## Integrated Analytical Strategies for Drug Discovery

de Vlieger, J.S.B.

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# Chapter 3

## Identification of trimethoprim phase-I metabolites using LC-high-resolution MS/MS and *in silico* prediction software.

**Abstract**

In this chapter we describe the identification of phase I metabolites of trimethoprim. Metabolites were generated using different enzyme systems, i.e. human liver microsomes, rat liver microsomes and a drug metabolizing mutant of cytochrome P450 BM3. Identification of the metabolites was done using LC-high resolution MS/MS experiments, facilitated by *in silico* metabolite prediction software. The MS/MS experiments on trimethoprim provided two marker fragments to be used for the identification of the sites of modification of the parent drug molecule. Significant fragmentation of trimethoprim was observed in ESI-MS/MS and ten metabolites were identified. Moreover we identified a synthesis side-product, being N-methylated on the (2,4-diaminopyrimidin-5-yl) ring. All metabolites were predicted by the SyGMA prediction software, although differences were observed in the prediction and actual experimental detection of several N-Oxides. Nevertheless, in this particular case, the use of a list of predicted metabolites is of added value to the identification process.

**Introduction**

Metabolite identification plays a major role in the drug discovery and development process. Metabolism of drugs and other xenobiotics increases the hydrophylicity of these compounds and alters their biologic activity. Therefore, the identification of drug-metabolites provides insight in the physiological behavior of the drug when it is administered to any kind of species. Early in vitro studies are frequently used to identify the 'soft spots' of drug candidates providing data to the medicinal chemist for rational synthesis of metabolic stable structure analogues [1]. Although infrared and nuclear magnetic resonance spectroscopy can both be applied for structure characterization, liquid chromatography-mass spectrometry (LC–MS) is the method of choice for most drug metabolism studies [2]. Analytical strategies and the need for metabolite identification using LC–MS are recently reviewed in several papers [3,4].

In addition to the instrumental analysis of metabolites, the prediction of biotransformation products by *in silico* metabolism studies is nowadays of increasing importance [5]. The combination of predictive software packages and the link to mass spectrometric experiments can significantly contribute to the speed of identification of metabolites in complex mixtures [6]. Several approaches

to integrate these processes have been described, also in combination with data processing software strategies [7,8]. The mainstream of predictive programs is rule-based and incorporate expert knowledge on metabolic pathways. An alternative approach is to apply statistical analysis on large databases for the implementation of probability scores. Combining the two, Ridder *et al.* recently published the new prediction tool SyGMa (Systematic Generation of potential Metabolites) to rank predicted metabolites based on empirical probability scores [9]. Trimethoprim (TMP) is a broad spectrum antibacterial agent, often used in combined therapy with sulfonamides in cotrimoxazole [10]. Previously, LC–MS studies were reported on the characterization of impurities in TMP tablets, using LC-UV, LC–(APCI)MS and NMR [11,12]. Eckers *et al.* discussed the fragmentation of TMP and other alkoxy-phenyl groups in considerable detail, concluding that radical cations are commonly observed in electrospray MS/MS spectra of this compound class [13]. At the same time, Eichorn *et al.* published the identification of microbial degradates of TMP by Hydrogen/Deuterium exchange experiments and accurate mass measurements [14].

In this chapter, we describe the application of *in silico* metabolite prediction in combination with the use of a hybrid ion-trap time-of-flight (IT-TOF) mass spectrometer for the identification of *in vitro* TMP phase-I metabolites generated with cytochrome P450s in various ways. LC-multistage MS ($MS^n$) experiments and accurate mass measurements of precursor and fragment ions were performed to identify the phase-I metabolites. Following this study, the identification of reactive intermediates of TMP upon bioactivation by mutant bacterial cytochrome P450s was performed. This is described in chapter 4 of this thesis [15].

