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Research Article

Development of a CD-MEKC method for investigating the metabolism of tamoxifen by flavin-containing monooxygenases and the inhibitory effects of methimazole, nicotine and DMXAA

A selective and low-cost CD-MEKC method under acidic conditions was developed for investigating the N-oxygenation of tamoxifen (TAM) by flavin-containing monooxygenases (FMOs). The inhibitory effects of methimazole (MMI), nicotine and 5,6-dimethylxanthenone-4-acetic acid (DMXAA) on the given FMO reaction were also evaluated; 100 mM phosphate buffer (pH 8.6) was used for performing the enzymatic reaction and the separation of TAM and its metabolite tamoxifen N-oxide (TNO) was obtained with a BGE consisting of 100 mM phosphoric acid solution adjusted to pH 2.5 with triethanolamine containing 50 mM sodium taurodeoxycholate, 20 mM carboxymethyl β-CD and 20% ACN. The proposed method was applied for the kinetics study of *FMO1* using TAM as a substrate probe. A Michaelis–Menten constant (K_m) of 164.1 μM was estimated from the corrected peak area of the product, TNO. The calculated value of the maximum reaction velocity (V_{max}) was 3.61 μmol/min/μmol *FMO1*; 50% inhibitory concentration and inhibition constant (K_i) of MMI, the most common alternate substrate FMO inhibitor, were evaluated and the inhibitory effects of two other important FMO substrates, nicotine and DMXAA, a novel anti-tumour agent, were investigated.

Keywords:
CD-MEKC / Enzyme inhibition / Flavin-containing monooxygenase / Tamoxifen
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1 Introduction

The importance of in vitro drug metabolism studies, performed in the early phase of drug development, for revealing potential metabolites of new drugs, allowing the elucidation of their toxicity as well as understanding and avoiding certain adverse drug reactions, is well known for many years. Most of these studies focus on the various cytochrome P450 (CYP450) isoforms, which are the primary oxidising enzymes and currently, flavin-containing monooxygenases (FMOs). The significance of FMOs has increased as more compounds have been recognised to be substrates of these enzymes [1].

FMOs represent a multi-gene family of microsomal enzymes involved in Phase I metabolism and oxygenate a wide range of nitrogen- and sulphur-containing xenobiotics and, in some cases, also oxygenate selenium, iodine, boron and even carbon. In general, FMOs convert lipophilic xenobiotics to more polar, oxygenated, readily excreted metabolites with reduced pharmacological and toxicological properties [1, 2]. To date, six FMO genes (*FMO1–6*) have been identified, although *FMO6* has been demonstrated to be a pseudogene. *FMO1* is the most prevalent FMO in the adult kidney and fetal liver, whereas *FMO3* is the main liver isoform.

Recent reports suggest that the significant contribution of FMOs to the metabolism of various drugs may lead to fewer adverse drug–drug interactions, because FMOs are not easily induced or readily inhibited [1, 2]. However, Tijet et al. reported that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin highly induced *FMO2* and *FMO3* in the mouse in an aryl hydrocarbon receptor-dependent manner [3]. Only a few potent competitive inhibitors of FMOs such as, indole-3-carbinol (I3C), (*N*,*N*-dimethylamino) stilbene carboxylates and 3,3′-diindolylmethane (DIM) have been reported [4–6]. Most

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Abbreviations: **CM-β-CD**, carboxymethyl β-CD; **CYP450**, cytochrome P450; **DMXAA**, 5,6-dimethylxanthenone-4-acetic acid; **EtOH**, ethanol; **FMO**, flavin-containing monooxygenase; **IC_{50}**, 50% inhibitory concentration; **I3C**, indole-3-carbinol; **IPA**, isopropanol; **IS**, internal standard; **MMI**, methimazole; **MAPS**, 3-(*N*,*N*-dimethylmyristyl-ammonium)propanesulphonate; **MeOH**, methanol; **NADPH**, nicotinamide adenine dinucleotide phosphate; **PAPS**, 3-(*N*,*N*-dimethylhexadecyl-ammonium)propanesulphonate; **STDC**, sodium taurodeoxycholate; **TAM**, tamoxifen; **TNO**, tamoxifen *N*-oxide; **TEA**, triethanolamine

