamine and serotonin molecularly imprinted polymer (DS-MIP) artificial receptor is designed and has been successfully used in fluorescent molecularly imprinted binding polymer assay for ergot binding studies. The dopamine and serotonin which are the printed molecules is bound by the DS-MIP and the ergot analyte

competes with dopamine/serotonin probe for the same binding site. The artificial receptor obtained showed to be highly specific and affinity was similar to natural receptors. A series of ergot derivatives (ergocryptine, ergocornine, ergocristine, ergonovine, agroclavine, pergolide and terguride) has been characterized as to their ligand binding activities with the DS-MIP. The affinity for the dopamine/serotonin binding site of the model ergots was measured by observing the increase of the free dopamine or serotonin in the presence of increasing concentrations of the ergot specie. The utilization of the DS-MIP in a competitive fluorescent ligand binding assay for ergot produced results which were comparable to those obtained using a competitive immunoassay data obtained using dopamine/serotonin receptors derived from the rat hypothalamus. These results indicate the applicability of the assay in characterizing the ligand binding characteristics of ergot derived molecules. The developed assay does not require the separation of free/bound ligands could be used to determine the binding specificities of possible new ergot derivatives.

บทคัดย่อ

ในงานวิจัยนี้ได้เตรียมพอลิเมอร์ที่มีรอยพิมพ์ประทับโมเลกุลโดปามีน (D-MIP) ที่ออกแบบให้มีคุณสมบัติไว ต่อการเปลี่ยนแปลงอุณหภูมิ จากปฏิกิริยาโคพอลิเมอไรเซชั่นระหว่างสารผสม methacrylic acid และ acrylamide ซึ่งทำ หน้าที่เป็น functional monomers และ *N,N'*-methylene-bis-acrylamide ทำหน้าที่เป็น cross-linker และใช้โดปามีนเป็น โมเลกุลตัวพิมพ์ ที่อุณหภูมิ 60 C ในตัวกลางผสมระหว่างเมธานอลและน้ำในสัดส่วน 1:4 พอลิเมอร์ที่มีรอยพิมพ์ ประทับโมเลกุลโดปามีนที่เตรียมได้นี้สามารถนำไปใช้ในการแยกโมเลกุลโดปามีนและสารสารอะดีเนอจิก (adrenergic agents) ที่อยู่ในสารละลายตัวอย่างที่อุณหภูมิห้องได้อย่างมีความจำเพาะเจาะจง D-MIP สามารถบวมตัว-หดตัวได้ใน ตัวกลางผสมระหว่างเมธานอล, น้ำ และในตัวกลางผสมระหว่าง methanol และน้ำ D-MIP มีความสามารถจดจำ โมเลกุลตัวพิมพ์ได้อย่างจำเพาะเจาะจงสูงสุดเมื่ออุณหภูมิเพิ่มขึ้นจนถึงอุณหภูมิ 35 C จากการตรวจ Binding isotherm และทำ Scatchard analysis ชี้ให้เห็นว่าเมื่ออุณหภูมิเพิ่มขึ้นมีผลทำให้ค่าสัมพันธภาพ (K_d) และปริมาณการจับ (B_{max}) ของ High-affinity binding site ของรีเซพเตอร์เพิ่มขึ้น แต่การเปลี่ยนแปลงอุณหภูมิจะไม่มีผลต่อ non-specific binding site ของรีเซพเตอร์ จากการประยุกต์ใช้พอลิเมอร์ D-MIP เป็นตัวดูดซับของคอลัมน์ SPE (solid phase extraction) เพื่อ แยกโมเลกุลโดปามีนและสารอะดีเนอจิกอื่นๆ จากตัวอย่างปัสสาวะ สารประกอบที่มีโครงสร้างคล้ายคลึงกับตัวพิมพ์ถูก ชะออกจากคอลัมน์ SPE ขณะที่โมเลกุลโดปามีนและซีโรโทนินยังคงถูกดูดซับไว้ในคอลัมน์ และการสกัดที่อุณหภูมิ ใกล้เคียง 35 C ให้ค่า recovery ของการสกัดดีกว่าอุณหภูมิอื่น ๆ จึงแสดงให้เห็นว่า D-MIP มีคุณสมบัติไวต่อการ เปลี่ยนแปลงอุณหภูมิ โดยใช้งานใน SPE มีประสิทธิภาพในการ clean-up สารสารอะดีเนอจิก และโมเลกุลตัวพิมพ์ ประทับ ที่อุณหภูมิแตกต่างกัน

นอกจากนี้ได้เตรียมพอลิเมอร์ที่มีรอยพิมพ์ประทับโมเลกุลของโดปามีนและซีโรโทนิน (DS-MIP) เพื่อใช้เป็น รีเซพเตอร์เลียนแบบของโดปามีนและซีโรโทนินรีเซ็พเตอร์ ในการศึกษาสัมพันธภาพของสารในกลุ่มเออกอต (ergots) กับโดปามีนและซีโรโทนินรีเซ็พเตอร์ เนื่องจากรีเซพเตอร์เลียนแบบนี้มีรอยพิมพ์โมเลกุลของทั้งโดปามีนและซีโรโท นิน สารเออกอตจะแข่งขันกับโดปามีนหรือซีโรโทนินจับที่ตำแหน่งของการจับยาเดียวกัน พอลิเมอร์ที่มีรอยพิมพ์ ประทับโมเลกุลของโดปามีนและซีโรโทนินที่สังเคราะห์ขึ้นนี้มีความจำเพาะเจาะจงสูงต่อโมเลกุลโดปามีนและซีโรโท นิน และมีการเลือกจดจำต่อสารที่มีโครงสร้างใกล้เคียงโดปามีนและซีโรโทนินได้บ้างเล็กน้อย เมื่อทำการตรวจลักษณะ การจับกับรีเซพเตอร์ระหว่างสาร ergot alkaloids หลาย ๆ ชนิดซึ่งได้แก่ ergocryptine, ergocornine, ergocristine, ergonovine, agroclavine, pergolide และ terguride) ด้วยวิธี competitive fluorescent molecularly imprinted polymer ที่ ใช้ DS-MIP เป็นรีเซพเตอร์ และใช้โดปามีนและซีโรโทนินเป็นโมเลกุลาโพรบ (molecular probe) และทำการวัดค่าสัม พันธภาพ (K_i) ระหว่างสารเออกอตกับ ตำแหน่งของการจับยาของโมเลกุลโดปามีนหรือซีโรโทนิน โดยการวัดปริมาณ โดปามีนหรือซีโรโทนินอิสระในสารละลายที่มีความเข้มข้นต่าง ๆ กันของ ergot alkaloids จากการเปรียบเทียบค่าสัม พันธภาพ (K_i) ที่ได้จากการวิเคราะห์กับพอลิเมอร์ DS-MIP พบว่ามีความสอดคล้องกับค่าสัมพันธภาพ (K_i) ที่ได้จากการ ทำ competitive immunoassay กับรีเซพเตอร์ธรรมชาติที่ได้จากไฮโปธาลามัสของหนู การศึกษานี้ชี้ให้เห็นว่าเทคนิค competitive fluorescent ligand binding assay กับพอลิเมอร์ที่มีรอยพิมพ์ประทับโมเลกุลของโดปามีนและซีโรโทนิน ก็ เป็นวิธีหนึ่งที่ใช้ในการตรวจคุณสมบัติการจับรีเซพเตอร์โดปามีนและซีโรโทนินของสารอนุพันธ์เออกอตใหม่ๆ ได้ และ วิธี competitive fluorescent ligand binding assay ที่พัฒนาขึ้นนี้ไม่จำเป็นต้องทำการแยกลิแกนด์อิสระและลิแกนด์ที่จับ กับรีเซพเตอร์ออกจากกันก่อนการวิเคราะห์

กุญแจคำ: พอลิเมอร์ที่มีรอยพิมพ์ประทับโมเลกุล; โดปามีน; ซีโรโทนิน; เออกอต; รีเซ็พเตอร์

1. ความสำคัญและที่มาของปัญหา

การจดจำโมเลกุล (Molecular recognition) เป็นปรากฏการณ์ที่พบได้ในสิ่งมีชีวิต เช่น เอนไซม์, แอนติบอดี, รีเซพเตอร์, เมมเบรน เป็นต้น โดยแนวคิดของการเลียนแบบการจดจำโมเลกุลนี้เริ่มต้นมาจาก Fisher ผู้เสนอแนวคิด "Lock-and-Key" ซึ่งหมายถึง molecular receptor (lock) ที่สามารถจับกับ substrate (key) ได้อย่างจำเพาะ ต่อมา Pederson, Lehn and Cram ได้รับรางวัลโนเบลในงานวิจัยเรื่อง "host-guest chemistry" ซึ่งเกี่ยวข้องกับการออกแบบรีเซพเตอร์เทียม (artificial receptor) ที่สามารถจับกับโมเลกุลขนาดเล็กได้อย่างจำเพาะ และกุญแจสำคัญที่ทำให้ซับสเตรท (substrate) สามารถจับกับรีเซพเตอร์ได้นั้นก็คือการมี binding group ที่สามารถเกิดปฏิสัมพันธ์ได้หลาย ๆ แบบ เช่น hydrogen bonding, electrostatic, hydrophobic interaction ดังนั้นรีเซพเตอร์เทียม (artificial receptor) จึงมีความสำคัญอย่างยิ่งต่อความเข้าใจกลไกการจำโมเลกุล (molecular recognition) นอกจากนี้การเกิดปฏิสัมพันธ์ระหว่างรีเซพเตอร์เทียมกับโมเลกุล guest สามารถอธิบายพันธะที่เกิดขึ้นในระดับโมเลกุลของขบวนการเกิดคอมเพล็กซ์ในสิ่งมีชีวิตได้ โดยเมื่อสร้างรอยพิมพ์ประทับ (imprint) ของโมเลกุล guest บนรีเซพเตอร์เทียมก็จะทำให้เกิด binding group ขึ้นบนรีเซพเตอร์อย่างเหมาะสมกับโมเลกุลของ guest เทคนิคการพิมพ์รอยโมเลกุล (molecular imprinting) เป็นวิธีหนึ่งที่สามารถใช้สร้างรีเซพเตอร์เทียมเพื่อเลียนแบบรีเซพเตอร์ธรรมชาติ โดยวิธีนี้จะเริ่มต้นจากการผสม functional monomer กับโมเลกุลตัวพิมพ์ (template) ซึ่ง monomer จะเกิดพันธะ covalent หรือ non-covalent ขึ้นหลังจากขบวนการรวมตัวกัน (self-assembly process) จากนั้นทำปฏิกิริยา copolymerization กับ cross-linking monomer ทำให้ได้พอลิเมอร์ที่แข็งและเมื่อสกัดเอาโมเลกุลของ guest ออกจากพอลิเมอร์แมททริกซ์นั้นก็จะได้พอลิเมอร์ที่มีรอยพิมพ์ประทับโมเลกุล (molecularly imprinted polymer) การนำพอลิเมอร์ที่มีรอยพิมพ์ประทับโมเลกุลมาใช้เป็นรีเซพเตอร์เลียนแบบมีประโยชน์อย่างมากต่อศึกษาความสามารถในการเลือกจดจำโมเลกุลของรีเซพเตอร์ธรรมชาติ รวมทั้งการศึกษาปฏิสัมพันธ์ระหว่างโมเลกุลยากับรีเซพเตอร์เพื่อทำให้เกิดความเข้าใจในลักษณะที่สำคัญของการจับของยากับรีเซพเตอร์และนำไปสู่การออกแบบยาให้ได้ยาที่มีฤทธิ์ดีและมีความจำ เพาะสูงนับว่าเป็นเรื่องที่น่าสนใจและมีประโยชน์อย่างมากต่องานด้านการออกแบบยาและพัฒนายา รีเซพเตอร์เทียมที่ถูกสร้างขึ้นมาแล้วด้วยเทคนิคการพิมพ์รอยโมเลกุล คือ estrogenic receptor, adrenergic receptor, cinchona alkaloid receptor, opioid receptor และ peptide receptor โดยทั่วไปข้อดีของพอลิเมอร์ที่มีรอยพิมพ์ประทับโมเลกุลในการเป็นรีเซพเตอร์เลียนแบบ ก็คือ สามารถเตรียมได้ง่าย และทนต่อสารเคมีและแรงกลได้ดี ขณะที่รีเซพเตอร์ธรรมชาติมีความคงตัวต่ำเมื่อถูกแยกออกมาจากสิ่งมีชีวิตและการแยกหรือการเก็บรีเซพเตอร์ธรรมชาติก็ทำได้ยากรวมทั้ง อาจมีปริมาณไม่เพียงพอที่จะใช้ศึกษา และนอกจากนี้จะมีประโยชน์อย่างมากในกรณีที่ไม่สามารถหารีเซพเตอร์ธรรมชาติได้

สาร ergot alkaloids ซึ่งแสดงคุณสมบัติทางเภสัชวิทยาที่หลากหลายและมีความสัมพันธ์กับ dopamine receptor และ serotonin receptor แต่ความหลากหลายของฤทธิ์ทางชีวภาพนั้นไม่ได้เกี่ยวข้องกับคุณสมบัติทางเภสัชวิทยาเพียงคุณสมบัติเดียว และยังไม่มีหลักฐานแสดงถึงฤทธิ์ทางชีวภาพที่เกี่ยวข้องกับกลไกในระดับเซลหรือระดับโมเลกุลของสารเหล่านี้เลย ดังนั้นการศึกษาปฏิสัมพันธ์ระหว่างโมเลกุลของ ergot กับรีเซพเตอร์เลียนแบบนั้นจะช่วยทำให้เกิดความเข้าใจในลักษณะการจับในระดับโมเลกุลของยา ergot alkaloids กับรีเซพเตอร์ธรรมชาติมากยิ่งขึ้น ดังนั้นจึงได้นำเทคนิคการพิมพ์โมเลกุลมาสร้างรีเซพเตอร์เทียมเพื่อเลียนแบบรีเซพเตอร์ธรรมชาติของสาร ergot alkaloids ซึ่งพบว่ายังไม่มีรายงานของการนำยาดังกล่าวมาศึกษาในทิศทางเดียวกันนี้เลย และนอกจากนี้มีข้อสันนิษฐานเกี่ยวกับฤทธิ์ทางชีวภาพของ ergot alkaloids ดังนี้ 1) สารประกอบ ergot สามารถเกิดปฏิสัมพันธ์กับ receptor site ได้มากกว่า 1 ชนิด, 2) จำนวน receptor site ที่

ergot เข้าไปจับ มีความแตกต่างกันตามชนิดของอวัยวะ และ 3) Affinity ของ receptor site และ intrinsic activity ก็มีความแตกต่างกันตามชนิดของ ergot ดังนั้นการศึกษาปฏิสัมพันธ์ระหว่างโมเลกุลของ ergot กับรีเซพเตอร์เลียนแบบนั้น จะช่วยทำให้เกิดความเข้าใจในลักษณะการจับในระดับโมเลกุลของยา ergot alkaloids กับรีเซพเตอร์ธรรมชาติมากยิ่งขึ้น และรีเซพเตอร์เลียนแบบที่จะเตรียมขึ้นจะถูกสร้างให้มีรอยจดจำที่จำเพาะต่อโมเลกุลของ dopamine และ serotonin เพื่อ ทำการศึกษาว่าสารประกอบ ergot alkaloids ต่าง ๆ เช่น pergolide, carbergoline, ergocristine, ergotamine และ ergocryptine มีการเลือกจับที่จำเพาะกับพอลิเมอร์ที่พิมพ์ประทับด้วย dopamine และ serotonin หรือไม่ รวมทั้ง ศึกษาลักษณะของการเกิดปฏิสัมพันธ์ระหว่างโมเลกุลของ ergot alkaloids กับรีเซพเตอร์เทียมที่สร้างขึ้นมาด้วย ส่วนเหตุผล ของการเลือกออกแบบรีเซพเตอร์เทียมสำหรับสารประกอบ ergot alkaloids ด้วยการสร้างรอยพิมพ์ประทับของโมเลกุล dopamine และ serotonin ลงบนพอลิเมอร์ก็เนื่องมาจากโครงสร้างของ ergot alkaloids ซึ่งเป็น ergoline ring system นั้นมีบางส่วนคล้ายคลึงกับโครงสร้างของ biogenic amines เช่น norepinephrine หรือ dopamine และ serotonin (ดังแสดงในรูปข้างล่าง) นอกจากนี้โมเลกุลของ ergot alkaloids สามารถจับกับรีเซพเตอร์ธรรมชาติได้ บางส่วนกับ dopamine receptor และ serotonin receptor อย่างที่ปรากฏหลักฐานจากฤทธิ์ทางเภสัชวิทยาของ ergot alkaloids ดังนั้นในการสังเคราะห์รีเซพเตอร์เทียมของ ergot alkaloids ด้วยเทคนิคการพิมพ์รอยโมลกุลนั้นจะใช้ dopamine และ serotonin เป็นตัวพิมพ์ (template)

2. วัตถุประสงค์

1. เพื่อศึกษาวิธีเตรียมรีเซพเตอร์เทียมที่มีความสามารถในการจดจำต่อโมเลกุลของ dopamine และ serotonin ด้วย เทคนิคการพิมพ์โมเลกุลบนพอลิเมอร์

2. เพื่อศึกษาความเป็นไปได้ของการสร้างความเลือกจำเพาะต่อสารหลายชนิด (multiple selectivity) ให้กับรีเซพ เตอร์เทียมโดยใช้ตัวพิมพ์ทั้งแบบเดี่ยวและแบบผสม