## Experimental

### *In silico* metabolite prediction

The *in silico* metabolite prediction of the phase I related metabolites of TMP was performed using the recently described SyGMa tool [9]. SyGMA predicts metabolites based on rules derived from expert knowledge and ranks these predicted metabolites on the basis of probability scoring. The chemical structure of TMP was processed and the predicted metabolites were subsequently used to facilitate metabolite identification. Probability scores were generated for rat and human *in vitro* and *in vivo* situations. The outcome of this prediction was kindly

provided by Dr. Lars Ridder (Molecular Design and Informatics department of Organon, now MSD Research Laboratories in Oss, the Netherlands).

**Enzymes and Reagents**

Rat liver microsomes (RLM) were prepared as described by Rooseboom *et al.* [16]. Preparation of the bacterial P450 BM3 mutant $M11_{his}$ was performed following the protocol described recently [17]. Both enzyme systems were kindly provided by Dr. J.N.M. Commandeur (section Molecular Toxicology, VU University Amsterdam). Human liver microsomes (HLM), pooled from 50 donors, and containing 20 mg/mL protein were obtained from Xenotech (Lot No. 0710619, Lenexa, USA). Acetonitrile, methanol and formic acid (ULC/MS grade) were obtained from Biosolve (Valkenswaard, The Netherlands), Water was prepared using a Millipore Milli-Q purification system (Milford, CT, USA). Trimethoprim (TMP) was supplied by Sigma Aldrich (Steinheim, Germany). Perchloric acid (70%), potassium mono- and dibasic phosphate were purchased from Riedel de Haën (Seelze, Germany). β-Nicotinamide adenine dinucleotide phosphate (NADPH) tetra sodium salt was obtained from Applichem (Lokeren, Belgium).

**Enzymatic incubations**

All enzymatic incubations were performed in 100 mM potassium phosphate buffer at pH 7.4 and had final volumes of 1 mL. The final substrate concentration present in the incubation was 100 µM. Metabolite production was initiated by the addition of 2 mM NADPH with a regenerating system containing 0.3 mM glucose-6-phosphate, and 0.4 units/mL glucose-6-phosphate dehydrogenase. RLM and HLM incubations contained 1 mg/mL protein and were incubated at 37°C. The BM3 $M11_{his}$ incubations contained 250 nM of purified enzyme and were performed at 24°C in a thermomixer. After 120 min incubation, the reactions were stopped by adding 100 µL of a 10 % solution of perchloric acid. Precipitated proteins were removed by centrifugation for 15 minutes at 6000 rpm and the supernatants were used for LC-$MS^n$ analyses.

**Apparatus and analytical conditions**

LC-$MS^n$ experiments were performed using a Shimadzu LC-IT-TOF-MS (‘s Hertogenbosch, the Netherlands). The LC-IT-TOF-MS system consisted of two LC20AD pumps, a SIL-20AD autosampler, a CTO-AC column oven, a DGU-3 solvent

degasser and a hybrid ion-trap time of flight mass spectrometer equipped with an ESI source. The system was controlled by a SCL-20ADvp system controller and the Shimadzu LC-MS Solutions software package (version 3.50). LC experiments were performed on a Phenomenex Luna 3 µm C18(2) 100 x 2 mm column using a flow rate of 200 µL/min for gradient elution. Solvent A consisted of 99% $H_2O$, 0.9 % of acetonitrile, and 0.1 % formic acid. Solvent B consisted of 99% acetonitrile, 0.9 % $H_2O$, and 0.1 % formic acid. The LC-gradient was as follows; 0 to 5 min isocratic 95% A; from 5 to 20 min a linear increase to 95 % B where kept constant for 5 min, and return to initial 95% A in 0.1 min for an equilibration step of 8 min. The MS was operated in positive ion mode with an interface voltage of 5 kV and nitrogen as drying gas at a flow of 1.5 L/min. The curved desolvation line (CDL) and heat block temperature was set at 200°C. For fragmentation experiments, the instrument was used in the data-dependent auto $MS^n$ mode. The first event was used for full scan mode from *m/z* 100 – 600. In the second event, the most abundant precursor ion between *m/z* 200 and *m/z* 400 was automatically selected and fragmented. External calibration of the instrument was performed with sodium TFA clusters allowing a mass accuracy of 5 ppm.