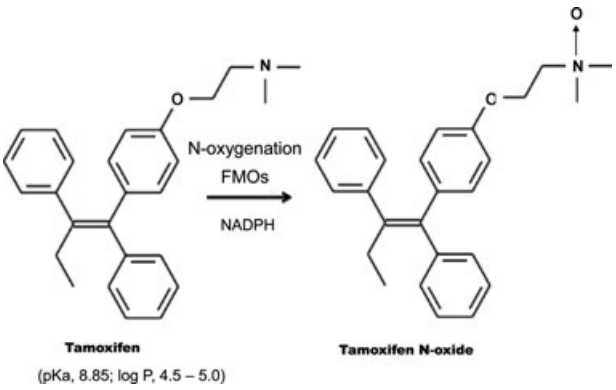

Figure 1. The FMO-catalysed N-oxygenation of TAM to TNO.

of the apparent FMO inhibition is due to alternate substrate competitive inhibition where a good nucleophile competes with the drug for FMO oxygenation. The most commonly used alternative substrate inhibitor of FMOs is methimazole (MMI) [7–10], whereas thiourea [11], trimethylamine, *N,N*-dimethylaniline and chlorpromazine [10, 12] have also been used. All these features of FMOs support an important role of FMOs in human drug metabolism and highlight the need for further studies.

Tamoxifen (TAM; trans-1-(4-β-dimethylaminoethoxyphenyl)-1,2-diphenylbut-1-ene), is a non-steroid anti-estrogen that has been widely used for the treatment of breast cancer. It has also been reported to have a role as a breast cancer chemopreventive agent. The major metabolites of TAM are derived from the CYP450 (α-hydroxylation, 4-hydroxylation, *N*-demethylation) or FMO (N-oxide)-dependent metabolism [13]. It is known that tertiary amines are often excellent substrates of FMOs and TAM is exclusively N-oxygenated to tamoxifen N-oxide (TNO) by FMOs (Fig. 1). Also, it has been reported that TAM is a better substrate for human *FMO1* than *FMO3* [9, 14].

The N-oxidation of TAM was investigated by TLC and the FMO involvement in this reaction was verified by the inhibitory effect of MMI [7], but kinetic parameters (i.e. Michaelis–Menten constant (K_m) and maximum reaction velocity (V_{max})) for TAM N-oxidation, 50% inhibitory concentration (IC_{50}) and inhibition constant (K_i) values of MMI were not mentioned [7, 9]. Hodgson et al. [13] reported the K_m value of TAM for mouse *FMO1* following the quantitation of TNO by the previous TLC method [7]. Also, the enzyme kinetics of TAM was determined using spectrophotometry [14]. Thus, there was a need to develop a selective and sensitive method to investigate the metabolism of TAM by FMOs and the inhibitory effects of MMI and other important FMO substrates like nicotine and 5,6-dimethylxanthenone-4-acetic acid (DMXAA).

CE is not a common separation technique in the determination of TAM and its metabolites, although it has many advantages, especially in the field of bioassays, such as automation and miniaturisation. Besides, being a highly efficient separation technique, CE offers good selectivity. It is a low-cost technique with low consumption of samples and solvents [15]. Several CE methods proposed for the determination of TAM and its metabolites are all NACE methods [16–19]. In two of these papers [17, 18], the separation and the quantification of TNO have not been reported while in the others, the separation of TNO has been shown but not the validation [16, 19]. Sanders et al. determined TAM and its metabolites, *N*-desmethyltamoxifen and 4-hydroxytamoxifen in serum and the sensitivity of the method (the LOD value for TAM was 800 amol) was improved by the use of UV absorbance detection at 214 nm which is not usually preferred, not to suffer from interferences [17]. In the other papers [16, 18, 19], MS is coupled to NACE systems which means more complex and less available instruments increasing the cost of the analysis. Carter et al. reported 11.5 min as the migration time of TNO [19], whereas Lu et al. detected TNO at a very long migration time (38.1 min) [16]. Also, considering the difficulties of working in non-aqueous solvent systems in many occasions, such as current problems due to water in the experimental materials or bubble formation in case of high voltage [20–22], the aim of this study is to develop an aqueous CE method as an alternative to previous NACE methods and to apply it for the *FMO1* study mentioned before.