3. เพื่อศึกษาความสามารถในการจับของ ergot alkaloids บนรีเซพเตอร์เลียนแบบ รวมทั้งศึกษาลักษณะการจับของ ergot alkaloids กับรีเซพเตอร์เลียนแบบที่เตรียมขึ้น

4. เพื่อเปรียบเทียบความสามารถในการจับของ ergot alkaloids บนรีเซพเตอร์เลียนแบบกับการจับของ ergot alkaloids บนรีเซพเตอร์ธรรมชาติ เช่น พลาสมาโปรตีน

5. เพื่อศึกษาการจับของ ergot alkaloids ที่ตำแหน่งการจับ (binding sites) บนรีเซพเตอร์โดยใช้แบบจำลอง โครงสร้างที่สร้างขึ้นมาจากโปรแกรม Computer

3. ระเบียบวิธีวิจัย

1. เตรียมรีเซพเตอร์เลียนแบบ ให้มีรอยจดจำตัวพิมพ์ต่าง ๆ 4 แบบด้วยกัน คือ

   a) พอลิเมอร์ที่มีรอยจดจำต่อ dopamineเพื่อเลียนแบบdopamine receptor โดยใช้ dopamine เป็นตัวพิมพ์

   b) พอลิเมอร์ที่มีรอยจดจำต่อ serotonin เพื่อเลียนแบบ serotonin receptor โดยใช้ 6-methoxytryptamine หรือ 6-hydroxytryptamine (serotonin) เป็นตัวพิมพ์

   c) พอลิเมอร์ที่ได้จากการผสมพอลิเมอร์ที่มีรอยจดจำต่อdopamineและพอลิเมอร์ที่มีรอยจดจำต่อ serotonin

   d) พอลิเมอร์ที่ได้จากการพิมพ์โมเลกุลบนพอลิเมอร์โดยใช้ serotoninและdopamineเป็นตัวพิมพ์ผสม

2. ตรวจคุณสมบัติทางเคมีและกายภาพของพอลิเมอร์ที่เตรียมได้ทั้งหมด ดังต่อไปนี้

   a) วัดขนาดอนุภาคของพอลิเมอร์ด้วยวิธี Light scattering

   b) ตรวจหาองค์ประกอบ C, H, N และ O ในพอลิเมอร์

   c) ตรวจการพองตัวของพอลิเมอร์ในตัวทำละลายชนิดเดียวกับที่ใช้ในการสร้างรอยจดจำ

   d) ตรวจขนาดของ pore ของพอลิเมอร์ที่มีความสามารถในการจดจำโมเลกุลตัวพิมพ์ดีที่สุด และศึกษาลักษณะ adsorption isotherm ของพอลิเมอร์ที่ได้

3. ตรวจความสามารถในการจดจำของพอลิเมอร์ที่มีรอยจดจำตัวพิมพ์ทั้ง 4 แบบ ด้วยวิธี batch rebinding assay

4. ตรวจ binding characteristics ของพอลิเมอร์ที่มีรอยจดจำต่อตัวพิมพ์ทั้ง 4 แบบ เพื่อตรวจ association constant (Ka) และจำนวน binding site ของพอลิเมอร์ (Bmax) และศึกษาปัจจัยต่าง ๆ เช่น ตัวทำละลาย อุณหภูมิ พี-เอช ความแรงของเกลือ ที่มีผลต่อการจับของตัวพิมพ์บนพอลิเมอร์

5. ศึกษาลักษณะ binding group ของรีเซพเตอร์เลียนแบบที่มีรอยจดจำต่อ dopamine และ serotonin ด้วยวิธี IR spectroscopy และ 1H-NMR spectroscopy

6. ตรวจ cross-reactivity ของพอลิเมอร์ที่มีรอยจดจำต่อตัวพิมพ์ทั้ง 4 แบบ ด้วยวิธี batch binding assay แบบ competitive binding และวิธี chromatography ชนิด thin-layer chromatography หรือ column chromatography ซึ่งเป็นการตรวจ cross-reactivity ของพอลิเมอร์ด้วยวิธีอ้อมโดยนำพอลิเมอร์ที่มีรอยพิมพ์ประทับด้วย dopamine หรือพอลิเมอร์ที่พิมพ์ประทับด้วย serotonin มาเตรียมเป็นเพื่อใช้เป็นเฟสอยู่กับที่ (stationary phase) แล้วตรวจหาสภาวะที่เหมาะสมในการแยกสาร โดยเลือกศึกษากับสารที่มีลักษณะโครงสร้างทางเคมีคล้ายคลึงกับตัวพิมพ์ เช่น พอลิเมอร์ที่พิมพ์กับ dopamine จะศึกษาการเลือกจับกับ epinephrine, norepineprine, phenylephrine, isopoterenol เป็นต้น ส่วนพอลิเมอร์ที่พิมพ์กับ serotonin จะศึกษาการเลือกจับกับ 5-methoxy-tryptamine และ histamine เป็นต้น และเปรียบเทียบผลที่ได้กับพอลิเมอร์ control ซึ่งถูกเตรียมขึ้นมาด้วยวิธีเดียวกับพอลิเมอร์ที่มีรอยพิมพ์ประทับแต่ไม่ใช้ตัวพิมพ์ในขั้นตอนการเตรียม

7. ศึกษาความสามารถในการจดจำของรีเซพเตอร์เลียนแบบที่เตรียมขึ้นต่อโมเลกุลของ ergot alkaloids โดยเลือก ergot alkaloids เช่น pergolide, carbergoline, ergocristine, ergotamine หรือ ergocryptine เป็นต้น มาทำการศึกษา ด้วยวิธี batch binding assay ทั้งแบบ competitive และ noncompetitive binding

8. ศึกษากลไกการจับของโมเลกุล ergot alkaloids ที่ตำแหน่งของการจับ (binding sites) บนรีเซพเตอร์เลียนแบบ ทำการศึกษาผลของปัจจัยต่าง ๆ เช่น ตัวทำละลาย อุณหภูมิ พี-เอช ความแรงของเกลือ ที่มีต่อการจับของโมเลกุล ergot alkaloids กับรีเซพเตอร์เลียนแบบที่เตรียมขึ้น

9. เปรียบเทียบความสามารถในการจับของโมเลกุล ergot alkaloids กับรีเซพเตอร์ธรรมชาติ และกับรีเซพเตอร์เลียนแบบด้วยการเปรียบเทียบค่า binding constant ของรีเซพเตอร์ธรรมชาติกับของรีเซพเตอร์เลียนแบบ

10. ศึกษาลักษณะการจับของ ergot alkaloids ที่ตำแหน่งของการจับ (binding sites) บนรีเซพเตอร์เลียนแบบที่เตรียมขึ้นเพื่อเลียนแบบ dopamine receptor และ serotonin receptor โดยใช้ computer modeling ทำการสร้างแบบจำลองของรีเซพเตอร์ และโมเลกุลยา ergot alkaloids ศึกษาสัมพันธภาพในการจับของสารหรือยาที่มีขนาดโมเลกุลใหญ่จากนั้นทำการศึกษาสัมพันธภาพในการจับของรีเซพเตอร์กับโมเลกุลของ ergot alkaloids จากแบบจำลองที่สร้างขึ้น

4. แผนการดำเนินงานวิจัย

แผนการดำเนินงานปีที่ 1

ผลงาน/กิจกรรม	เดือนที่											
	1	2	3	4	5	6	7	8	9	10	11	12
ผลงานที่ 1: ได้รีเซพเตอร์เทียมที่มีรอยจดจำต่อ dopamine หรือ serotonin และ รีเซพเตอร์เทียมที่มีรอยจดจำทั้ง dopamine และ serotonin	↔											
ศึกษาวิธีการเตรียมพอลิเมอร์ที่มีรอยพิมพ์โมเลกุล			↔									
เตรียมพอลิเมอร์ที่มีรอยจดจำต่อ dopamine				↔								
ตรวจความสามารถในการจดจำของพอลิเมอร์ที่เตรียมขึ้นต่อ dopamine ด้วยการทดสอบแบบ binding assay						↔						
เตรียมพอลิเมอร์ที่มีรอยจดจำต่อ serotonin							↔					
เตรียมพอลิเมอร์ที่มีรอยจดจำต่อสารหลายชนิด โดยใช้ serotonin และ dopamine เป็นตัวพิมพ์ผสม							↔					
ผลงานที่ 2: ได้ข้อมูลเกี่ยวกับคุณสมบัติทั่วไปของพอลิเมอร์ที่เตรียมได้							↔					
ตรวจภาพพื้นผิวของพอลิเมอร์ด้วยกล้องจุลทรรศน์อิเล็กตรอน							↔					
ตรวจวัด particle size ของพอลิเมอร์ด้วยวิธี Light scattering โดยใช้เครื่อง Laser diffraction							↔					
ทำ Nitrogen adsorption เพื่อตรวจวัด pore size, pore volume, และ pore distribution								↔				
ตรวจการพองตัวของพอลิเมอร์									↔			
ตรวจความสามารถในการจดจำของพอลิเมอร์จดจำด้วยวิธี batch rebinding assay ภายใต้สภาวะอิ่มตัว										←	→	
ตรวจความสามารถในการจดจำ (recognition ability) ของพอลิเมอร์ที่มีรอยจดจำต่อตัวพิมพ์ทั้ง 4 แบบ เพื่อ											↔	
ตรวจหา Association constant (Ka) และจำนวนของ binding site บนพอลิเมอร์ (Bmax)											↔	

แผนการดำเนินงานปีที่ 2

ผลงาน/กิจกรรม	เดือนที่											
	1	2	3	4	5	6	7	8	9	10	11	12
ผลงานที่ 3: ได้ข้อมูลเกี่ยวกับลักษณะ binding group ของรีเซพเตอร์เลียนแบบ ศึกษา interaction ระหว่างโมเลกุลของ functional monomer กับตัวพิมพ์ด้วย FT-IR ศึกษา interaction ระหว่างโมเลกุลของ functional monomer กับตัวพิมพ์ด้วย 1H-NMR ศึกษา interaction ระหว่างโมเลกุลของ functional monomer กับตัวพิมพ์ด้วย computer modeling ศึกษาการเปลี่ยนแปลง conformation ของ binding site ในตัวทำละลายต่าง ๆด้วย Particle size analyser	↔											
ผลงานที่ 4: ได้ข้อมูลในคุณสมบัติการเลือกจำ และกลไกการเลือกจำโมเลกุลของรีเซพเตอร์เลียนแบบ 1 ตรวจ Cross-reactivity ของพอลิเมอร์ที่มีรอยจดจำต่อตัวพิมพ์ทั้ง 4 แบบ โดยใช้วิธี batch re-binding assay 2. ตรวจ Cross-reactivity ของพอลิเมอร์ที่มีรอยจดจำต่อตัวพิมพ์ทั้ง 4 แบบ	←→											
ผลงานที่ 5: ได้ผลการเลือกจับรวมทั้งลักษณะการเลือกจำของรีเซพเตอร์เลียนแบบต่อโมเลกุ ergot ศึกษาความสามารถในการจดจำของรีเซพเตอร์เลียนแบบที่เตรียมขึ้นสำหรับโมเลกุลของ ergot alkaloids ศึกษาการเลือกจับของ ergot alkaloids กับรีเซพเตอร์เลียนแบบที่เตรียมขึ้นด้วยวิธี chromatography						←→			←→			

แผนการดำเนินงานปีที่ 3

ผลงาน/กิจกรรม	เดือนที่											
	1	2	3	4	5	6	7	8	9	10	11	12
ผลงานที่ 6: ได้ผลการเลือกจับรวมทั้งลักษณะการเลือกจำ ของรีเซพเตอร์เลียนแบบต่อโมเลกุล ergot เพิ่มเติม ศึกษากลไกการจับของโมเลกุล ergot alkaloids บนรีเซพ เตอร์เลียนแบบที่สร้างขึ้น จากการศึกษาผลของปัจจัยต่าง ๆ เช่น ตัวทำละลาย อุณหภูมิ พี-เอช ความแรงของเกลือ เป็น ต้น ที่มีต่อการจับของโมเลกุล ergot alkaloids กับรีเซพ เตอร์เลียนแบบ ศึกษาเปรียบเทียบความสามารถในการจับของโมเลกุลยากับรี เซพเตอร์ธรรมชาติ เช่น พลาสมาโปรตีน กับการจับของยา ergot alkaloidsบนรีเซพเตอร์เลียนแบบ ศึกษาลักษณะการจับของยา ergot alkaloids กับรีเซพ เตอร์เลียนแบบที่เตรียมขึ้นเพื่อเลียนแบบ dopamine receptor และ serotonin receptor โดยใช้ computer modeling และประเมินผลที่ได้ร่วมกับผล การทดลองที่ได้จาก binding assay	←———————→			←———————→		←———————→		←→				
สรุปและอภิปรายผล เขียนรายงาน								←→	←———————→			

Chapter 1

Temperature sensitive dopamine-imprinted (*N,N*-methylene-bis-acrylamide cross-linked) polymer and its potential application to the selective extraction of adrenergic drugs from urine

1. Introduction

Natural receptors have evolved so as to achieve molecular recognition of ligands with high specificity and often efficiently bind complex molecules such as proteins. Many studies have investigated the design and the construction of synthetic receptors to mimic the selectivity of such natural receptors. In particular preparations of molecularly imprinted polymers (MIPs) have been investigated as a convenient and applicable means of creating three-dimensional networks with a cavity capable of memorizing the shape and functional group positions, complementary to the template molecule [1,2]. MIP receptors of this kind offer much potential in a number of application areas including analytical chemistry, separation science, sensor construction and drug design [3,4,5]. This is because of the potentially high selectivity and excellent stability of such polymers. For example, many kinds of MIP receptors have been prepared for the selective separation of some target compounds from liquid matrices, and these have been employed in clean-up procedures in highly sensitive analyses of such compounds in environmental and/or biological samples [6,7]. Generally, the preparation of MIP for organic compounds has been based on the hydrogen bonding interactions which occur between polymer and substrate in non-polar solvents and due to this it is much more difficult to prepare MIPs for polar compounds. Consequently, MIPs prepared for use in the sample preparation of biological and environmental samples do not usually allow the processing of samples in aqueous media.

Typically conventional molecular imprinting technology deals with highly cross-linked materials having relatively rigid structures, whereas natural receptors in contrast possess a more flexible and conformationally adaptable structure. The rigidity limits the number of binding sites available to the target molecule. Many studies have shown that lightly crosslinked polymer gels can undergo reversible swelling and shrinking under an external stimulus, which increases the number of binding interactions with the target molecule [8,9,10]. Cross-linked N-substituted polyacrylamides are among the most widely-studied polymeric materials used for the molecular imprinting of biomolecules such as protein and DNA [11,12]. These polymers continue to receive much attention in the field of controlled drug delivery [13,14] because they can undergo a temperature-controlled volume phase transition in aqueous solution [15]. Combining the properties of a thermosensitive polymer with molecular imprinting techniques may provide a promising strategy for ensuring the system responds more rapidly to an external temperature change. In the present study, the copolymerisation of acrylamide with a cross-linker and additional monomers, in the presence of a template, was used to synthesize imprinted polymers which might exhibit reversible phase transition based phenomena at ambient temperature.

Dopamine was the compound of interest employed in this study, as the template molecule. It is a naturally occurring catecholamine, which can bind to adrenergic receptors and its hydrochloride salt is used in the treatment of acute congestive heart failure and renal failure [16]. The analytical detection of dopamine in urine has been reported to provide a valuable diagnosis of neuroblastoma in patients. Several methods have been described including a fluorescence-based method [17] and ion-exchange chromatography [18] both of which allow for precise measurement of the drug. Urine samples containing dopamine and its analogs (epinephrine and norepinephrine) have been analyzed by HPLC with electrochemical detection after isolation of the compounds

using ion exchange resins [19]. Integrated MIP and temperature-controllable mediated transitions could enable a novel selective extraction method to be developed.

The aim of the current study was to seek to prepare thermoresponsive imprinted polymers and compare their recognition ability to that obtained from structurally rigid polymers, prepared using ethylene glycol dimethacrylate (EDMA) as a cross-linking monomer. Dopamine (Figure 1), a polar compound which is not soluble in any organic solvents, was used as a template molecule. It was planned to prepare the polymers in an aqueous methanol solvent, with a view to strengthening any hydrogen bond interactions between dopamine and the chosen functional monomer. The temperature-dependence of the recognition property of the prepared thermoresponsive polymer and its application as an adsorption phase for the selective extraction of dopamine and other adrenergic compounds (Figure 1) from the spiked human urine samples were also to be investigated.

2. Experimental

2.1. Materials

Ethylene glycol dimethacrylate (EDMA), *N,N'*-methylenebisacrylamide (MBAA), methacrylic acid (MAA) and acrylamide (ACM) were obtained from Aldrich Chemical Company (Milwaukee, WI, USA). 2,2'-Azobis-(isobutyronitrile) (AIBN) was purchased from Janssen Chimica (Geel, Belgium). EDMA and MAA were purified by distillation under reduced pressure. Dopamine hydrochloride, isoproterenol, serotonin hydrochloride, salbutamol sulfate, histamine, methyldopa, epinephrine and ascorbic acid (Figure 1) were obtained from Aldrich Chemical Company (Milwaukee, WI, USA). *O*-phthalaldehyde was supplied by Fluka (Buchs, Switzerland). Working standard solutions were prepared daily. All solvents were analytical grade and were dried with a molecular sieve prior to use. The extraction of urine samples using the MIP cartridge

was performed by first dissolving the drugs of the interest in the drug-free urine of healthy volunteers.

