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Distinct urinary metabolite profiles of two pharmacologically active *N*-methylanthranilates: Three approaches to xenobiotic metabolite identification

Niko S. Radulović [a, *], Ana B. Miltojević [b], Nikola M. Stojanović [c], Pavle J. Randjelović [d]

[a] Department of Chemistry, Faculty of Science and Mathematics, University of Niš, Višegradska 33, 18000 Niš, Serbia
[b] Faculty of Occupational Safety, University of Niš, Čarnojevića 10a, 18000 Niš, Serbia
[c] Faculty of Medicine, University of Niš, Zorana Đinđića 81, 18000 Niš, Serbia
[d] Institute of Physiology, Faculty of Medicine, University of Niš, Zorana Đinđića 81, 18000 Niš, Serbia

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ABSTRACT

Two volatile alkaloids, isopropyl *N*-methylanthranilate (IMA) and methyl *N*-methylanthranilate (MMA), present in the human diet and cosmetic products, were recently demonstrated to possess important pharmacological activities. While MMA is considered to be phototoxic, there is scarce data on the toxicity of IMA. Herein, we analyzed urinary metabolites of IMA and MMA in rats (200 mg kg^{-1}, *i.p.*, 7 days) by combining three different approaches: 1) preparative chromatography, 2) synthesis, and 3) SPR. The preparative approach, Sephadex LH-20 chromatography of the extract of urine samples of IMA treated animals, in conjunction with NMR, enabled the identification of 16 different anthranilate derivatives, among which products of aromatic core hydroxylation (isopropyl 5-hydroxy-*N*-methylanthranilate, isopropyl 5-hydroxyantranilate, isopropyl 3-hydroxyantranilate) were the major ones. The first application of the synthetic/combinatorial approach led to a successful identification of MMA metabolites, where 2-(methylamino)benzamide and *N*-methylanthranilic acid were the principal ones, among 14 others. Generally, MMA and IMA undergo analogous biotransformation pathways; however, MMA predominantly underwent chemical conversions of the ester group, i.e. transformation into derivatives of anthranilamide and anthranilic acid, while the major metabolic pathway of IMA was hydroxylation of the aromatic core. Additionally, pathohistological examinations revealed no signs of liver toxicity, or other signs of toxicity.

1. Introduction

Methyl *N*-methylanthranilate (MMA) is a part of human everyday diet as it naturally occurs in various fruits and is used as a food flavor; also, it is added to cosmetic products, detergents, etc. (Radulović et al., 2011; SCCS, 2011). An estimated daily oral intake of MMA in Europe is 60 μg day^{-1} (1 μg kg^{-1} bodyweight (bw) day^{-1}), while cutaneous exposure to MMA is estimated to be 2.7 μg kg^{-1} bw day^{-1} (SCCS, 2011). The acute oral LD_{50} of MMA in rats was reported to be 3.7 g kg^{-1} bw (SCCS, 2011). In a range finding study for a subchronic study, there was no mortality at 2.25 g kg^{-1} bw (*p.o.*) or less, but 100% mortality at the next higher dose of 3.38 g kg^{-1} bw (Gaunt et al., 1970). In a 13-week feeding study in rats, NOAEL (no observed adverse effect level) of MMA was reported to be *ca.* 20 mg kg^{-1} bw day^{-1}, based on increased absolute and relative kidney weights at 80 mg kg^{-1} bw (Gaunt et al., 1970; Oser et al., 1965). MMA has an established phototoxic potential; 1.0% solution MMA was considered to be phototoxic and produced reactions in 14/35 humans (SCCS, 2011).

Methyl (MMA) and isopropyl (IMA) esters of *N*-methylanthranilic acid have been recently identified in the essential oil of *Choisya ternata* Kunth (Rutaceae) (Radulović et al., 2011). Both of these volatile alkaloids have been proven to possess diverse pharmacological activities, including antinociceptive (Gomes Pinheiro et al., 2014; Radulović et al., 2011), anti-inflammatory (Gomes Pinheiro et al., 2015), gastro-, hepato- and nephroprotective activities (Radulović et al., 2013a, 2013b; 2015a), anxiolytic and antidepressant properties, as well as an effect on diazepam-induced sleep (Radulović et al., 2013c). Although the toxicity of MMA has

* Corresponding author.
E-mail address: nikoradulovic@yahoo.com (N.S. Radulović).

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been previously investigated (Gomes Pinheiro et al., 2014; SCCS, 2011), there are only scarce data on the toxicity of IMA. In one of our previous studies (Gomes Pinheiro et al., 2014), no signs of toxicity of IMA and MMA in mice (100 mg kg^{-1}, *p.o.*, 5-day study) were observed.

Metabolites of pharmacologically active compounds could be more or less active than the parent compound (Mitra et al., 2014) or even toxic (Macherey and Dansette, 2008). Generally, the major metabolic pathway of aromatic compounds is an oxidation resulting in the formation of arenol metabolites. This process takes place via an epoxide intermediate (arene oxide), catalyzed by cytochrome P450 enzymes, followed by the NIH (1,2-hydride) shift. Typically, the products are phenolic compounds, where the position of the newly introduced hydroxyl group depends on the type of the substituent already on the ring (electron-donating substituents direct *o*- and *p*-hydroxylation, Williams, 2012). In the case of anthranilic acid, hydroxylation is known to yield 3-hydroxyanthranilic and 5-hydroxyanthranilic acids, whereas the latter is the dominant metabolite (Ueda et al., 1978). Furthermore, another metabolic pathway leads to the formation of anthranilamide (Sutamihardja et al., 1972). Ishiguro et al. (1974) discovered that anthranilamide could be further hydroxylated to 3- and 5-hydroxyanthranilamides by hepatic microsomal enzymes. The metabolism of methyl *N*-methylanthranilate is consistent with that of anthranilic acid esters. In rats and humans, the main metabolic pathway is the hydrolysis of the ester function to *N*-methylanthranilic acid which can, to a small extent, be further *N*-demethylated to yield anthranilic acid. Both processes occur principally in the liver and the two metabolites are excreted via urine (SCCS, 2011). Yamaori et al. (2005) revealed that MMA could also initially undergo *N*-demethylation to methyl anthranilate by guinea pig liver homogenates that can subsequently be hydrolyzed to anthranilic acid.

Urine is commonly used in the drug safety/toxicity assessments because it allows continuous noninvasive monitoring (Jiye et al., 2008). Hitherto, many analytical techniques have been applied to the metabolomic analysis of urine, but nuclear magnetic resonance (NMR) and mass spectrometry (MS) coupled to gas chromatography (GC), liquid chromatography (LC), and capillary electrophoresis (CE) are the most commonly used in this sense (Barding et al., 2013; Emwas, 2015; Jiye et al., 2008). Because GC-MS and NMR can give extensive structural information on multiple chemical classes in a single analytical procedure and taking into account their inherent analytical strengths and weaknesses (Barding et al., 2013; Emwas, 2015; Jiye et al., 2008; Pasikanti et al., 2008), both spectroscopic platforms were used in this study to query the metabolites of *N*-methylanthranilic acid esters in the urine of rats treated with IMA and MMA.

GC-MS can be expected to work perfectly in the case of metabolic studies when there are available standards or library (mass spectral and retention) data of the metabolites, if these are known at all. However, it can fail completely if one has to identify a compound based solely on its mass spectrum or has to differentiate among isomers of compounds, as is frequently encountered in the analysis of metabolites (e.g. among regioisomeric hydroxyanthranilic acid derivatives). We believe that there are three possible approaches in such situations: 1) preparative chromatography followed by NMR, 2) synthesis of the presumed metabolites, and 3) prediction of retention data of the possible xenobiotic metabolites.

Ideally, for approach No. 1 to be successful, one has to have a sufficient amount of the metabolites within the biological matrix, and an efficient chromatographic technique to obtain pure samples of the metabolites for NMR. Usually, both prerequisites are hard to achieve. The amount of the metabolites could in theory always be increased by a higher number of experimental organisms utilized, though, this is ethically non-acceptable. This means that the chromatography must not only be highly efficient but also has to allow maximal recovery. Today, an array of SiO_2-based stationary phases is available for this purpose, and this caused other sorbents to become obsolete, despite their possible advantages over silica materials. One of them is Sephadex LH-20 that is known not only for its size exclusion chromatographic properties (Salituro and Dufresne, 1998), but also for an unusual affinity of this type of gel towards phenolic and heteroatomic compounds, and practically 100% recovery of the chromatographed material, ensuring no losses of the metabolites.

The synthetic approach, No. 2, seems feasible if approach No. 1 fails. Recently, synthetic libraries of natural compounds were prepared in order to facilitate their identification and have proved to be particularly useful in structure-activity/property studies (Radulović et al., 2013d, 2014; 2015b, 2015c). This approach appears to be suitable in the case of regioisomeric *in vivo* hydroxylation of aromatic compounds. Such a library could be the starting point for approach No. 3, since it enables a collection of retention properties (for example, gas chromatographic retention indices) of compounds with relevant (for identification purposes) structural variation.

The mentioned three approaches combined are expected to significantly facilitate urine metabolite identification and represent either a revival of an almost forgotten application of Sephadex LH-20, or are completely novel (synthetic libraries and SPR of xenobiotic metabolite retention properties) in the field of metabolite analysis. Having in mind that the peak research activities on the metabolism of anthranilic acid and its derivatives took place almost half a century ago, in this work we report on the analysis of urine metabolites of two pharmacologically active *N*-methylanthranilic acid esters, IMA and MMA, in rats, by the application of the three approaches. The preparative approach utilizing Sephadex LH-20 was applied in the case of IMA due the fact that no previous reports on its metabolism exist. For MMA, the synthetic/combinatorial approach, which should provide both standards and a means for SPR analysis, was explored. This meant that we could reduce the number of experimental animals in the second case to a minimum that could allow a routine GC-MS analysis and consequently the total duration of the experiments.

2. Materials and methods

2.1. General

Methyl and isopropyl esters of *N*-methylanthranilic acid were synthesized as described before (Radulović et al., 2011). All other reagents and solvents were obtained from commercial sources (Sigma-Aldrich, St. Louis, Missouri, USA; Merck, Darmstadt, Germany; Carl Roth, Karlsruhe, Germany) and used as received, except the solvents were purified by distillation.

