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**Identification of *in vitro* metabolites of ethylphenidate by liquid chromatography
coupled to quadrupole time-of-flight mass spectrometry**

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Abstract

Ethylphenidate is a new potent synthetic psychoactive drug, structurally related to methylphenidate. Using human liver microsomes and cytosol, we have investigated for the first time the Phase-I and Phase-II *in vitro* metabolism of ethylphenidate. The structure of the metabolites was elucidated by hybrid quadrupole time-of-flight mass spectrometry. Overall, seven Phase-I, but no Phase-II metabolites were detected. Ethylphenidate underwent hydroxylation forming two primary mono-hydroxylated metabolites and, subsequently, dehydration and ring opening with an additional hydroxylation, forming secondary metabolites. The involvement of different human cytochrome P450 (CYP) enzymes in the formation of ethylphenidate metabolites was investigated using a panel of human recombinant CYPs (rCYPs). rCYP2C19 was the most active recombinant enzyme involved in the formation of all seven ethylphenidate metabolites detected, although other rCYPs (rCYP1A2, rCYP2B6, rCYP2C9, rCYP2D6, and rCYP3A4, but not rCYP2E1) played a role in the metabolism of ethylphenidate. All metabolites identified in the present study can be considered as potential specific biomarkers of ethylphenidate in toxicological studies. Additionally, ritalinic acid and methylphenidate were formed by non-enzymatic hydrolysis and trans-esterification, and, therefore, they cannot be considered as (oxidative) metabolites of ethylphenidate. The presence of methylphenidate and ritalinic acid cannot be exclusively associated to the use of ethylphenidate, since methylphenidate is a drug itself and ritalinic acid can be formed from both ethylphenidate and methylphenidate.

Keywords: ethylphenidate, high-resolution mass spectrometry, *in vitro* metabolism, methylphenidate, new psychoactive substances.

Introduction

The presence of new psychoactive substances (NPS) has recently increased on the drug market through various modes of distribution, including the Internet and the so-called “smart shops” [1]. Ethylphenidate has recently appeared as a recreational drug of abuse and it was already found responsible of a human fatality [2]. The legal status of this commercially available NPS remains ambiguous. The Federal Analogue Act classifies an analogue of an existing schedule I or II controlled substance (ethylphenidate is the ethylated analogue of methylphenidate) as illicit if the new compound exhibits chemical and pharmacological properties “substantially similar” to the controlled substance [3]. Methylphenidate is a prescription stimulant commonly used to treat Attention Deficit Hyperactivity Disorder (ADHD) in children and adults [4, 5]. The drug is often abused and sold on the black market to adolescents, particularly in high schools and on college campuses in order to enhance their energy and attention [6]. However, methylphenidate has been shown to present cardiovascular side effects (e.g. tachycardia and arrhythmia) [6, 7]. It is also known that methylphenidate in combination with the intake of ethanol generates ethylphenidate [3, 8], which was detected in blood and liver samples from two victims who had ingested large quantities of methylphenidate together with alcohol [9].

The identification of human metabolites is a necessary task during the drug development process, as well as in a clinical and forensic context, where specific metabolites are used to confirm the consumption of drug [10]. Knowledge about the drug-specific metabolites can be obtained by analyzing urine or blood samples from a drug user or by conducting *in vitro* metabolism experiments with the drug of interest. To our knowledge, no human *in vivo* metabolism study of ethylphenidate has been performed until now. Also, only one study investigated the human *in vitro* metabolism of ethylphenidate [11]. However, in that study, only the ethylphenidate metabolism mediated by human carboxylesterase enzymes was investigated. The metabolite formed from ethylphenidate (namely ritanilic acid) was also formed from methylphenidate, suggesting that ritanilic acid is not specific enough to

distinguish between the consumption of methylphenidate and ethylphenidate. The chemical structure of ethylphenidate suggests that it can be metabolized by cytochrome P450 (CYP) enzymes producing ethylphenidate-specific metabolites, which can be further conjugated by Phase-II enzymes. In addition, Phase-II metabolites are more polar than Phase-I metabolites, therefore being more likely to be excreted in the urine, a key characteristic for markers of drug exposure to be monitored not invasively using urine samples.

The aim of this study was to investigate the *in vitro* metabolism of ethylphenidate using human liver microsomes and cytosol to monitor the formation of Phase-I and Phase-II metabolites. The influence of different experimental variables (incubation time, enzyme and substrate concentrations) was also studied to assess the consistency and reproducibility of the formation of the metabolites detected. The metabolites formed were chromatographically separated by liquid chromatography (LC) and their structures elucidated by tandem mass spectrometry (MS/MS) using a quadrupole time-of-flight (QTOF) as analyzer.