2.2. Instrumentation

UV absorbance measurements and spectra were recorded using a Hewlett-Packard diode array spectrophotometer Series 8452A (CS, USA). Fluorescence measurements were performed with a LS50B Perkin Elmer luminescence spectrometer equipped with a 150 W xenon lamp (CT, USA). Proton NMR spectra were obtained using a Varian 500 MHz FT-NMR spectrometer (CA, USA). The solid phase extraction study was developed in off-line mode using a Supelco vacuum manifold (PA, USA) connected to a vacuum pump. High-performance liquid chromatography (HPLC) was carried out using an Agilent 1100 system consisting of a quaternary pump, an autosampler, a thermostated column compartment with a built-in-six-port switching valve and fluorescence detector (CA, USA).

2.3. Polymer synthesis

In this study, four molecularly imprinted polymers (MIP1, MIP2, MIP3 and MIP4) and corresponding non-imprinted polymers (NIP1, NIP2, NIP3 and NIP4) were prepared using a thermal method involving free radical polymerization, according to that reported previously [2]. For the preparation procedure of these polymers, the polymerizing compositions listed in Table 1 were dissolved in 25 ml of methanol/water (4:1, v/v) mixture. Subsequently, the polymeric mixtures were sonicated under vacuum, purged with nitrogen for 5 min and polymerised by heating in a hot-air oven at 60 C for 24 h. The resulting polymers were crushed, ground and sieved through a 100 mesh-sieve.

The print molecule was eluted from the polymer particles by washing with three 500 ml portions 10% v/v acetic acid in methanol and subsequently with three portions of 500 ml methanol. Complete extraction of the template molecule from polymer was confirmed by the absence of dopamine in a methanol rinse of polymer, as verified using the fluorescence spectroscopic assay described in section 2.8. Finally, the polymer particles were dried under vacuum and stored at ambient temperature until required.

2.4. Characterisation methods

The mean size as well as the size distribution of the prepared particles was determined at 25 C using laser diffraction (Malvern Mastersizer, Worcester, UK) and water as the suspending medium. The mean of the triplicate measurements on the same batch was determined. The degree of swelling of the polymers was determined from the ratio between the volume of the swollen polymer and the volume of the dry polymer in each of four solvents; water, methanol, a methanol/water (4:1 v/v) mixture and phosphate buffer (pH 7.4), using calibrated measuring cylinder. A total of three replicates was used for such test. The determination of pore volume and specific surface area was carried out by nitrogen adsorption/desorption techniques using a Coulter SA3100 series surface area and pore analyzer (Coulter, USA) which enables pores between 0.3-200 nm to be measured. The samples were degassed at 120 C and a 50-point pressure table was used. The surface area was determined from a Brunauer, Emmett and Teller (BET) plot whilst the average pore diameter and the cumulative pore volume were obtained using a Barrett, Johner and Halenda (BJH) model of the adsorption isotherm.

2.5. Binding experiments

The ability of the MIP prepared to selectively recognise the template molecule in comparision to the prepared NIPs was evaluated in four different solvents; water, methanol, a methanol/water mixture (4;1 v/v) and pH 7.4 phosphate buffer after equilibration of the polymers with a dopamine solution. In a typical binding assay, the powdered polymer (50 mg) was added to 5 ml of the solvent containing 5 g.ml^{-1} of dopamine or 5 ml of the pure solvent (blank), and the suspension stirred for 24 h at room temperature (30±1 C). The polymer particles were then filtered off and the filtrate was analysed for dopamine using a fluorescence spectroscopy method. The quantity of drug in solution was determined by reference to a calibration curve. The amount of bound drug was obtained by subtracting the amount of free drug from the total amount of the drug added. The imprinting factor ( ), which represented the effect of the imprinting process, was the ratio of the amount of substrate bound by the MIP to that bound by the corresponding NIP.

The binding of dopamine to the thermoresponsive imprinted polymer (MIP4) and corresponding non-imprinted polymer (NIP4) was examined at six different temperatures (25, 30, 35, 40, 45 and 70 C) using the binding assay protocol.

2.6. Determination binding characteristics of the thermoresponsive polymers

The binding characteristics of the thermoresponsive imprinted polymer (MIP4) as well as the control polymer were further examined at three different temperatures (25, 35 and 45 C) using 50-mg samples of polymer with dopamine solutions ranging in concentration from 0.1 to 100 g.ml^{-1}, using methanol/water (4:1, v/v) mixture as medium. The amount bound (Q) was determined at each drug/polymer molar ratio (R). The binding parameters were determined from the equation, Bound/Free = (B_{max}-B)/K_d, where K_d is the equilibrium dissociation constant, and B_{max} is the maximum number of

binding sites which were obtained from the slope and intercept on *x*-axis of the straight line of the Scatchard plot, respectively. The association constant (K_a) value was obtained as the reciprocal of the K_d value. The mean dopamine binding constants calculated from triplicate independently derived results.

2.7. Selectivity evaluation

In order to verify selective recognition of the thermoresponsive MIP, the equilibrium binding analysis was examined using both a non-competitive and competitive ligand-binding assay.

2.7.1. Non-competitive ligand binding assay

Non-competitive ligand binding analysis of the polymers was determined by a saturation binding experiment using serotonin, salbutamol, isoproterenol, epinephrine and methyldopa as the related probes and histamine and ascorbic acid as non-related probes (Figure 1). Particulate polymer (50 mg) was stirred into 5 ml methanol/water (4:1 v/v) solution containing 5 g.ml^{-1} of each analyte of interest at room temperature. After 24 h, the filtrate was analysed for the amount of unbound analyte. The amount of each compound bound was calculated by subtraction of the concentration in the filtrate from the concentration in the original stock solution. The selectivity (%) was obtained by determining each specific the amount of compound sorbed per unit weight of MIP relative to the amount of dopamine sorbed.

2.7.2. Competitive ligand binding assay.

In this experiment, the putative binding sites of dopamine on the polymer were identified by a displacement assay, using the same molecular probes as those used in

the non-competitive ligand binding analysis. Particulate polymer (50 mg) was incubated with 5 ml of methanol/water (4:1 v/v) containing 5 g.ml^{-1} of dopamine and a test probe within the concentration range 0.02-20 g.ml^{-1}, for 24 h at room temperature. The changes in fluorescence intensity of dopamine in solution were monitored at 320 nm following excitation at 279 nm. The binding of dopamine in the presence of substrates was calculated and reported as the % binding of dopamine to sites on the polymer. Each experiment was repeated three times.

2.8. Analysis method

Dopamine, serotonin, histamine or salbutamol in the samples was quantified using a fluorescence spectroscopic method. A sample (1 ml) containing dopamine was transferred to a 10.0 ml volumetric flask containing 2.0 ml of pH 3.6 acetate buffer solution, and diluted to volume with methanol. The fluorescence intensity of the solution was measured at 320 nm using an excitation wavelength of 279 nm (limit of quantitation (LOQ) = 7.3 ng.ml^{-1}). The serotonin sample was determined by adding the sample (1 ml) to phosphate buffer pH 6.5 (2 ml), diluting with methanol (7 ml) and measuring the fluorescence intensity at wavelength of 335 nm with excitation wavelength at 300 nm (LOQ = 22.4 ng.ml^{-1}). The assay of histamine in samples was carried out using 0.2% *o*-phthalaldehyde as a derivertising agent. The sample (1 ml) containing histamine was transferred to 200 l of 1 M sodium hydroxide contained in a 25.0 ml volumetric flask. Hydrochloric acid (100 l, 3 M) and 50 l of 0.2% *o*-phthalaldehyde was added and the solution diluted to volume with 4:1 v/v methanol/water mixture. The fluorescence intensity of the solution was measured at 430 nm using an excitation wavelength of 330 nm against a reagent blank, prepared using the same reagent concentrations but containing no histamine (LOQ = 9.0 ng.ml^{-1}). For the assay of salbutamol-containing

samples, the fluorescence intensity was measured at a wavelength of 309 nm with the excitation wavelength fixed at 218 nm (LOQ = 0.8 µg.ml^{-1})

Isoproterenol, epinephrine or methyldopa in the samples was assayed by UV spectroscopy using a wavelength of 279 nm (LOQs of isoproterenol, epinephrine and methyldopa were 12.0 ng.ml^{-1}, 3.0 µg.ml^{-1} and 4.6 µg.ml^{-1}, respectively).

The assay of ascorbic acid-containing samples was performed using a potentiometric titration method.   A 2.0 ml sample was placed in a 150 ml vessel and 2 M potassium chloride (10 ml) added. The solution was diluted to 100.0 ml with distilled water before titration with a standard solution of 0.01 M sodium hydroxide.   The pH values were recorded after the addition of each 0.1 ml titrant added. The plot of the titrant volume *vs* pH was made to determine the end-point (LOQ = 0.5 µg.ml^{-1}).

2.9. Solid-phase extraction experiments

Twenty-five milligrams of the particulate polymer suspended in water was packed into a home-built SPE cartridge comprising a borosilicate glass tube (0.5 cm in internal diameter, 5 cm in length) with a double-walled water jacket for controlling temperature. Studies were conducted into the ability of thermoresponsive MIP to selectively extract dopamine and the other adrenergic compounds present in the mixture by evaluating the efficiency of SPE at three different extraction temperatures (25ºC, room temperature (30ºC) and 40 C).

To determine the recovery of bound material from urine samples at three different temperatures, aqueous solution (1 ml) including 5 µg.ml^{-1} of dopamine and 1.25 µg.ml^{-1} of each adrenergic compound presented as a mixture (serotonin, isoproterenol, epinephrine and salbutamol) was added to 4.5 ml of methanol and this solution was then diluted with human urine to 10 ml, before filtering to remove any insoluble material. A sample (1 ml) of filtrate was loaded onto the SPE cartridge containing either MIP or

NIP and the eluant collected for analysis. The column was then washed with 5 ml of 4:1 v/v methanol/water mixture and the analytes finally eluted with 5 ml of 1% acetic acid in methanol. Each experiment was run three times, using three different cartridges. The fractions eluted from each cartridge were collected separately and the amounts of recovered dopamine and other adrenergic compounds present in the mixture were quantified.

A reversed phase HPLC method was used for the quantitative analysis of dopamine and other adrenergic compounds after solid-phase extraction, using a method adapted from that reported by Wood and Hall [20]. Briefly, mobile phase A comprising 0.05% aqueous trifluoroacetic acid (THF)-methanol (97.5:2.5, v/v) and mobile phase B consisting of 0.05% aqueous TFA-methanol (40:60, v/v) were used for elution. An injection volume of 20 l was employed and the analytical column was a Luna 5 C_{18}, 25 cm x 0.46 cm (Phenomenex, USA). A flow-rate of 1.0 ml min^{-1} was used over 20-min with the following gradient: 0.00 min, 100% A; 1.00 min, 100%A; 16.00 min, 50% A and 50% B (linear gradient from 1 to 16 min); 16.05 min, 100% A to return column to initial condition by 20 min. The fluorescence detector used was set at $_{ex}$ 220 nm and $_{em}$ 320 nm.

3. RESULTS AND DISCUSSION

3.1. Synthesis and characterisation of polymers

Putative thermoresponsive dopamine-imprinted polymers were synthesised using two functional monomers, MAA and ACM, such that the acid or amide group of the monomers might interact with the hydroxyl groups of the dopamine template, together with MBAA cross-linker. A mixture of methanol and water (4:1, v/v) was chosen as the porogen solvent since the dopamine (HCl) template is soluble only in polar solvent and it was the aim of this work to generate a MIP for use in an aqueous environment.

Figure 1: Structure of dopamine (template molecule), other adrenergic compounds and non-related probes used in this study.

In the present work, the binding of dopamine to the thermosensitive imprinted polymer was compared to its binding to the structurally rigid polymers. The latter were prepared by using EDMA, a cross-linking monomer either singly or combined with MBAA monomer. Normally, EDMA generates an imprinted polymer that is compact,

inert and highly stable with respect to rigidity of polymer structure. Molecularly imprinted polymers and corresponding non-imprinted polymers, consisting of MBAA and/or EDMA as cross-linker, were created following a common protocol for MIP synthesis using the compositions listed in Table 1. The physical characteristics of the polymers were examined and the data are summarised in Table 2. In general, the MBAA cross-linked polymers (MIP4, NIP4) were found to have both a large pore size and pore volume compared with the more structurally rigid polymers (MIP1, MIP2, MIP3, NIP1, NIP2 and NIP3). Both NIP4 and MIP4 particles possessed micropores in the polymer network, and also exhibited specific swelling properties that were different to the structurally more rigid EDMA-cross-linked polymers. These observations show that the physical properties of the prepared polymers were markedly dependent upon the cross-linking monomer employed. It is apparent that the pore diameter and specific surface area of the MBAA cross-linked NIP (NIP4) were almost twice as large as those of the corresponding MIP (MIP4). Also, the pore volume of NIP4 was larger than that of MIP4. By contrast, pore diameter, pore volume and specific surface area of the NIPs and MIPs of the structurally more rigid EDMA cross-linked polymers were not significantly different. Indeed, either MIP4 or NIP4 was prepared with the same polymer component and the same polymerising conditions except the print molecule was present in the polymerising phase only when the MIP4 was synthesized. Hence, the smaller pore size of MIP4 in comparison to that of NIP4 must be related to the presence of dopamine during the preparation process. Probably, the dopamine template present in the lightly MBAA-cross-linked polymer gels causes a compactness of the size of the cavities, within gelling network, during the change of temperature from polymerising temperature (60 C) to extraction temperature (room temperature). This may have accounted for the specific surface area of MIP4 being lower than the specific surface area of the control.

Table 1: Polymer composition.

Composition	MIP1	NIP1	MIP2	NIP2	MIP3	NIP3	MIP4	NIP4
Dopamine HCl (g)	0.62	-	0.62	-	0.62	-	0.62	-
MAA (g)	1.12	1.12	0.56	0.56	-	-	0.56	0.56
ACM (g)	-	-	0.46	0.46	0.46	0.46	0.46	0.46
EDMA (g)	9.72	9.72	6.48	6.48	6.48	6.48	-	-
MBAA (g)	-	-	-	-	1.10	1.10	5.04	5.04
AIBN (g)	0.71	0.71	0.71	0.71	0.71	0.71	0.71	0.71

Table 2: Characteristics of synthesised polymers.

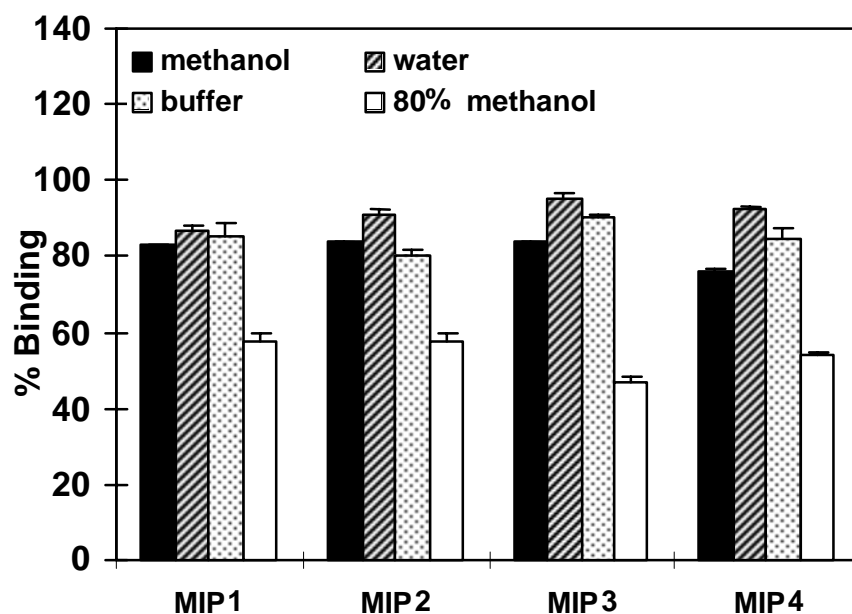| Polymer	Functional monomer	Cross-linking monomer	Elemental analysis (%)				Particle size[a] (μm)	Pore diameter (nm)	Pore volume[b] (ml/g)	BET Surface area[b] (m^2/g)	Specific swelling (ml/ml)			
			C	H	O	N					MeOH	Water	Mixture[c]	buffer
MIP1	MAA	EDMA	0.66	7.2	26.8	-	8.26	15.91	0.0010	3.61	-	-	-	-
NIP1	MAA	EDMA	65.8	7.1	27.1	-	12.75	14.42	0.0007	2.17	-	-	-	-
MIP2	MAA-ACM	EDMA	65.7	7.3	26.7	0.4	9.36	8.96	0.0011	2.93	-	-	-	-
NIP2	MAA-ACM	EDMA	66.1	7.1	26.5	0.3	10.29	15.21	0.0006	2.63	-	-	-	-
MIP3	ACM	MBAA-EDMA	53.4	7.2	37.9	1.5	5.85	18.72	0.0015	6.29	-	-	-	-
NIP3	ACM	MBAA-EDMA	52.7	7.3	38.5	1.5	6.58	18.31	0.0016	5.72	-	-	-	-
MIP4	MAA-ACM	MBAA	55.1	7.4	24.3	13.3	19.93	30.17	0.2369 (0.014)	130.41 (34.37)	1.12	1.00	0.87	0.80
NIP4	MAA-ACM	MBAA	55.7	7.6	23.4	13.3	36.72	54.55	0.2633 (0.0068)	213.32 (20.78)	0.62	0.57	0.25	0.27

a) Approximate mean in particle size

b) The micropore surface and pore volume (values in parenthesis) from a *t*-plot using Harkins-Jula average thickness.

c) Refer to 4:1, v/v methanol:water solvent.

