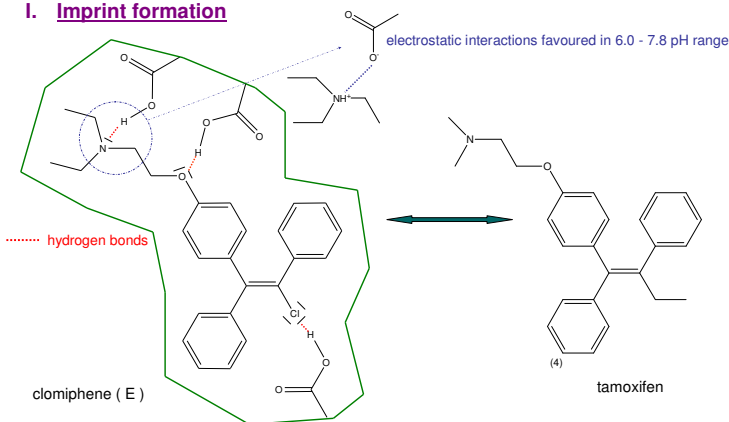


Introduction

Tamoxifen is normally used for the treatment of breast cancer, and it displays anti-oestrogenic activity. In males, anti-oestrogens induce a decrease in side effects after abusive consumption of anabolic androgenic agent. Since 2000, tamoxifen and related anti-oestrogens have been banned by the International Olympic Committee. The presence of tamoxifen metabolites in male urines is a proof of doping.

A molecularly imprinted polymer (MIP) has been prepared in our laboratory to selectively extract tamoxifen and metabolites from urine by molecularly imprinted solid-phase extraction (MISPE) before HPLC-UV analysis. Clomiphene, a chlorinated tamoxifen analogue, was selected as template for MIP synthesis by thermal polymerisation of methacrylic acid as functional monomer and ethylene glycol dimethacrylate as cross-linking agent, with acetonitrile as porogenic solvent.

I. Imprint formation



II. MIP evaluation by Freundlich isotherm and affinity distribution

Equilibrium binding experiments have been performed with tamoxifen (0.013 – 2.7 mM) in acetonitrile in order to plot Freundlich isotherm (Figure a):

$$B = a \cdot F^m \quad \text{or} \quad \log B = \log a + m \log F$$

B: concentration of bound analyte ($\mu\text{mol/g}$) and F: concentration of free analyte (mol/L), a and m are fitting parameters; m: heterogeneity index with $0 < m < 1$.

The binding equilibrium equation is related to K (affinity constant).

$$S_{\text{MIP}} + A \rightleftharpoons [A-S_{\text{MIP}}]$$

A: analyte (tamoxifen)
 S_{MIP} : binding site
 $[A-S_{\text{MIP}}]$: analyte bound to binding site

$$K = \frac{a_{[A-S_{\text{MIP}}]}}{a_{S_{\text{MIP}}} a_A} = \frac{1}{F} \quad (\text{L/mol}) \quad a_A = F \quad (\text{mol/L}), a_{[A-S_{\text{MIP}}]} = 1, a_{S_{\text{MIP}}} = 1.$$

Freundlich isotherm is based upon a continuous distribution model assuming a continuous range of affinity constants for the adsorption of the analyte by the polymer. For three subsets of the entire distribution of K, $N_{\text{MIP}}/N_{\text{NIP}}$ ratio is calculated (Figure b).

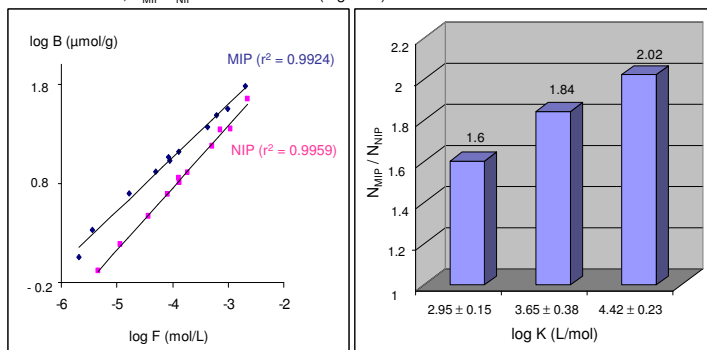


Figure a

Figure b

Figure a:

r^2 (> 0.99) values demonstrate the ability of Freundlich isotherm to accurately model the isotherm of MIP (and NIP) with 10 data points.

m_{MIP} (0.5373) $<$ m_{NIP} (0.6282) means that MIP is more heterogeneous than NIP. Imprinting effect is characterized by an increase in the number of sites relative to non-imprinted polymer. Thus, heterogeneity is higher for MIP than for NIP.

Figure b:

$N_{\text{MIP}} / N_{\text{NIP}}$ (> 1) increases with binding affinity (log K). These results reveal selectivity between MIP and NIP versus tamoxifen. Moreover, selectivity depends on high binding affinity sites.