Material and methods

Chemicals and reagents

Ethylphenidate was obtained from LGC Standards (Molsheim, France) as neat powder (purity>99%) and it was dissolved in methanol (purity $\geq$ 99.9%) purchased from Merck (Darmstadt, Germany). The internal standard, theophylline, was obtained as powder (anhydrous, purity>99%) from Sigma-Aldrich (Diegem, Belgium). Pooled human liver microsomes (HLMs, mix gender, n=200) were purchased from Tebu-Bio (Boechout, Belgium). Pooled human liver cytosol (HLCYT, mix gender, n=50), chemical standards for 2,6-uridinediphosphate glucuronic acid (UDPGA), alamethicin (neat, purity>99%), adenosine 3'-phosphate 5'-phosphosulfate (PAPS; neat, purity>60%) lithium salt hydrate, 4-nitrophenol (4-NP), 4-nitrophenolglucuronide (4-NP-Gluc; neat, purity>99%), 4-nitrophenolsulfate (4-NP-Sulf; neat, purity>99%) and NADPH (neat, purity>99%) were purchased from Sigma-Aldrich. Baculovirus-insect cell microsomes containing expressed human recombinant CYP enzyme (rCYP1A2, 2B6, 2C9, 2C19, 2D6, 2E1 or 3A4) co-expressed with human CYP

oxidoreductase and human cytochrome b5 were purchased from BD Biosciences (Erembodegem, Belgium) and Tebu-Bio. Ultrapure water was prepared using a Purelab flex water system by Elga (Tienen, Belgium). Acetonitrile and formic acid were purchased from Merck. All organic solvents were HPLC grade.

***In vitro* metabolism assays**

The reaction mixture (final volume: 1 mL), consisting of 100 mM TRIS buffer (pH adjusted to 7.4 at 37 °C), HLM (final concentration: 0.5 mg/mL) and ethylphenidate (final concentration: 10 µM), was pre-incubated for 5 min in a shaking water bath at 37 °C. The percentage of organic solvent was kept below <1% to minimize its inhibitory effect towards CYPs catalytic activity [12]. The reaction was initiated by addition of 10 µL of NADPH solution (final concentration: 1 mM) in the mixture. To stop the reaction after 3 h, 250 µL of ice-cold acetonitrile containing 1% formic acid and 5.0 µg/mL of theophylline (used as internal standard) was added to each sample, which was then vortex-mixed for 30 s and centrifuged at 8,000 rpm for 5 min. The supernatant was transferred to a glass tube, concentrated to near dryness under nitrogen at 60 °C and reconstituted with 200 µL ultrapure water.

Generation of Phase-II metabolites was investigated in two major steps. At first, Phase-I metabolites of ethylphenidate were produced by incubating ethylphenidate with HLMs and NADPH as described above. The reaction was quenched by placing the samples on ice for 5 min. Samples were then centrifuged at 8,000 rpm for 5 min. Then, 940 µL of the supernatant containing the fraction of non-metabolized ethylphenidate and its metabolites generated by CYP enzymes were transferred to a new tube which contained a fresh aliquot of pooled HLM or pooled HLCYT (final concentration: 0.5 mg/mL) for the samples investigating uridinediphosphate glucuronosyl transferase (UGT) or sulfotransferase (SULT) enzymes mediated metabolism, respectively. Secondly, for incubations with UGT enzymes, the reaction mixture was prepared as described above for CYP enzyme samples, but adding also a 10 µL aliquot of alamethicin (final concentration: 10 µg/mL) dissolved in dimethyl sulfoxide before pre-incubating the samples. The addition of 1% dimethyl sulfoxide does not

affect the UGTs activity [13]. Also, the reaction was started by addition of UDPGA or PAPS cofactors (1 mM, final concentration) to activate UGTs and SULTs, respectively. The samples were incubated for 3 h and prepared as described above. This experimental design was adopted to maximize the concentration of the Phase-I metabolites present in the samples investigating the formation of the corresponding glucuronidated and sulfated metabolites.

Further experiments were carried out by incubating ethylphenidate with HLM over a range of incubation times (10, 20, 40, 60, and 90 min), enzyme concentrations (0.2, 0.4, 0.6, and 0.8 mg/mL) and substrate concentrations (1, 3, 5, and 10 μ M) to monitor the consistent formation of ethylphenidate metabolites produced by CYPs. The reaction mixtures were prepared as described above.

Positive and negative control samples for each family of enzymes were incubated in parallel under the same conditions described above. In the positive control samples for UGT and SULT activity, 4-nitrophenol (10 μ M, final concentration) was selected as the substrate and the formation of 4-nitrophenolglucuronide and 4-nitrophenolsulfate, respectively, was monitored [14, 15]. No positive control samples for CYP activity were prepared, because data about the catalytic activity of major human liver CYPs was provided by the HLM vendor. For each family of enzymes, negative control samples were prepared as described above but omitting the enzymes, substrate or the cofactor in the reaction mixture in order to ensure that no false-positive metabolite formation occurred.

The role of individual human CYP enzymes in the formation of the metabolites detected incubating ethylphenidate with HLM was investigated using a panel of human recombinant CYPs (rCYPs), including human rCYP1A2, 2B6, 2C9, 2C19, 2D6, 2E1, and 3A4. Reaction mixtures were prepared as described above for Phase-I experiment, but using one human rCYP (20 pmol/mL, final concentration) per sample instead of HLMs. The reaction was allowed to proceed for 1 h. Enzyme negative control samples were prepared omitting the human rCYP.