The ion-accumulation time was set to 10 ms, collision energy and gas to 50%. Fragmentation of the parent drug molecule was done by direct infusion at 10 µL/min of a 10 µM standard in 50% acetonitrile with 0.1 % formic acid using a Hamilton Model 22 syringe pump (Harvard Apparatus, Hollister, USA).

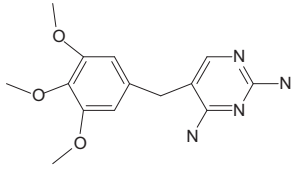
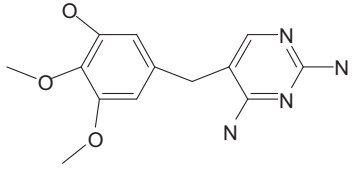
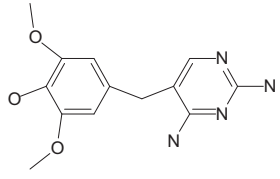
**Results and discussion**

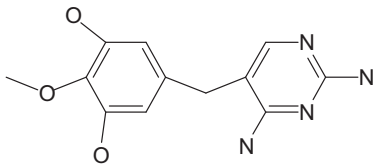
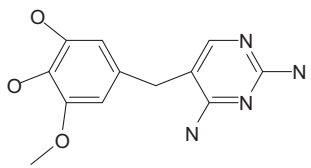
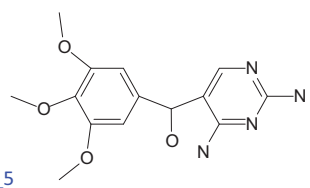
***In silico* prediction of phase-I metabolites**

Results from the *in silico* prediction of the phase-I metabolites of TMP are depicted in Table 1. For the first 10 predicted metabolites, Table 1 shows the probability of the metabolites to be formed in human and rat *in vitro* and *in vivo* experiments. The metabolites are ranked by the probability of occurrence in an *in vivo* human study but the ranking for an *in vitro* incubation is not significantly different. For each predicted metabolite, the accurate mass and the change in the molecular formula relative to the parent drug is provided as well as the reaction steps required for the formation of the metabolite. The mass information is provided for the molecule itself but can be easily transformed to *m/z* values of $[M+H]^+$ or $[M-H]^-$, depending on the preferred ionization mode.

I

**Table 1:** Top ten ranked SyGMA predicted phase I metabolites of trimethoprim

| CHEMISTRY | Reaction Sequence | Human vivo Probability | Human vitro Probability | Rat vivo Probability | Rat vitro Probability | Molecular Mass | Rel. Mol. Formula |
|---|---|---|---|---|---|---|---|
|  trimethoprim | 1. PARENT: trimethoprim | 1 | 1 | 1 | 1 | 290.1379 | $C_{14}H_{18}N_4O_3$ |
|  trimethoprim_1 | 1. O-demethylation | 0.2713 | 0.3912 | 0.2837 | 0.3518 | 276.1223 | $-CH_2$ |
|  trimethoprim_2 | 1. O-demethylation | 0.2713 | 0.3912 | 0.2837 | 0.3518 | 276.1223 | $-CH_2$ |

| CHEMISTRY | Reaction Sequence | Human vivo Probability | Human vitro Probability | Rat vivo Probability | Rat vitro Probability | Molecular Mass | Rel. Mol. Formula |
|---|---|---|---|---|---|---|---|
|  trimethoprim_3 | 1. O-demethylation<br>2. O-demethylation | 0.0736 | 0.1531 | 0.0805 | 0.1237 | 262.1066 | $-C_2H_4$ |
|  trimethoprim_4 | 1. O-demethylation<br>2. O-demethylation | 0.0736 | 0.1531 | 0.0805 | 0.1237 | 262.1066 | $-C_2H_4$ |
|  trimethoprim_5 | 1. benzylic hydroxylation<br>(c-CH2-CR) | 0.0719 | 0.1103 | 0.0787 | 0.1426 | 306.1328 | +O |