III. Clean-up of urine samples by MISPE

Two SPE cartridges were filled with 60 mg of MIP and 60 mg of NIP. Then, conditioning step was performed with CH_3OH (4 mL), CH_3CN (4 mL) and $\text{NH}_2\text{OH}-\text{CH}_3\text{COOH}$ (pH 7, $I = 10 \text{ mM}$) buffer.

loading	2 mL tamoxifen dissolved in $\text{NH}_2\text{OH}-\text{CH}_3\text{COOH}$ (pH 7, $I = 10 \text{ mM}$) buffer
washing 1	2 mL H_2O
washing 2	2 mL $\text{CH}_3\text{OH} / \text{H}_2\text{O}$ (50/50, v/v)
washing 3	2 mL CH_3CN
washing 4	3 mL $\text{CH}_3\text{CN} / \text{CH}_3\text{COOH}$ (99.5/0.5, v/v)
elution	4 mL $\text{CH}_3\text{OH} / \text{CH}_3\text{COOH}$ (90/10, v/v)

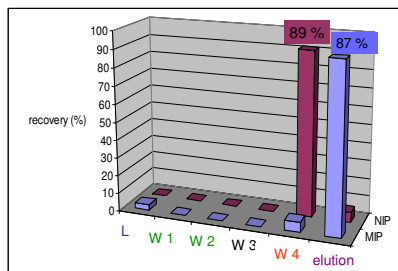
Electrostatic and hydrophobic interactions between polymer and tamoxifen.

Elution of mineral salts (NaCl, KCl) and polar molecules (urea, urobilins). Development of non-specific interactions between polymer and tamoxifen.

Hydrophobic molecules elution (hormones).

Disruption of non-specific interactions. Development of specific interactions between tamoxifen and polymer in the cavities of the MIP.

a) Standard aqueous solution of tamoxifen (0.5 $\mu\text{g/mL}$):



Tamoxifen was eluted from NIP with recovery of 89% (and only 4% from MIP).

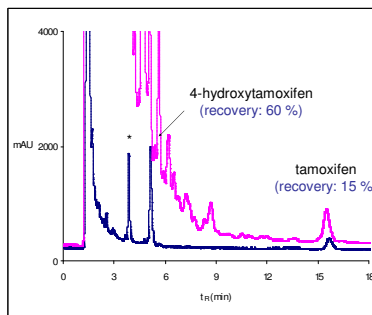
Tamoxifen was eluted from MIP with recovery of 87%.

Fractions analysed by HPLC with Zorbax-XDB C₈ Eclipse, 125 mm x 4 mm i.d. (5 μm) column.
 Eluent: $\text{CH}_3\text{CN} / \text{CH}_3\text{COOH}-\text{NH}_3$ (pH 4, $I = 20 \text{ mM}$) 60/40 (v/v).
 Detection 240 nm.

The optimized MISPE induced good recoveries and MIP/NIP selectivity values for tamoxifen aqueous samples.

b) Hydrolysed urine sample spiked with tamoxifen and its main metabolite:

The previous protocol was applied to 8 mL of hydrolysed urine sample spiked with tamoxifen and 4-hydroxytamoxifen (0.125 $\mu\text{g/mL}$). Before loading, β -glucuronidase was used to hydrolyse β -glucuronide metabolites. The ionic strength of the loading buffer was increased to match high concentrations of salts in urine.



The clean-up of the urine sample is clearly efficient compared to liquid-liquid extraction.

But low experimental recoveries are obtained. Remaining salts are supposed to weaken interactions between analytes and polymer. The first washing (H_2O) is unable to fully eliminate urinary salts.

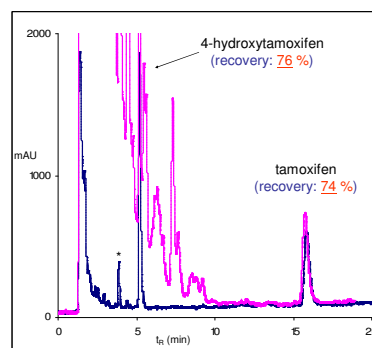
Those results have been further improved by suppression of urinary salt interferences before MISPE.

— liquid-liquid extraction — MIP elution
 (* unidentified peak)

IV. Clean-up of urine samples by consecutive HLB-SPE and MISPE

Hydrolysed urine was first extracted on a polymeric support (HLB, 1 mL, 30 mg, Waters) to eliminate endogenous salts. Furthermore, the aqueous matrix of urine was replaced by CH_3CN which is the porogenic solvent used during MIP synthesis.

The protocol was the same as previously (except for the washing 1 suppressed).



The clean-up of the urine by consecutive HLB-SPE and MISPE is successful.

Recoveries are suitable.

MIP/NIP selectivity is satisfactory because the tamoxifen is totally eliminated from the NIP during the washing steps.

— SPE-HLB extraction — MIP elution
 (* unidentified peak)

Conclusion

The MIP of tamoxifen has been successfully synthesized and characterized by its Freundlich isotherm. The high number and binding affinity of MIP sites allow to retain selectively tamoxifen and metabolites by this support.

Nevertheless, urinary matrix decreases the retention of the analytes by MIP. Therefore, tamoxifen and its metabolite recoveries are improved by elimination of endogenous salts on a first cleaning step on HLB support prior MISPE.