LC-QTOF-MS analytical method

Metabolite identification was performed with LC-MS which consisted of a 1290 Infinity LC system (Agilent Technologies, Wilmington, DE, USA) connected to a 6530 Accurate-Mass QTOF-MS (Agilent Technologies, Wilmington, DE, USA) with a heated-electrospray ionization source (JetStream ESI). Chromatographic separation was performed on a C₈ Zorbax Eclipse Plus column (150 x 2.1 mm, 3.5 µm) from Agilent Technologies, maintained at 30 °C, using a mobile phase consisting of 0.1% of formic acid in ultrapure water (A) and acetonitrile (B) with the following gradient: 0-5 min, 3% B; 30 min, 50% B; 31-33 min, 100% B; 34-40 min: 3% B. The flow rate and the injection volume were set at 0.18 mL/min and 5 µL, respectively.

The QTOF-MS instrument was operated in the 2 GHz (extended dynamic range) mode, which provides a full width at half maximum (FWHM) resolution of approximately 4700 at m/z 118 and 10000 at m/z 922. Both polarity ESI modes were used under the following specific conditions: gas temperature 300 °C; gas flow 8 L/min; nebulizer pressure 40 psi; sheath gas temperature 325 °C; sheath gas flow 11 L/min. Capillary and fragmentor voltages were set to 3500 and 100 V, respectively. A calibration solution was continuously sprayed in the source of the QTOF-MS system during sample analysis. The ions selected for (re)calibrating the mass axis, ensuring the accuracy of mass assignments throughout the chromatographic run were m/z 121.0508 and 922.0097 for positive mode and m/z 112.9856 and 966.0007 for negative mode. The QTOF-MS device was acquiring from m/z 50 to 1,000 in MS mode and from m/z 40 to 500 in data-dependent acquisition mode (auto-MS/MS) using three different collision energy values (10, 20, and 40 eV) for the fragmentation of the parent ions. The maximum number of precursor ions per MS cycle was set to three with minimal abundance of 2,000 counts. In addition, precursor ions were excluded after every three spectra and released after 0.6 min. An exclusion list with ions present in the substrate negative control was also added. For some metabolites, additional injections in targeted MS/MS were necessary in order to obtain proper MS/MS fragmentation data.

Data processing and analysis

Metabolite identification was based on their accurate masses, isotopic abundances obtained in the MS mode, as well as on their MS/MS fragmentation patterns and the accurate masses of the resulting products ions. In order to increase the throughput and facilitate the detection and identification of the metabolites, two strategies were carried out.

The first strategy consisted on the use of the metabolomics software MZmine 2.10 [16]. This tool performs a differential analysis of several sets of samples discarding automatically spectral features also present in the control sample set (i.e. negative controls), increasing the confidence and the throughput for complex experiments and decreasing the chances of false positive results. Exact masses of the peaks detected by this software were extracted using MassHunter and a mass window of 10 ppm around the ionized precursor ion to confirm or discard their identity.

The second strategy employs a metabolism-specific software Nexus (v1.5, Lhasa Limited). After selecting the substrate, the families of enzymes and the species of interest, Nexus software produces a list of candidate metabolites and their structures. The likelihood of metabolite formation is also indicated as probable, plausible or equivocal. For ethylphenidate, Nexus predicted 5 plausible metabolites and 29 equivocal metabolites.

Results

Detection and identification of ethylphenidate transformation products and metabolites

The incubation of ethylphenidate with HLMs resulted in the formation of seven Phase-I metabolites (Table 1). All detected metabolites were ionized in positive mode due to the presence of an amino group in their structures. The tentative metabolite structures were postulated based on the product ions generated from auto-MS/MS experiments (Fig S1a). Some product ions present in the MS/MS spectra of the parent compound were also present in the MS/MS spectra of the metabolites making thus the identification of the fragments reliable. For the parent compound, the first loss corresponded to an ethyl group leading to

the fragment at m/z 220.1356 ($\Delta m = 10.90$ ppm), followed by the loss of a carboxyl group leading to the fragment at m/z 174.1274 ($\Delta m = -1.72$ ppm) (Fig. S1a). Then, the ring was opened losing the amino group (m/z 157.1003, $\Delta m = -5.73$ ppm) and additional losses of ethene and pentadiene from the alkyl chain led to the fragments at m/z 129.0709 ($\Delta m = 7.90$ ppm) and 91.0546 ($\Delta m = 4.06$ ppm), respectively. The fragment at m/z 84.0815 ($\Delta m = 8.56$ ppm) corresponds to the piperidine ring and the fragment at m/z 56.0502 ($\Delta m = 12.85$ ppm) to the empirical formulae $[C_3H_6N]^+$.

The two major transformation products detected were at m/z 220.1335 (TP1) and 234.1506 (TP2) and had similar fragmentation to ethylphenidate (Fig. S1a), except for the deviation of 28.0314 and 14.0143 u from the precursor ion, which corresponded to the loss of an ethyl group leading to the formation of ritalinic acid (TP1) and trans-esterification leading to methylphenidate (TP2).

However, the amount of TP1 found in the negative controls in absence of cofactor for CYPs (NADPH) or enzyme (HLM) was similar to the amount present in the metabolism incubations, suggesting that no (microsomal) enzyme was involved in its formation. On the other hand, TP1 was not detected in the substrate negative control, suggesting that TP1 might be an impurity of the ethylphenidate standard. TP2 was detected also in the NADPH negative control samples. The amount of TP2 detected in NADPH negative control sample incubated for 3 h was similar to the amount detected in the incubation samples, suggesting that TP2 was not produced by CYP enzymes. The structural elucidation based on the fragmentation pattern unequivocally showed that TP2 corresponded to methylphenidate, the methyl ester of ethylphenidate.