Nuclear magnetic resonance (NMR) spectra were recorded at 25 °C on a Bruker Avance III 400 MHz NMR spectrometer (^{1}H at 400 MHz, ^{13}C at 100 MHz) using $CDCl_3$ as the solvent. Chemical shifts were expressed as δ (ppm) using tetramethylsilane as an internal standard. 2D experiments (^{1}H-^{1}H COSY, NOESY, TOCSY, HSQC and HMBC), as well as DEPT-90 and DEPT-135, were run on the same instrument with usual pulse sequences. UV spectra (in acetonitrile) were measured using a UV-1800 Shimadzu spectrophotometer (Tokyo, Japan). Infrared (IR) measurements (attenuated total reflectance) were carried out using a Thermo Nicolet 6700 FTIR spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Chromatographic separations were carried out using Sephadex LH-20 (Sigma-Aldrich, St. Louis, Missouri, USA) using a mixture of methanol and chloroform (1: 1, v/v) as the eluent. For

analytical thin layer chromatography (TLC) silica gel 60 on Al plates (Kieselgel 60 F_{254}, layer thickness 0.2 mm; Merck, Darmstadt, Germany) were used; the spots on TLC were visualized by UV light (254 nm) and by spraying with 10% (w/v) ethanolic solution of phosphomolybdic acid, followed by heating. Microanalyses of carbon, hydrogen and nitrogen were carried out on a Carlo Erba Elemental Analyzer model 1106 (Carlo Erba Strumentazione, Italy); their results agreed favorably with the calculated values.

2.2. GC and GC-MS analyses

Gas chromatography-mass spectrometry (GC-MS) analyses were repeated three times for each sample using an HP 6890N gas chromatograph coupled with an HP 5975B mass-selective detector (Hewlett-Packard, Palo Alto, California, USA). The gas chromatograph was equipped with a DB-5MS fused silica capillary column (5% phenylmethylsiloxane, 30 m × 0.25 mm, film thickness 0.25 μm; Agilent Technologies, Palo Alto, California, USA). The oven temperature was raised linearly from 70 to 315 °C at a heating rate of 5 °C min^{-1} and then held isothermally for 10 min. Helium at a flow rate of 1 ml min^{-1} was used as a carrier gas. The injector and interface were maintained at 250 and 320 °C, respectively. The samples, 1 μl of the solutions of urine extracts, the corresponding chromatographic fractions or pure compounds in diethyl ether (*ca.* 1 mg in 1 ml of Et_2O), were injected in a pulsed split mode (the flow rate was 1.5 ml min^{-1} for the first 0.5 min and then set to 1 ml min^{-1} for the remainder of the analysis; split ratio 40:1). The mass-selective detector was operated at the ionization energy of 70 eV in the *m/z* 35–500 range with a scanning speed of 0.34 s.

The GC analyses were carried out using a Agilent 7890A GC system equipped with a single injector, one flame ionization detector (FID) and a fused silica capillary column DB-5MS (5% phenylmethylsiloxane, 30 m × 0.32 mm, film thickness 0.25 μm; Agilent Technologies). The oven temperature was programmed from 150 to 300 °C at 15 °C min^{-1} and then held isothermally at 300 °C for 5 min; the carrier gas was nitrogen at 3 ml min^{-1}; the injector temperature was held at 250 °C. The samples, 1 μl of the mentioned corresponding solutions, were injected in a splitless mode. The parameters of the FID detector were as follows: heater temperature, 300 °C; H_2 flow, 30 ml min^{-1}; air flow, 400 ml min^{-1}; makeup flow, 23.5 ml min^{-1}; signal, 20 Hz.

The percentage composition was computed from GC-FID peak areas without the use of correction factors. The linear retention indices relative to the retention times of C_{10}–C_{21} *n*-alkanes on the DB-5MS column were calculated according to Van Den Dool and Kratz (1963). Qualitative analyses of the mentioned samples were firstly based on the comparison of their mass spectra with those of authentic standards as well as those from Wiley 10 and NIST2017, as well as on the analysis of the fragmentation patterns from their mass spectra. Finally, wherever possible, identification was achieved by GC co-injection with an authentic sample.

2.3. Animals

Adult male and female Wistar rats weighing 200–250 g, obtained from the Vivarium of the Scientific Research Center for Biomedicine, Faculty of Medicine, University of Niš, were maintained under standard laboratory conditions: 22 ± 2 °C, 60% humidity and 12/12 (light/dark) cycle, with food and water available *ad libitum*. All experimental procedures were conducted in accordance with the principles of care and use of laboratory animals in research and approved by the local Ethics committee. The approval of the committee (number 01-7289-11) was given on 14th October 2011. All efforts were made to minimize animal suffering and reduce the number of animals used (3R).

2.4. Experimental design

After a week of adaptation, the animals were randomly divided into three groups, two experimental and one control. Each animal was housed individually in a metabolic cage (designed as to provide a complete separation of urine and feces. i.e. prevent urine from washing over and entering the feces tube) with food and water provided *ad libitum*. Group I, consisting of 8 animals, and group II, consisting of 4 animals, were treated with IMA and MMA, respectively, dissolved in olive oil, at a dose of 200 mg kg^{-1}. The control group, 4 animals, received only olive oil (1 ml kg^{-1}). All substances were administrated daily, for seven consecutive days, by an intraperitoneal injection. Urine samples were collected daily, minimum 4 h after the administration of the substances, and pooled with urine samples collected from animals within the same group. In this way, in total, *ca.* 400 ml of urine (on average 3.1 ml of urine/100 g bw/day) from IMA treated group, *ca.* 185 ml (on average 3.0 ml of urine/100 g bw/day) from MMA treated group and *ca.* 205 ml (on average 3.4 ml of urine/100 g bw/day) of urine from the control group were obtained. After daily collection, the urine samples were frozen and kept at −80 °C until analyses.

2.5. Biochemical measurements

The blood obtained from rats by cardiac puncture was centrifuged at 2000 rpm at 4 °C for 15 min to obtain the serum in which aspartate transaminase (AST), alanine transaminase (ALT), cholesterol (Cho), total (TB) and direct bilirubin (DB) were assayed by Olympus AU680® Chemistry-Immuno Analyzer.

2.6. Histopathological observation

The liver tissue specimens separated for histopathological examination were fixed in neutral formaldehyde solution (10%, w/v), dehydrated with ethanol solutions of differing concentration (50–100%, v/v), embedded in paraffin, cut into 4–5 μm thick sections, stained with hematoxylin and eosin (HE) and further examined with an Olympus BH2 light microscope.

2.7. Preparation of urine samples for analyses

The hydrolysis of glucuronides present in the urine samples was effectuated in two ways, enzymatically or chemically (hydrolysis by a mineral acid). The enzymatic hydrolysis of the samples was performed as previously described by Naito et al. (1984): the urine samples (60 ml in the case of IMA treated group and 30 ml in the case of MMA treated group and the control group, two repetitions), adjusted to pH 4.9 with an 0.1 mol l^{-1} acetate buffer, were incubated with β-glucuronidase at 37 °C for 2 h. Otherwise, concentrated HCl (15 ml) was added to a sample of urine (60 ml in the case of IMA treated group and 30 ml in the case of MMA treated group and the control group, two repetitions) and the obtained mixture was heated for 2 h at 50 °C with stirring, after which it was neutralized with 10 mol l^{-1} KOH (Feng et al., 2001).

To the hydrolyzed urine samples, both from enzymatic and acidic hydrolyses, 1 ml of 40% phosphate buffer was added (pH 9) and the samples were exhaustively extracted with diethyl ether. After the organic layers were washed with water and dried over anhydrous $MgSO_4$, the solvent was evaporated under a stream of dry nitrogen. Non-hydrolyzed urine samples (60 ml in the case of IMA treated group and 30 ml in the case of MMA treated group and the control group, two repetitions) were extracted directly with diethyl ether and treated in the same manner as described above. In this way, in total, 18 samples were obtained that were further analyzed by GC and GC-MS.

2.8. Sephadex LH-20 chromatography of the diethyl ether extracts of urine samples

Following the GC-MS analyses, the extracts of both hydrolyzed and non-hydrolyzed urine samples, originating from IMA, possessing matching TICs, were pooled. The collective sample of IMA urine metabolites was subjected to an isocratic column chromatography on Sephadex LH-20 (Sigma-Aldrich, St. Louis, Missouri, USA) using a mixture of methanol and chloroform (1: 1, v/v). The progress of the chromatography was easily monitored by exposing the column to UV light (254 nm) for a short period. Brightly fluorescent bands of different metabolites could be noted in different segments of the column. This provided the means to reduce the number of individual fractions collected. Similar fractions were subsequently additionally analyzed by TLC. The pooled fractions were further analyzed by GC-MS and NMR.

2.9. Methylation of 3-hydroxyanthranilic and 5-hydroxyanthranilic acids

Commercially available 3-hydroxyanthranilic and 5-hydroxyanthranilic acids were methylated by an excess of ethereal diazomethane, which was previously synthetized following the procedure of Arndt (1935). GC-MS analysis revealed that both reaction mixtures consisted of mono-, di-, tri- and tetramethylated products, which were further separated in an analogous way to urine extract (an isocratic column chromatography on Sephadex LH-20 using a mixture of methanol and chloroform (1: 1, v/v)). The progress of the chromatography, fraction collection and analysis were done as described before.

2.10. Spectral characterization of the isolated compounds

2.10.1. Isopropyl 3-hydroxyanthranilate

Isopropyl 3-hydroxyanthranilate (isopropyl 2-amino-3-hydroxybenzoate, compound **1**): $C_{10}H_{13}NO_3$; yellowish liquid; 33 mg; UV (CH_3CN) λ_{max} (log ε) 340 (4.47), 218 (5.18) nm; FTIR (neat) ν_{max} 3371.6, 2918.5, 2849.7, 1682.6, 1550.2, 1489.5, 1464.1, 1372.7, 1283.5, 1235.2, 1097.3, 949.2, 746.3 cm^{-1}; Rt 21.811 min, RI (DB-5MS) 1726; EIMS *m/z* (rel.int.): 195 $[M]^+$ (53.9%), 153 $[M-H_2C{=}CH{-}CH_3]^+$ (53.2%), 136 $[M-(CH_3)_2CHO]^+$ (34.7%), 135 $[H_2C{=}CH{-}CH_3{-}H_2O]^+$ (100%), 134 $[M-H_2C{=}CH{-}CH_3{-}H_2O{-}H]^+$ (9.8%), 108 $[M-COOCH(CH_3)_2]^+$ (12.4%), 107 $[M-H_2C{=}CH{-}CH_3{-}H_2O{-}CO]^+$ (79.2%), 106 (9.2%), 80 (11.6%); 52 (8.8%); Calc. for $C_{10}H_{13}NO_3$: C 61.53, H 6.71, N 7.18, O 24.59%; Found: C 61.52, H 6.68, N 7.16, O 24.64%; ^1H and ^{13}C NMR data, see Table 4.