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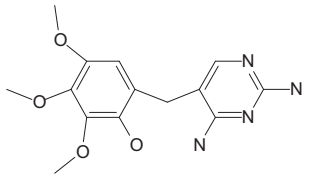
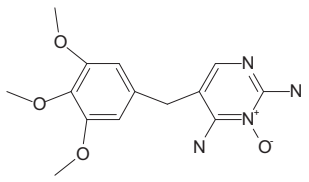
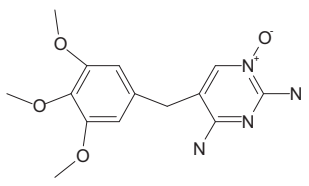
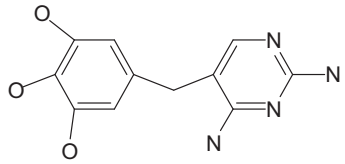
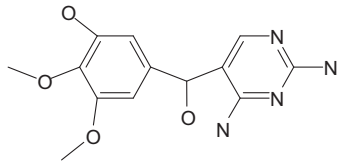
| CHEMISTRY | Reaction Sequence | Human vivo Probability | Human vitro Probability | Rat vivo Probability | Rat vitro Probability | Molecular Mass | Rel. Mol. Formula |
|---|---|---|---|---|---|---|---|
|  | 1. aromatic hydroxylation | | | | | | |
| trimethoprim_6 | (para to oxygen) | 0.0631 | 0.0891 | 0.0565 | 0.0866 | 306.1328 | +O |
|  | | | | | | | |
| trimethoprim_7 | 1. N-oxidation (-N=) | 0.0333 | 0.0475 | 0.0302 | 0.0453 | 306.1328 | +O |
|  | | | | | | | |
| trimethoprim_8 | 1. N-oxidation (-N=) | 0.0333 | 0.0475 | 0.0302 | 0.0453 | 306.1328 | +O |

| CHEMISTRY | Reaction Sequence | Human vivo Probability | Human vitro Probability | Rat vivo Probability | Rat vitro Probability | Molecular Mass | Rel. Mol. Formula |
|---|---|---|---|---|---|---|---|
|  trimethoprim_9 | 1. O-demethylation<br>2. O-demethylation<br>3. O-demethylation | 0.02 | 0.0599 | 0.0228 | 0.0435 | 248.0909 | $-C_3H_6$ |
|  trimethoprim_10 | 1. O-demethylation<br>2. benzylic hydroxylation (c-CH2-CR) | 0.0195 | 0.0432 | 0.0223 | 0.0502 | 292.1172 | $+O -CH_2$ |

**Fragmentation of trimethoprim**

The fragmentation of TMP with ion-trap MS was previously described by Eichorn *et al.* [14]. The fragments proposed were confirmed by the accurate-mass data generated with the IT-TOF MS. In general, TMP is extensively fragmented in ESI-MS/MS experiments, which is an important characteristic to facilitate the identification of metabolites.

Figure 1 shows the MS-MS spectrum of TMP. The modification site of the parent molecule is easier to indicate if the parent drug molecule shows good marker fragments. In the case of TMP, two useful marker fragments can be recognized in the MS-MS spectrum, which are the ion with *m/z* 123.0664 due to the (2,4-diaminopyrimidin-5-yl)methylium ion and the ion with *m/z* 181.0847 due to the (3,4,5-trimethoxyphenyl)methylium ion (the fragments B and A, respectively, in Figure 1) [11-14]. Other fragment ions include the losses from the 3,4,5-trimethoxyphenyl ring, *i.e.*, $CH_3^{\bullet}$ to an ion with *m/z* 276, of $CH_4$ from two adjacent methoxy-groups to *m/z* 275, the loss of $C_2H_6$ to *m/z* 261 (rather than the loss of $H_2C{=}O$, [13]) and the combined loss of $H_2C{=}O$ and $CH_3O^{\bullet}$ to *m/z* 230 [11-14].