2 Materials and methods

2.1 Chemicals and reagents

The human FMO (*FMO1* and *FMO3*) supersomes with a concentration of 5.0 mg/mL were obtained from BD Gentest™ (Woburn, MA, USA). TAM and TNO were supplied by Cayman Chemical (Ann Arbor, MI, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. SDS, sodium dihydrogen phosphate, disodium hydrogen phosphate, methanol (MeOH), DMSO, α-CD, β-CD hydrate, γ-CD and hydroxypropyl-β-CD were purchased from Acros Organics (Geel, Belgium). Tween 20 was supplied by AppliChem GmbH (Darmstadt, Germany). MMI, nicotine, 3-(*N,N*-dimethylmyristyl-ammonium)propanesulphonate (MAPS), 3-(*N,N*-dimethylhexadecyl-ammonium)propanesulphonate (PAPS), nicotinamide adenine dinucleotide phosphate (NADPH), sodium taurodeoxycholate (STDC), anhydrous magnesium chloride ($MgCl_2$), DMXAA, carboxymethyl β-CD (CM-β-CD) and sulphated-β-CD sodium salt were purchased from Sigma-Aldrich (Steinheim, Germany). Carboxymethylated-γ-CD and 2-hydroxypropyl-γ-CD were obtained from Cyclolab (Budapest, Hungary). ACN, isopropanol (IPA) and sodium hydroxide (NaOH) were supplied by Fisher Scientific (Loughborough, UK). Ethanol (EtOH) and phosphoric acid (H_3PO_4) were obtained from VWR International (Leuven, Belgium). Triethanolamine (TEA) was purchased from Chem-Lab (Zedelgem, Belgium). All chemicals and reagents were of analytical grade. Water was purified with a Milli-Q Gradient system (Millipore, Molsheim, France). The

pH was measured using a Metrohm 691 pH meter (Metrohm, Herisau, Switzerland).

2.2 CE

Experiments were carried out on a P/ACE MDQ CE system (Beckman, Fullerton, CA, USA) equipped with a diode array UV-Vis detector. Instrument control and data evaluation were performed using 32 Karat software (Beckman Coulter, version 5.0). CZE separation was performed on fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) of 75 μm id and 60 cm total length (50 cm effective length). New capillaries were activated as follows: after filling with 1 M NaOH, the capillary was stored for 2 h at room temperature and finally rinsed with water for 10 min. At the start of each day, the capillary was conditioned by consecutive 10 min rinsing steps with 0.1 M NaOH, water and BGE. Between runs, the capillary was rinsed with water, 0.1 M NaOH and water for 1.0, 3.0 and 1.0 min, respectively, and then 5.0 min with BGE. For storage overnight, the capillary was additionally washed with water for 10 min. All solutions were filtered through 0.2 μm RC filters (Whatman, Dassel, Germany). A pressure of 20 psi was applied for all rinsing steps. The injection of standards was performed at 0.5 psi for 5 s. The voltage was set at –20 kV, reversed polarity, to perform the separation with a BGE of 100 mM phosphoric acid solution adjusted to pH 2.5 with TEA containing 50 mM STDC, 20 mM CM-β-CD and 20% ACN. The capillary was thermostated at 25°C and the sample tray was kept at 10°C. In addition, the detection was carried out at 240 nm.

2.3 Enzyme reaction monitoring

The reaction mixture contained 5 μL FMO protein (2075 nM; *FMO1* or *FMO3*), 80 μL NADPH (2 mM), 10 μL $MgCl_2$ (5 mM) and 103 μL incubation buffer (100 mM phosphate buffer, pH 8.6). It is important to mention that the concentrations in parentheses are the final concentrations. The FMOs and NADPH were dissolved and/or diluted in incubation buffer. TAM stock solution, prepared in EtOH, was diluted with incubation buffer to final organic solvent contents lower than 1% to sustain FMO activity. Enzyme, TAM and NADPH solutions were prepared freshly every day. The enzyme was stored at −80°C and before use it was thawed rapidly and stored on ice. All the other stock solutions were stored at −20°C until use.

After pre-incubation of the reaction mixture in a thermomixer (Eppendorf, Germany) for 5 min at 37°C, the reaction was initiated by the addition of TAM and allowed to incubate for 15 min at 37°C. The final concentrations of TAM were varied in the range of 10.62–255 μM for drawing the Michaelis–Menten curve. After incubation, the reaction was stopped by adding 200 μL EtOH and 15 μL H_3PO_4. The mixture was centrifuged for 10 min. Then, the supernatant fluid was transferred to mini-CE vials and analysed by the proposed method.

2.4 Inhibition studies

MMI stock solution (1.5 mM), dissolved in water, was diluted and added to incubation mixture to obtain seven different concentrations of MMI in the range of 0–1493.5 μM. After pre-incubation at 37°C for 5 min, the reaction was started by the addition of 43.0 μM TAM. The reaction was stopped and processed as described in Section 2.3. For investigating the type of inhibition and calculating the K_i value of MMI, three different MMI concentrations (0, 213.4 and 746.9 μM) were used and the TAM concentration was varied in the range of 10.62–255 μM ($n = 8$) in each experiment. The IC_{50} and K_i values were evaluated by GraphPad Prism version 5.0 software (San Diego, CA, USA). The kinetic constants were calculated using non-linear regression, also with GraphPad Prism version 5.0.