Injection of a solution of ethylphenidate in ultrapure water of the same ethylphenidate stock solution used for the metabolism experiments did not show any ritalinic acid (TP1), neither methylphenidate (TP2), see Fig. S2. However, the same solution injected after five months of storage contained both TP1 and TP2 (Fig. S2). Therefore, these data strongly suggested that TP1 and TP2 can be formed from ethylphenidate via a non-enzymatic route. To check this hypothesis, incubations with HLMs were repeated using this “degraded” standard solution

and compared with a negative control without cofactor (NADPH). Fig. S2 shows the extracted ion chromatogram for ritalinic acid and methylphenidate in both standard solutions (degraded and not degraded) prepared in ultrapure water, in the negative control and in the real experiment. The signal for ritalinic acid and methylphenidate was almost the same in the three chromatograms confirming that TP1 and TP2 were formed non-enzymatically.

An additional injection of the same ethylphenidate stock solution, but diluted directly in methanol, confirmed the degradation of the stock solution after five months of its preparation (Fig. S3). Whereas the response of ethylphenidate and ritalinic acid was the same, methylphenidate was present by about 30% of the signal of ethylphenidate. Clearly, ethylphenidate was non-enzymatically transformed into TP1 and TP2.

M_{TP1} with empirical formulae $C_{13}H_{15}NO_2$ and m/z 218.1175 ($\Delta m = 0.46$ ppm) was also detected, corresponding to a dehydrogenation of TP1 (m/z 220.1335) or to the loss of water after TP1 hydroxylation. Therefore, TP1 is not a Phase-I metabolite of ethylphenidate. The MS/MS spectrum for M_{TP1} (Fig. S1b) showed fragments at m/z 172.1119 ($\Delta m = -1.16$ ppm) and 82.0653 ($\Delta m = 2.07$ ppm) instead of m/z 174.1277 and 84.0808 as observed for ethylphenidate and TP1, suggesting the presence of a double bond in the piperidine ring (m/z 82.0653) yielding to the dihydropyridine. The MS/MS spectrum did not provide the exact position of the double bond.

All other detected peaks were not present in the negative control samples, suggesting that they are all oxidative metabolites of ethylphenidate (Table 1). Fig. 1 shows the extracted ion chromatograms for the metabolites detected. For discussion purposes, logD values at the pH of the mobile phase (pH 2.5) were calculated (Table 1). Three peaks with m/z deviation of 0.87 to 1.63 ppm to that of hydroxylated metabolites of ethylphenidate (m/z 264.1589) were detected (M1a, M1b, and M1c). In the three MS/MS spectra (Fig. S1b), the major ion at m/z 100.0757 ($[C_5H_{10}NO]^+$) can be formed by the addition of a hydroxyl group to the corresponding fragment at m/z 84.0808 of ethylphenidate, indicating hydroxylation of the piperidine ring. The position of the hydroxyl group in the piperidine group could not be

deduced from the obtained fragmentation patterns, but one of them (M1c) should be in alpha to the nitrogen atom, leading to the formation of a lactam followed by ring opening to the respective aliphatic aldehyde. In this way, the newly formed alkyl chain was exposed to a stronger interaction with the stationary phase of the column and, therefore, resulting in a longer elution time (21.75 min). The fragment at m/z 246.1500 ($\Delta m = 4.63$ ppm) corresponded to a loss of water and can be explained by the formation of a six-member ring between the primary amine and the carbon of the aldehyde. Subsequent losses of the ethyl and carboethoxy moieties led to the ions at m/z 218.1200 ($\Delta m = 11.19$ ppm) and 173.1216 ($\Delta m = 9.82$ ppm), respectively.

Although the mass spectrometric data (Fig S1b,c) could not confirm the structures of M2a and M2b, the lack of a second loss of water in both MS/MS spectra suggested that only one hydroxyl group was present in the structures. On the other hand, if only the dihydroxylation happened, the retention times should be shorter than M1a and M1b, so the only explanation was a ring opening mechanism exposing the newly formed alkyl chain to a stronger interaction with the stationary phase and, therefore, resulting in a longer elution time. Although the position of the hydroxyl group along the newly formed alkyl chain could not be determined, the most probable position should be between the nitrogen and the carbonyl group due to the presence of a double bond in the fragments at m/z 188 and 171.

The MS/MS spectrum of m/z 246.1485 (M3) was similar to that of ethylphenidate (Fig. S1d). The precursor ion value of M3 and that of ethylphenidate differed only by 2.016 u and the ion at m/z 172.1091 ($\Delta m = -17.43$ ppm) indicates a double bond in the piperidine ring. This result suggests that M3 was the dehydrogenation product of ethylphenidate or the product of M1 dehydration. The MS/MS data (Fig S4d) suggests that M4 could be formed from M2 by a loss of water or from M3 by a hydroxylation on the carbon adjacent to the nitrogen atom followed by an oxidation and ring opening.

No glucuronidated or sulfated metabolites could be detected incubating ethylphenidate and its Phase-I metabolites. In the positive control samples, the glucuronidated and the sulfated metabolites of 4-NP produced by UGTs and SULTs, respectively, were detected in large

amounts. Therefore, the positive control samples substantiated the lack of formation of glucuronidated and sulfated metabolites of ethylphenidate and its Phase-I metabolites under the experimental conditions tested.