2.10.2. Isopropyl 5-hydroxyanthranilate

Isopropyl 5-hydroxyanthranilate (isopropyl 2-amino-5-hydroxybenzoate, compound **2**): $C_{10}H_{13}NO_3$; yellowish liquid; 28 mg; UV (CH_3CN) λ_{max} (log ε) 340 (4.62), 218 (5.28) nm; FTIR (neat) ν_{max} 3178.7, 2978.0, 1679.1, 1550.0, 1489.9, 1465.7, 1355.1, 1232.6, 1096.9, 1019.7, 948.0, 794.9, 746.1 cm^{-1}; Rt 22.521 min, RI (DB-5MS) 1760; EIMS *m/z* (rel.int.): 195 $[M]^+$ (31.2%), 153 $[M-H_2C{=}CH{-}CH_3]^+$ (50.5%), 136 $[M-(CH_3)_2CHO]^+$ (23.4%), 135 $[H_2C{=}CH{-}CH_3{-}H_2O]^+$ (100%), 134 $[M-H_2C{=}CH{-}CH_3{-}H_2O{-}H]^+$ (3.5%), 108 $[M-COOCH(CH_3)_2]^+$ (12.8%), 107 $[M-H_2C{=}CH{-}CH_3{-}H_2O{-}CO]^+$ (30.7%), 80 (6.5%), 68 (5.3%), 52 (7.9%); Calc. for $C_{10}H_{13}NO_3$: C 61.53, H 6.71, N 7.18, O 24.59%; Found: C 61.50, H 6.76, N 7.23, O 24.51%; ^1H and ^{13}C NMR data, see Table 4.

2.10.3. Isopropyl 5-hydroxy-N-methylanthranilate

Isopropyl 5-hydroxy-*N*-methylanthranilate (isopropyl 5-hydroxy-2-(methylamino)benzoate, compound **3**): $C_{11}H_{15}NO_3$; yellowish liquid; 26 mg; UV (CH_3CN) λ_{max} (log ε) 376 (4.06), 257 (4.38), 220 (4.84) nm; FTIR (neat) ν_{max} 3382.5, 2978.7, 2931.7, 1674.6, 1582.4, 1515.0, 1455.4, 1358.8, 1205.8, 1166.1, 1103.7, 1080.7, 1051.5, 955.4, 907.8, 811.8, 752.9 cm^{-1}; Rt 23.685 min, RI (DB-5MS) 1815; EIMS *m/z* (rel.int.): 209 $[M]^+$ (55.4%), 167 $[M-H_2C{=}CH{-}CH_3]^+$ (90.5%), 150 $[M-(CH_3)_2CHO]^+$ (24.0%), 149 $[M-H_2C{=}CH{-}CH_3{-}H_2O]^+$ (42.2%), 148 $[M-H_2C{=}CH{-}CH_3{-}H_2O{-}H]^+$ (21.8%), 122 $[M-COOCH(CH_3)_2]^+$ (17.9%), 121 $[M-H_2C{=}CH{-}CH_3{-}H_2O{-}CO]^+$ (100%), 120 $[M-H_2C{=}CH{-}CH_3{-}H_2O{-}CO{-}H]^+$ (58.4%), 94 (13.4%), 80 (4.4%), 65 (14.7%); Calc. for $C_{11}H_{15}NO_3$: C 63.14, H 7.23, N 6.69, O 22.94; Found: C 63.17, H 7.20, N 6.61, O 23.02; ^1H and ^{13}C NMR data, see Table 4.

2.10.4. N-methylanthranilic acid amide

N-methylanthranilic acid amide (2-(methylamino)benzamide, compound **4**): $C_8H_{10}N_2O$; yellowish liquid; 9 mg; UV (CH_3CN) λ_{max} (log ε) 340 (4.76), 257 (5.11), 217 (5.63) nm; FTIR (neat) ν_{max} 3354.8, 3167.3, 2918.5, 2850.3, 2811.1, 1652.9, 1614.6, 1578.0, 1508.9, 1434.4, 1404.3, 1238.5, 1155.2, 1115.8, 1027.9, 829.1, 746.6 cm^{-1}; Rt 19.139 min, RI (DB-5MS) 1604; EIMS *m/z* (rel.int.): 150 $[M]^+$ (100%), 133 $[M-NH_2-H]$ (59.6%), 132 (24.6%), 105 $[M-NHCH_3-NH]$ (81.1%), 104 $[M-NHCH_3-NH_2]$ (96.6%), 92 $[M-NHCH_3-CO]$ (7.8%), 91 (6.0%), 78 (27.4%), 77 (37.9%), 63 (6.1%), 51 (9.5%); Calc. for $C_8H_{10}N_2O$: C 63.98, H 6.71, N 18.65, O 10.65%; Found: C 63.90, H 6.68, N 18.78, O 10.64%; ^1H and ^{13}C NMR data, see Table 4.

IR, ^1H NMR and MS spectroscopic data are in accordance with literature (Bertini et al., 2005), while MS data are in accordance with Selva et al. (2005).

2.10.5. Methyl 3-hydroxyanthranilate

Methyl 3-hydroxyanthranilate (methyl 2-amino-3-hydroxybenzoate): $C_8H_9NO_3$; yellowish liquid; yield 67.7%; UV (CH_3CN) λ_{max} (log ε) 340 (4.67), 244 (4.79), 224 (5.37) nm; FTIR (neat) ν_{max} 3409.0, 3321.3, 3023.6, 2951.5, 1699.7, 1393.9, 1287.5, 1271.7, 1081.1, 994.4, 740,0 cm^{-1}; Rt 19.748 min, RI (DB-5MS) 1632; EIMS *m/z* (rel.int.): 167 $[M]^+$ (64.5%), 136 $[M-CH_3O]^+$ (24.1%), 135 $[M-CH_3OH]^+$ (66.7%), 108 $[M-COOCH_3]^+$ (15.9%), 107 $[M-CH_3OH-CO]^+$ (100%), 106 $[M-CH_3-H_2O-CO]^+$ (15.2%), 79 (21.9%), 63 (5.3%); Calc. for $C_8H_9NO_3$: C 57.48; H 5.43; N 8.38; O 28.71; Found: C 57.39; H 5.46; N 8.45; O 28.70; ^1H and ^{13}C NMR data, see Table 6.

^1H and ^{13}C NMR spectroscopic data are in accordance with literature (Fielder and Collins, 1995; Huang et al., 2006; Moon et al., 2000; Tipparaju et al., 2008), except Moon and his workers (2000) assigned signal at 146.2 ppm to C(2). MS data are in accordance with Fielder and Collins (1995), while IR spectroscopic data are in accordance with Moon et al. (2000) and Huang et al. (2006).

2.10.6. Methyl 3-hydroxy-N-methylanthranilate

Methyl 3-hydroxy-*N*-methylanthranilate (methyl 3-hydroxy-2-(methylamino)benzoate): $C_9H_{11}NO_3$; yellowish liquid; yield 24.4%; UV (CH_3CN) λ_{max} (log ε) 340 (4.60), 248 (4.78), 226 (5.31) nm; FTIR (neat) ν_{max} 3492.7, 3373.4, 2949.4, 1720.5, 1686.5, 1434.0, 1241.6, 1223.5, 1084.3, 903.3, 743.7 cm^{-1}; Rt 15.249 min, RI (DB-5MS) 1439; EIMS *m/z* (rel.int.): 181 $[M]^+$ (100%), 166 $[M-CH_3]^+$ (26.4%), 149 $[M-CH_3OH]^+$ (33.1%), 148 $[M-CH_3-H_2O]^+$ (74.8%), 134 $[M-CH_3OH-CH_3]^+$ (18.6%), 121 $[M-CH_3OH-CO]^+$ (94.1%), 120 $[M-CH_3-H_2O-CO]^+$ (47.8%), 107 (10.1%), 106 (47.6%), 93 (58.6%), 78 (19.5%), 65 (40.3%); Calc. for $C_{11}H_{15}NO_3$: C 59.66, H 6.12, N 7.73, O 26.49; Found: C 59.58, H 6.10, N 7.76, O 26.56; ^1H and ^{13}C NMR data, see Table 6.

2.10.7. Methyl 3-methoxyanthranilate

Methyl 3-methoxyanthranilate (methyl 2-amino-3-methoxybenzoate): $C_9H_{11}NO_3$; yellowish liquid; yield 1.2%; UV (CH_3CN) λ_{max} (log ε) 338 (4.45), 247 (4.66), 224 (5.18) nm; FTIR

(neat) ν_{max} 3494.6, 3375.4, 2950.9, 1719.5, 1687.6, 1549.8, 1434.8, 1242.4, 1224.5, 1085.0, 904.3, 744.2 cm^{-1}; Rt 18.012 min, RI (DB-5MS) 1555; EIMS *m/z* (rel.int.): 181 $[M]^+$ (100%), 166 $[M-CH_3]^+$ (52.7%), 149 $[M-CH_3OH]^+$ (28.6%), 148 $[M-CH_3-H_2O]^+$ (22.6%), 135 $[M-CH_3O-CH_3]^+$ (10.1%), 134 $[M-CH_3OH-CH_3]^+$ (69.4%), 121 $[M-CH_3OH-CO]^+$ (56.8%), 120 $[M-CH_3-H_2O-CO]^+$ (8.8%), 107 (22.5%), 106 (62.3%), 91 (17.4%), 78 (24.9%), 65 (14.0%); Calc. for $C_9H_{11}NO_3$: C 59.66, H 6.12, N 7.73, O 26.49; Found: C 59,74, H 6.09, N 7.72, O 26.45; ^1H and ^{13}C NMR data, see Table 6.

IR, ^1H and ^{13}C NMR spectroscopic data are in accordance with Schuler et al. (2006).

2.10.8. Methyl 3-methoxy-N-methylanthranilate

Methyl 3-methoxy-*N*-methylanthranilate (methyl 3-methoxy-2-(methylamino)benzoate): $C_{10}H_{13}NO_3$; yellowish liquid; yield 6.6%; UV (CH_3CN) λ_{max} (log ε) 304 (4.25), 221 (5.00), 195 (5.17) nm; FTIR (neat) ν_{max} 3365.6, 2949.1, 1721.0, 1434.7, 1241.1, 1096.6, 1057.3, 943.4, 747.6 cm^{-1}; Rt 14.887 min, RI (DB-5MS) 1424; EIMS *m/z* (rel.int.): 195 $[M]^+$ (59.3%), 180 $[M-CH_3]^+$ (100%), 164 $[M-OCH_3]^+$ (62.5%), 162 $[M-CH_3-H_2O]^+$ (51.5), 149 $[M-CH_3O-CH_3]^+$ (17.8%), 148 $[M-CH_3OH-CH_3]^+$ (93.1%), 135 $[M-CH_3OH-CO]^+$ (10.1%), 134 $[M-CH_3-H_2O-CO]^+$ (34.1%), 121 (11.7%), 120 (34.6%), 107 (20.0%), 92 (19.1%), 77 (13.4%), 65 (34.0%); Calc. for $C_{11}H_{15}NO_3$: C 61.53, H 6.71, N 7.18, O 24.59; Found: C 61.57, H 6.64, N 7.19, O 24.60; ^1H and ^{13}C NMR data, see Table 6.