Nicotine, dissolved in EtOH, and DMXAA, dissolved in DMSO, were used at the concentration ranges of 0–1493.5 μM ($n = 8$) and 0–238.4 μM ($n = 6$), respectively, to investigate their effect on the N-oxygenation of TAM by both *FMO1* and *FMO3*. The other conditions were the same as mentioned above for MMI.

3 Results and discussion

3.1 Separation conditions

Oxidative metabolism generally produces more polar metabolites with only minor structural modifications of the parent compounds. The separation of these metabolites from the parent compounds has often been a difficult task. Besides separation, the determination of TAM and TNO has also been very challenging since these compounds have a very hydrophobic character.

Initially, pH 2.5–3.5 phosphate buffer and pH 4–5.5 acetate buffer were tried as BGE. Good resolution was achieved between TAM and TNO with phosphate buffer in the pH range of 2.5–3.5, but peak shapes were not promising. This was probably because of analyte interactions with the capillary wall. Adding CTAB to the BGE to dynamically coat the capillary, improved the speed of analysis but not the overall peak shapes. Then, the use of different organic solvents (i.e. MeOH, ACN and IPA) and CDs (i.e. α-, β-, γ-CDs and their derivatives) were investigated separately and in combination. Unfortunately, none of these strategies were successful.

In a previous study, it was suggested to use MEKC with non-ionic (Tween 20) and/or zwitterionic surfactants (MAPS and PAPS) under acidic conditions to separate basic drugs that have very similar structure [23]. Non-ionic or zwitterionic surfactants have been preferred because they do not change the ionic strength of the buffer, whereas cationic and anionic surfactants increase the conductivity of the buffer and limit

the use of higher voltages. Different surfactants including MAPS, PAPS and Tween 20 were investigated for their ability to enhance separation of TAM and TNO with good peak shapes. The resolution was not achieved with these surfactants alone or with their mixtures unless an anionic surfactant was added. The separation of the analytes was not obtained with SDS alone, because of the undesired strong electrostatic interactions with TAM, which is a basic molecule with a pKa of 8.85 [23]. Nevertheless, promising results were obtained when SDS was combined with a non-ionic surfactant in a BGE consisting of 25 mM SDS and 10 mM Tween 20 in the reversed mode of MEKC. Another system with separation was anionic surfactant and zwitterionic surfactant mixture consisting of 10 mM heptanesulphonic acid and 25 mM PAPS in normal mode of MEKC. Although different concentrations of all reagents in both systems and different chain lengths of heptanesulphonic acid were tried, the baseline was not promising with distorted and broad peak shapes.

In literature, the separation of highly hydrophobic drugs using MEKC with bile salts was reported. Bile salts, natural chiral surfactants, have a relatively weak solubilisation power compared with long-alkyl-chain surfactants and they have been found to be effective for drugs that were almost totally solubilised in SDS micelles because of their high hydrophobicity and were not separated by MEKC with SDS [24]. We could not try all of the bile salts because non-conjugated types must be used at pH >5, whereas taurine-conjugated ones are applicable at lower pHs. STDC at the reversed mode of MEKC, gave promising results for the separation of TAM and TNO but a complete separation could not be obtained.

CEC has been previously reported for difficult separations of isomers [25, 26]. As an alternative, the CD-MEKC technique in which surfactants are coupled with CDs, has been used to improve the separation of compounds with hydrophobic, isomerous or chiral properties [27]. Different α-, β-, γ-CDs and their derivatives were screened combined with STDC and the best separation of TAM and TNO was observed in the mixed system of STDC and CM-β-CD. Different concentrations of both agents, in the range of 50–150 mM STDC and 10–30 mM CM-β-CD, were tried and the optimum system was obtained with 50 mM STDC and 20 mM CM-β-CD. There was a need of adding organic solvent to this CD-MEKC system to improve TAM solubility, because of its hydrophobic character. After investigating MeOH, ACN and IPA in the concentration range of 5–20%, an optimised system was obtained with the addition of 20% ACN.

Although phosphate buffer (pH 2.5) containing 50 mM STDC, 20 mM CM-β-CD and 20% ACN was adopted as the optimal separation system, there was peak tailing of TAM because of basic analyte interaction with the capillary wall. In previous studies, this kind of peak tailing was minimised with a TEA-phosphate buffer and the EOF was also reversed in this way [28, 29]. Therefore, TEA was used to prepare the phosphate buffer. Different concentrations of the buffer, 50, 75 and 100 mM, at different pHs in the range of 2–3.5 were tried and a good peak symmetry with reasonable migration times was obtained with 100 mM phosphoric acid buffer adjusted to pH 2.5 with TEA containing 50 mM STDC, 20 mM CM-β-CD and 20% ACN (Fig. 2).

