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SHORT COMMUNICATION

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The metabolic fate of two new psychoactive substances – 2-aminoindane and *N*-methyl-2-aminoindane – studied in vitro and in vivo to support drug testing

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Abstract

The aim of this study was to characterize the in vitro and in vivo metabolism of 2-aminoindane (2,3-dihydro-1*H*-inden-2-amine, 2-AI), and *N*-methyl-2-aminoindane (*N*-methyl-2,3-dihydro-1*H*-inden-2-amine, NM-2-AI) after incubations using pooled human liver microsomes (pHLMs), pooled human liver S9 fraction (pS9), and rat urine after oral administration. After analysis using liquid chromatography coupled to high-resolution mass spectrometry, pHLM incubations revealed that 2-AI was left unmetabolized, while NM-2-AI formed a hydroxylamine and diastereomers of a metabolite formed after hydroxylation in beta position. Incubations using pS9 led to the formation of an acetyl conjugation in the case of 2-AI and merely a hydroxylamine for NM-2-AI. Investigations on rat urine showed that 2-AI was hydroxylated also forming diasteromers as described for NM-2-AI or acetylated similar to incubations using pS9. All hydroxylated metabolites of NM-2-AI except the hydroxylamine were found in rat urine as additional sulfates. Assuming similar patterns in humans, urine screening procedures might be focused on the parent compounds but should also include their metabolites. An activity screening using human recombinant *N*-acetyl transferase (NAT) isoforms 1 and 2 revealed that 2-AI was acetylated exclusively by NAT2, which is polymorphically expressed.

KEYWORDS

aminoindanes, in vitro, in vivo, LC–HRMS/MS, NPS

1 | INTRODUCTION

2-Aminoindane (2,3-dihydro-1*H*-inden-2-amine, 2-AI), and *N*-methyl-2-aminoindane (*N*-methyl-2,3-dihydro-1*H*-inden-2-amine, NM-2-AI) are emerging new psychoactive substances (NPS) that were recently offered in online shops.[1] 2-AI and NM-2-AI are indirectly sympathomimetic acting drugs causing a transporter mediated increased release of monoamines in the synaptic cleft.[2,3] Unlike substituted aminoindanes, the selective inhibition of 2-AI is merely limited to the norephedrine reuptake transporter (NET); it also releases norephedrine (NE) and dopamine (DA).[4] NM-2-AI has more stimulant properties but a lower potency than 2-AI. It inhibits the NET and the DA transporter (DAT) selectively and causes an efflux of NE and DA leading to their accumulation in the synaptic cleft. Both aminoindanes influence the serotonergic neurotransmission system.[4] Typical symptoms after overdosing are tachycardia, hyperpnoea, increased transpiration, and

dehydration as well as anxiety, depression, and paranoia.[5] Consumption of 5-Methoxy-2-aminoindane (MEAI) resulted in a mild euphoric, alcohol-like tipsy experience and a reduced desire to consume alcoholic beverages. Cytotoxic effects were also demonstrated in concentrations of 500 and 1000 mg/L with calculated IC50 value of approximately 400 mg/L for rat brain striatum primary neurons and human primary healthy hepatocytes.[6]

Before analyzing urine samples for the presence of NPS such as 2-AI and NM-2-AI, suitable analytical targets need to be defined. As these targets are often unknown, metabolism studies are necessary[7] Different in vitro and in vivo models are available for this purpose.[8]

However, it is well known that (human) in vitro models are only partially able to simulate metabolic processes that may occur in a living system. Identification of metabolites in vivo that have not been detected in vitro is therefore not uncommon.[8] Thus, the complementary use of in vitro and in vivo methods to predict human metabolism should be preferred. The aim of this study was therefore to characterize the metabolism of 2-AI and NM-2-AI using pooled human liver microsomes (pHLMs), pooled human liver S9 fraction (pS9), and rat urine after oral administration.