2.10.9. Methyl 5-hydroxyanthranilate

Methyl 5-hydroxyanthranilate (methyl 2-amino-5-hydroxybenzoate): $C_8H_9NO_3$; yellowish liquid; yield 54.3%; UV (CH_3CN) λ_{max} (log ε) 371 (4.50), 256 (4.59), 220 (5.15) nm; FTIR (neat) ν_{max} 3381.7, 3288.3, 2950.6, 1678.5, 1429.3, 1201.9, 1085.4, 980.7, 762.9 cm^{-1}; Rt 20.461 min, RI (DB-5MS) 1664; EIMS *m/z* (rel.int.): 167 $[M]^+$ (83.1%), 136 $[M-CH_3O]^+$ (25.8%), 135 $[M-CH_3OH]^+$ (100%), 108 $[M-COOCH_3]^+$ (28.4%), 107 $[M-CH_3OH-CO]^+$ (79.1%), 106 $[M-CH_3-H_2O-CO]^+$ (9.6%), 80 (20.0%), 68 (14.7%); Calc. for $C_8H_9NO_3$: C 57.48; H 5.43; N 8.38; O 28.71; Found: C 57.40; H 5.44; N 8.42; O 28.74; ^1H and ^{13}C NMR data, see Table 7.

IR and ^1H NMR spectroscopic data are in accordance with Hara et al. (2004).

2.10.10. Methyl 5-hydroxy-N-methylanthranilate

Methyl 5-hydroxy-*N*-methylanthranilate (methyl 5-hydroxy-2-(methylamino)benzoate): $C_9H_{11}NO_3$; yellowish liquid; yield 18.1%; UV (CH_3CN) λ_{max} (log ε) 378 (4.66), 257 (4.74), 220 (5.28) nm; FTIR (neat) ν_{max} 3395.2, 3302.8, 2951.9, 1676.0, 1526.4, 1428.2, 1293.6, 1198.7, 1084.9, 980.7, 809.7, 762.1 cm^{-1}; Rt 21.718 min, RI (DB-5MS) 1722; EIMS *m/z* (rel.int.): 181 $[M]^+$ (100%), 166 $[M-CH_3]^+$ (2.6%), 149 $[M-CH_3OH]^+$ (24.2%), 148 $[M-CH_3-H_2O]^+$ (48.7%), 121 $[M-CH_3OH-CO]^+$ (76.1%), 120 $[M-CH_3-H_2O-CO]^+$ (91.6%), 107 (6.5%), 94 (21.9%), 80 (8.4%), 65 (26.1%); Calc. for $C_{11}H_{15}NO_3$: C 59.66, H 6.12, N 7.73, O 26.49; Found: C 59,70, H 6.05, N 7.72, O 26.53; ^1H and ^{13}C NMR data, see Table 7.

2.10.11. Methyl 5-methoxyanthranilate

Methyl 5-methoxyanthranilate (methyl 2-amino-5-methoxybenzoate): $C_9H_{11}NO_3$; yellowish liquid; yield 13.8%; UV (CH_3CN) λ_{max} (log ε) 377 (4.59), 257 (4.69), 220 (5.23) nm; FTIR (neat) ν_{max} 3394.3, 3300.2, 3003.4, 2952.0, 1676.6, 1526.1, 1433.1, 1296.3, 1200.0, 1164.6, 1083.0, 979.0, 761.7 cm^{-1}; Rt 18.678 min, RI (DB-5MS) 1584; EIMS *m/z* (rel.int.): 181 $[M]^+$ (100%), 166 $[M-CH_3]^+$ (2.6%), 149 $[M-CH_3OH]^+$ (24.2%), 148 $[M-CH_3-H_2O]^+$ (48.7%), 121 $[M-CH_3OH-CO]^+$ (76.1%), 120 $[M-CH_3-H_2O-CO]^+$ (91.6%), 107 (6.5%), 94 (21.9%), 80 (8.4%), 65 (26.1%); Calc. for $C_9H_{11}NO_3$: C 59.66, H 6.12, N 7.73, O 26.49; Found: C 59,70, H 6.05, N 7.72, O 26.53; ^1H and ^{13}C NMR data, see Table 7.

^1H NMR spectroscopic data are in accordance with Patterson et al. (2004).

2.10.12. Methyl 5-methoxy-N,N-dimethylanthranilate

Methyl 5-methoxy-*N,N*-dimethylanthranilate (methyl 2-(dimethylamino)-5-methoxybenzoate): $C_{11}H_{15}NO_3$; yellowish liquid; yield 5.2%; UV (CH_3CN) λ_{max} (log ε) 352 (4.10), 256 (4.34), 210 (4.83), 194 (4.90) nm; FTIR (neat) ν_{max} 2948.4, 2839.6, 1689.3, 1500.0, 1433.2, 1208.4, 1070.8, 818.6, 785.7 cm^{-1}; Rt 18.935 min, RI (DB-5MS) 1595; EIMS *m/z* (rel.int.): 209 $[M]^+$ (96.3%), 194 $[M-CH_3]^+$ (81.2%), 178 $[M-OCH_3]^+$ (73.9%), 176 $[M-CH_3-H_2O]^+$ (57.4%), 163 $[M-CH_3O-CH_3]^+$ (26.7%), 162 $[M-CH_3OH-CH_3]^+$ (100%), 148 (14.6%), 135 $[M-CH_3OH-CO]^+$ (28.1%), 134 $[M-CH_3-H_2O-CO]^+$ (23.3%), 120 (28.8%), 106 (14.5%), 92 (18.3%), 77 (14.5%), 65 (11.7%); Calc. for $C_{11}H_{15}NO_3$: C 63.14, H 7.23, N 6.69, O 22.94; Found: C 63.19, H 7.22, N 6.59, O 23.00; ^1H and ^{13}C NMR data, see Table 7.

3. Results and discussion

3.1. Study design and toxicity evaluation

Our experiments were commenced by the investigation of the metabolism of IMA and MMA in rats that were intraperitoneally injected with a high (respective to its pharmacologically active) dose of this substance (200 mg kg^{-1}). This mode of application, number of animals, dose, and duration of urine collection (7 days), was chosen: (a) to insure a "good yield" of the metabolites of this xenobiotic since a preparative separation was envisioned in the subsequent analysis in the case of IMA (8 animals); (b) to provide only the minimal amount of the metabolites for GC and GC-MS analyses in the case of MMA (4 animals).

As the liver plays a central role in xenobiotic biotransformations and can be susceptible to possible toxic effects either from the xenobiotics, or from their metabolites, in parallel with the chemical analyses of urine metabolites, we evaluated the influence of IMA and MMA application on rat liver tissue morphology and function by means of standard biochemical and histopathological analyses. Serum levels of two transaminases (aspartate AST, and alanine ALT), cholesterol (Cho), and, total (TB) and direct bilirubin (DB), in rats treated with IMA and MMA, did not significantly differ from the control group levels (Table 1). Such findings indicated a preserved liver function, while a previous study revealed that the two tested compounds also have no influence on the kidney function (they had no effect on serum urea and creatinine levels) (Radulović et al., 2015a). Furthermore, liver wet weight was not affected by the one-week-long exposure to these anthranilates. Histopathological appearance of liver sections of the control and experimental animals are shown in Fig. 1. Microscopic evaluation of liver sections from the vehicle, as well as IMA and MMA, treated groups displayed normal morphology of liver tissue with characteristic hepatic architecture: the central veins, portal tracts, hepatocytes and sinusoids appeared normal (Fig. 1). The administration of IMA or MMA did not cause changes in liver morphology and function and no other signs of toxicity were observed in the treated animals, which was in accordance with previous studies (Gomes Pinheiro et al., 2014; Radulović et al., 2013b; SCCS, 2011). These findings are important in connection with the possible future usage of IMA or MMA as analgesics and/or anxiolytics (Gomes Pinheiro et al., 2014; Radulović et al., 2011, 2013c), since numerous drugs, specifically analgesics and anxiolytics, are known to cause liver and kidney damage after a prolonged or intensified consumption (Ritter et al., 2008).

Table 1
Serum biochemical parameters and relative liver weight of rats after seven days exposure to IMA and MMA.

	AST ($U\ l^{-1}$)	ALT ($U\ l^{-1}$)	Cho ($mmol\ l^{-1}$)	TB ($mmol\ l^{-1}$)	DB ($mmol\ l^{-1}$)	Relative liver weight (% to bw)
Control	176 ± 32	37 ± 2	1.5 ± 0.3	2.1 ± 0.1	0.4 ± 0.1	3.4 ± 0.3
MMA	232 ± 38	39 ± 2	1.5 ± 0.3	2.0 ± 0.2	0.4 ± 0.2	2.8 ± 0.3
IMA	130 ± 23	27.2 ± 0.5	1.6 ± 0.1	1.8 ± 0.2	0.6 ± 0.3	3.9 ± 0.4

Values are presented as means ± SD. Mean values within columns did not significantly differ ($p > 0.05$). AST - aspartate transaminase, ALT - alanine transaminase, Cho – cholesterol, TB - total bilirubin and DB - direct bilirubin.

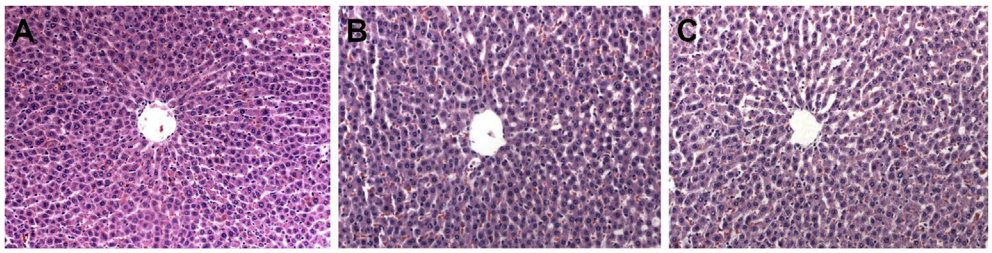


Fig. 1. Histopathological observations of liver sections stained with HE (× 200): (A) control group (olive oil); (B) MMA treated group; (C) IMA treated group. Characteristic hepatic architecture and preserved liver tissue can be seen in all three groups.