Profiling of ethylphenidate metabolites

To provide further evidence of the metabolic nature of the peaks detected and to distinguish them from abiotic products and impurities, the formation of Phase-I metabolites was monitored over different incubation times (Fig. 2A,B), enzyme concentrations (Fig. 2C,D) and substrate concentrations (Fig. 2E,F). The amount of metabolite formed is reported as ratio between the peak area of the metabolite and the area of the IS. The amounts of TP1 and TP2 detected clearly increased with increasing incubation time (Fig. 2A) and increasing ethylphenidate concentration (Fig. 2E), which can be explained by either an enzymatic or a non-enzymatic reaction. However, the amount of TP1 and TP2 present in the NADPH negative controls was similar to the amount present in the metabolism incubations (see previous section) and the response of TP1 and TP2 showed only a very moderate increase with respect to increasing enzyme concentrations (Fig. 2C). These data suggest that no (microsomal) enzyme is involved in the formation of TP1 and TP2, which are not metabolites, but transformation products of ethylphenidate. .

All other metabolites were not detected in any negative control sample and the amount formed increased with respect to increasing incubation time (Fig. 2B), enzyme (Fig. 2D) and substrate concentrations (Fig. 2F). The only exception was M3, which was formed in a sharply increasing amount when incubation time was increased, but only up to 40 min (Fig. 2B) and after that, a decrease was observed because possibly of its involvement in the subsequent formation of M4. Overall, the response values associated with the metabolites detected in Phase-I experiments suggested that they were similarly minor in comparison with the transformation products TP1 and TP2.

Human CYP enzymes involved in the metabolism of ethylphenidate

The seven most abundant human hepatic rCYPs (rCYP1A2, 2B6, 2C9, 2C19, 2D6, 2E1, and 3A4) were tested for their ability for catalyzing the formation of the *in vitro* metabolites of ethylphenidate. As shown in Fig. 3, several human rCYP enzymes catalyzed the formation of M1, M2, M3, and M4. rCYP2C19 was the most important rCYP tested in the formation of M1a, M1b, M2a, M2b and M2c. In contrast, no differences were observed in the contribution of rCYP1A2, 2B6, 2C9, 2C19, 2D6 in the formation of M3. M4 was catalyzed in the same extent by rCYP2C19 and 3A4. rCYP2E1 did not catalyze the formation of any metabolite of ethylphenidate in detectable amount. TP1 and TP2 were present in the same amount in all samples, including the rCYP negative control (data not shown). This data provides further evidence that formation of TP1 and TP2 does not require enzymes, further supporting the hypothesis that they are products of non-enzymatic transformation of ethylphenidate.

Proposed metabolic pathway

The proposed *in vitro* metabolism pathway of ethylphenidate is presented in Fig. 4. CYP-mediated metabolism of ethylphenidate produced two primary mono-hydroxylated metabolites by hydroxylation of the piperidine ring in two different positions (M1a and M1b). Subsequently, one of these mono-hydroxylated metabolites (M1a or M1b) was transformed into M3 by a loss of water. Ethylphenidate also underwent ring opening due to hydroxylation of the alpha carbon to the nitrogen atom and subsequent oxidation to the respective aliphatic aldehyde (M1c). The formed alkyl chain was newly hydroxylated leading to M2a and M2b. Subsequent loss of water of one of those isomers resulted in the formation of M4, which could also be produced from M3 by hydroxylation followed by ring opening. For each metabolite, the major CYP enzyme(s) catalyzing its formation are indicated.

Discussion

Identification of ritalinic acid and methylphenidate

The main transformation products of ethylphenidate were formed predominantly by hydrolysis (de-esterification) to the ritalinic acid (TP1) and trans-esterification to

methylphenidate (TP2) of the ester group. Incubation at pH 7.4 and 37 °C promotes the hydrolysis of ethylphenidate and its conversion into ritalinic acid increases with time and concentration of ethylphenidate. This fact is not surprising taking into account that esters are hydrolyzed to acids even at neutral conditions by a nucleophilic attack on the carbonyl moiety [17] and this reaction is enhanced by temperature [18]. However, ritalinic acid was unexpectedly present also in the ethylphenidate standard solution in methanol after several months of storage at -20 °C in the dark. This finding is consistent with the findings of Scarth et al. [19], who associated the ritalinic acid observed in their *in vitro* experiments with a possible impurity in the standard.

Negative control samples produced the same results for the formation of TP2 (methylphenidate), suggesting a direct trans-esterification of ethylphenidate in the presence of methanol in spite of that only 1% v/v of methanol was used during the microsomal incubations. The degradation of ethylphenidate into methylphenidate in the standard solution in methanol after 5 months confirmed this hypothesis. However, further experiments showed that the amount of TP2 formed depends on incubation time, protein concentration and substrate concentration, suggesting that TP2 is a true metabolite of ethylphenidate. Since abiotic and biotic reactions can occur simultaneously, our results suggest that TP2 is formed abiotically (breakdown product), but also enzymatically (metabolite) from ethylphenidate. Methanol has been used in the preparation of ethylphenidate solutions, as recommended by many vendors. However, these data suggest that methanol is probably not an appropriate solvent for the storage and/or dilution of ethylphenidate.