2 | EXPERIMENTAL

2.1 | Chemicals and reagents

2-AI was purchased at Sigma-Aldrich (Taufkirchen, Germany) and NM-2-AI was ordered from GR8 Research Chemicals-EU (https://www.gr8researchchemicals-eu.com). Acetyl coenzyme A (AcCoA), acetylcarnitine, acetylcarnitine transferase (AcT), dithiotreitole (DTT), reduced glutathione (GSH), 3′-phosphoadenosine-5′-phosphosulfate (PAPS), S-(5′-adenosyl)-L-methionine (SAM), $MgCl_2$, K_2HPO_4, KH_2PO_4, superoxide dismutase (SOD), isocitratedehydrogenase (IDH), isocitrate, tris (hydroxymethyl)-aminomethane (TRIS), ammoniumformate, and formic acid were received from Sigma-Aldrich (Taufkirchen, Germany). NADP-Na_2, acetonitrile [liquid chromatography–mass spectrometry (LC–MS) grade], ammonium formate (analytical grade), formic acid (LC–MS grade), methanol (LC–MS grade), ethylenediaminetetraacetic acid (EDTA), triethanolamine (TEA), and all other chemicals and reagents (analytical grade) were obtained from VWR (Darmstadt, Germany). Water was purified using a Millipore filtration unit (18.2 Ω × cm water resistance). pHLM (20 mg microsomal protein/mL, 330 pmol total CYP/mg protein, from 34 individual donors), pS9 (20 mg protein/mL, from 30 individual donors), UGT reaction mix solution A (25mM UDP-glucuronic acid), and UGT reaction mix solution B (250mM Tris–HCl, 40mM $MgCl_2$, and 0.125 mg/mL alamethicin) were obtained from Corning (Amsterdam, Netherlands). Baculovirus infected insect cell-expressed human arylamine N-acetyltransferase 1 (NAT1, 1*4, wild type allele) and NAT2 (2*4, wild type allele) were from BD Biosciences (Heidelberg, Germany). After delivery, the enzymes and liver preparations were thawed at 37°C, aliquoted, snap-frozen in liquid nitrogen, and stored at −80°C until use.

2.2 | Liquid chromatography–high resolution tandem mass spectrometry apparatus for identification of metabolites

Analysis was performed according to a previously published study.[9] Details can be found in the Supporting Information.

2.3 | Incubations using pHLM, pS9, and recombinant NAT isoenzymes and collection of rat urine samples

Conditions were similar to previous studies and further details can be found in the Supporting Information.[10-12]

2.4 | Sample preparation for identification of phase I and II metabolites in rat urine by liquid chromatography–high resolution tandem mass spectrometry

As described elsewhere,[13] a 100-µL aliquot of rat urine was vortexed for 2 minutes after addition of 500 µL acetonitrile. The mixture was centrifuged for 2 minutes at 10,000 rpm. The supernatant was transferred into a glass vial and evaporated to dryness at 70°C under a stream of nitrogen. The residue was dissolved in 50 µL of a mixture of eluent A and B (1:1; *v*/v) and a 1-µL aliquot was injected onto the Orbitrap-based liquid chromatography–high resolution tandem mass spectrometry (LC–HRMS/MS) system. The second urine sample preparation was performed as described in previously published literature.[14] Five-hundred µL urine was mixed with 2 mL deionized water. The sample was loaded on a HCX solid phase extraction (SPE) cartridge, which had previously been conditioned with 1 mL methanol and 1 mL aqua destillata. Washing steps were performed with 1 mL aqua destillata, 1 mL 0.01 M hydrochloric acid and methanol after the sample passed through the cartridge. Remaining basic compounds were eluted by using 1 mL of a freshly prepared mixture of methanol and 32% aqueous ammonia (98:2, v/v). The eluates were evaporated to dryness under a gentle stream of nitrogen at 70°C. After reconstitution of residues with 50 µL methanol, 1 µL of the sample was injected onto the LC–HRMS/MS system for analysis.

3 | RESULTS AND DISCUSSION

3.1 | Proposed fragmentation patterns after LC–HRMS/MS and tentative identification of metabolites

Positive electrospray ionization (ESI^+) spectra of the metabolites are given in Figures 1 and 2, ordered by m/z and retention time. Metabolites were identified by comparing the MS^2 spectra of the metabolite with those of the parent compounds. In case of 2-AI (Figure 1A), cleavage of the amino group lead to the indan fragment ion with m/z 117.0698 (C_9H_9) and the indan fragment ion with m/z 115.0542 (C_9H_7). Further fragmentation of the ring system led to