3.2. Chemical analyses of urine metabolites

Rat urines were daily collected and pooled to obtain two samples from the animals treated with IMA and MMA (200 mg kg^{-1}, *i.p.*), for seven consecutive days. The pooled urine samples were subjected to two different work-ups, a hydrolytic one, followed by extraction of aglycones by Et_2O, or a direct extraction with Et_2O. Both of these opalescent extracts, although differing in yield, were initially analyzed by GC-MS and were found to be nearly identical (and were not treated separately further on). Detailed analysis which included the mentioned three approaches (chromatographic separation, synthesis and SPR) enabled the identification of 16 different anthranilate derivatives, representing 70.5% of the total extract in the case of IMA and 14 different anthranilate derivatives, representing 68.9% of the total extract in the case of MMA. The remainder of the detected peaks (up to 30%) corresponded to ubiquitous rat urine metabolites (phenol, *p*-cresol, *p*-ethylphenol (Bakke, 1969)) detected in the urine sample originating from the control group. The identified constituents (only the ones related to anthranilic acid), their relative amount in the extracts, their structures, Rt and RI values and methods of identification are combined in Tables 2 and 3. The following passages provided the detailed account of the application and general usefulness of the mentioned three approaches.

3.2.1. Approach No. 1 - preparative chromatography of the urine of rats treated with IMA

Three major metabolites of IMA were detected by GC-MS analysis (at RI (DB5-MS) 1726, 1760 and 1815, corresponding to compounds **1**, **2** and **3**, respectively) of the diethyl ether extracts of the urine of rats treated with IMA. Mass spectra of the first two compounds (**1** and **2**) were mutually very similar (Fig. S1, Supplementary material), with the highest value of *m/z* 195. Molecular ion peak of compound **3** was observed at *m/z* 209, *i.e.* 14 amu higher, indicating the presence of an extra methyl group in the third molecule. The comparison of the mass spectra of these compounds with those from the available commercial MS libraries (Wiley10-NIST2017) suggested that they could represent derivatives of hydroxyanthranilic acid(s). An additional common feature of all three compounds was the intense peak that corresponded to a McLafferty fragmentation of the isopropyl ester moieties $[M - H_2C{=}CH{-}CH_3]^+$ giving the ions of hydroxyanthranilic (*m/z* 153, compounds **1** and **2**) and hydroxy-*N*-methylanthranilic acids (*m/z* 167, compound **3**). Based on this, the first two eluting compounds (**1** and **2**) were tentatively identified as mutually isomeric isopropyl hydroxyanthranilates, while the slowest eluting one (**3**), most probably represented one of the regioisomers of isopropyl hydroxy-*N*-methylanthranilates.

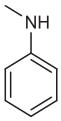
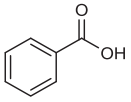
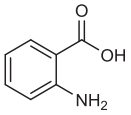
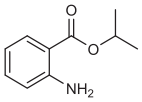
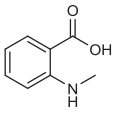
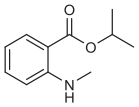
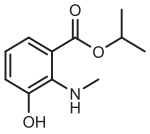
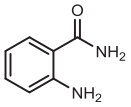
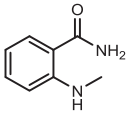
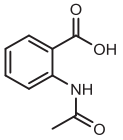
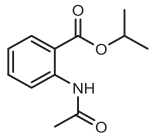
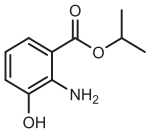
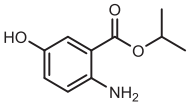
To determine the position of the hydroxyl groups in the before mentioned compounds, we opted for NMR. We decided to perform a preparative Sephadex LH-20 chromatography of the extract of the aglycones hoping that this stationary phase would enable an otherwise possibly difficult separation of positional isomers and homologues of similar polarity. Following a short (compared to the SiO_2 counterpart), straightforward, isocratic chromatography on Sephadex LH-20, we arrived at five different fractions, pooled based on TLC and most conveniently on an intense fluorescence emitted by the analytes when exposed to UV light (the course of the chromatography could be monitored in this way). According to GC-MS, principal constituents of chromatographic fractions 1, 2 and 3, were compounds **3** (RI 1815), **2** (RI 1760) and **1** (RI 1726), respectively. These turned out to be sufficiently pure to allow an unobstructed structural elucidation by NMR. A combination of 1D and 2D NMR experiments led to a complete assignment of ^1H and ^{13}C resonances of compounds **1**, **2** and **3** which were unambiguously identified as isopropyl 3-hydroxyanthranilate, isopropyl 5-hydroxyanthranilate and isopropyl 5-hydroxy-*N*-methylanthranilate, respectively (Fig. 2, Table 4). Details on the NMR spectral elucidation of these compounds are given in the Supplementary Material.

Chromatography on Sephadex LH-20 allowed us to concentrate a minor constituent, compound **4** (RI 1604). First clues on the identity of this compound came from a GC-MS analysis and the existence of amide bands in its IR spectrum. This data coupled with NMR permitted us to assign compound **4** as 2-(methylamino)benzamide (amide of *N*-methylanthranilic acid) (Fig. 2). Complete NMR data are collected in Table 4.

3.2.2. Approach No. 2 - synthetic approach

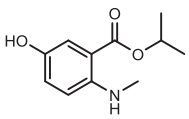
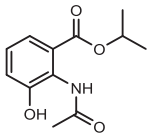
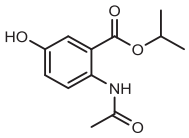
As mentioned above, due to ethical reasons, the number of

Table 2
Percentage composition of the diethyl ether extract of the urine of rats treated with IMA.

No[a]	Rt [min]	RI	%	IdenMeth[b]	Constituent structure	Constituent name
1.	6.534	1081	tr	RI, MS, CoI		*N*-Methylaniline
2.	8.655	1169	tr	RI, MS, CoI		Benzoic acid
3.	14.850	1423	§	RI, MS, CoI		Anthranilic acid
4.	15.573	1452	3.3	RI, MS, CoI		Isopropyl anthranilate
5.	15.900	1466	2.3	MS		*N*-Methylanthranilic acid
6.	17.129	1517	0.7	RI, MS, CoI		Isopropyl *N*-methylanthranilate
7.	17.555	1536	0.3	MS		Isopropyl 3-hydroxy-*N*-methylanthranilate
8.	17.881	1550	tr	MS		Anthranilamide
9.	19.139	1604	5.7	MS, NMR		2-(Methylamino)benzamide
10.	20.625	1671	tr	RI, MS		*N*-Acetylanthranilic acid
11.	20.745	1677	tr	MS*		Isopropyl-2-acetamidobenzoate
12.	21.811	1726	21.7	MS*, NMR		Isopropyl 3-hydroxyanthranilate
13.	22.521	1760	18.6	MS*, NMR		Isopropyl 5-hydroxyanthranilate

(*continued on next page*)

Table 2 (*continued*)

N°[a]	Rt [min]	RI	%	IdenMeth[b]	Constituent structure	Constituent name
14.	23.685	1815	17.2	MS*, NMR		Isopropyl 5-hydroxy-*N*-methylanthranilate
15.	23.806	1822	0.4	MS*		Isopropyl 2-acetamido-3-hydroxybenzoate
16.	28.136	2040	0.3	MS*		Isopropyl 2-acetamido-5-hydroxybenzoate
Total identified			70.5			

[a] Compounds listed in order of elution on DB-5MS column (Rt: retention time (in min) and RI: experimentally determined retention indices on the mentioned column by co-injection of a homologous series of *n*-alkanes C_{10}–C_{21}).

[b] RI, constituent identified by retention index matching; MS, constituent identified by mass spectra comparison; MS*, identification based on mass spectral fragmentation pattern; Col, constituent identity confirmed by GC co-injection of authentic sample; NMR, structure of an isolated pure constituent confirmed by ^{1}H and ^{13}C NMR spectral analysis; tr, trace amount (less than 0.05%); §, percentage was not determined due to peak tailing.

experimental animals treated with MMA was reduced to a minimum, but that still allows a routine GC-MS analysis (the number of animals was halved, 4 instead of 8). This deemed to provide sufficient material and data since the metabolism of MMA was studied previously (SCCS, 2011). However, due to scarce literature (spectral and retention) data, we were faced with a problem to differentiate and identify regioisomeric hydroxy derivatives.

Specifically, compounds eluting at RI 1632 and 1664, tentatively identified as methyl esters of hydroxyanthranilic acids, and at RI 1439 and 1722, tentatively identified as methyl esters of hydroxy-*N*-methylanthranilic acids, could not be differentiated based on mass spectral comparison with the MSes from the Wiley-NIST database and the analysis of the fragmentation patterns in their mass spectra. Since, we possessed only minute amounts of the xenobiotic metabolite mixture, the isolation of these compounds from the diethyl ether extract of the urine was not an option.

Knowing that IMA metabolites possessed either 3- or 5-hydroxyl groups on the anthranilic core, most probably MMA metabolites also represent analogous regioisomeric hydroxy derivatives. This situation appeared perfect for the application of the second approach. The corresponding 3- and 5-hydroxyanthranilic acids are commercially available and we had two reliable tools at our hands that would permit us to synthetize the target xenobiotic metabolites. One is the reaction that would produce all desired isomers in a single step (CH_2N_2 methylation) and the other one is a working chromatographic set-up that would furnish pure regioisomers.

Excess ethereal diazomethane effectuated methylation of carboxylic acid and amino groups in the two commercial hydroxyanthranilic acids, and, undesirably, the phenolic functionality. Thus, in a simple and fast methylation step, we ended up with a mixture of mono-, di-, tri- and tetramethylated products, according to a GC-MS analysis. The low chemoselectivity of methylation with CH_2N_2, though it might appear contradictory at first sight, turned out to be particular fruitful regarding Approach No. 3.