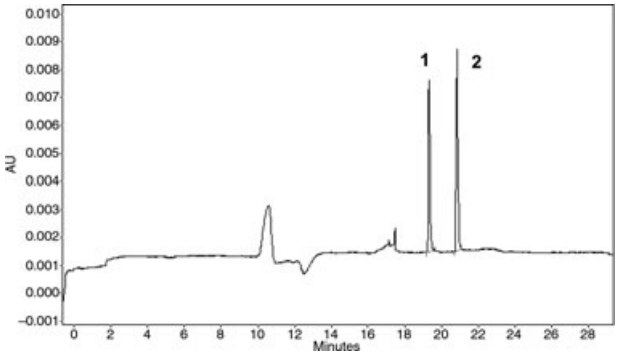

Figure 2. A typical electropherogram of standard TAM and TNO under optimised conditions (1: TNO; 2: TAM).

More than 14 different compounds were tested for possible use as an internal standard (IS), for example, small molecules, hydrophobic molecules or large acid molecules, which do not interact with the micelles. All small molecules and hydrophobic molecules used, had longer migration times than TAM, and thus extended the analysis time. Acid molecules exhibited reasonable migration times around 20 min, but they interfered with TNO. Only 3,6-dihydroxynaphthalene-2,7-disulphonic acid had a suitable migration time (15.7 min), but unfortunately it degraded when it was added to a sample of TAM and TNO also containing 15 μL H_3PO_4 like an incubation sample. So, as no proper IS was found, the experiments were performed without IS, using corrected peak area (the peak area divided by the migration time).

3.2 Method validation

The calibration curves of TAM and TNO standards were obtained by triplicate analysis of six different solutions containing 2.45–979.2 μM TAM and of six different solutions containing 1.86–621.8 μM TNO, using the established method. The method showed to be linear over the tested concentration range of TAM and TNO with a determination coefficient of 0.997 and 0.998, respectively. The LODs were 0.53 μM for TAM and 0.38 μM for TNO (S/N = 3). The limits of quantitation were found as 1.76 μM for TAM and 1.29 μM for TNO (S/N = 10). The repeatability was measured for two concentration levels of both TAM and TNO, at 24.5 and 245 μM TAM, 31.1 and 311 μM TNO, by injecting each solution eight times. The RSD for the corrected peak area is lower than 5.8% and for the migration time it does not exceed 2.6%. All results are summarised in Table 1.

3.3 *FMO1* kinetics

Initially, the aim of this study was to develop an electrophoretically mediated microanalysis method, in which the

Table 1. Method validation for TAM and TNO

	TAM		TNO	
	24.5 μM	245 μM	31.1 μM	311 μM
Repeatability of migration time in % (RSD, $n = 8$)	1.9	1.8	1.8	2.6
Repeatability of corrected peak area in % (RSD, $n = 8$)	3.8	2.7	5.8	5.6
Linearity (μM)	2.45–979.2		1.86–621.8	
Equation[a)]	$y = 37.01x - 287.15$		$y = 24.18x + 5.98$	
Determination coefficient (R^2)	0.997		0.998	
LOD (μM)	0.53		0.38	
LOQ (μM)	1.76		1.29	

a) x is the concentration of TAM or TNO in μM, y is the corrected peak area of TAM or TNO.

reaction is initiated inside the capillary and the capillary is used as a separation tool, and a micro-reactor to investigate the metabolism of TAM by FMOs. In this approach, the main advantages are extremely minute sample consumption (nL) and complete automation by the integration of all the assay steps. Recently, several in-line CE methods have been reported for FMO studies [30–32]. Unfortunately, it was not possible to work in-line because of the excessive hydrophobic character of TAM. The solubility of TAM was assured only in organic solvents, while the percentage of organic solvent must be less than 1% for enzyme activity as mentioned before. Then, the metabolism of TAM by FMOs was investigated with an off-line method.

The Pharmaceutical Research and Manufacturers of America Drug Metabolism and Clinical Pharmacology Technical Working Groups define a minimal best practice for in vitro and in vivo kinetic studies [33]. Although this practice was based on CYP450, the principles can also be applied to other drug-metabolising enzymes such as FMOs. As mentioned, the following experiments are needed to establish accurate kinetic parameters. First, a reaction time course experiment should be performed in which the incubation is conducted at a single concentration of protein and substrate, with the metabolite formation measured at several time points. Second, the relationship between enzyme concentration and reaction velocity at one particular incubation time, determined in the former experiment, should be established.