Identification of ethylphenidate oxidative metabolites

Using HLMs, ethylphenidate was metabolized into hydroxylated metabolites (M1a and M1b), including oxidation and ring opening (M1c), additional hydroxylation (M2a and M2b) and losses of water (M3, M4), resulting in seven identified Phase-I metabolites, including isomers, formed by CYP enzymes. The results with rCYPs showed that rCYP1A2, 2B6, 2C9,

2C19, 2D6, and 3A4 were involved in the formation of the seven metabolites of ethylphenidate formed in incubations containing HLM.

Based on the MS/MS spectra, it was possible to reveal the moieties at which the alteration of the ethylphenidate molecule took place during Phase-I metabolism, but the exact position of hydroxyl groups could not be determined. However, there is a strong evidence that one of the hydroxyl groups of M1 should be in alpha position to the nitrogen atom in the piperidine ring, leading to the formation of a lactam followed by ring opening to the respective aliphatic aldehyde (intermediate of M2), which is very common in the metabolism of other piperidine/pyrrolidine compounds [20].

Nexus metabolite prediction

Metabolite prediction with Nexus software definitely speeded up the creation of a list of possible metabolites and it showed a high confidence in the precursor formulas for the generated metabolites. The Nexus software predicted two plausible isomers out of the three M1 detected, with the hydroxyl groups in the aromatic ring and in the ethyl chain as the most probable sites. For M2, the Nexus software predicted a carboxylic acid as plausible and an additional hydroxylation. In addition, Nexus predicted the formation of a lactam, not observed in our study but ring opening of this lactam was observed (M1c). M3 was not predicted by Nexus, and neither the non-oxidative transformation products, ritalinic acid and methylphenidate.

Comparison of ethylphenidate and methylphenidate metabolism

Apart from our study, no data about the oxidative metabolism of ethylphenidate is available in the literature so far. Due to similar structure with methylphenidate, its metabolism pathways should show similarities, such as metabolism by human esterases [11].. In fact, ethylphenidate is a unique biomarker of the combination of methylphenidate and ethanol [21], while in our work, methylphenidate (TP2) was found to be a transformation product of ethylphenidate.

Oxidative metabolites were reported in the literature for methylphenidate. Faraj et al. [22] observed oxo-methylphenidate, *p*-hydroxy-methylphenidate, oxo-ritalinic acid, *p*-hydroxy-ritalinic acid, as well as the glucuronide of *p*-hydroxy-ritalinic acid in human urine, but these metabolites were excreted <2% of the dose of methylphenidate in humans [22]. In addition, Patrick et al. [23] demonstrated that *p*-hydroxy-methylphenidate was not a significant pharmacologically active metabolite of methylphenidate in humans. In our *in vitro* work, the hydroxylation reactions in ethylphenidate were found to take place only at the piperidine ring and in the alkyl chain when the ring was opened. We could also demonstrate two dehydrations for ethylphenidate.

Until now, the presence of ritalinic acid was investigated in urine [24] and in aquatic systems [4, 25] as the main biomarker for the consumption of methylphenidate. With our work, we demonstrate that ritalinic acid is not specifically formed from methylphenidate, since it can also be formed by de-esterification of ethylphenidate. Therefore, more specific metabolites of ethylphenidate (Fig. 4) should be monitored in biological samples to demonstrate specific consumption of ethylphenidate.

Conclusions

To the best of our knowledge, this is the first study characterizing the CYP-mediated *in vitro* metabolism of ethylphenidate. The results show that ethylphenidate is transformed into seven Phase-I metabolites produced by CYP enzymes. The identified metabolites are potential specific biomarkers of ethylphenidate to be included in drug screening programs. The data further show that ethylphenidate is quickly transformed into ritalinic acid and methylphenidate through non-enzymatic hydrolysis and trans-esterification in presence of methanol. However, the presence of these two compounds in human matrices is not exclusive to the use of ethylphenidate, since ritalinic acid is also a metabolite of methylphenidate. Therefore, ritalinic acid cannot be used as specific biomarker of ethylphenidate.

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Figure captions

Fig. 1. Extracted ion chromatograms of ethylphenidate Phase-I metabolites after 3 h of incubation.

Fig. 2. Metabolic profiles of ethylphenidate abiotic transformation products **(A)** and Phase-I metabolites **(B)** at different times (Enzyme and substrate concentration were kept at 0.8 mg/mL and 10 μ M, respectively). Metabolic profiles of ethylphenidate abiotic transformation products **(C)** and Phase-I metabolites **(D)** at different enzyme concentrations (Time and substrate concentration were kept at 90 min and 10 μ M, respectively). Metabolic profiles of ethylphenidate abiotic transformation products **(E)** and Phase-I metabolites **(F)** at different substrate concentrations (Time and enzyme concentration was kept at 90 min and 0.8 mg/mL, respectively).

Fig. 3. Ratio response of ethylphenidate Phase-I metabolites formed after incubation of 10 μ M ethylphenidate (37 °C, 1 h) using a panel of seven human recombinant CYP enzymes (20 pmol/mL). Ratio response was calculated as the peak area of the compound corrected with the area of the IS peak.

Fig. 4. Proposed CYP-mediated metabolism pathway of ethylphenidate.

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Table 1: Postulated structures for ethylphenidate and its metabolites/transformation products after incubation with HLM.