3.2. Media effects on ligand-binding of MIPs

The influence of various binding media parameters (the type of the solvent, pH and ionic strength) was studied further so as to provide optimised parameters for binding in aqueous media, with a view to developing a thermoresponsive imprinted polymer adsorbent suitable for SPE analysis. Initially, the influence of the solvent on the recognition properties of MIP4 was studied and the binding of dopamine for this polymer was compared with that of the more structurally rigid polymers (MIP1, MIP2 and MIP3). Figure 2 shows the binding of dopamine to the various MIPs and corresponding NIPs when solvated in water, methanol, methanol/water (4:1 v/v) mixture and pH 7.4 buffer. Dopamine was bound in lowest amount when present in methanol/water (4:1 v/v) solvent compared to the other solvents. The non-specific adsorption of dopamine to all MIPs from methanol, buffer and water was very high. However the methanol/water (4:1 v/v) mixture which was used as the preparation phase of the MIPs was found to be a better solvent for selectively binding of dopamine to the polymers, as the imprinting effect was found to be most marked in this solvent. In general, the imprinting factor of the MIPs prepared using mixed functional monomers was higher than that to the MIPs prepared using a single functional monomer. The selective adsorption of dopamine to the MIP prepared using MBAA when equilibrated in methanol/water (4:1 v/v) was higher than that to the MIP prepared using EDMA (= 0.98 or 1.16) or mixed EDMA/MBAA (= 1.39). This demonstrates that MIP4, prepared with MBAA as the cross-linking monomer, (= 1.87), provided better recognition than the more structurally rigid MIPs prepared using EDMA as the cross-linking monomer. This could be explained by the rigidity of the EDMA polymer preventing the cavity having sufficient flexibility to orientate so that maximum binding occurs within the polymer matrix. Also, the hydrophobic properties of the EDMA-containing polymer may promote a higher non-specific adsorption of the drug to the polymer when placed in an aqueous medium.
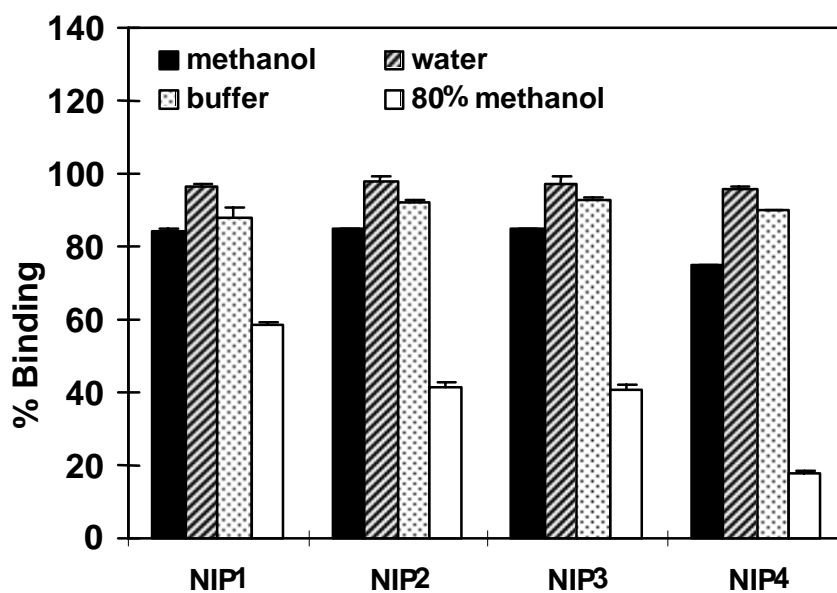
(a)

(b)

Figure 2: Effect of solvent on the % dopamine (employed as the template molecule) bound to various (a) MIPs and (b) corresponding NIPs synthesized in this study.

MAA and ACM, employed as functional monomers for imprinting in this study contain both amide and carboxylic acid functional groups which can interact with the hydroxyl group of dopamine *via* non-covalent bonding. The amide group of ACM is not ionizable, whereas the carboxylic group of MAA monomer can ionize and hence a change in non-specific adsorption of the dopamine to the polymer can occur in aqueous medium as a function of pH. In fact medium pH had a large effect on the binding of dopamine to MIP4 and NIP4 (Figure 3) with a low pH (pH 3 and 4) to much less binding of dopamine to the polymers than occurring at low pH (pH 3 and 4) compared to when the latter polymers were incubated with drug at a high pH (pH 5-7).
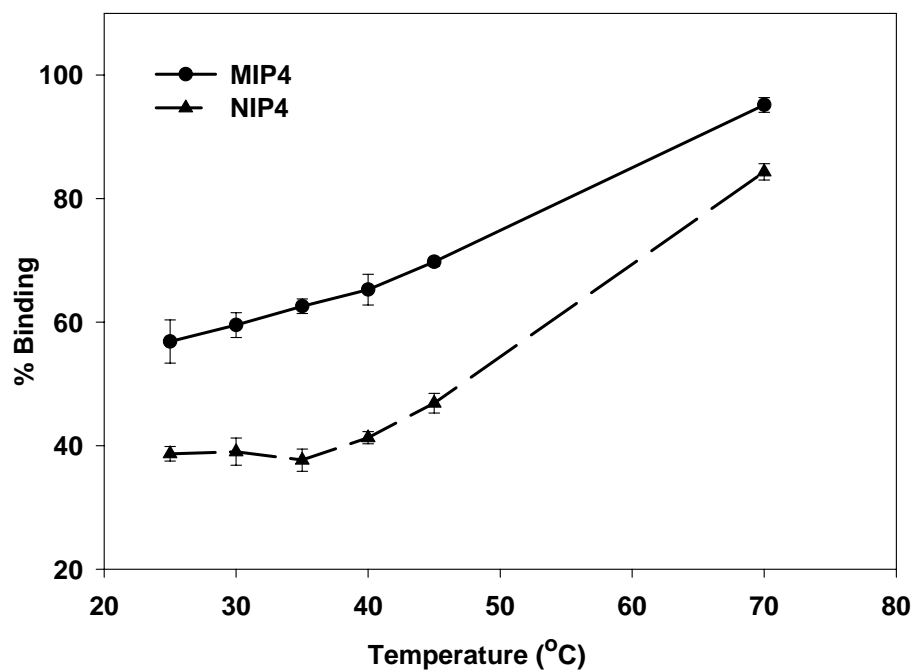

Figure 3: Effect of medium pH on the % dopamine (employed as the template molecule) bound to MIP4 and NIP4.

Dopamine (pK_a = 10.6) will be charged positively over the whole pH working range (pH 3-7), whereas at the higher pH values the thermoresponsive polymer will be effectively negatively charged. Thus non-specific electrostatic interaction between drug and polymer might be expected to occur and indeed the amount bound of dopamine
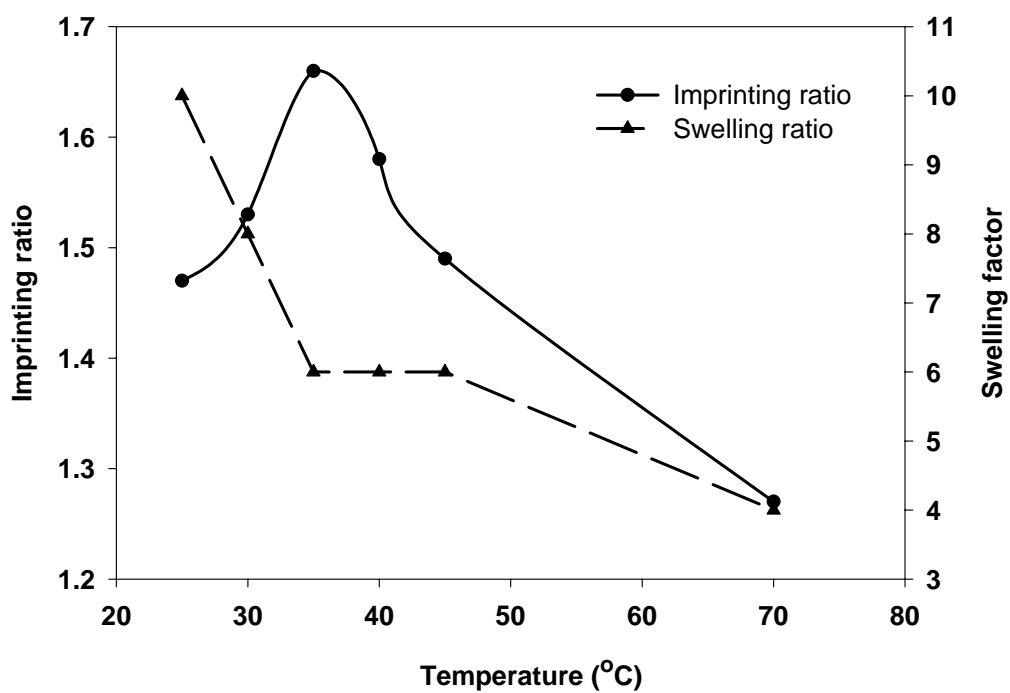
bound to the MIP and NIP was not significantly different at any of the pH values studied. In addition the NIP synthesized in this study had a larger specific surface area than the corresponding MIP providing a greater area for a higher non-specific binding of dopamine from solution. Since the selectivity of binding between MIP4 and dopamine was greatest from the methanol/water (4:1, v/v) mixture, it was thought that this solvent would be suitable to be employed in any SPE employing MIP4 as the imprinted polymer.

3.3. Temperature effect on recognition ability

The ability of the thermoresponsive MIP (MIP4) to recognize template molecule after a dynamic change in swelling was evaluated by equilibrium binding analysis. The experiment was performed at temperatures ranging from 25-70 C. Figure 4(a) shows effect of temperature on % binding of dopamine to MIP4 and control polymer (NIP4) in methanol/water (4:1, v/v) mixture. The temperature dependent swelling of the thermoresponsive MIP is also shown in Figure 4(b). The adsorption pattern of dopamine to the MIP varies with temperature, with sorption increasing as a function of temperature (see Figure 4a). A temperature increase from 25-35 C promoted binding of the template molecule to the MIP, whilst binding to the corresponding NIP scarcely changed. The imprinting factor of the MIP was highest at a temperature of 35 C and this appeared to correspond to the transition temperature of the thermoresponsive MIP. At temperatures beyond 35 C, it was found that binding of dopamine to both the MIP and NIP gradually increased but that the increase in the binding to the non-selective polymer was greater than to the MIP (Figure 4a), resulting in a decrease in the imprinting factor (Figure 4b). This result suggests that the higher binding to the thermoresponsive MIP at high temperatures (45 C) is likely to be primarily the consequence of increasing non-specific adsorption of the template

(a)

(b)

Figure 4: Effect of temperature on (a) the % dopamine to MIP4 and NIP4 and (b) binding affinity and swelling ratio of polymer of MIP4 in methanol/water (4:1 v/v) (mean ± SE, n=3).

molecules. The % change in binding as a function of temperature also allows the activation energy of dopamine binding to sites within thermosensitive MIP to be determined and the latter was found to be 9.83 Kcal.mol^{-1}. The results show that binding to MIP4 was temperature sensitive and although a degree of molecular selectivity was apparent either in the more swollen or collapsed states, at the transition temperature, 35°C, the recognition of dopamine by the MIP was maximal.
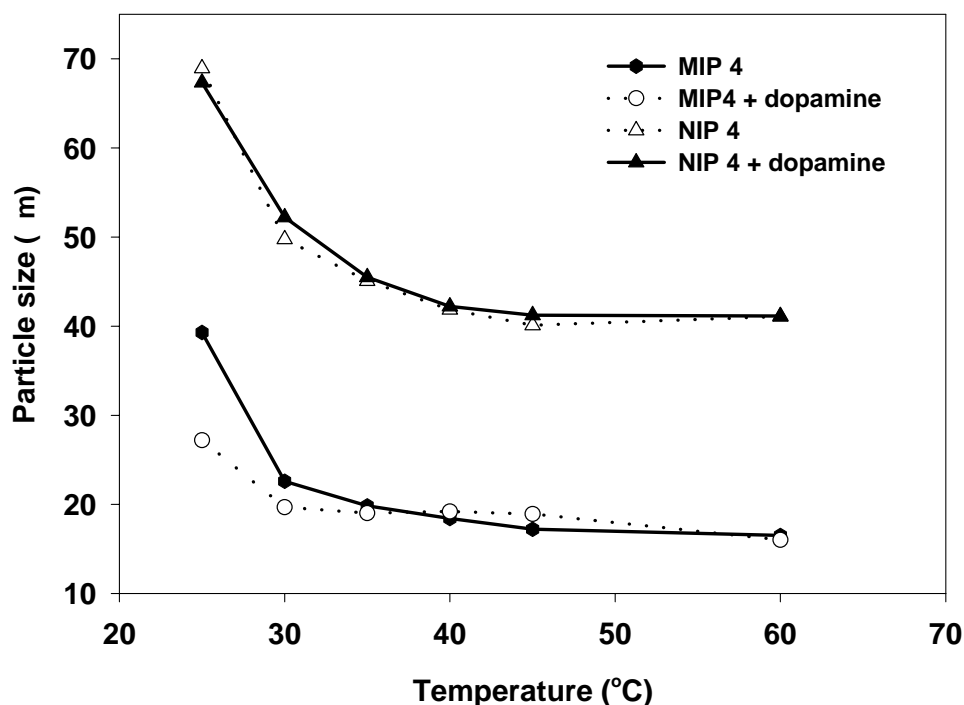

Figure 5: Effect of temperature on the mean volume diameter of polymer particles of the thermoresponsive imprinted polymer (MIP4) and non-selective polymer (NIP4), in the presence and absence of dopamine template dissolved in methanol/water (4:1 v/v) (mean ± SE, n=3).

The size of either MIP or NIP particles was measured before and after 30 min-exposure to dopamine in methanol/water (4:1 v/v) solution at temperatures ranging from 25 - 60 C. As seen in Figure 5, the thermoresponsive MIP decreased in size in the presence of template molecule, although this effect was only apparent at temperatures

below 35 C. In contrast, the change in the size of polymer particle of the corresponding NIP exposed to dopamine was relatively little. This result suggests that the shrinkage of the swollen MIP at fixed temperatures below 35 C which occurs in the presence of dopamine is a consequence of the binding of the template molecule at the imprint sites. However, at temperatures over 35 C the size of polymer exposed to dopamine did not change as a function of temperature. It is apparent that the polymer shrinking/swelling in response to the presence of template was affected by temperature. At higher temperatures the MIP forms a highly compact polymer structure in which binding within the polymer may be hindered. This could account for the decrease in selectivity of the thermoresponsive MIP at higher temperatures (40 C). It would appear that the selectivity of the thermoresponsive MIP is controlled by the size of the cavities in the polymer, and that conformational changes may be required to open the cavity to enable greater binding to occur. A previous study has demonstrated that ligand binding can affect the geometry of a protein binding site, with significant rearrangements occurring upon ligand binding [21]. An approach towards the handling of ligand-induced domain movements has been reported by Sandak and co-workers [22]. The flexibility of the thermoresponsive MIP may enable movement of the polymer domains forming the binding cavity, which in turn affects the degree of molecular recognition between the ligand and receptor.

Previous studies have demonstrated that a change in pH, ionic strength, solvent or temperature can alter the conformation of polymer chains within structure of MIPs and that these have a strong effect on the polymer recognition properties [23, 24]. Turner et al. [25] showed that any factors that alter the surface potential and conformation of polymer chains will change the size and shape of template-complementary binding pockets thereby disrupting binding. These latter workers also

reported that a high buffer concentration can increase the recognition of the MIP such that a compactness in the structure of the polymer is promoted. The results obtained in the present study suggest that thermal-stimuli are capable of changing the binding properties of the thermoresponsive MIP, which is factor that may be exploitable for the selective extraction of the target compound from aqueous media by the polymer.