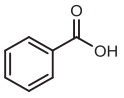
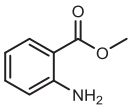
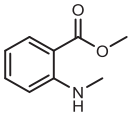
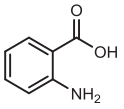
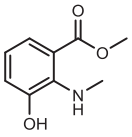
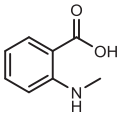
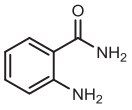
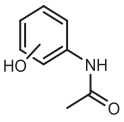
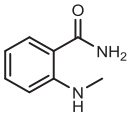
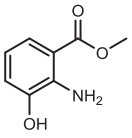
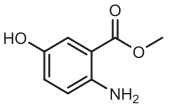
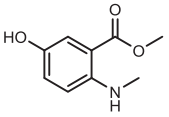
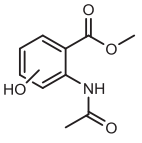
Both reaction mixtures were subjected to isocratic chromatography on Sephadex LH-20. The course of the chromatography was monitored by an intense fluorescence emitted by the analytes when exposed to UV light. In both cases four major fractions were obtained (initially pooled based on TLC). The fractions were initially analyzed by GC–MS. In this way, pure samples of methyl 3-hydroxyanthranilate, methyl 3-hydroxy-*N*-methylanthranilate, methyl 3-methoxyanthranilate, methyl 3-methoxy-*N*-methylanthranilate, methyl 5-hydroxyanthranilate, methyl 5-hydroxy-*N*-methylanthranilate, methyl 5-methoxyanthranilate, methyl 5-methoxy-*N,N*-dimethylanthranilate were obtained (Table 5), whose identities were confirmed by spectral means (MS, IR, 1D and 2D NMR, UV-VIS). Completely assigned NMR data are collected in Tables 6 and 7. GC co-injection experiments of the synthetic methyl 3-hydroxy- and 5-hydroxyanthranilates, as well as methyl 3-hydroxy- and 5-hydroxy-*N*-methylanthranilates with the diethyl ether extract of the urine of rats treated with MMA enabled an unambiguous identification of these compounds (Table 3). Although it might appear that such a synthetic approach is feasible only in this or related situations, one should consider that chemists have an arsenal of biomimetic transformations that might offer a pathway to the xenobiotic metabolites via, for example, an oxidation of the starting xenobiotic.

3.2.3. *Approach No. 3—structure-property relationship analysis*

Approaches Nos. 1 and 2 were the starting points for the approach No. 3. The first approach, i.e. preparative chromatography, yielded pure urine metabolites of IMA (isopropyl esters of 3- and 5-hydroxyanthranilic and 5-hydroxy-*N*-methylanthranilic acids). The synthetic approach, specifically, the methylation of 3- and 5-hydroxyanthranilic acids with CH_2N_2, provided pure synthetic samples of methyl 3-hydroxyanthranilate, methyl 3-hydroxy-*N*-methylanthranilate, methyl 3-methoxyanthranilate, methyl 3-methoxy-*N*-methylanthranilate, methyl 5-hydroxyanthranilate, methyl 5-hydroxy-*N*-methylanthranilate, methyl 5-methoxyanthranilate, and methyl 5-methoxy-*N,N*-dimethylanthranilate. A careful consideration of the retention indices of the isolated/synthetized compounds led us to following conclusions:

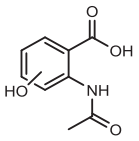
(1) Retention indices of isopropyl and methyl *N*-methylanthranilates were 65 RI units higher than the ones of isopropyl- and methyl anthranilates, in other words, the

Table 3
Percentage composition of the diethyl ether extract of the urine of rats treated with MMA.

No[a]	Rt [min]	RI	%	IdenMeth[b]	Constituent structure	Constituent name
1.	8.655	1169	tr	RI, MS, CoI		Benzoic acid
2.	13.092	1350	0.7	RI, MS, CoI		Methyl anthranilate
3.	14.655	1415	3.9	RI, MS, CoI		Methyl *N*-methylanthranilate
4.	14.850	1423	§	RI, MS, CoI		Anthranilic acid
5.	15.249	1439	tr	RI, MS*, CoI		Methyl 3-hydroxy-*N*-methylanthranilate
6.	15.900	1466	13.0	MS		*N*-methylanthranilic acid
7.	17.881	1550	1.3	MS		Anthranilamide
8.	18.465	1575	tr	MS		Acetamidophenol
9.	19.139	1604	36.8	MS, NMR		2-(Methylamino)benzamide
10.	19.748	1632	3.4	MS*, CoI		Methyl 3-hydroxyanthranilate
11.	20.461	1664	1.2	MS*, CoI		Methyl 5-hydroxyanthranilate
12.	21.718	1722	8.6	MS*		Methyl 5-hydroxy-*N*-methylanthranilate
13.	23.591	1811	tr	MS*		Methyl 2-acetamido-3(or 5)-hydroxybenzoate

(*continued on next page*)

Table 3 (*continued*)

N°[a]	Rt [min]	RI	%	IdenMeth[b]	Constituent structure	Constituent name
14.	25.748	1920	tr	MS*		2-(Acetylamino)-3(or 5)- hydroxybenzoic acid
Total identified			68.9			

[a] Compounds listed in order of elution on DB-5MS column (Rt: retention time (in min) and RI: experimentally determined retention indices on the mentioned column by co-injection of a homologous series of *n*-alkanes C_{11}–C_{20}).

[b] RI, constituent identified by retention index matching; MS, constituent identified by mass spectra comparison; MS*, identification based on mass spectral fragmentation pattern; CoI, constituent identity confirmed by GC co-injection of authentic sample; NMR, structure of an isolated pure constituent confirmed by ^{1}H and ^{13}C NMR spectral analysis; tr, trace amount (less than 0.05%); §, percentage was not determined due to peak tailing.

Table 4
NMR spectroscopic data (400 MHz, $CDCl_3$) for compounds **1**–**4** isolated from the diethyl ether extract of the urine of rats treated with IMA.

Position	Isopropyl 3-hydroxyanthranilate (1)		Isopropyl 5-hydroxyanthranilate (2)		Isopropyl 5-hydroxy-*N*-methylanthranilate (3)		2-(Methylamino) benzamide (4)	
	δ_C, mult.	δ_H (*J*, Hz)	δ_C, mult.	δ_H (*J*, Hz)	δ_C, mult.	δ_H (*J*, Hz)	δ_C, mult.	δ_H (*J*, Hz)
1	112.4 C	–	112.3 C	–	110.8 C	–	108.7 C	–
2	140.8 C	–	144.8 C	–	147.2 C	–	152.8 C	–
3	143.1 C	–	118.2 CH	6.60, d (8.7)	112.1 CH	6.59, d (9.0)	111.3 CH	6.69, dd (8.7, 1.0)
4	117.8 CH	6.82, dd (7.6, 1.3)	122.8 CH	6.88, dd (8.7, 3.0)	123.1 CH	6.99, dd (9.0, 3.0)	135.5 CH	7.42, ddd (8.7, 7.1, 1.6)
5	114.8 CH	6.50, dd (8.2, 7.6)	146.1 C	–	144.7 C	–	114.3 CH	6.61, ddd (8.1, 7.1, 1.0)
6	123.5 CH	7.50, dd (8.2, 1.3)	116.2 CH	7.35, d (3.0)	117.0 CH	7.42, d (3.0)	132.4 CH	7.94, dd (8.1, 1.6)
C=O	168.0 C	–	167.2 C	–	167.7 C	–	172.3 C	–
$CH(\underline{CH_3})_2$	22.0 CH_3	1.35, d (6.3)	22.0 CH_3	1.35, d (6.3)	22.0 CH_3	1.34, d (6.3)	–	–
$\underline{CH}(CH_3)_2$	67.7 CH	5.22, septuplet (6.3)	67.9 CH	5.20, septuplet (6.3)	67.7 CH	5.18, septuplet (6.3)	–	–
$NH\underline{CH_3}$	–	–	–	–	30.1 $\underline{C}H_3$	2.90, s, $NHCH_3$	31.9 $\underline{C}H_3$	2.93, s, $NHCH_3$
$N\underline{H}R/O\underline{H}/CON\underline{H_2}$	–	5.63, br s 7.60, br s	–	very broad signals	–	5.20, br s 7.30, br s	–	4.72, br s 7.70, br s

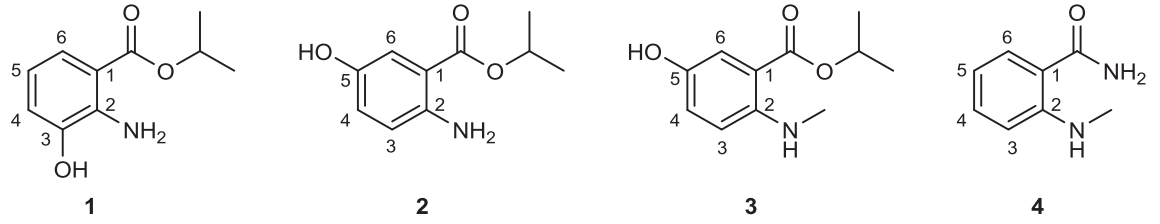


Fig. 2. Structures of compounds **1**–**4**.

introduction of a methyl group onto the nitrogen atom of anthranilate esters increased the value of the parent compound for 65 units;

(2) An analogous situation was encountered in the case of the retention indices of isopropyl and methyl 5-hydroxy-*N*-methylanthranilates, where the *N*-methylated derivatives possessed *ca.* 55 units higher RIs. Thus, for 5-hydroxyanthranilic acid esters, the introduction of the *N*-methyl group led to a similar increases retention index as for anthranilate esters;

(3) On the other hand, RI value of methyl 3-hydroxy-*N*-methylanthranilate was 193 units lower than the one of methyl 3-hydroxyanthranilate. Since the first two approaches did not permit us to detect the corresponding *N*-methylated isopropyl ester, we were drawn to test the regularity of RI-structure relationship and methodically combed through the initial TICs of the ether extracts of the urines of rats treated with IMA. Hence, if isopropyl 3-hydroxy-*N*-methylanthranilate were present in this sample, it would elute at RI *ca.* 1533 (=1726 (RI of isopropyl 3-hydroxyanthranilate)−193 (the mentioned increment)). Indeed, a peak at RI 1536 that displayed a mass spectrum that corresponds to a methylated derivative of an isopropyl ester of hydroxyanthranilic acids was noted and in this way assigned to isopropyl 3-hydroxy-*N*-methylanthranilate.

Such RI-structure relationship values could be rationalized in the following way: addition of a methyl group increases the

Table 5
Percentage composition of the crude products from the methylation of 3- and 5-hydroxyanthranilic acids.