FMO1 and *FMO3* have been compared according to TNO formation with the same concentrations of enzyme and substrate. The results, which are in agreement with previous papers [9, 14], indicate that TAM is a markedly better substrate for *FMO1* than *FMO3*. TNO formation by *FMO3* was less than one fifth of TNO formation by *FMO1* (data not shown). So, the following experiments were performed with *FMO1*.

3.3.1 Dependence of *FMO1* reaction on incubation time

The incubation time of the enzymatic reaction should be within the linear range in relation with the metabolite production to properly reflect reaction rates. The linearity of TNO production was investigated at five different time points (0–20 min). The concentration of TAM was kept at 0.51 mM and the concentration of enzyme used was 8300 nM. Each time point was analysed in triplicate. The corrected peak area of TNO (y) was plotted as a function of incubation time (x) and subjected to linear regression analysis. The regression equation is $y = 70.42x + 164.2$ and the product amount is linear up to 20 min of incubation time.

In theory, any time point within the linearity range could be chosen as incubation time for further investigations. However, after testing with several different substrate and enzyme concentrations, 15 min was chosen as the incubation time, so that even at the lowest concentration of substrate or enzyme investigated, TNO peaks would still be quantifiable.

3.3.2 Dependence of *FMO1* reaction on enzyme amount

The effect of the enzyme amount on the *FMO1* reaction was assessed by investigating six different enzyme concentrations (500–8300 nM). Following 15 min incubation of 0.51 mM TAM, the mixture was analysed in triplicate. No TNO peak could be detected when the enzyme concentration was 500 nM. The corrected peak area of TNO (y) was plotted as a function of *FMO1* concentration in the range of 830–8300 nM (x) and subjected to linear regression analysis. The regression equation is $y = 0.72x - 266.54$ with a determination coefficient of 0.9975 indicating sufficient linearity. Then, it was decided to use an enzyme concentration of 2075 nM to obtain sufficient product using less enzyme.

3.3.3 K_m determination

The K_m is the substrate concentration at half the V_{max} and is determined by non-linear regression of a plot of product formation versus substrate concentration. To obtain an accurate value, substrate concentrations should span a range of at least 1/5 K_m to 5 K_m with at least six concentrations. The kinetic parameters of *FMO1* were determined from eight

different TAM concentrations ranging from 10.62 to 255 μM. The incubation time of 15 min and the enzyme concentration of 2075 nM were kept constant. Each substrate concentration point was measured in duplicate. The K_m and V_{max} values were derived using non-linear regression by GraphPad Prism version 5.0 as 164.1 μM and 3.61 μmol/min/μmol *FMO1*, respectively, for the formation of TNO by *FMO1*. It has been noted that calculated K_m values are well above the plasma concentration of TAM [34]. In literature the K_m value was reported as 43 μM for human *FMO1* [14] and 1.2 mM for mouse *FMO1* [13]. As K_m values vary depending on the origin of the *FMO1* enzyme and the method conditions, the K_m for TAM cannot be compared exactly with the K_m values from other studies. Nevertheless, the K_m value obtained is in the range of previously reported data.

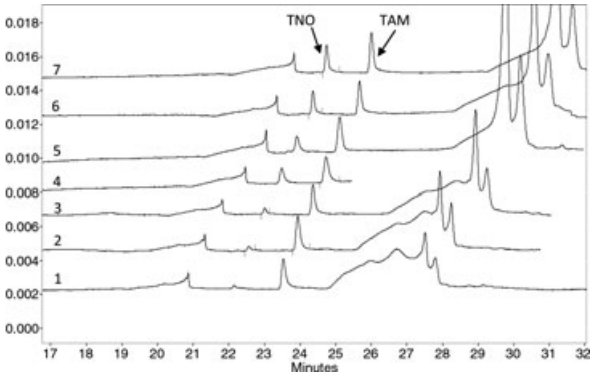

Figure 3. The overlapped electropherograms of incubation samples inhibited with different concentrations of MMI in the range of 0–1493.5 μM (1: 1493.5, 2: 746.74, 3: 373.37, 4: 186.68, 5: 93.34, 6: 46.67 and 7: 0 μM MMI).

3.4 Inhibition studies

In previous papers, MMI [7, 9] and octylamine, were used to indicate the involvement of FMOs in N-oxidation of TAM. They act, respectively, as inhibitor and as a positive effector of FMOs in several species [7]. Also, Katchamart et al. reported that dietary I3C and 3,3′-diindolylmethane reduced *FMO1* protein levels in rats and they suggested the potential for altered toxicity of TAM and nicotine due to the alteration of FMO- and CYP-mediated drug metabolism [6]. In another study, they demonstrated that *FMO1* downregulation by I3C may not be common to other mammals (i.e. guinea pigs, mice and rabbits) for which *FMO1* is the major isoform in the liver [35]. Jordan et al. found that the production of TNO was completely inhibited in the presence of peroxidase inhibitors such as reduced glutathione, ascorbate and sodium azide. Also, it was observed that TNO formation was completely inhibited when superoxide dismutase and catalase were preincubated for 30 min with rat liver homogenate at 37°C [36].