The influence of temperature on the binding characteristics of the thermoresponsive MIP was examined further. It was found that the adsorption isotherms of the imprinted polymer fitted well to the bi-Langmuir model with a predominance of high-affinity binding sites but with a low fraction of low-affinity binding sites being present. This suggests that the recognition sites of the MIP are heterogeneous. The association constant (K_a) and binding capacity (B_{max}) values of recognition sites at various temperatures (25, 35 and 45 C) are shown in Table 3. Increasing temperature from 25 C to 45 C greatly increased the K_a value of high-affinity binding site of the MIP, while the K_a values for the high and low-affinity binding sites of the corresponding NIP were also increased but to a lesser extent. A significant decrease in binding capacity with increasing temperature was observed in the case of high-affinity binding site for MIP. The results show that an increase in temperature increases the efficiency of the binding of the template with the polymers. The great increase in the binding affinity of high-affinity binding site of the MIP when temperature increases is most likely due to the increased the strength of the interactions between complementary functionalities in the template and polymer, within imprint cavity. It is possible that the polymer having shrunken dimensions at higher temperatures facilitates a higher order of molecular association. A greater dominance of hydrophobic forces within a more dehydrated polymer matrix might also promote binding affinity.
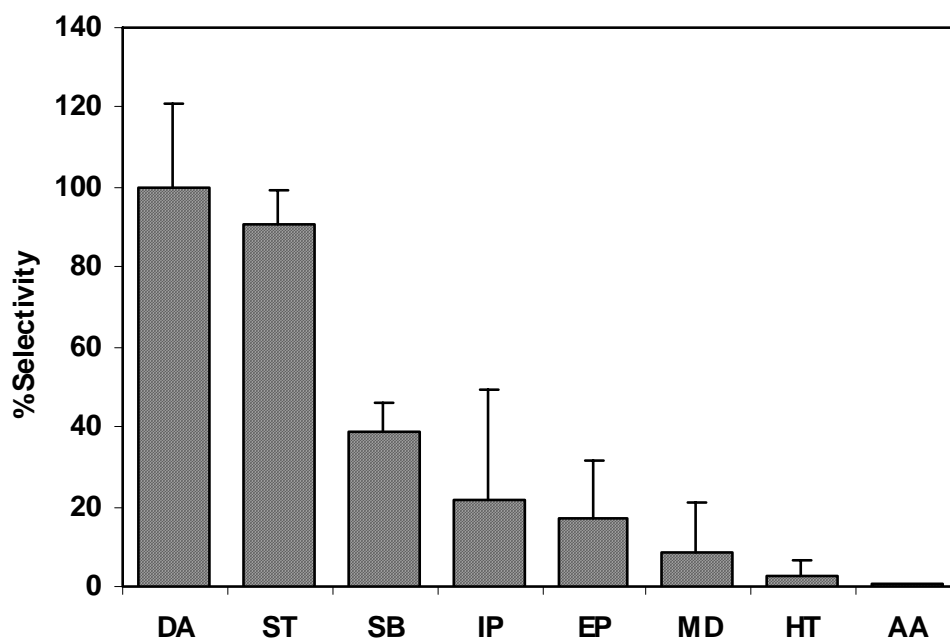
Figure 6: The selectivity of polymer for binding of dopamine and analogs. (mean ± SE , n=3). DA = dopamine, ST = serotonin, SB = salbutamol, IP = isoproterenol, EP = epinephrine, MD = methyldopa, HT = histamine, AA = ascorbic acid

Table 3: The binding characteristics of the thermo-responsive polymers at various temperatures.

Temp. (ºC)	MIP4				NIP4			
	High affinity site		Low affinity site		High affinity site		Low affinity site	
	K_a (mM^{-1})	B_{max} (µmol g^{-1})	K_a (mM^{-1})	B_{max} (µmol g^{-1})	K_a (mM^{-1})	B_{max} (µmol g^{-1})	K_a (mM^{-1})	B_{max} (µmol g^{-1})
25	1.70 ± 0.25	61.17 ± 34.2	0.84 ± 0.36	1.59 ± 1.02	0.70 ± 0.21	0.15 ± 0.10	0.42 ± 0.05	1.37 ± 0.18
35	9.09 ± 0.92	3.48 ± 0.70	0.80 ± 0.31	0.89 ± 0.02	0.71 ± 0.61	0.39 ± 0.15	1.35 ± 0.52	2.71 ± 1.06
45	49.70 ± 7.93	1.22 ± 0.20	4.35 ± 0.80	0.77 ± 0.30	3.85 ± 0.65	0.80 ± 0.20	7.69 ± 1.79	3.12 ± 1.35

K_d = Dissociation constant
B_{max} = Binding capacity

3.4. The specific binding site of the thermal-responsive polymer

Molecular selectivity in imprinted polymers is often demonstrated by comparing the extent of binding of the template molecule in comparison to the binding of molecules with similar features. This affords an indication of the extent of cross-reactivity between the selected molecules and the polymer. The binding selectivity of the prepared thermoresponsive MIP for its template and a range of structural analogues (serotonin, salbutamol, isoproterenol, epinephrine and methyldopa) as well as non-related compounds (histamine and ascorbic acid) (Figure 1) was determined. The results suggest that structurally related compounds bound more effectively to the MIP than non-related compounds, such as histamine and ascorbic acid (see Figure 6). This indicated that it was possible to produce a temperature sensitive imprinted polymer with selectivity towards dopamine but with a reasonable cross-reactivity to dopamine analogs which contained the catecholamine structure.
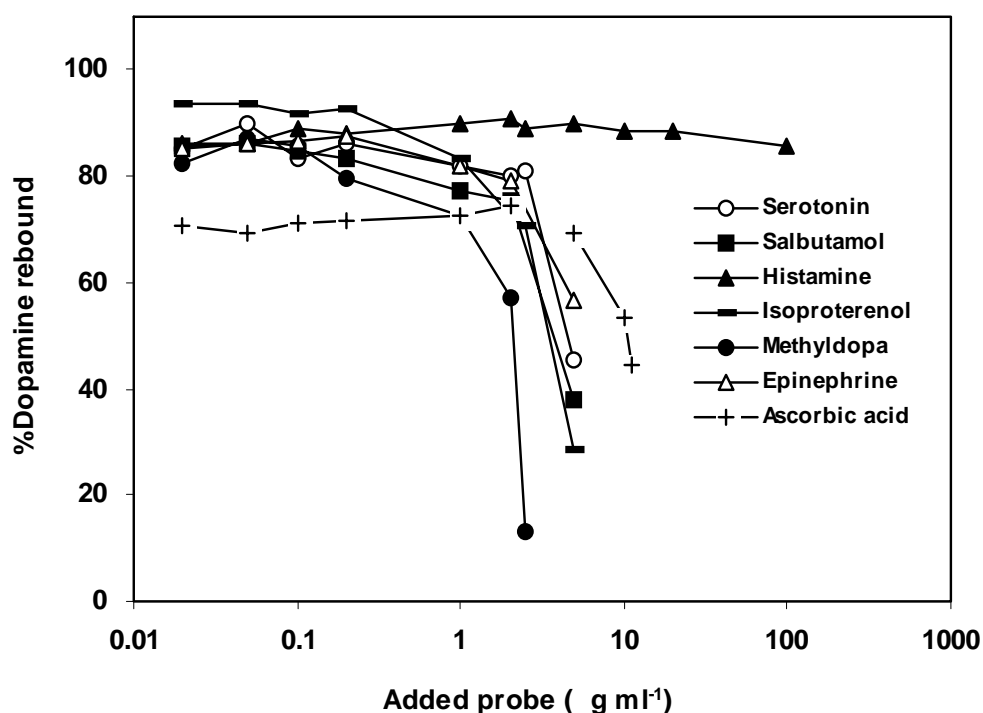

Figure 7: Displacement of dopamine by other compounds.

Subsequent competitive ligand binding tests showed that the relative affinity of the thermoresponsive polymer for molecules related to dopamine was greater than for non-related dopamine compounds, with histamine and ascorbic acid in particular showing very poor dopamine displacement characteristics (Figure 7). Competitive binding for the selected probes occurred only at high concentrations. Non-specific probes would be expected to bind to low-affinity sites with a 'less good' template complementary only when present at higher concentrations. These results confirm that the thermoresponsive MIP binds the template molecule strongly but that there is partial cross-reactivity to structurally closely related compounds. This specificity of the thermoresponsive MIP might be explained on the basis of the molecular recognition, which relates to binding sites having shape and size selection as well as the correct spatial orientation of the functional groups in the MIP binding sites [26,27].

3.5. Application of the thermal-responsive polymer to SPE

The feasibility of using the generated thermoresponsive MIP in an SPE column to recover dopamine and related compounds from a mixture in urine was examined. The influence of temperature on polymer capacity when employed in the SPE protocol was also determined since use of an elevated temperature does potentially offer an elegant approach to promoting specific adsorption, which may increase the selectivity of the polymer. Table 4 shows % recovery of compounds from a mixture of compounds using the thermoresponsive MIP and corresponding NIP at various temperatures. The template was not detectable in the initial breakthrough samples from the MIP loaded SPE column at any of the temperatures studied, indicating that dopamine remained selectively bound through specific interactions with the imprinted binding sites within the polymer. In contrast dopamine was detected in the breakthrough sample of the NIP loaded column particularly at temperature 25 C but also at room temperature (30±1 C),

although at higher temperatures (40 C) dopamine remained bound to the NIP. More of the template molecule was retained on the MIP other than NIP and more was eluted by the 9:1, v/v methanol:acetic acid solvent. In addition to the template, the MIP displayed selectivity in retaining serotonin, which has structural similarities to that of the template (Figure 1). The recovery of serotonin from the column was also temperature dependent (see Table 4).
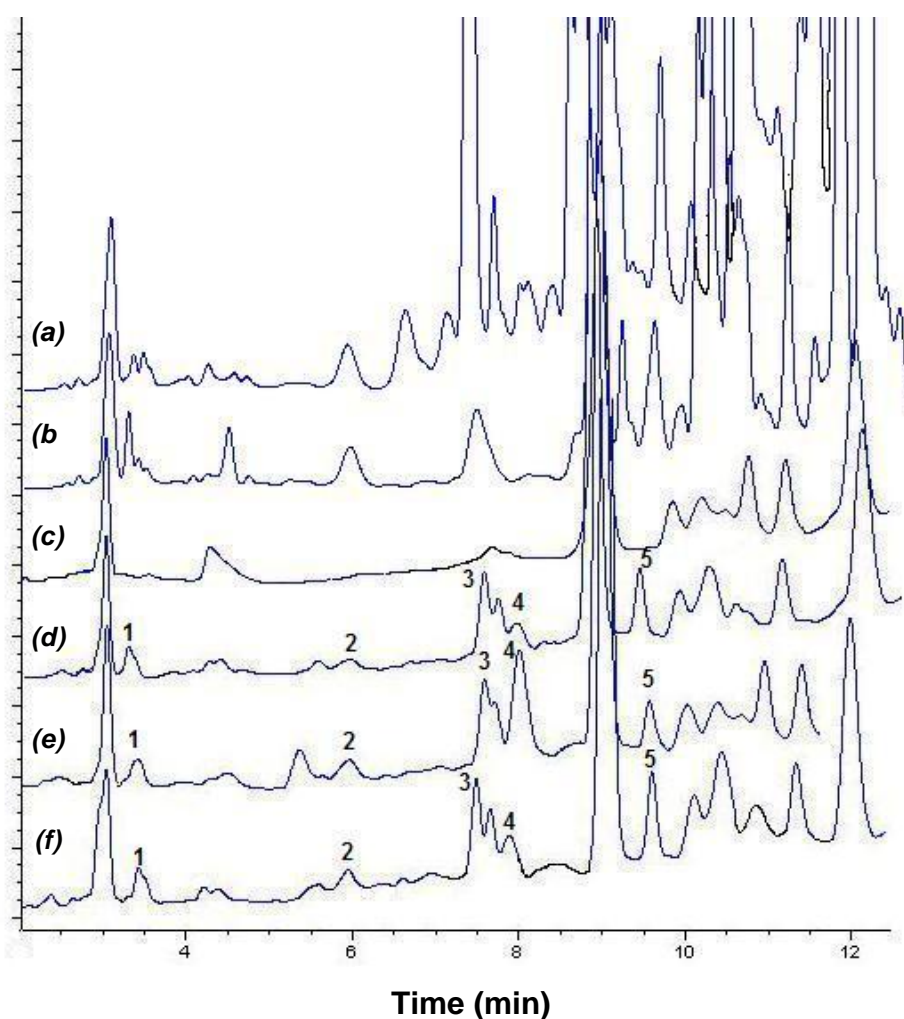

Figure 8: HPLC chromatograms of urine blank and spiked urine sample before and after extraction of the various analogues after elution through a thermoresponsive dopamine-imprinted polymer cartridge (using methanol/water, 4:1 v/v and methanol/acetic acid, 9:1 v/v as the elution solvents) at different temperatures. *(a)* urine blank; *(b)* spiked urine sample; *(c)* urine blank after extracting by MIP cartridge at room temperature; *(d)* spiked urine sample after extraction at 40°C; *(e)* spiked urine sample after extraction at room temperature (~30°C); *(f)* spiked urine sample after extraction at 25°C. 1 = epinephrine, 2 = dopamine, 3 = isoproterenol, 4 = serotonin, 5 = salbutamol.

Table 4: Mean recovery (%) of dopamine (0.5 µg.ml^{-1} in urine) and other adrenergic compounds (0.125 µg.ml^{-1} in urine) after solid-phase extraction through MIP and NIP containing cartridges at various temperatures[*].

Fractions	Temperature (ºC)	Recovery (%)									
		Dopamine		Serotonin		Salbutamol		Isoproterenol		Epinephrine	
		MIP	NIP	MIP	NIP	MIP	NIP	MIP	NIP	MIP	NIP
Breakthrough (1 ml)	25	0	13.5	13.4	11.0	27.7	37.1	30.1	37.1	19.8	18.0
	30	0	17.2	12.1	34.0	37.1	25.7	31.9	25.7	20.3	19.1
	40	0	0	14.4	5.7	45.9	41.2	47.3	31.9	29.3	31.2
Methanol:water, 4:1 v/v (5 ml)	25	33.3	74.4	14.6	70.4	77.6	69.8	76.0	69.8	82.5	84.2
	30	24.3	72.3	31.6	59.7	63.6	77.6	72.4	71.4	82.2	84.9
	40	14.2	63.7	10.1	77.9	60.1	61.3	42.4	52.3	72.3	79.2
Methanol:acetic acid, 9:1 v/v (5 ml)	25	36.6	12.1	25.8	22.6	0	0	0	0	0	0
	30	46.7	10.5	59.6	6.4	0	0	0	0	0	0
	40	51.3	36.2	21.7	4.4	0	0	0	0	0	0
Total recovery	25	69.9	87.9	53.8	104.0	105.3	106.9	106.1	106.9	102.3	102.2
	30	71.0	89.5	101.3	101.1	100.7	103.3	104.3	97.1	102.5	104.0
	40	65.5	99.9	46.2	88.0	106.0	102.5	89.7	84.2	101.6	110.4

[*] The relative standard deviations (RSDs) were 2-10% (n = 3)

The elution of human urine spiked with dopamine and analogues through the MIP columns when employing the SPE protocol at different temperatures (Figure 8) resulted in different enrichment profiles of the various compounds. At room temperature (30±1ºC) the clean-up and enrichment of the sample in dopamine and serotonin using the thermoresponsive MIP was greater than that achieved at the other temperatures (25 and 40ºC). Marked interference with the absorbance due to the analyte peaks was found to occur due to absorbing compounds within the urine. However even though some of these interfering compounds still co-eluted with the analytes after the SPE, the feasibility of carrying out an assay based on this method was proven. The pretreatment of human urine using SPE with the thermoresponsive MIP enabled total recoveries of dopamine and other adrenergic compounds to be achieved with the values in this study ranging 70-106%, depending on extraction temperature.

4. CONCLUSION

The design and synthesis of thermal-responsive materials for separation process was demonstrated in this study. An adsorption phase consisting of molecular recognition and thermal-responsive elements has been developed and evaluated for application in the separation of dopamine and analogues contained in urine samples, using SPE. The results in the present study demonstrated that combining the thermosensitive polymer with molecular imprinting techniques generated a molecular recognition material which could respond more rapidly to an external temperature change. The material could be employed in aqueous environments and enabled a selective recognition of dopamine and its analogues to be produced. The potential application of this material as a selective sorbent for SPE in the assay of dopamine in human urine has been urine has been demonstrated with some degree of success, although a fully validated assay has not established. Further investigation and development of the system is warranted with

a view to developing a thermoresponsive MIP material having high selectivity and suitable properties for applying as selective sorbent phase of SPE or even as recognition material in other uses, *e.g.* chromatographic separation, sensor and immunoassay.

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Chapter 2

"A Molecularly Imprinted Artificial Receptor for the Screening of Ergot Family"

1. Introduction

Ergots are a type of ergoline derivatives which possess a wide and different spectrum of pharmacological activities including central, peripheral and neurohumoral effects, due to their capability to bind unselectively to adrenergic, dopaminergic and serotonergic receptor sites[1-3]. Compounds of this class (see general structure in Figure 1) may act as agonists or antagonists at the receptor sites of biogenic amine neurotransmitters, and may be also assume a partial agonist and antagonist role. Interesting for drug discovery and development is the dopamine agonist activity of ergolines, which has many important clinical implications such as treatment in Parkinson's disease and hyperprolactinaemia[4-5]. Several chemical modification and synthetic variation of the ergot compounds have been done with the aim of finding compounds with a narrower range of activity with more selective, more specific effect [6-7], which modification of the ergoline skeleton, is shared by all the ergots present many challenges in the development of new dopaminergic agents as well as the identification of new series of serotonergic agents[8-9]. Study in binding affinity of this class of compounds with either dopaminergic or serotonergic receptor is required, in addition a number of research groups are generating large number of ergot-derived compounds to test structure activity relationships.

A high-throughput screening assay system is necessary for large scale screening for ligands of dopaminergic and serotonergic receptors. To facilitate the determination of ligand binding specificity and affinity several biochemical assays have been developed.