N°[a]	Rt[a] [min]	RI	%	IdenMeth[b]	Constituent name
Products of methylation of 3-hydroxyanthranilic acid					
1.	14.887	1424	6.6	MS*, NMR, CoI	Methyl 3-methoxy-*N*-methylanthranilate
2.	15.249	1439	24.4	MS*, NMR, CoI	Methyl 3-hydroxy-*N*-methylanthranilate
3.	16.126	1475	tr	MS*	Methyl 3-methoxy-*N,N*-dimethylanthranilate
4.	16.440	1488	tr	MS*	Trimethylated derivative
5.	18.012	1555	1.2	MS, NMR, CoI	Methyl 3-methoxyanthranilate
6.	18.446	1574	tr	MS*	Trimethylated derivative
7.	19.748	1632	67.7	MS*, NMR, CoI	Methyl 3-hydroxyanthranilate
Total			99.9		
Products of methylation of 5-hydroxyanthranilic acid					
1.	18.678	1584	13.8	MS*, NMR, CoI	Methyl 5-methoxyanthranilate
2.	18.935	1595	5.2	MS*, NMR, CoI	Methyl 5-methoxy-*N,N*-dimethylanthranilate
3.	20.072	1646	3.4	MS*	Trimethylated derivative
4.	20.461	1664	54.3	MS*, NMR, CoI	Methyl 5-hydroxyanthranilate
5.	20.866	1682	5.1	MS*	Trimethylated derivative
6.	21.718	1722	18.1	MS*, NMR, CoI	Methyl 5-hydroxy-*N*-methylanthranilate
7.	22.901	1778	tr	MS*	Trimethylated derivative
Total			99.9		

[a] Compounds listed in order of elution on DB-5MS column (Rt: retention time (in min) and RI: experimentally determined retention indices on the mentioned column by co-injection of a homologous series of *n*-alkanes C_{13}–C_{18}).
[b] RI, constituent identified by retention index matching; MS, constituent identified by mass spectra comparison; MS*, identification based on mass spectral fragmentation pattern; CoI, constituent identity confirmed by GC co-injection of authentic sample; NMR, structure of an isolated pure constituent confirmed by ^{1}H and ^{13}C NMR spectral analysis; tr, trace amount (less than 0.05%).

molecular weight consequently increasing the boiling point and the value of RI. However, in this particular case, the hydrogen that is being substituted for the methyl group can form intermolecular hydrogen bonds factor that determine the boiling point, thus, this formal methyl-addition also decreases the value of RI. The overall effect in still an increase in RI. One more point that needs to be taken into account is the onset of the intramolecular hydrogen bonding between the second NH proton and the ester carbonyl oxygen. This hydrogen bonding reflects itself on the value of RI by keeping the COOR and NHR groups in a constant relative position mutually and with respect to the aromatic core, causing the dipole moment of the anthranilate derivatives not to change significantly. The introduction of a 5-hydroxyl group changes insignificantly the mentioned hydrogen bonding or relative arrangement of COOR and NHR groups, while it engages in intermolecular hydrogen bonding by itself.

The intermolecular forces change dramatically in the case of 3-hydroxy derivatives since now the major hydrogen donor for hydrogen bonding with other molecules is the phenolic OH at position 3 (both NH_2 are involved in intramolecular hydrogen bonds HO—HNH—O=COR). Methylation of the nitrogen atom in 3-hydroxy derivatives leads to the disruption of one of these intramolecular hydrogen bonds and additionally hinders the intermolecular ones by sheer proximity. Overall, *N*-methylation decreases the mentioned intermolecular forces, leading to a significant decrease of RI.

(4) No matter the number of the substituents on the nitrogen atom of anthranilic acid esters, an introduction of a hydroxyl group at position 5 increases the retention index for *ca.* 310 units due to the fact that OH group participates in the formation of intermolecular hydrogen bonding.
(5) On the other hand, an introduction of a hydroxyl group at position 3 variably influences the retention index value of anthranilic and *N*-methylanthranilic acid esters. For example, the introduction of the 3-hydroxy group in the molecules of isopropyl or methyl anthranilates increases RI around 280 units, due to the mentioned intermolecular hydrogen bonding. When comparing the RI data for *N*-methylanthranilates and 3-hydroxy-*N*-methylanthranilates, one can see that there is an RI increase of only some 20 units, as explained above;
(6) The introduction of a methoxy group at position 5 of methyl esters of anthranilic and *N,N*-dimethylanthranilic acids increased the values of their retention index for 234 and 189 units, respectively.
(7) Similarly, the introduction of a MeO-group at position 3 of the methyl ester of anthranilic acid increased the retention index *ca.* 200 units. Expectedly from the discussion above, the introduction of a methoxy group at position 3 of the methyl ester of *N*-methylanthranilic and *N,N*-

Table 6
NMR spectroscopic data (400 MHz, $CDCl_3$) for selected methylated 3-hydroxyantranilic acid derivatives.

Position	Methyl 3-hydroxyanthranilate		Methyl 3-hydroxy-*N*-methylanthranilate		Methyl 3-methoxyanthranilate		Methyl 3-methoxy-*N*-methylanthranilate	
	δ_C, mult	δ_H (*J* in Hz)	δ_C, mult	δ_H (*J* in Hz)	δ_C, mult	δ_H (*J* in Hz)	δ_C, mult	δ_H (*J* in Hz)
1	111.5 C	–	120.7 C	–	110.1 C	–	114.4 C	–
2	140.4 C	–	139.9 C	–	141.7 C	–	144.8 C	–
3	143.4 C	–	150.7 C	–	147.1 C	–	150.5 C	–
4	118.1 CH	6.82, dd (7.6, 1.1)	120.0 CH	7.09, dd (8.0, 1.3)	112.9 CH	6.85, dd (7.8, 1.2)	116.3 CH	6.93, dd (7.9, 1.4)
5	115.4 CH	6.50, pseudo t (7.9)	122.9 CH	6.92, pseudo t (8.0)	114.6 CH	6.58, pseudo t (8.0)	116.7 CH	6.67, pseudo t (8.0)
6	123.2 CH	7.47, dd (8.2, 1.1)	122.5 CH	7.48, dd (8.1, 1.3)	122.5 CH	7.47, dd (8.2, 1.2)	123.5 CH	7.51, dd (8.1, 1.4)
C=O	168.9 C	–	168.1 C	–	168.7 C	–	169.1 C	–
$COOCH_3$	51.7 CH_3	3.87, s	52.1 CH_3	3.92, s	51.5 CH_3	3.86, s	52.3 CH_3	3.93, s
NR_1R_2	–	5.65, br s, NH_2, $R_3 = H$	42.8 CH_3 $R_1 = CH_3$, $R_2 = H$	2.81, s, $NHCH_3$	–	6.06, br s, NH_2	34.1 CH_3 $R_1 = CH_3$, $R_2 = H$	3.05, s, $NHCH_3$, 5.99, br s, $NHCH_3$
OR_3	– $R_3 = H$		– $R_3 = H$	very br s, $NHCH_3$, $R_3 = H$	55.7 $R_3 = CH_3$	3.85, s $R_3 = CH_3$	56.1 $R_3 = CH_3$	3.83, s, $R_3 = CH_3$

Table 7
NMR spectroscopic data (400 MHz, $CDCl_3$) for selected methylated 5-hydroxyantranilic acid derivatives.

Position	Methyl 5-hydroxyanthranilate		Methyl 5-hydroxy-*N*-methylanthranilate		Methyl 5-methoxyanthranilate		Methyl 5-methoxy-*N*,*N*-dimethylanthranilate	
	δ_C, mult	δ_H (*J* in Hz)	δ_C, mult	δ_H (*J* in Hz)	δ_C, mult	δ_H (*J* in Hz)	δ_C, mult	δ_H (*J* in Hz)
1	111.1 C	–	109.9 C	–	111.1 C	–	123.9 C	–
2	144.9 C	–	147.2 C	–	145.1 C	–	147.0 C	–
3	118.2 CH	6.61, d (8.8)	112.2 CH	6.60, d (8.9)	118.3 CH	6.65, d (8.9)	118.7 CH	6.99, m*(8.9, 0.9)
4	123.1 CH	6.89, dd (8.8, 3.0)	123.5 CH	7.01, dd (8.9, 2.9)	123.3 CH	6.96, dd (8.9, 3.0)	119.3 CH	6.97, m*(8.9, 3.0)
5	146.0 C	–	144.7 C	–	150.5 C	–	153.3 C	–
6	116.0 CH	7.33, d (3.0)	116.8 CH	7.39, d (2.9)	113.0 CH	7.35, d (3.0)	115.6 CH	7.23, dd (3.0, 0.9)
C=O	168.1 C	–	168.6 C	–	168.3 C	–	168.4 C	–
$COOCH_3$	51.7 CH_3	3.87, s	51.6 CH_3	3.84, s	51.7 CH_3	3.88, s	52.2, CH_3	3.91, s
NR_1R_2	–	5.41, br s, $N\underline{H}_2$	30.1 CH_3 $R_1 = CH_3$, $R_2 = H$	2.87, s, $NH\underline{CH}_3$ 4.93, br s, $N\underline{H}CH_3$	–	4.93, br s, $N\underline{H}_2$	44.8, CH_3 R_1,$R_2 = CH_3$	2.77, s, R_1,$R_2 = C\underline{H}_3$
OR_3	–	4.37, br s, $R_3 = H$	–	7.0, br s, $R_3 = H$	55.9, $R_3 = \underline{C}H_3$	3.77, s, $R_3 = C\underline{H}_3$	55.7, $R_3 = \underline{C}H_3$	3.79, s, $R_3 = C\underline{H}_3$

m* high-order multiplicity; coupling constant values were obtained using a spectral simulation.

dimethylanthranilic acid increased the retention index for only 9 and 69 RI units, respectively. In this case three voluminous groups sterically hindered efficient intermolecular interactions.

Such structure-gas chromatographic considerations and the generated small library of such data are expected to make future identifications of related xenobiotic/naturally-occurring metabolites more facile.

3.3. Metabolism of isopropyl and methyl esters of N-methylanthranilic acid

The urinary metabolite profiles of IMA and MMA were qualitatively analogous (*i.e.* differed only in the alcohol moiety of the metabolites). Both profiles displayed several products of hydroxylation of the aromatic core of anthranilic acid derivatives. These hydroxylated metabolites were the major ones in the case of IMA, comprising *ca.* 60% of the total extracted GC-MS analyzable compounds (Table 2), while in the case of MMA they comprised only around 13% (Table 3). The products of hydroxylation could be divided into three groups of 3- and 5-hydroxy derivatives of esters of: (1) *N*-methylanthranilic, (2) anthranilic, and (3) *N*-acetylanthranilic acids. Generally, the introduction of a 3- or 5-hydroxyl group is expected from the fact that aromatic compounds are hydroxylated by cytochrome P450 enzymes via epoxide (arene oxide) intermediates. Electron-donating ($-NH_2$) groups direct the opening of the epoxide to form *ortho*- and *para*-hydroxy-derivatives with respect to that group (Williams, 2012) (Fig. 3). 5-Hydroxy derivatives were predominant over the 3-hydroxy counterparts most probably due to steric reasons (isopropyl and methyl 3-hydroxy-*N*-methylanthranilates were present in low amounts, 0.3% and trace amounts, respectively, Tables 2 and 3).