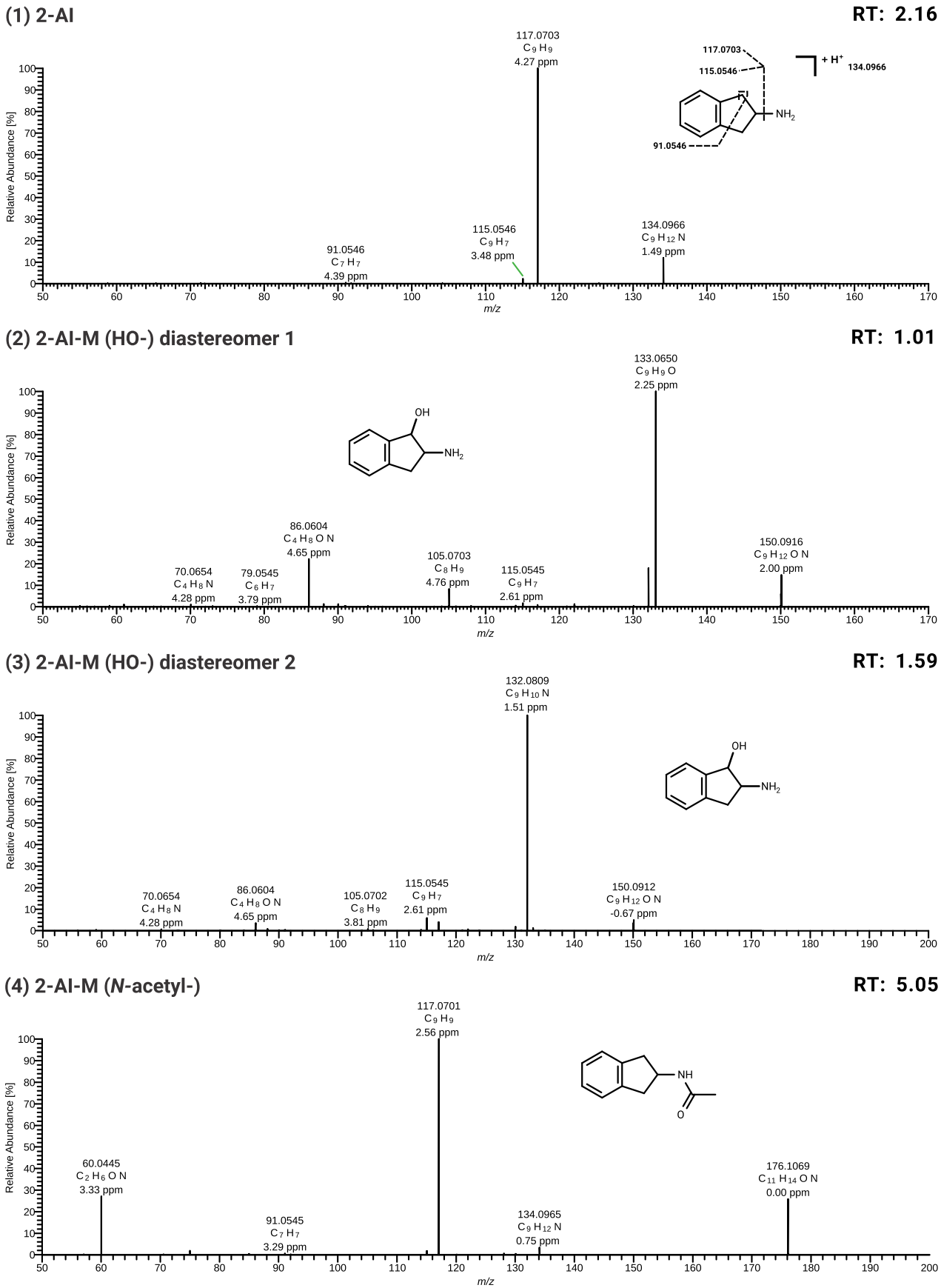

FIGURE 1 ESI$^+$ spectra of 2-AI and its phase I and II metabolites sorted by mass of protonated molecule. Fragments with accurate mass, calculated elemental formula, and mass error value in parts per million (ppm)