Traditional dopamine or serotonin receptor assays for analysis of involve filtration, transfer and washing, and the use of radiolabeled probes[10]. Some studies sought to examine equilibrium binding constants using chromatography using resins to separate the protein-bound and unbound forms of radiolabeled probes[11-13]. However, chromatography and other techniques which utilize physical separation of bound from free ligand often perturb the equilibrium, a process which can result in dramatically altered affinity constant[14]. Chromatography is also limited by its reliance on radiolabeled ligands, a requirement that can prove to be bothersome when one considers the costs of using radioactive materials and the difficulties in obtaining the compound. Another approach has been the examination of binding interactions through the use of fluorescence probes. However, the probes often contain large, hydrophobic moieties that are necessary for their fluorescent character. The hydrophobic moieties change the chemical nature of the probes so that they exhibit properties that may not be identical to their native counterparts. Therefore, the use of this type of probes to obtain physiological relevant equilibrium constant must be made with caution. For study in an affinity binding of ergot compound with the receptors, the isolated dopamine and serotonin receptors of sacrificed animals *e.g.* rats[15] or some other rodent species[16] are used for this purpose. However, the limited operation and storage stability of the native receptors, along with the difficulties associated with their preparation and isolation, are among the drawbacks that have limited their use for study. In addition, the variation in result with different species of the tested animal can be reluctant for discussion. All these make it difficult to adapt the assays into high-throughput screening assay for dopamine and serotonin receptors

The molecularly imprinted polymer which can be applied to use in a competitive radioligand-binding assay, or so-called molecularly imprinted polymer sorbent assay (MIA), is similar to solid-phase radioimmunoassay, but the immobilized antibody is

replaced by an imprinted polymer[17]. This method can distinguish between the bound and free forms of a ligand-receptor complex without physical separation have proven to be very useful. A competitive fluorescent ligand binding analysis technique is among the best adapted for use with imprinted polymers, because it allows highly sensitive detection, depending on the fluorescent label, be performed both in aqueous and organic solvents.

In this article, we describe a fluorescent molecularly imprinted sorbent assay to study receptor-ligand binding of ergots by use a displacement assay of unlabeled dopamine/serotonin as probes and present the application of the molecularly imprinted sorbent assay in affinity screening of the ergot-derived compounds. In this study, dopamine and serotonin that are the endogenous dopaminergic agonist and serotoninergic agonist of biological dopamine and serotonin receptors were employed as the printed molecules for creating the selectivity to molecularly imprinted polymer adsorbent phase. Also these compounds were adopted as the fluorescence probes in a competitive molecularly imprinted binding assay. The affinity of ergot for the dopamine and serotonin binding site can then be assessed by observing the increase of the free dopamine or serotonin probe in the presence of increasing concentrations of ergot compound. In generating mixed dopamine and serotonin-binding site of a molecularly imprinted polymer, a molecular imprinting using multiple-template technique was applied with a free-radical cross-linking copolymerization of two functional monomers, methacrylic acid (MAA) and acrylamide (ACM) with *N,N*-methylene-bis-acrylamide (MBAA) as cross-linker in the presence of dopamine and serotonin as the template mixture in 80% aqueous methanol solution. The ability of the model ergot compounds (i.e. ergocryptine, ergocornine, ergocristine, ergonovine, agroclavine, pergolide and terguride) to displace bound dopamine (D) and serotonin (S) from the binding sites using a developed competitive fluorescent ligand binding assay was measured and

compared to those obtained using an ELISA-based immunoassay data obtained using D/S receptors derived from the rat hypothalamus. The results indicate the applicability of the assay in characterizing the ligand binding characteristics of ergot derived molecules. The molecularly imprinted sorbent assay with DS-MIP is present for application ergot binding studies using displacement of unlabeled-(radioligand) dopamine/serotonin which this can be used without physical separation.

2. Experimental

2.1. Reagents and chemicals

Dopamine hydrochloride, isoproterenol, serotonin hydrochloride, salbutamol sulfate, histamine, methyldopa) epinephrine and epinine purchased from Sigma-Aldrich (Milwaukee, WI, USA). Ergocryptine, ergocornine, ergocristine, ergonovine, agroclavine, pergolide and terguride were obtained from Sigma-Aldrich (Milwaukee, WI, USA). *N,N'*-methylenebisacrylamide (MBAA), methacrylic acid (MAA) and acrylamide (ACM) were purchased from Aldrich Chemical Company (Milwaukee, WI, USA). 2,2'-Azobis-(isobutyronitrile) (AIBN) was purchased from Janssen Chimica (Geel, Belgium). Sodium heptanesulfonate and pentafluoropropionic anhydride were purchased from Sigma-Aldrich (Milwaukee, WI, USA). MAA was purified by distillation under reduced pressure. Working standard solutions were prepared daily. All solvents were of either analytical or HPLC grade.

2.2. Apparatus

UV absorbance measurements and spectra were recorded using a Hewlett-Packard diode array spectrophotometer Series 8452A (CS, USA). Fluorescence measurements were performed with a LS50B Perkin Elmer luminescence spectrometer

equipped with a 150 W xenon lamp (CT, USA). C,H,N analysis of polymers was made on the CE Instrumentals Flash 1112 (Milan, Italy). An Agilent 1100 HPLC system (Hewlett-Packard, CS, USA) consisting of a G1322A quaternary pump, G1322A in-line solvent degasser, G1313A auto injector (20 l injection loop), equipped with a Hewlett-Packard 1049A programmable electrochemical detector by a 35900E Hewlett-Packard interface was employed. HPLC data were collected and analysed on a personal computer using HP ChemStation (Hewlett-Packard, CS, USA).

2.3. Polymer Synthesis

The DS-MIP was prepared by in situ polymerization of MAA, ACM and MBAA at 1:2:2:10 mole-ratio of template:MAA:ACM:MBAA, but in the presence of *D* and *S* as mixed template at 1:1 mole ratio. In study selectivity of the DS-MIP, the dopamine and serotonin molecularly imprinted polymer made by using single template polymerization was used as the reference polymer. The single-recognition MIPs were prepared by using *D* and *S* as single template, using 2 mole-ratio of the template and the same monomeric ratio as that used for preparation of the multiple-recognition material, DS-MIP. A non-imprinted polymer (NIP), which was included as the control, was prepared in the same way as the MIPs but omitting the template molecules. The polymers were synthesized and characterized by using the procedure described previously[18]. In the typical polymer synthesizing process, the monomeric components were dissolved in 25 ml of a methanol/water (4:1 v/v) mixture. Subsequently, the solution was purged with a stream of nitrogen gas for 5 min to remove the radical scavenger oxygen. The polymerisation was carried out at 60ºC for 18 h in a hot-air oven. The resulting polymers were crushed, ground and sieved through a 100 mesh-sieve. The polymer particles were washed with many portions of first 10% v/v acetic acid in methanol (500 ml) and methanol (500 ml). Complete extraction of the template molecule from the

polymer was confirmed by the absence of the template in a methanol rinse of polymer, as verified using the fluorescence spectroscopic method same as that in the binding analysis study. Finally, the polymer particles were dried under vacuum and stored at ambient temperature until required. The dopamine and serotonin imprinted polymer of single polymerization was achieved by mixing an equal amount of two individual recognition MIP at 1:2:2:10 mole ratio of template:MAA:ACM:MBAA. The physical properties of the DS-MIP: mean size, size distribution, pore volume, surface area and degree of swelling were determined[18].

2.4. Determination in Selectivity of DS-MIP

The recognition property of the DS-MIP , NIP and the reference polymers (1:1 w/w D-MIP and S-MIP mixture, D-MIP and S-MIP) was evaluated in a various solvents : acetonitrile, water, methanol and methanol/water mixture (4;1 v/v) in parallel experiments with two individual recognition MIP (D-MIP, S-MIP)and the dopamine and serotonin imprinted polymer artificial receptor prepared using a single polymerization method after equilibration of the polymers with a solution of print molecules at room temperature (30 1 C). In a typical binding assay, the polymer powder (50 mg) was added to 5 ml of the solvent containing 5 g.ml^{-1} of the printed molecules 5 ml of the pure solvent (blank), and the suspension stirred for 24 h at room temperature (30±1 C). The polymer particles were then filtered off and the filtrate was analysed for the printed molecules by fluorescence spectroscopy. The measurement in fluorescence intensity of the tested filtrate was conducted at 320 nm emission wavelength following excitation at 279 nm for dopamine analysis and at 335 nm emission wavelength after the excitation at 330 nm for serotonin analysis. The quantity of printed molecules in the solution was determined by reference to a calibration curve. The amount of bound drug was obtained

by subtracting the amount of free drug from the total amount of the drug added. Recognition data as shown by selectivity factor ( ) value, which was the ratio of the amount of substrate bound by the MIP to that bound by the NIP.

2.5. Determination in Binding Characteristics of DS-MIP.

The binding characteristics of the DS-MIP as well as that of the related single-recognition MIPs (D-MIP and S-MIP) and of the corresponding non-imprinted polymer were examined at room temperature using 25-mg samples of polymer with template solutions ranging in concentration from 0.1 to 100 g.ml^{-1}, using methanol/water (4:1, v/v) mixture as medium. The amount bound (Q) was determined at each print molecule/polymer molar ratio. The binding parameters were determined from the equation, Bound/Free = $(B_{max}-B)/K_d$, where K_d is the equilibrium dissociation constant, and B_{max} is the maximum number of binding sites which were obtained from the slope and intercept on x-axis of the straight line of the Scatchard plot, respectively. The mean drug binding constants calculated from triplicate independently derived results.

2.6. Determination in Specificity of DS-MIP

The DS-MIP was evaluated for recognition ability using the templates and analogs such as salbutamol (*SB*), isoproterenol (*IP*), epinephrine (*EP*), methyldopa (*MD*) and histamine (*HT*) as the substrates, in saturation binding experiments. To verify cross-selectivity of the DS-MIP, saturation binding experiment of the related single-recognition MIPs (D-MIP and S-MIP) was also performed. In a typical binding assay, the polymer (25 mg) was added to 5 ml of methanol/water (4:1, v/v) mixture containing 2.5 µg/mL of analyte of interest or 5 ml of the pure solvent (blank), and stirred overnight at room temperature for equilibrium to be established. The polymer particles were then filtered off and the filtrate was analysed for the analyte using fluorescence and UV

spectroscopy as previously reported[18]. The quantity of drug in the solution was determined by reference to a calibration curve. The amount of bound drug was obtained by subtracting the amount of free drug from the total amount of the drug added. The specificity was shown as cross-reactivity (%CR) value obtained by determining each specific the amount of compound sorbed per unit weight of MIP related to the amount of printed molecule sorbed.

2.7. Determination in Binding Reaction of Ergots to DS-MIP

DS-MIP particles (2.5 mg/mL) were incubated between 0 and 12 h in 10-ml vials at room temperature by agitation with a 1.5 g/mL of the tested ergot in 5 ml of methanol/water (4:1 v/v). At 0, 0.5, 1, 3, 6 and 12 h, the filtrate was analysed for the amount of unbound analyte by HPLC method described below. The percentage of each ergot bound to the polymer was plotted as a function of the incubation time. All solutions were prepared in triplicate and each ergot sample was analyzed three times.

A reversed phase HPLC-ECD detection method was used for the quantitative analysis of all ergot samples, except agloclavine sample. Mobile phase comprising 34:66 (v/v) a 15 mM KH_2PO_4, 3.75 mM, sodium heptanesulfonate and 7.5 mM KCl aqueous solution adjust pH 4 by phosphoric acid : methanol was used for elution. The analytical column was a Luna 5 C_{18}, 25 cm x 0.46 cm (Phenomenex, USA). A flow-rate of 1.0 ml min^{-1} was used. The electrochemical detector was set at 0.8 V potential. The analysis of the agroclavine content of the samples was performed using a HPLC-fluorescence detection method. The column was a Luna 5 C_{18}, 25 cm x 0.46 cm (Phenomenex, USA). The mobile phase was 25:75 (v/v) 1 M ammonium acetate:acetonitrile and a flow rate of 1.0 ml/min was employed. The fluorescence detector was set at $_{ex}$ 310 nm and $_{em}$ 410 nm. Correlation coefficients for the calibration

curves of the ergots in the range 2-25 g.ml^{-1} were greater than 0.999. The sensitivity of detection was 1.3 g.ml^{-1} and the reproducibility of the peak areas of analytes was more than 95%.

2.8. Fluorescent Competitive Molecularly Imprinted Ligand Binding Assay

The chosen ergoline-related compounds were assessed for their ability to displace *D* or *S* bound to the MIP. A twenty-five milligram of DS-MIP was incubated with 2.5 mL of methanol/water (4:1 v/v) containing 2.5 g/mL of either *D* (or *S*) and 2.5 mL of methanol/water (4:1 v/v) containing ergot within the concentration range 0.02-1000 g/mL, for 6 h at room temperature (28±1 C). The changes in fluorescence intensity of *D* in solution were monitored at 320 nm following excitation at 279 nm or *S* in solution at 335 nm after the excitation at 330 nm. The decay in corrected fluorescence intensity as a function of competitor concentration was used to determine the midpoint of the competition (*IC$_{50}$*). An apparent (*K$_i$*) value was calculated using $K_i = [IC_{50}/(1+[L]/K_d)$, where *K$_i$* = apparent inhibitor constant, [L] = free concentration of dopamine or serotonin, and *K$_d$* = apparent dissociation constant of a given MIP for dopamine (or serotonin). The binding of original probes in the presence of competitors was calculated and reported as the % binding of printed molecule to sites on the DS-MIP. Each experiment was repeated three times.

2.9. Determination in Affinities of the Ergots on Natural Receptor

The tested ergots were assessed for dopamine and serotonin binding affinities in immunocompetitive experiment using serotonin and dopamine-discarded receptor of male-Wistar rats with the exogenously added dopamine (or serotonin) as the molecular probe. The rat hypothalami receptors were isolated using the procedure described

previously[19], and followed by washing with 50 mM Tris HCl, until the endogenous dopamine and serotonin are no longer found in the rinse as verified by the fluorescence method that was described in the competitive fluorescence imprinted polymer binding experiment. The resulting pellet was examined for protein content by using the procedure of Bradford[20]. The pellets containing the discard receptors were re-suspended in 50 mM Tris HCl with 0.5 mM Na_2EDTA, 0.1% Na ascorbate before use. Saturation experiments for the extract rat hypothalamus receptor were carried out by varying the concentration of D or S ligand using 50 mM Tris HCl with 0.5 mM Na_2EDTA, 0.1% Na ascorbate as medium, at 37°C. The Scatchard plot analysis revealed that the binding sites of the extract rat hypothalamus receptor constituted a single population. The dissociation constant (K_d) and receptor binding (B_{max}) values were also determined to be 121.9 $\pm$ 3.69 nM (n =3) and 360 $\pm$ 11 pmol/mg protein (n =3), respectively for the D, and a K_d of 53.18 $\pm$ 2.89 nM (n=3) and 370 $\pm$ 40 pmol/mg protein (n=3), respectively for the S. The ability of 6-8 concentrations of test ergot to displace 0.2 µmole D or S probe was measured in drug displacement studies. The D/S binding was saturated with high affinity. To verify the equilibrium binding constant for the ergots, the unbound forms of the D and S probes was analysed by using GC-MS analysis method with the use of cation exchange resin to separate the protein bound and unbound of the probes. All experiments were performed with triplicate determinations using 50 mM Tris HCl with 0.5 mM Na_2EDTA, 0.1% Na ascorbate as medium, to which 10 mg of protein was added giving a final volume of 1 ml. The tubes were allowed to equilibrate for 30 min at 37°C before filtering with a 0.45 µm cellulose acetate syringe filter (Whatman, NJ, USA) followed by two 5 ml washes using ice-cold Tris-buffer. Filters were lyophilized and analysed for free probes. The supernatant aliquots were then passed through small columns of Amberlite CG-50 as previously reported[21]. The 3 ml of 1:3, v/v formic acid-ethanol eluates containing the analytes were evaporated to dryness in a polyethylene

vial and the residues were reconstituted with methanol before assay with fluorescence spectrometer. The binding of D and S probes in the presence of the ergots was calculated and plotted to the ergot concentrations for determination of IC_{50}. An apparent K_i value was calculated same as that in the competitive fluorescent molecularly imprinted polymer ligand binding assay.

3. Results and discussion

3.1. The MIP and characteristics

A dopamine and serotonin molecularly-imprinted polymer (DS-MIP) artificial receptor was synthesized by the thermal polymerization of methacrylic acid and acrylamide using N,N-methylene-bis-arylamide as cross-linking monomer in the presence of dopamine and serotonin (see chemical structure in Figure 1) as the template in aqueous methanol solution. MAA and ACM were adopted as the functional monomer mixture for imprinting of the polymer with dopamine and serotonin templates, because the carboxylic group or the amide group of these monomers is capable of interacting with the acid group and amino group of either two printed molecules. This could give a great variety of chemical properties, which could be useful to exploit many different interactions such as ionic, hydrogen bond and charge transfer interactions, with the templates. MBAA was employed as cross-linking monomer of this work, due to its anticipated property of giving a flexible and conformationally adaptable to the polymer as the protein-based natural recognition systems. The chosen functional monomers and cross-linker were constructed into the MIP structure with a view to increasing the possibility of obtaining an efficient recognition system in a polar medium, thus mimicking the binding environment of natural receptors. A mixture of methanol and water (4:1, v/v) was chosen as the porogen solvent since the templates are soluble only in polar solvent.
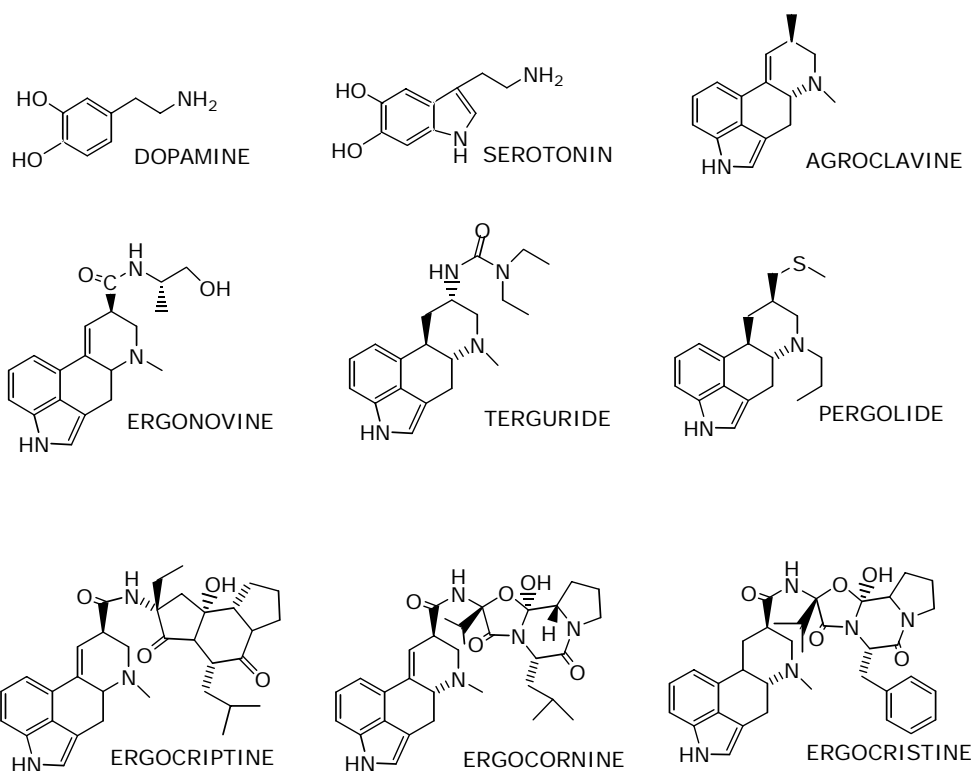
Figure 1: Structure of templates and ergot compounds studied in this work.