	Precursor ion (MS-TOF)				Product ions (MS/MS-TOF)				³ Proposed structure
	Measured <i>m/z</i> [M+H] ⁺	Expected <i>m/z</i> [M+H] ⁺	¹ Δ <i>m</i> (ppm)	² RT (min)	Measured <i>m/z</i> [M+H] ⁺	Expected <i>m/z</i> [M+H] ⁺	¹ Δ <i>m</i> (ppm)	Tentative formula [M+H] ⁺	
EP	248.1649	248.1645	1.57	19.15	220.1356	220.1332	10.90	[C ₁₃ H ₁₈ N ₃ O ₂] ⁺	<p>C₁₅H₂₁NO₂ logD -0.63</p>
					174.1274	174.1277	-1.72	[C ₁₂ H ₁₆ N] ⁺	
					157.1003	157.1012	-5.73	[C ₁₂ H ₁₃] ⁺	
					129.0709	129.0699	7.90	[C ₁₀ H ₉] ⁺	
					91.0546	91.0542	4.06	[C ₇ H ₇] ⁺	
					84.0815	84.0808	8.56	[C ₅ H ₁₀ N] ⁺	
					56.0502	56.0495	12.85	[C ₃ H ₆ N] ⁺	

¹ *m/z* measurement error; ² Retention time; ³ logD at pH 2.5 calculated by Chemicalize.org by ChemAxon (<http://www.chemaxon.com>)

Table 1cont. Postulated structures for ethylphenidate and its metabolites/transformation products after incubation with HLM.

	Precursor ion (MS-TOF)				Product ions (MS/MS-TOF)				³ Proposed structure
	Measured <i>m/z</i> [M+H] ⁺	Expected <i>m/z</i> [M+H] ⁺	¹ Δ <i>m</i> (ppm)	² RT (min)	Measured <i>m/z</i> [M+H] ⁺	Expected <i>m/z</i> [M+H] ⁺	¹ Δ <i>m</i> (ppm)	Tentative formula [M+H] ⁺	
TP1	220.1335	220.1332	1.09	15.23	202.1241	202.12264	7.22	[C ₁₃ H ₁₆ NO] ⁺	C ₁₃ H ₁₇ NO ₂ logD -1.03
					174.1277	174.1277	0.00	[C ₁₂ H ₁₆ N] ⁺	
					146.0983	146.0964	13.01	[C ₁₀ H ₁₂ N] ⁺	
					129.0701	129.0699	1.70	[C ₁₀ H ₉] ⁺	
					91.0554	91.0542	12.85	[C ₇ H ₇] ⁺	
					84.0816	84.0808	9.75	[C ₅ H ₁₀ N] ⁺	
					56.0501	56.0495	11.06	[C ₃ H ₆ N] ⁺	
TP2	234.1506	234.1489	7.43	17.28	202.1184	202.1226	-20.78	[C ₁₃ H ₁₆ NO] ⁺	C ₁₄ H ₁₉ NO ₂ logD -0.99
					174.1249	174.12773	-16.25	[C ₁₂ H ₁₆ N] ⁺	
					146.0943	146.0964	-14.37	[C ₁₀ H ₁₂ N] ⁺	
					129.0683	129.06988	-12.24	[C ₁₀ H ₉] ⁺	
					91.0536	91.0542	-6.92	[C ₇ H ₇] ⁺	
					84.0802	84.0808	-6.90	[C ₅ H ₁₀ N] ⁺	
					56.0492	56.0495	-5.00	[C ₃ H ₆ N] ⁺	
M _{TP1}	218.1175	218.1176	-0.46	14.99	172.1119	172.1121	-1.16	[C ₁₂ H ₁₄ N] ⁺	C ₁₃ H ₁₅ NO ₂ logD -1.01
					129.0712	129.0699	10.07	[C ₁₀ H ₉] ⁺	
					103.0548	103.0542	5.53	[C ₈ H ₇] ⁺	
					91.0549	91.05423	7.36	[C ₇ H ₇] ⁺	
					82.0653	82.06513	2.07	[C ₅ H ₈ N] ⁺	
					70.0638	70.06513	-18.98	[C ₄ H ₈ N] ⁺	
					55.0547	55.05423	8.54	[C ₄ H ₇] ⁺	

¹ *m/z* measurement error; ² Retention time; ³ logD at pH 2.5 calculated by Chemicalize.org by ChemAxon (<http://www.chemaxon.com>)

Table 1cont. Postulated structures for ethylphenidate and its metabolites/transformation products after incubation with HLM.