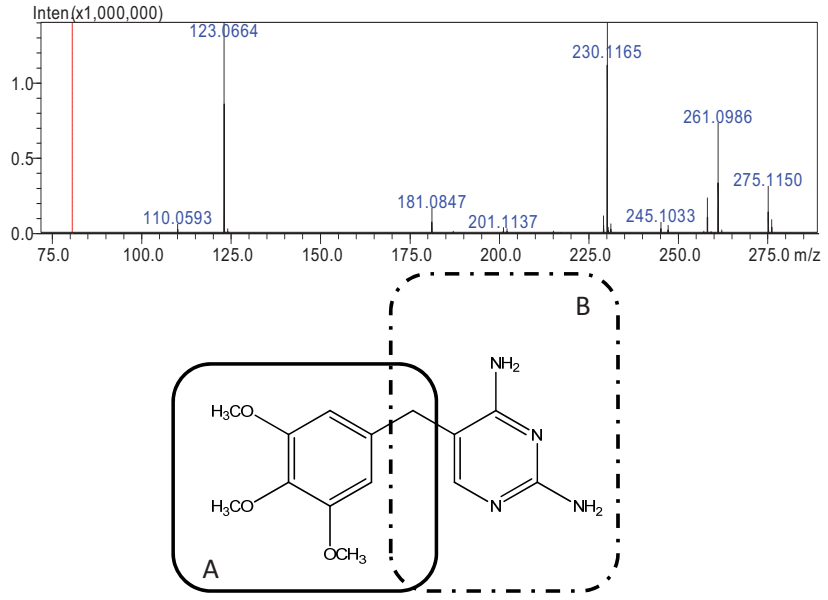



**Figure 1:** ESI-MS-MS spectrum of Trimethoprim and fragmentation of Trimethoprim in two parts (A *m/z* 181 and B *m/z* 123) indicating site of modification in the metabolites.

**Metabolite identification**

Table 2 summarizes the results of the *in vitro* enzymatic incubations using the three enzymatic systems, RLM, HLM and BM3 M11, showing the phase-I metabolites formed. Two metabolites (TMP-1 and TMP-2) were found with $[M+H]^+$ of *m/z* 277.1295, corresponding to demethylation of TMP. MS-MS analysis resulted in identical fragmentation spectra for both compounds, featuring the presence of the ion with *m/z* 123. This indicates *O*-demethylation in the 3,4,5-trimethoxyphenyl ring. *O*-Demethylation of the 3'-methoxy or the 5'-methoxy group results in identical structures. *O*-Demethylation of the 4'-methoxy group results in the other structure. Based on the other fragmentation data, no distinction can be made between 3'- and 5'- demethylated or 4'-demethylated TMP. Both demethylated metabolites were predicted with highest probability by SyGMa (*cf.* Table 1). Four chromatographic peaks were detected in the rat and human liver microsomal incubations with *m/z* 307.1401, indicating the presence of four hydroxylated or *N*-oxidated TMP metabolites. Only one of these metabolites, TMP-4b, showed the unmodified characteristic B fragment with *m/z* 123, whereas two others, TMP-4a and TMP-5, showed a +16-Da modified B fragment with *m/z* 139.0610, indicating an additional oxygen atom in the B fragment, *i.e.*, in the (2,4-diaminopyrimidin-5-yl) ring. Finally, TMP-3 showed neither the characteristic A nor B fragment but did show the loss of $H_2O$, indicating hydroxylation at an aliphatic carbon, *i.e.*, the exocyclic $CH_2$ is hydroxylated. Other fragments of TMP-3 involve the cleavage of $CH_3^{\bullet}$ from the trimethoxyphenyl ring, which is consistent with the previously reported spectrum where the hydroxyl position was determined with an H/D exchange experiment [14]. The hydroxyl metabolite having the unmodified B fragment, TMP-4b, showed the loss of $OH^{\bullet}$, indicating that the hydroxylation most likely occurred at the aromatic ring. For the modified B ring metabolites, TMP-4a and TMP-5, there are several structures possible: *N*-oxidation, hydroxylation at an aromatic carbon, or the formation of a hydroxylamine at the exocyclic $NH_2$ groups. Because in TMP-5 the loss of $OH^{\bullet}$ is observed, we propose hydroxylation at the aromatic carbon of the (2,4-diaminopyrimidin-5-yl) ring. The MS/MS spectrum of TMP-4a showed the loss of both $H_2O$, $OH^{\bullet}$, and O which is reported to be characteristic of an *N*-oxide [18]. *Hydrogen/Deuterium* exchange experiments [19] could provide more information on this, but were not performed. Due to its low abundance, TMP-5 showed no clear further fragmentation.