The physical characteristics of the DS-MIP was examined, which the DS-MIP had particle sizes in the range of 20-37 m as determined by a laser diffraction (Malvern Mastersizer; Worcester, UK) and pore diameters of the polymers were inspected by nitrogen adsorption/desorption techniques using a Coulter SA3100 series surface area and pore analyzer (Coulter, USA), which the range of 18-55 nm and pore volumes in the range of 0.21-0.31 ml/g were found (see table 1). The polymer particles possessed micropores in the polymer network and swelled in several aqueous solvents (i.e. acetonitrile, methanol, water and 4:1 (v/v) methanol:water). The DS-MIP was found to have swollen to a greater extent than the non-imprinted polymer in all the solvents studied.

Table 1: Characteristics of polymers.

Polymer	Print molecule	Elemental analysis (%)				Particle size[a) (μm)	Pore diameter (nm)	Pore volume[b) (ml/g)	BET Surface area[b) (m²/g)	Specific swelling (ml/ml)			
		C	H	O	N					Acetonitrile	MeOH	Water	Mixture[c)
NIP	-	55.7	7.6	23.4	13.3	36.72	54.55	0.26 (0.007)	213.32 (20.78)	0.9	0.6	0.6	0.3
D-MIP	D	55.1	7.4	24.3	13.3	19.93	25.21	0.24 (0.014)	130.41 (34.37)	0.2	1.1	1.0	0.9
S-MIP	S	53.6	7.2	25.4	13.8	19.43	21.09	0.31 (0.005)	158.41 (14.76)	0.4	1.4	2.0	2.2
DS-MIP	D and S	53.7	7.1	25.7	13.5	22.23	18.36	0.21 (0.006)	133.35 (17.46)	0.6	1.4	2.0	1.6

d) Approximate mean in particle size.

e) The micropore surface and pore volume (values in parenthesis) from a *t*-plot using Harkins-Jula average thickness.
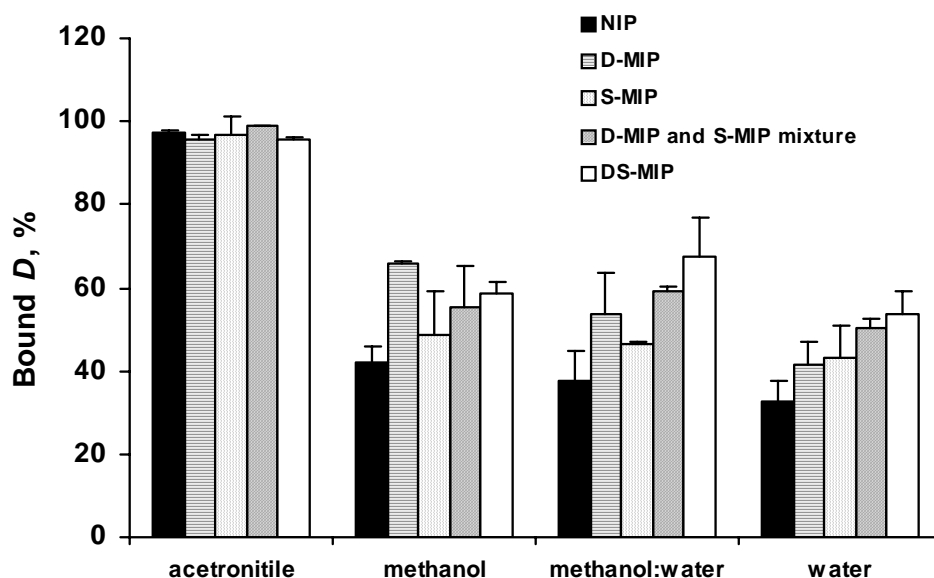
f) Refer to 4:1, v/v methanol:water solvent.

The selectivity of DS-MIP was generated by the multiple-template imprinting approach to create multiple dopamine and serotonin-binding site in the imprinted receptor. For this method, it offers a rapid and easy means capable of providing a multiple-recognition for a MIP. However when making a MIP with different combination of templates, a selectivity pattern can be generated with selectivity pattern having broad recognition range for compounds. The selectivity pattern achieved with the DS-MIP that was obtained by multiple-template polymerization may be different, so that the selectivity profiles and screening outputs of the DS-MIP were verified upon comparison study with the MIP made as single-recognition material either as individual or mixture.

3.2. The selectivity of DS-MIP

An evaluation of the presence of an imprinting effect in DS-MIP was performed in a various aqueous media using batch binding assay in parallel experiment with the reference polymers (1:1 w/w D-MIP and S-MIP mixture, D-MIP and S-MIP) and corresponding non-imprinted polymer. Figure 2 show the binding of *D* and *S* to the DS-MIP and reference polymers when incubated in acetonitrile, methanol, methanol/water (4:1 v/v) mixture and pure water.   The %binding of *D* and *S* were highest in acetonitrile and lowest in the mixture of methanol and water solvent. However, the non-specific adsorption of *D* and *S* binding sites were high in acetonitrile as well as methanol and water. The template recognition of DS-MIP and that of all the reference receptors is highest in the mixture of methanol and water solvent, showing that this solvent used as the synthesizing medium of the MIP receptors was good solvent for selectively binding of either *D* or *S* to the polymer receptors. The binding results for *D* and *S* of either related single-recognition MIPs (D-MIP, S-MIP) were corresponded to those of the DS-MIP. Apart from this, %binding of either *D* or *S* for DS-MIP was not to be different from that on the dopamine and serotonin imprinted polymer made as single recognition

material (1:1, w/w D-MIP and S-MIP mixture). The results showed that it is possible to prepare DS-MIP with a good recognition towards *D* and *S* in an aqueous solution.
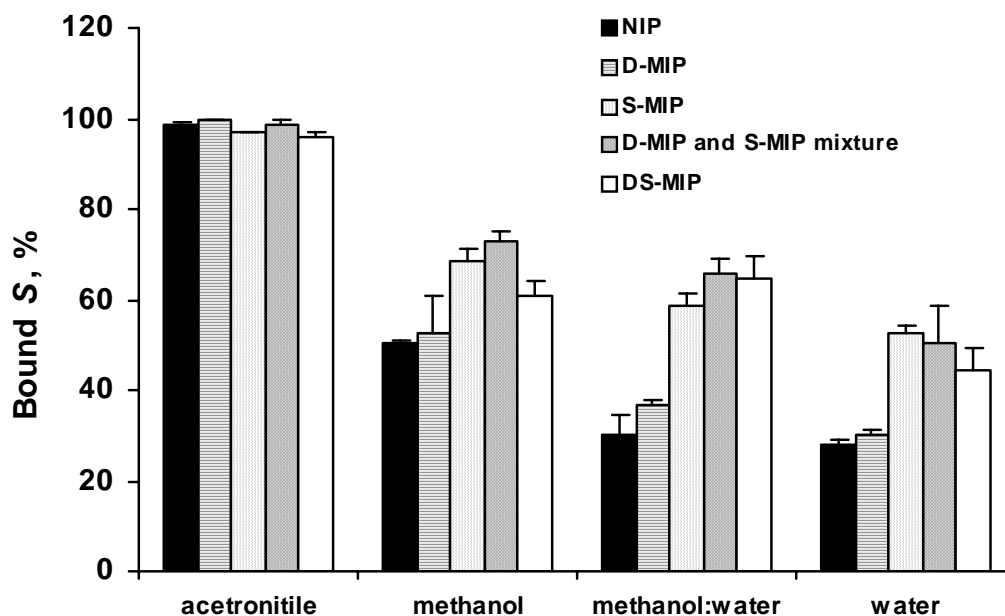
A

B

Figure 2: Effect of solvent on the % binding of the print molecules bound to various molecular artificial imprinted receptors and corresponding NIP synthesized in this study. The values shown are mean ± SE of 3 replicate specimens.

3.3. Binding characteristics of DS-MIP

Table 2 shows dissociation constant (K_d) and binding capacity (B_{max}) values of the higher and lower affinity binding sites of the imprinted and non-imprinted polymers. The Scatchard analysis of DS-MIP and the single-recognition polymers as well as the non-imprinted polymer showed that the recognition sites of the polymers prepared are heterogeneous. The imprinted polymers contain high proportion of high-affinity binding sites but that minor low-affinity binding site being present. It can be observed that binding affinity of the *D*/*S* probe to the control polymer was very low in all cases. Generally, the binding of *S* ligand on DS-MIP and the reference MIP (D-MIP, S-MIP) is greater than that for *D* ligand. Moreover, the binding affinity of DS-MIP with *D* and *S* ligands is higher than that of the single-recognition MIPs (D-MIP, S-MIP), whilst binding capacity of these two MIPs are not different for both *D* and *S*. The non-printed molecule *D* and *S* can also bind to the single-recognition MIPs, S-MIP and D-MIP, but that having less affinity than that of the printed molecule. This means that the single-recognition MIPs exhibit cross-reactivity to the compound that is structurally closely related to the template. DS-MIP which was prepared with the use of *D* and *S* as the mixed template gave higher affinity to both *D* and *S* than the single-recognition MIPs did. The selectivity generated into DS-MIP is likely derived both by imprinting and cross-reactive effects of the imprinted polymer.

Table 2: Dissociation constant (K_d) and binding capacity (B_{max}) of the polymers. The values shown are mean ± SE of 3 replicate specimens.

Polymer	Ligand	High-affinity binding site		Low-affinity binding site	
		K_d (10^{-4}M)	B_{max} (µmol/g)	K_d (10^{-4}M)	B_{max} (µmol/g)
DS-MIP	*D*	0.7 ± 0.0	3.1 ± 0.9	100.0 ± 2.1	9.4 ± 3.3
	S	0.3 ± 0.0	3.6 ± 0.1	16.7 ± 1.3	11.9 ± 5.7
D-MIP	*D*	1.1 ± 0.3	3.5 ± 0.7	12.5 ± 6.0	0.9 ± 0.0
	S	2.0 ± 1.2	5.1 ± 2.9	35.3 ± 21.0	3.1 ± 2.2
S-MIP	*D*	2.8 ± 1.6	3.6 ± 1.7	100.0 ± 3.6	7.4 ± 0.8
	S	0.7 ± 0.1	4.7 ± 0.3	144.9 ± 45.3	12.4 ± 0.9
NIP	*D*	14.1 ± 3.5	0.4 ± 0.2	7.4 ± 4.1	2.7 ± 1.1
	S	50.0 ± 23.3	1.5 ± 0.6	1.6 ± 1.3	15.1 ± 1.2

3.4. The specificity of the DS-MIP

The dopamine and serotonin selectivity of DS-MIP was generated by multiple-template imprinting technique using template mixture molecules. Using multiple-template imprinting method, it offers a rapid and easy means capable of providing a multiple-recognition for a MIP. However when making a MIP with different combination of templates, a selectivity pattern can be generated with selectivity pattern having broad recognition range for compounds. The selectivity pattern achieved with the DS-MIP that was obtained by multiple-template polymerization may be different, so that the selectivity profiles and screening outputs of the DS-MIP were verified upon comparison study with the corresponding single-recognition MIPs and a mixture of these single-recognition MIPs.

The selectivity range of DS-MIP was examined with respect to recognition selectivity of various adrenomimetic ligands (i.e. dopamine serotonin, salbutamol, isoproterenol, epinephrine and methyldopa) (*MD*) and serotomimetic ligands (i.e. serotonin and histamine) by using batch binding experiments. A control experiment was carried out using the relative single-recognition MIPs, D-MIP and S-MIP as reference polymers. Figure 3 shows %binding of various adrenergic ligands and serotonergic ligands to DS-MIP and the reference MIPs. The results showed that %binding value of the printed species on either DS-MIP or either two single-recognition MIPs was higher than that of other ligand species. In addition, the results suggest that the closely structure to the templates bound more effectively for either DS-MIP or the single-recognition MIPs. The cross-reactivities shown by DS-MIP and the reference polymers for various ligands indicate that a basic structure theme is sufficient for recognition. Since the common demomination of the structure of the ligands studied is the phenylethylamine unit, substituents on this structure lead to variations in recognition. Both *D* and *S* printed molecules which did not have hydroxyl group at -position of the phenylethylamine skeleton. It was found to be most favourably bound by the DS-MIP and the reference polymers compare to that of other structurally related compounds. The binding of either -OH-phenethylamine or methyl-substituted phenethylamine derivative (*i.e.* methyldopa) was similar order in the single-recognition MIPs and the DS-MIP. The single-recognition MIPs showed high cross-reactivies with a wide range of adrenomimetic and serotomimetic ligands than the DS-MIP did. The DS-MIP prepared by using multiple-template imprinting technique presents a great specificity to *D* and *S* ligands with partial cross-reactivity due to template mixture.

The DS-MIP and the corresponding single-recognition MIPs selectively recognised the *D* and/or *S* print species which has a phenethylamine structure better than -OH-phenethylamine compounds, epinephrine, salbutamol, isoproterenol and

methyldopa. This may be due to steric constraints imposed by the hydroxyl group in three-dimensional arrangement of the compounds, such that they interact less favourably with the -hydroxyl group. Histamine which has an imidazole ring in its structure, favourably bind to the S-MIP, but give less affinity for the D-MIP. This indicates that the catechol structure is necessary for ligand binding of the D-MIP, whilst this is not crucial in case of S-MIP. The DS-MIP prepared in this study demonstrates the recognition patterns for dopaminergic and serotonergic agents similarly to the native receptors. Indeed, the biogenic amines, norepinephrine, dopamine, and serotonin can be viewed as structural elements of the ergoline ring system that is shared by all the ergot alkaloids. Therefore, the DS-MIP produced in this work is able to be used as a receptor for preliminary screening of new dopamine/serotonin agents and for determination receptor-ligand binding of new ergot-derived molecules.
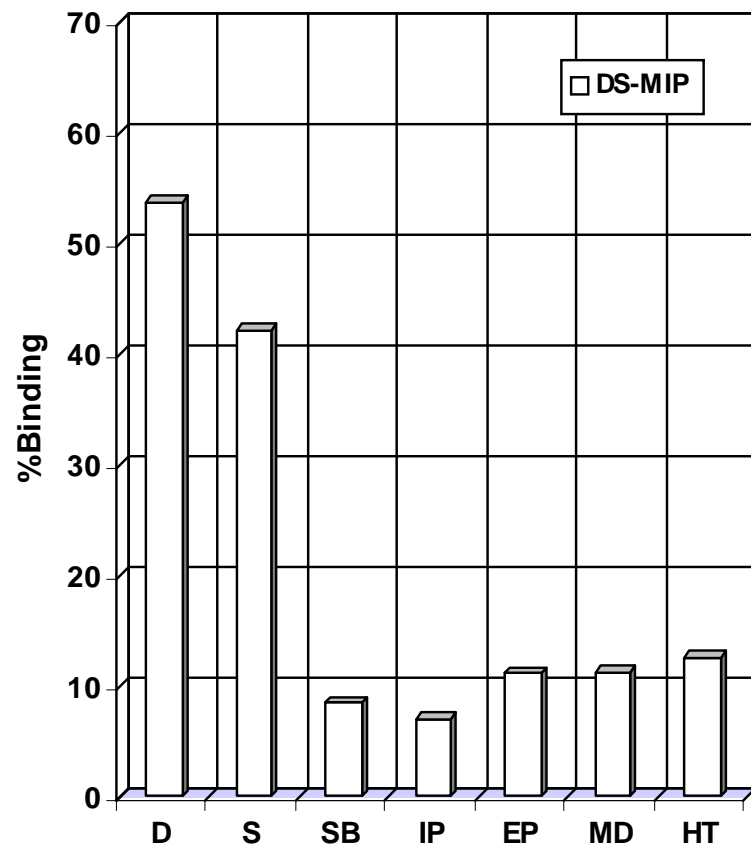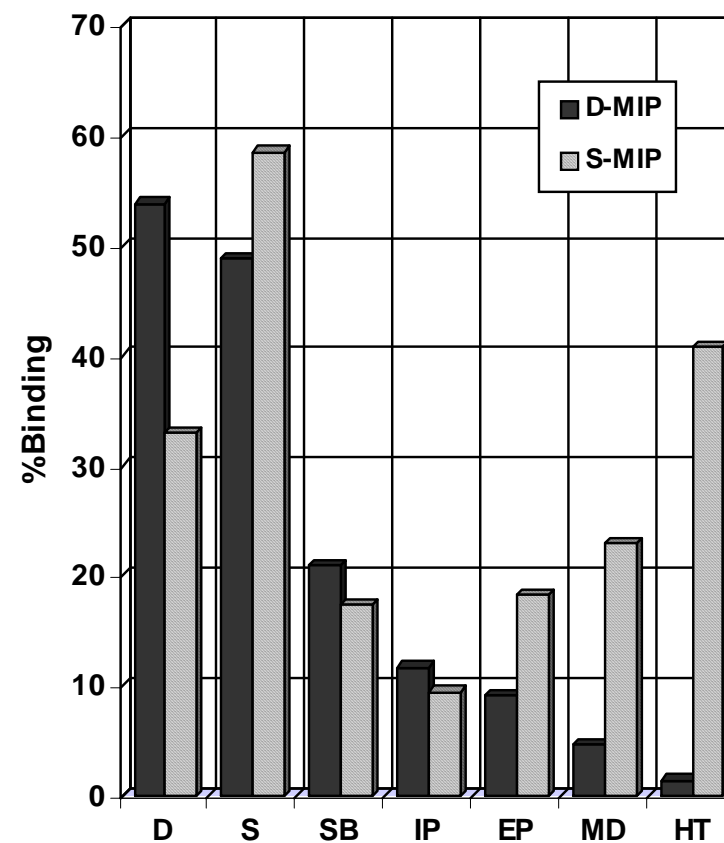
Figure 3: The % binding of various adrenergic ligands and serotonergic ligands for *D* and *S* binding site on (a) the DS-MIP and (a) the related single-recognition MIPs (mean ± SE, n=3). dopamine (*D*), serotonin (*S*), salbutamol (*SB*), isoproterenol (*IP*), epinephrine (*EP*) methyldopa (*MD*), histamine (*HT*). The values shown are mean ± SE of 3 replicate specimens.