In this work, the inhibitory effects of MMI, nicotine and DMXAA on the N-oxygenation of TAM by FMOs were investigated.

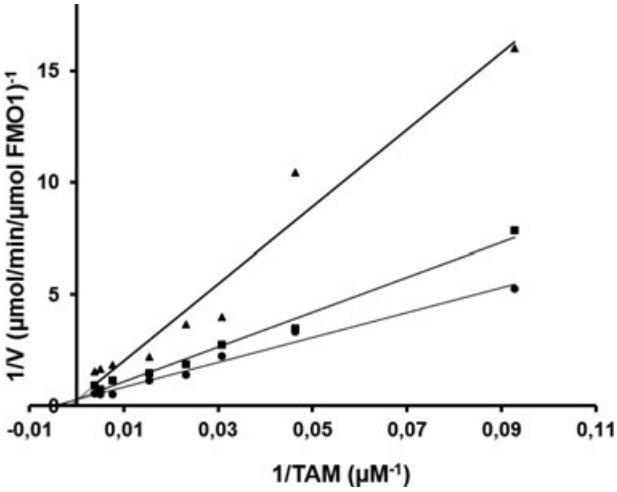

Figure 4. The overlapped Lineweaver–Burk plots for the enzymatic reaction of *FMO1* at a concentration of 2075 nM, incubation time 15 min. The line (▲) is the plot for inhibitor MMI at 746.9 μM and varying TAM from 10.62 to 255 μM. The line (■) is the plot for MMI at 213.4 μM and varying TAM in the same concentration range. The line (●) is the plot for no MMI with varying TAM in the same concentration range.

3.4.1 MMI effect

It is the most common approach to inhibit FMO-mediated drug metabolism with MMI, although it is not specific for FMO inhibition. In fact, MMI is used clinically as an anti-thyroid drug as it inhibits thyroid peroxidase [2]. It is S-oxygenated by FMOs and it acts as an alternate substrate inhibitor [7–10].

One of the most important characteristics of a given inhibitor from a pharmaceutical point of view is the IC_{50}. Its determination was performed by changing the concentration of MMI from 0 to 1493.5 μM (0, 46.7, 93.3, 186.7, 373.4, 746.7 and 1493.5 μM) in a reaction mixture containing fixed concentrations of TAM (43.0 μM) and *FMO1* (2075 nM); the incubation was performed for 15 min (Fig. 3). Besides, K_i for MMI was also determined by measuring the initial velocities of the *FMO1* reaction at varying concentrations of the substrate and the inhibitor, each time analysed in duplicate. The Lineweaver–Burk plots of the *FMO1* reaction at two MMI concentrations are intersecting the $1/V$ axis in the point $1/V_{max}$ and show that MMI is a competitive inhibitor with respect to the TAM N-oxygenation reaction (Fig. 4). The IC_{50} and the apparent K_i values evaluated by the given software were 266.2 ± 1.13 μM and 159 ± 40.46 μM, respectively. According to our knowledge, it is the first time these values have been determined for TAM N-oxygenation.

3.4.2 DMXAA effect

DMXAA is a novel anti-tumour agent, developed by the Auckland Cancer Society Research Centre in New Zealand.

It has an unusual activity, compared to other cytotoxic anti-tumour agents. It induces rapid vascular collapse and necrosis in transplantable murine tumours. Immune modulation by the induction of cytokines (e.g. tumour necrosis factor, interferons, serotonin and nitric acid), as well as anti-vascular and anti-angiogenic effects are considered to be the major mechanisms of action [37]. DMXAA alone does not show striking anti-tumour activity in patients. This is consistent with the results obtained for many other biological response modifiers. However, co-administration of DMXAA with other drugs has been shown to result in enhanced anti-tumour activity and alterations in pharmacokinetics [38].

Multiple studies on the pharmacokinetics of DMXAA using rodent models and in vitro human liver microsomes have indicated that DMXAA is metabolised to DMXAA 1-O-acyl glucuronide (DMXAA-G) and 6-hydroxymethyl-5-methylxanthenone-4-acetic acid (6-OH-MXAA). DMXAA-G results from conjugation to glucuronic acid and this is the major metabolic pathway (70%). 6-OH-MXAA on the other hand results from 6-methyl hydroxylation by CYP1A2. *FMO3* has also exhibited catalytic activity towards the formation of 6-OH-MXAA and MMI has inhibited 35% of the hydroxylation verifying this activity [39].