**table 2:** TMP phase I metabolites formed by in vitro incubation using RLM, HLM and BM3 M11, annotation of metabolites is related to chapter 5

| Phase I Metabolites | Measured $[M+H]^+$ (*m/z*) | Molecular formula | Proposed structure | Fragmentation A (*m/z* 181) | B (*m/z* 123) | Additional Marker fragments | RLM | HLM | $M11_{his}$ | SyGMa rank |
|---|---|---|---|---|---|---|---|---|---|---|
| **TMP** | 291.1451 | $C_{14}H_{18}N_4O_3$ | - | | | | | | | |
| **TMP-1** | 277.1304 | $C_{13}H_{16}N_4O_3$ | TMP-$CH_2$ | -14 | √ | - | √ | √ | √ | **1-2** |
| **TMP-2** | 277.1301 | $C_{13}H_{16}N_4O_3$ | TMP-$CH_2$ | -14 | √ | - | √ | √ | √ | **1-2** |
| **TMP-3** | 307.1394 | $C_{14}H_{18}N_4O_4$ | TMP+O | - | - | - $H_2O$ (*m/z* 289) | √ | √ | √ | **5** |
| **TMP -4a** | 307.1401 | $C_{14}H_{18}N_4O_4$ | TMP+O | √ | + 16 | -•OH (*m/z* 290) | √ | √ | √ | **7/8/13** |
| **TMP-4b** | 307.1409 | $C_{14}H_{18}N_4O_4$ | TMP+O | - | √ | -•CH3 -•OH (*m/z* 292 – 275) | √ | √ | √ | **6** |
| **TMP-5** | 307.1403 | $C_{14}H_{18}N_4O_4$ | TMP+O | - | +16 | -•OH (*m/z* 290) | √ | √ | - | **21** |
| **TMP-6** | 263.1149 | $C_{12}H_{14}N_4O_3$ | TMP-2x$CH_2$ | - | √ | - | √ | √ | √ | **3** |
| **TMP-7** | 323.1348 | $C_{14}H_{18}N_4O_5$ | TMP+2xO | - | - | -$CH_4O$ (*m/z* 291) | - | - | √ | **39/40** |
| **TMP-8** | 323.1351 | $C_{14}H_{18}N_4O_5$ | TMP+2xO | - | - | -$CH_4O$ (*m/z* 291) | - | - | √ | **39/40** |
| **TMP -9** | 293.1245 | $C_{13}H_{16}N_4O_4$ | TMP- $CH_2$ + O | - | - | -$H_2O$ (*m/z* 275) | - | - | √ | **10/11** |
| **IMP-1** | **305.1601** | **$C_{15}H_{19}N_4O_3$** | **TMP + $CH_2$** | **+14** | **√** | **-** | **-** | **-** | **-** | **-** |