The DS-MIP was adopted for a preliminary screen of a group of ergoline derivatives, initially the specific sensitivity for binding of seven tested ergots (ergocryptine, ergocornine, ergocristine, ergonovine, agroclavine, pergolide and terguride) were assessed in the absence of *D* or *S* probe in 4:1 (v/v) methanol/water at room temperature (28±1 C), using a MIP slurry of 5 mg/mL. The percentage binding of the 2.5 µg/mL of ergots as a function of time is depicted in Figure 4. The %binding of ergots on DS-MIP when measuring in the absence of *D/S* was constant within 3 hr. The binding value of the ergopeptine compounds (i.e. ergocryptine, ergocristine, ergocornine) is about two times lower than that of clavine derivative, agroclavine. By contrast, the lysergic acid derivatives (*i.e.* ergonovine, pergolide, and terguride) have percentage binding lower than that of the clavine but higher than that of the ergopeptines.
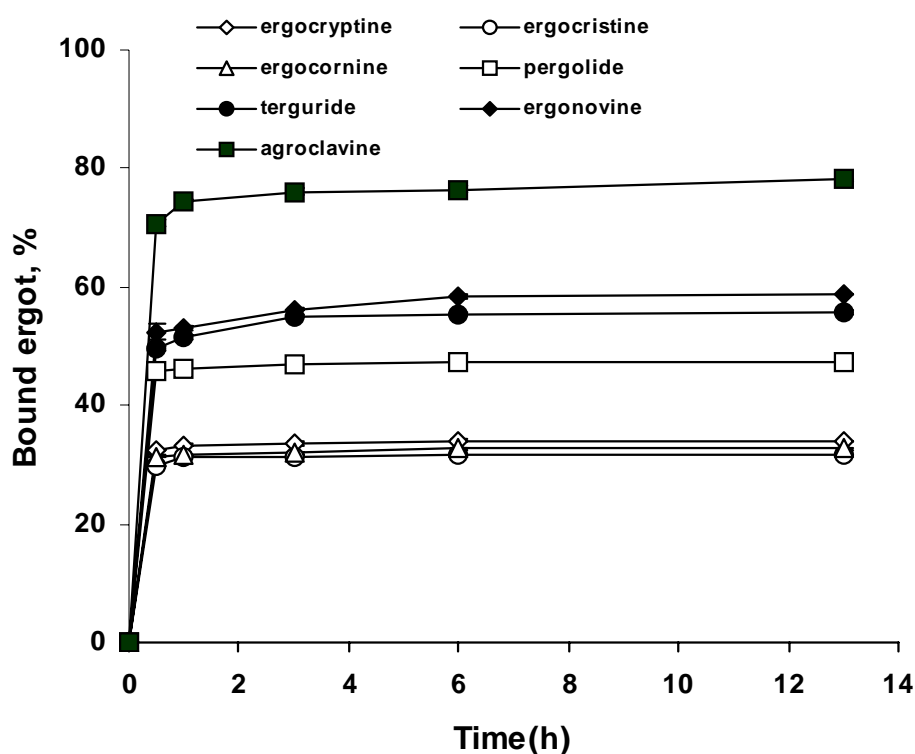

Figure 4: Time course for the binding of ergots on the DS-MIP at room temperature. The values shown are mean ± SE of 3 replicate specimens.
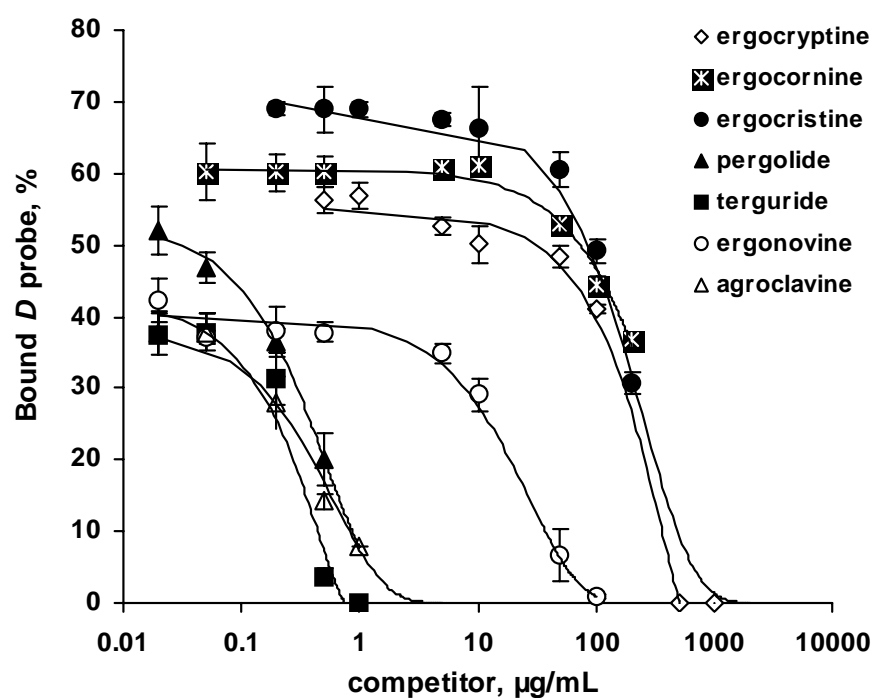
The %binding obtained from this study reflects to the specific binding of the ergots with the DS-MIP, which three libraries of the tested ergots were achieved, and this correlated to their molecular structure.

Preliminary studies was carried out to determine binding reactions of the ergots (ergocryptine, ergocornine, ergocristine, ergonovine, agroclavine, pergolide and terguride) on DS-MIP binding sites when incubated either with D or S probe, which %binding of the ergots was analysed by using a competitive assay which measured the ability of various ergoline compounds to displace bound D or S probe with the use of chromatographic method for analysis of the unbound ergot in incubation medium. With the addition of D probe, %binding of pergolide and tergolide change in the concentration range between 0.1 and 5 μg/mL %binding of ergonovine changes in the concentration range between 1 and 100 μg/mL and %binding of ergocornine, ergocriptine and ergocristine change in the concentration range between 1 and 500 μg/mL. In contrast, the displacement of S probe yields the lower change in binding of the ergot than that of of D probe and generally the change of binding occurred in the range of 1-50 μg/mL for pergolide, tergolide, ergonovine or agroclavine and 1-500 μg/mL for ergocriptine, ergocornine or ergocristine. The result suggests the ability of the ergots to act as the competitors of D/S bound to DS-MIP and also the differences in competition reaction of various ergots with the D/S ligand. In addition, the increasing amounts of the ergot enabling increased ergot-binding in the medium containing D or S indicates reversibility of ligand-receptor interaction in the DS-MIP as well as having high specific activity to D and S ligand of the DS-MIP, which would be a suitable indicator of ligand binding activity of ergots for this receptor system.

3.5. Screening of Dopamine/Serotonin Binding Receptor of the Ergots by DS-MIP

The greatest utility of the displacement assay with DS-MIP using D/S as a fluorescent probe may be in its ability to serve as a screen for novel ligands of the dopamine and/or serotonin binding receptor. The determination in ligand binding activity of ergots by chromatography had proven to be expensive and time consuming, which is not suitable for high-throughput screening of drug development. This work demonstrates an application of DS-MIP, which adapted to fluorescence assay study. A group of ergot derivatives, comprising with four ergopeptine derivatives such as ergocryptine, ergocornine, ergocristine, ergonovine, one clavine agroclavine, and two amide of lysergic acids ; pergolide and terguride has been characterized as to their ligand binding activities on competitive fluorescence imprinted polymer binding assay protocol performing in presence of *D* or *S* as a fluorescent probe. Figure 6(a-b) shows competitive binding assays with the various ergoline compounds and the different probes (*D* and *S*) when conducted with the competitive fluorescent molecularly imprinted assay using DS-MIP as adsorbent phase. Typical sigmoid calibration curves similar to those by competitive immunoassays were obtained with both *D* and *S* probe. The apparent inhibition constants (K_i) obtained from these experiments are listed in Table 3. The lower the K_i, the greater receptor binding. The related affinity of DS-MIP for molecules related to the template molecules was greater than for ergot in particular showing modest either dopamine receptor or serotonin receptor displacement characteristics. There are differences of each ergot for competitive binding with *D* or *S*. All ergopeptines displaced *D* binding with affinities about four fold lower than the lysergic acid derivative, which had affinity about four and two fold lower than the clavine derivative for *D* and *S* binding site, respectively. Both lysergic acid derivatives and clavine derivatives bound to *D* with higher affinities than those bound to *S*. All ergopeptines has low affinity at both of *D* binding sites and *S* binding sites.

A
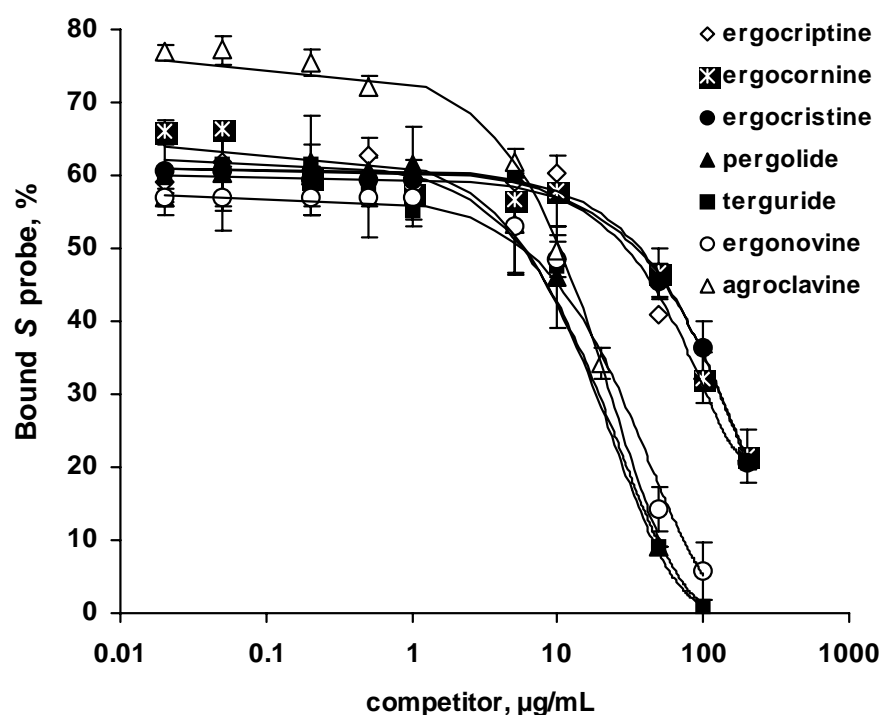

B

Figure 5: Calibration curves for ergot compounds obtained from competitive fluorescent molecularly imprinted assay with DS-MIP competitive binding assays using (a) *D* and (b) *S* as a probe. The values shown are mean ± SE of 3 replicate specimens.

All of the lysergic acid derivatives bound to *D* binding sites and *S* binding sites with affinities similar to each other. Therefore, this assay demonstrates that all the ergot studied act as a dopamine/serotonin binding receptor and that the binding characteristics of the compounds are similar, but not identical. These experiments also confirm the ability of this assay using the DS-MIP as a receptor mimic to identify new ligands for the dopamine/serotonin binding receptor. The affinity of all those ergots on natural receptor was evaluated to compare in the ligand binding activity of the tested ergots. As seen in Table 3, the DS-MIP in a competitive fluorescent ligand binding assay for ergot produced results which were comparable well to those obtained using a competitive immunoassay data obtained using dopamine/serotonin receptors derived from the rat hypothalamus, although the natural receptor showed very high affinity to each of the ergots. The current study shows that the assay with DS-MIP is highly specific and affinity is similar to natural receptors, is applicable for screening the ergot family.

Table 3: Binding affinities (inhibition constant [K_i]) for DS-MIP (mean ± SE, n=3) and rat hypothalamus receptor (mean ± SE, n=4) of the ergots.

Ergot [a]	The DS-MIP		The natural receptor	
	D ligand, µmol/L	*S* ligand, µmol/L	*D* ligand , nmol/L	*S* ligand , nmol/L
ergocryptine	111.60 ± 0.99	103.30 ± 0.57	106.30 ± 1.06	312.2 ± 7.78
ergocristine	130.80 ± 1.20	93.30 ± 0.50	285.20 ± 2.12	195.5 ± 4.81
ergocornine	229.80 ± 2.12	110.0 ± 0.64	388.50 ± 3.25	58.9 ± 1.48
pergolide	0.45 ± 0.01	17.0 ± 0.07	262.90 ± 3.82	187.2 ± 4.67
terguride	0.59 ± 0.01	21.60 ± 0.14	1.87 ± 0.07	36.60 ± 0.92
ergonovine	44.80 ± 0.42	33.30 ± 0.24	97.85 ± 2.26	33.90 ± 0.85
agroclavine	0.79 ± 0.01	19.16 ± 0.18	58.5 ± 1.13	33.50 ± 0.85

[a] See Figure and text for the structure of the ergots.

As previously stated, ergot derivatives usually contain dopaminergic/adrenergic pharmacophore and serotonergic pharmacophore, and their pharmacological activities are responses mediated by adrenergic, serotonin or dopamine receptors. Either dopamine or serotonin is an important neurotransmitter, acts on those receptor types. The competitive fluorescent ligand binding data achieved with using DS-MIPs may be useful for evaluating in binding competition of ergot derived molecule with one of the dopamine and serotonin neurotransmitters. The study of biomolecular binding events in areas such as proteomics, neuroscience, cancer research, developmental cell biology, structural biology and immunology is essential for the understanding of the fundamental mechanisms of living cells. The fluoresecent molecularly imprinted binding assay with DS-MIP offers a direct detection and monitoring of biomolecular binding events in real time without labeling and without purification of the substances involved to determine the binding specificities of possible new ergot derivatives. The assay could provide the way to measure the speed of binding events. Indeed the dopamine and serotonin receptor of biological sources has many subtype and the assay with DS-MIP can not distinguish between agonist and antagonist agents either of the subtypes of receptors, but that this technique has been useful in describing the binding specificities for a newly described member of the ergot family, as particularly when combines the experimental studies with molecular modeling. Even though the basis of the affinity for the biological dopamine and serotonin receptor and the DS-MIP sorbent phase were completely different, but that this technique can be used to preliminary screening of ergot family, and to identify high affinity ligand and to discriminate for the dopamine/serotonin specific receptor. In addition because the DS-MIP does not derive from biological origin, there is no problem as the among class variation among biological receptors.

4. Conclusion

In this report, we have shown that the prepared dopamine and serotonin molecularly imprinted polymer artificial receptor bind dopamine and serotonin with high affinity and that this assay can be used to examine the dopamine/serotonin binding characteristics of several ergot molecules. Furthermore, this technique has been useful to serve as a screen and/or describe specificity for a newly described member of the ergot family. We conclude that the assay with the dopamine and serotonin molecularly imprinted polymer provides a fast and inexpensive means of analyzing the ligand binding characteristics for virtually any available ligands. Because the number of ergoline compounds being modified and reported is increasing rapidly, the dopamine and serotonin displacement assay can be used as a general tool for the evaluation of ligand binding affinity and specificity for dopaminergic and serotonergic receptors, i.e. various members of the ergot derived compounds. In addition, the advantage of screening of ergot ligands with the MIP artificial receptor by competitive fluorescent ligand binding assay method is a possible of screening library for both dopamine and serotonin receptors binding mimics to replace their biological counterpart concurrently in radio-immuno ligand binding assay, accelerating drug discovery and development.

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