The effects of various anti-cancer drugs (i.e. vinblastine, vincristine, amsacrine, daunorubicin, *N*-[2-(dimethylamino)-ethyl]acridine-4-carboxamide (DACA), 5-fluorouracil, paclitaxel, tirapazamine and methotrexate) on the major metabolic pathways (glucuronidation and 6-methyl hydroxylation) of DMXAA in human liver microsomes were investigated [40]. However, according to our knowledge there is no study reporting its effect on the metabolism of another anti-cancer drug. Also, it has not been used as a possible alternate substrate inhibitor of FMOs.

The DMXAA effect on N-oxygenation of TAM by *FMO1* was investigated by changing its concentration from 0 to 238.4 μM in the reaction mixture. Higher concentrations of DMXAA could not be used due to its low solubility, even in DMSO. The other conditions were the same as mentioned for MMI.

Interestingly, DMXAA inhibited the reaction of TAM catalysed by *FMO1*, although no activity of *FMO1* containing microsomes was reported towards the DMXAA hydroxylation [39]. The inhibition increased up to 51.1 μM DMXAA and then started to decrease and inhibition was almost the same at DMXAA concentrations of 119.2 and 238.4 μM. DMXAA at 51.1 μM inhibited TNO formation by 52.2 ± 5.0%, whereas the inhibition was 13.8 ± 4.5% at a concentration of 238.4 μM, possibly because of the atypic metabolism of DMXAA by FMOs. Zhou et al. observed a reduction in the rate of DMXAA 6-methyl hydroxylation in cDNA-expressed *FMO3* at DMXAA concentrations above 100 μM according to a substrate inhibition model [39].

As mentioned before, TAM is a better substrate for human *FMO1* than *FMO3* [9,14]. Because of this, when the effect of DMXAA on TNO formation by *FMO3* was investigated, the TNO peak areas were very small and it was impossible to draw conclusions about the effect of DMXAA on this reaction.

3.4.3 Nicotine effect

Smoking is the leading cause of preventable mortality in the world and the use of tobacco is still rising globally. Nicotine is not a direct cause of most tobacco-related diseases, but it is highly addictive. The addictiveness of nicotine results in the continuing use of tobacco products and exposure to the diverse array of carcinogens and other bioactive compounds in tobacco, making tobacco use the leading cause of premature deaths in developed countries [41].

P450 CYP2A6 is considered the primary catalyst of nicotine metabolism, but also FMOs play an important role in nicotine metabolism [42]. Until recently, *FMO3* has been known as the main enzyme responsible for nicotine N′-oxide formation and the formation of (S)-nicotine N′-oxide has been reported as a highly stereoselective probe of human *FMO3* function [43,44]. Recently, Hinrichs et al. determined the catalytic efficiency of nicotine N-oxidation by *FMO1* to be higher than *FMO3*, although *FMO1* was not known to metabolise nicotine [42].

Because of these features, the aim was to investigate for the first time the effect of nicotine on the N-oxygenation of TAM and the possible behaviour of nicotine as an alternate substrate FMO inhibitor.

The effect of nicotine was investigated by changing its concentration from 0 to 1493.5 μM as mentioned for MMI and DMXAA. Interestingly, no inhibitory effect of nicotine on the *FMO1* reaction was found in this concentration range; 1493.5 μM is quite above the plasma concentration of nicotine [41], so there is no need to check for a possible inhibitory effect of nicotine at an even higher concentration.

The result of the experiments with *FMO3* was difficult to interpret for nicotine, as no comparative data were obtained because of the very small TNO peaks.

4 Concluding remarks

Although many types of enzymes have been characterised by in vitro drug metabolism studies, there is not as great an appreciation for other drug-metabolising enzymes such as FMOs in comparison with cytochromes P450. In this work, a selective and sensitive CD-MEKC method in aqueous media has been developed for the first time to investigate TAM metabolism by *FMO1*. The inhibitory effect of MMI, a well known alternate substrate FMO inhibitor, on this reaction has been investigated exhaustively and the possible effects of nicotine and DMXAA as alternate substrate FMO inhibitors have been evaluated for the first time. Obtained *FMO1* kinetic parameters are in good agreement with the literature values. No inhibitory effect of nicotine on TAM N-oxygenation has been observed, whereas DMXAA inhibited this reaction by 52%. Also, IC_{50} and K_i values of MMI have been evaluated.

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