Alongside, isopropyl and methyl esters of 3-hydroxy- and 5-hydroxy-*N*-methylanthranilic acids, the corresponding *N*-demethylated metabolites were identified. Two sequences of events could have led to these metabolites: hydroxylation followed by *N*-demethylation, or the order of these metabolic steps could be inversed. Also both sequences could operate simultaneously. The presence of the *N*-methyl group appears to have hindered the introduction of a hydroxyl group in position 3, since there was an approximately equal chance of hydroxylation in positions 3 and 5 of anthranilic acid derivatives without the *N*-methyl group, although the overall extent of the hydroxylation was significantly different for the methyl and isopropyl esters (Tables 2 and 3). Based on the low relative abundance of isopropyl and methyl 3-hydroxy-*N*-methylanthranilates, we argue that the demethylation step occurred prior to hydroxylation in position 3 of anthranilic acid derivatives. This is substantiated by the identification of isopropyl (3.3%) and methyl anthranilates (0.7%). The process of *N*-demethylation is known to be catalyzed by cytochrome P450 in guinea pigs (Yamaori et al., 2005). In fact, cytochrome P450 initially hydroxylates the *N*-methyl group to form an aminal, which subsequently decomposes to give *N*-demethylated products (Fig. 4).

As opposed to the xenobiotic metabolism of IMA, the urinary metabolite profile of MMA displayed 2-(methylamino)benzamide (36.8%), a product of transformation of the ester group into an amide, and *N*-methylanthranilic acid (13.0%), a product of hydrolysis, as the major metabolites (in the case of IMA they comprised only 5.7% and 2.3%, respectively). The biotransformation of IMA and MMA to *N*-methylanthranilic acid is consistent with the metabolism of anthranilic acid esters; the ester function undergoes hydrolysis, principally in the liver, followed by excretion of *N*-methylanthranilic acid in the urine (SCCS, 2011). Hydrolysis of anthranilic and *N*-methylanthranilic acid esters is most likely catalyzed by carboxylesterases located in various tissues, being most abundant in hepatocytes (Heymann, 1980). Up to now, in rats and humans, the main metabolic pathway of methyl *N*-methylanthranilate known is its hydrolysis to *N*-methylanthranilic acid, followed by *N*-demethylation, to yield anthranilic acid (SCCS, 2011; Yamaori et al., 2005), whether the order of metabolic steps could be inversed.

2-(Methylamino)benzamide and 2-aminobenzamide (anthranilamide) are both found as the metabolites of IMA and MMA. Naito et al. (1984) investigated the formation of anthranilamide from anthranilic acid in the isolated perfused liver of rats. With respect to the finding of this metabolite in the bile, but not in the liver perfusate, they supposed that the amide is non-enzymatically produced in the bile from anthraniloyl glucuronide. As bile metabolites are generally eliminated via feces, but we found the mentioned two metabolites in the urine, we assumed that the bile is not the only place of this transformation and that besides non-enzymatically, the enzymatically catalyzed counterpart occurs as well. There are two possible orders of metabolic steps:

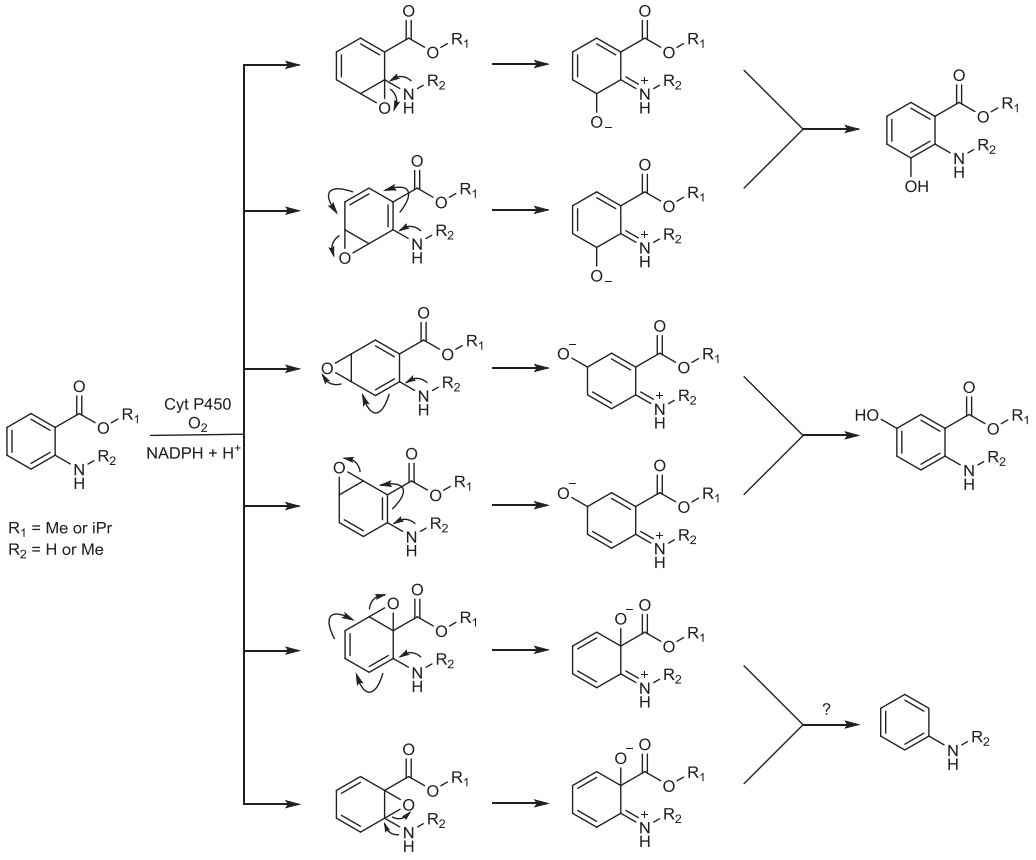


Fig. 3. Proposed cytochrome P450-catalyzed hydroxylation of anthranilic esters.

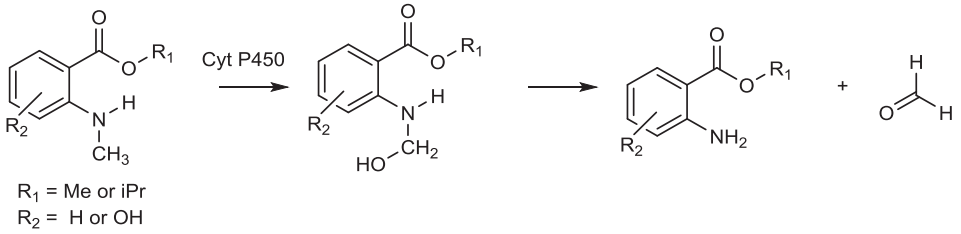


Fig. 4. Proposed cytochrome P450-catalyzed *N*-demethylation of *N*-methylanthranilic acid derivatives.

transformation of an ester into an amide – *N*-demethylation or vice versa, or both steps could occur simultaneously.

The urinary metabolite profiles of IMA and MMA displayed several products of *N*-acetylation. In the case of IMA, isopropyl 2-acetamidobenzoate (tr), isopropyl 2-acetamido-3-hydroxybenzoate (0.4%), isopropyl 2-acetamido-5-hydroxybenzoate (0.3%) and *N*-acetylanthranilic acid (tr) were identified, while in the case of MMA, hydroxyl derivatives of acetanilide, methyl 2-acetamidobenzoate and 2-acetylaminobenzoic acid (single regioisomers) were detected. *N*–acetylation of aromatic amines is recognized as a major detoxification pathway in arylamine metabolism in humans and experimental animals (Hein et al., 2000), effectuated by arylamine *N*-acetyltransferases that utilize acetyl coenzyme A as a cofactor (Riddle and Jencks, 1971; Dupret and Rodrigues-Lima, 2005).

Considering that MMA is a part of human diet and the fact that both tested *N*-methylanthranilic acid esters possess a number of pharmacological activities and that they could be potentially used in pharmacological purposes, it is of great importance to envisage their metabolism in humans. Although there are several similarities between rat and human cytochrome P450 isoform expression, one cannot overlook the dissimilarities that exist as well (Martignoni et al., 2006). These differences can reflect on both the metabolism intensity (lower/higher expression in various species) and the metabolism itself (presence/absence of the isoform). However, it is known that very different cytochrome P450 enzymes can catalyze the same reactions, thus, the interspecies differences are not absolute (Guengerich, 1997). Since at the present time the exact cytochrome P450 enzymes involved in MMA and IMA metabolism are not identified, and that differences in the enterohepatic recirculation between rodents and humans have not been taken into account, we cannot directly translate the herein studied metabolism in rats to humans.

4. Conclusion

To summarize, urine metabolites of two pharmacologically active *N*-methylanthranilic acid esters, isopropyl and methyl *N*-

methylanthranlates (IMA and MMA, respectively), in rats, were analyzed by three different approaches–1) preparative chromatography, 2) synthesis, and 3) SPR. The preparative approach, employed in the case of IMA metabolites, represents a revival of an almost forgotten application of Sephadex LH-20, while the synthetic/combinatorial approach used in the case of MMA metabolites, that provided both standards and a means for SPR analysis, is a novelty in the field of xenobiotic metabolite analysis. The urinary metabolite profiles of the two esters were qualitatively analogous (*i.e.* they differed only in the alcohol moiety of the metabolites) indicating that they both undergo analogous biotransformation pathways. However, in the case of IMA, among 16 different anthranilic acid related metabolites, products of hydroxylation of the aromatic core (isopropyl 5-hydroxy-*N*-methylanthranilate, isopropyl 5-hydroxyanthranilate, and isopropyl 3-hydroxyanthranilate) were the major ones. On the other hand, 2-(methylamino)benzamide and *N*-methylanthranilic acid were identified as the principal metabolites of MMA, among in total 14 metabolites identified. The relative ratio and the structures of the identified IMA and MMA metabolites led us to conclude that MMA predominantly undergoes reactions of the ester group, transformation into an amide and hydrolysis, while for the isopropyl ester, the major metabolic pathway is hydroxylation, probably due to steric hindrance imposed by the isopropyl group on the ester carbonyl. Additionally, IMA and MMA, turned out not to be hepatotoxic, and no other signs of toxicity were observed. The results are of interest due to the presence of natural and synthetic MMA in the human diet and the fact that IMA and MMA possess diverse pharmacological activities (antinociceptive, anti-inflammatory, gastro-, hepato- and nephroprotective activities, anxiolytic and antidepressant properties, as well as an effect on diazepam-induced sleep). Additionally, the novel combination of the identification approaches allows implementation of the 3R-strategy in metabolite analysis.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fct.2017.09.006.

Transparency document

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