	Precursor ion (MS-TOF)				Product ions (MS/MS-TOF)				³ Proposed structure
	Measured <i>m/z</i> [M+H] ⁺	Expected <i>m/z</i> [M+H] ⁺	¹ Δ <i>m</i> (ppm)	² RT (min)	Measured <i>m/z</i> [M+H] ⁺	Expected <i>m/z</i> [M+H] ⁺	¹ Δ <i>m</i> (ppm)	Tentative formula [M+H] ⁺	
M1a	264.1591	264.1589	0.87	15.81	172.1106	172.1121	-8.72	[C ₁₂ H ₁₄ N] ⁺	<p>C₁₅H₂₁NO₃ logD -2.16 or -1.70</p>
					130.0638	130.0651	-10.23	[C ₉ H ₈ N] ⁺	
					100.0751	100.0757	-5.90	[C ₅ H ₁₀ NO] ⁺	
					82.0644	82.0651	-8.90	[C ₅ H ₈ N] ⁺	
					56.0491	56.0495	-6.78	[C ₃ H ₆ N] ⁺	
M1b	264.1593	264.1589	1.63	17.55	172.1128	172.1121	4.07	[C ₁₂ H ₁₄ N] ⁺	<p>C₁₅H₂₁NO₃ logD -2.16 or -1.70</p>
					100.0752	100.0757	-4.90	[C ₅ H ₁₀ NO] ⁺	
					82.0659	82.0651	9.38	[C ₅ H ₈ N] ⁺	
					55.0543	55.0542	1.27	[C ₄ H ₇] ⁺	
M1c	264.1593	264.1589	1.63	21.75	246.1500	246.1489	4.63	[C ₁₅ H ₂₀ NO ₂] ⁺	<p>C₁₅H₂₁NO₃ logD -1.41</p>
					218.1200	218.1176	11.19	[C ₁₃ H ₁₆ NO ₂] ⁺	
					173.1216	173.1199	9.82	[C ₁₂ H ₁₅ N] ⁺	
					130.0666	130.0651	11.53	[C ₉ H ₈ N] ⁺	
					100.0778	100.0757	21.08	[C ₅ H ₁₀ NO] ⁺	
					83.0739	83.0730	10.83	[C ₅ H ₉ N] ⁺	
					55.0559	55.0542	30.33	[C ₄ H ₇] ⁺	

¹ *m/z* measurement error; ² Retention time; ³ logD at pH 2.5 calculated by Chemicalize.org by ChemAxon (<http://www.chemaxon.com>)

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M2a	280.1552	280.1543	3.11	16.77	262.1445	262.1438	2.67	[C ₁₅ H ₂₀ NO ₃] ⁺	<p>C₁₅H₂₁NO₄ logD -2.29 or -2.72</p>
					188.1058	188.1070	-6.33	[C ₁₂ H ₁₄ NO] ⁺	
					160.1099	160.1121	-13.62	[C ₁₁ H ₁₄ N] ⁺	
					117.0687	117.0699	-10.08	[C ₉ H ₉] ⁺	
					98.0599	98.0600	-1.43	[C ₅ H ₈ NO] ⁺	
					70.0648	70.0651	-4.28	[C ₄ H ₈ N] ⁺	
M2b	280.1537	280.1543	-2.25	18.08	262.1467	262.1438	11.06	[C ₁₅ H ₂₀ NO ₃] ⁺	<p>C₁₅H₂₁NO₄ logD -2.29 or -2.72</p>
					171.0810	171.0804	3.51	[C ₁₂ H ₁₁ O] ⁺	
					143.0821	143.0855	-23.76	[C ₁₁ H ₁₁] ⁺	
					129.0704	129.0699	4.03	[C ₁₀ H ₉] ⁺	
					116.0697	116.0706	-7.75	[C ₅ H ₁₀ NO ₂] ⁺	
					98.0589	98.0600	-11.63	[C ₅ H ₈ NO] ⁺	

¹ *m/z* measurement error; ² Retention time; ³ logD at pH 2.5 calculated by Chemicalize.org by ChemAxon (<http://www.chemaxon.com>)

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	Measured m/z [M+H] ⁺	Expected m/z [M+H] ⁺	¹ Δm (ppm)	² RT (min)	Measured m/z [M+H] ⁺	Expected m/z [M+H] ⁺	¹ Δm (ppm)	Tentative formula [M+H] ⁺	
M3	246.1485	246.1489	-1.30	19.11	186.1263	186.1277	-7.52	[C ₁₃ H ₁₆ N] ⁺	C ₁₅ H ₁₉ NO ₂ logD -0.84
					172.1091	172.1121	-17.43	[C ₁₂ H ₁₄ N] ⁺	
					155.0812	155.0855	-27.73	[C ₁₂ H ₁₁] ⁺	
					129.0682	129.0699	-13.17	[C ₁₀ H ₉] ⁺	
					105.0671	105.0699	-26.65	[C ₈ H ₉] ⁺	
					84.0816	84.0808	9.75	[C ₅ H ₁₀ N] ⁺	
					56.0501	56.0495	11.06	[C ₃ H ₆ N] ⁺	
M4	262.1432	262.1438	-2.02	22.99	216.1015	216.1019	-1.85	[C ₁₃ H ₁₄ NO ₂] ⁺	C ₁₅ H ₁₉ NO ₃ logD -0.62 or -1.42 or -1.62
					188.1060	188.1070	-5.26	[C ₁₂ H ₁₄ NO] ⁺	
					155.0836	155.0855	-12.25	[C ₁₂ H ₁₁] ⁺	
					129.0686	129.0699	-10.07	[C ₁₀ H ₉] ⁺	
					117.0668	117.0699	-26.31	[C ₉ H ₉] ⁺	
					91.0531	91.0542	-12.41	[C ₇ H ₇] ⁺	
					55.0543	55.05423	1.27	[C ₄ H ₇] ⁺	

¹ m/z measurement error; ² Retention time; ³ logD at pH 2.5 calculated by Chemicalize.org by ChemAxon (<http://www.chemaxon.com>)