TMP-7 and TMP-8 were only formed by the BM3 mutant. The observed *m/z* of the $[M+H]^+$ is consistent with double-oxygenated molecules. In MS/MS, both TMP-7 and TMP-8 lack the characteristic A or B fragments, which indicates they are most likely hydroxylated at the exocyclic $CH_2$. Without the A and/or fragments, it is difficult to decide where the second oxidation took place. TMP-7 and TMP-8 showed a fragment ion with *m/z* 291.1091, consistent with the loss of $CH_4O$, most likely due to the loss of O from an N-oxide and the loss of $CH_4$ from two adjacent methoxy-groups in the A fragment. The discrimination between the two is not possible based on this data since the fragmentation spectra are identical. The *m/z* of protonated TMP-9 is consistent with demethylation and oxygenation. In MS/MS, no characteristic A or B fragments are observed, but a loss of $H_2O$ as well as of $CH_3^{\bullet}$ is observed. This leads to the assumption that the demethylated metabolite is hydroxylated at the exocyclic $CH_2$ group.

Finally, an impurity with *m/z* 305.1608 (IMP-1 in Table 2) was detected in all samples, i.e., the active incubations, the controls, and the standard sample. This indicates an impurity with an additional $CH_2$ group. A reaction side-product with one ethoxy and two methoxy ring substituents, rather than three methoxy ring substituents, was reported by Lehr *et al.* [12]. However, the impurity found here showed a +14-Da shift in the *m/z* values of the B fragment (*m/z* 123 to 137), indicating *N*-methylation at one of exocyclic $NH_2$ groups.

**Correlation with *in silico* predicted metabolites**

All metabolites detected in the *in vitro* systems were also predicted by the *in silico* prediction tool. Comparing the experimental results with the predicted list of metabolites, their rankings and the actual metabolites detected in the samples shows that the tool in this case certainly can be of added value to deliver input to MS experiments. There are also some differences, especially in the prediction and actual detection of *N*-oxides, but it is complicated to determine the causes of such difference. The formation of *N*-oxides in the (2,4-diaminopyrimidin-5-yl) ring is not unexpected from a metabolism point-of-view and has been reported in humans before [20]. In addition, the fragmentation spectra obtained on all oxidated metabolites do not readily provide strong confirmation on the detection of the *N*-oxides in the present study. The doubly oxidized metabolites formed by the BM3 mutants, however, could well be *N*-oxides as well, given the loss of O and additional loss of $CH_4$ from adjacent methoxy-groups. Although not detected in

RLM or HLM experiments, these metabolites were predicted by the SyGMa algorithm, but ranked at low positions (39 and 40, *i.e.*, not shown in Table 1). The aromatic hydroxylation of the B ring in TMP-5, being observed in HLM and RLM, is ranked at place 21. This is rather low given the fact that the probability for 2 or 3 subsequent metabolic reactions is higher according to the prediction. This can also be related to the high probability of *N*-oxide formation in the B part of this molecule.

**Conclusions**

The identification of phase-I biotransformation products of drugs and drug candidates is important for the evaluation of potential pharmacologically or toxicologically active metabolites. Moreover, it can provide information on the clearance of a drug from the body. The importance of these studies is reflected in the development of new analytical strategies for this purpose. *In silico* metabolism prediction tools are developed to facilitate drug metabolite identification. For the identification of phase-I metabolites of Trimethoprim, the SyGMa program predicted all observed metabolites, including additional *N*-oxide metabolites of which we were not completely certain. We were able to identify four hydroxylated metabolites, one on the trimethoxy ring, two on the diaminopyrimidine ring, and one hydroxylation on the exocyclic $CH_2$. Demethylated and double demethylated metabolites were observed as well. Although theoretically a difference in the MS-MS spectra between the 3'- and 5'demethylated at one hand and the 4'demethylated TMP should be possible, we did not observe this and therefore cannot make a distinction between the two. In this study, we observed that the *in silico* prediction of the metabolites was of added value in the identification process.

**Acknowledgements**

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