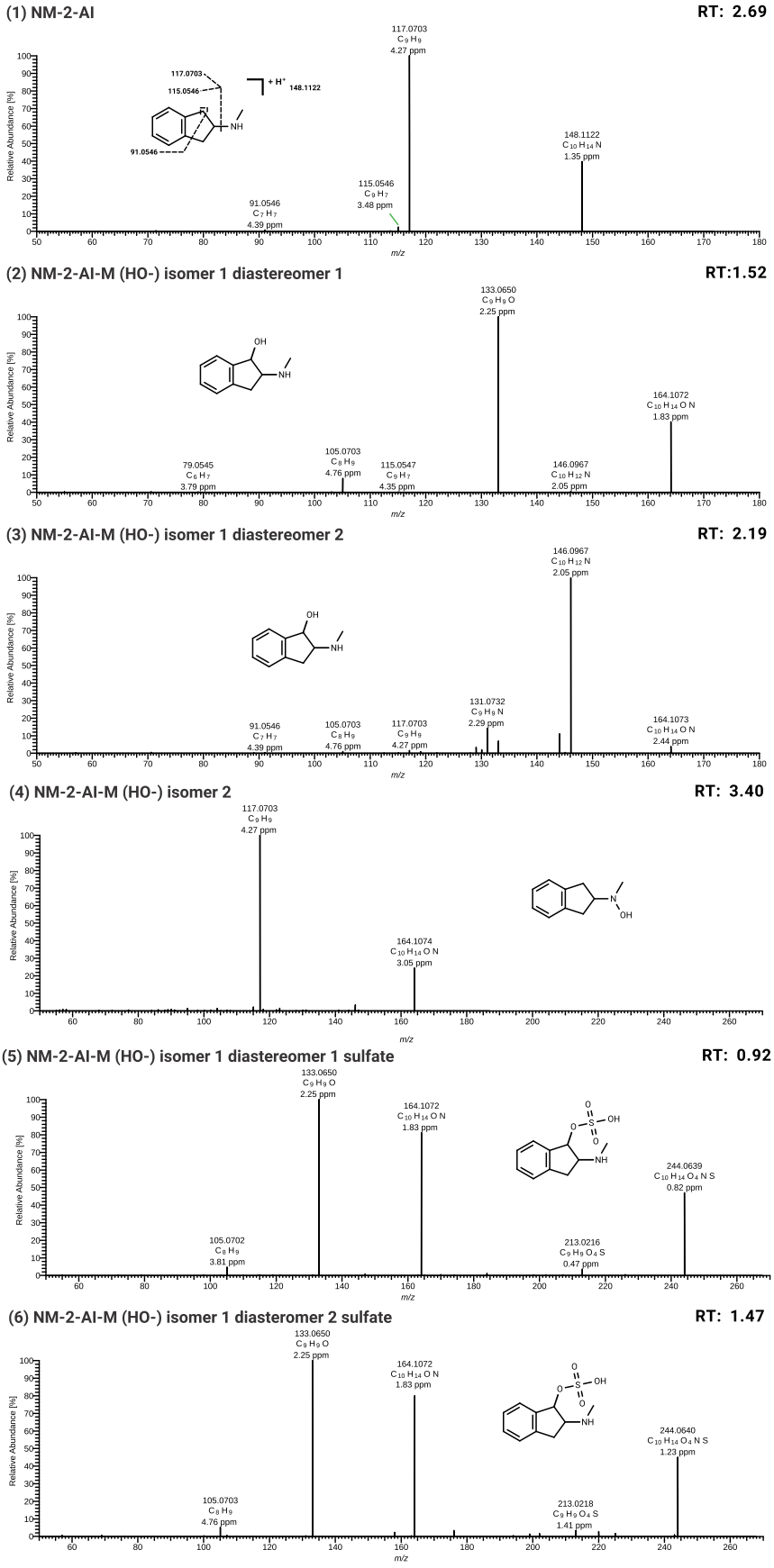

FIGURE 2 ESI^+ spectra of NM-2-AI and its phase I and II metabolites sorted by mass of protonated molecule. Fragments with accurate mass, calculated elemental formula, and mass error value in parts per million (ppm)

the formation of a tropylium ion with m/z 91.0542 (C_7H_7). 2-AI formed diastereomers after hydroxylation in beta position of the amino group (2 and 3 in Figure 2) with m/z 150.0913 ($C_9H_{12}ON$). The applied method could not determine the exact configuration of each metabolite and only diasteromers could be distinguished. Thus, they are discussed in the following. It seemed that for (1S,2S)- and (1R,2R)-isomers the elimination of the amino group was preferred, forming the corresponding fragment ion with m/z 133.0647 (C_9H_9O). This elimination showed a characteristic shift of the parent ion by −17.0266 u (NH_3). Ring opening resulted in the fragment ion with m/z 105.0698 (C_8H_9). Further fragmentation led to the fragment ion with m/z 86.0600 (C_4H_8ON) and the fragment ion with m/z 79.0542 (C_6H_7). For the (1S,2R)- and (1R,2S)-isomers, the elimination of the hydroxy group was preferred, forming the corresponding fragment ion with m/z 132.0807 ($C_9H_{10}N$). Further fragmentation was similar to the aforementioned (1S,2S)- and (1R,2R)-isomers. One possible explanation for this observation might be that the protonated hydroxy group is a better leaving group than the protonated amine. Since the elimination of the hydroxy group is only preferred in (1S,2R)- and (1R,2S)-isomers, it is likely that the hydroxy group forms a hydrogen bond with the protonated amine enabling the molecule to eliminate water. In (1S,2S)- and (1R,2R)-isomers the formation of such a leaving group by hydrogen bonds is not possible leaving the protonated amine ion as the prioritized leaving group for elimination reactions. For *N*-acetyl-2-AI (Figure 2D), which was detected with m/z 176.1069 ($C_{11}H_{14}ON$), the fragmentation was almost similar to the parent compound after cleavage of the acetyl group indicated by a shift of −42.0104 u (C_2H_2O). Additionally, an acetamide ion with m/z 60.0443 (C_2H_6ON) was detected that was not present in any other spectra.

NM-2-AI (Figure 2A) showed a similar fragmentation compared to 2-AI. After elimination of the amino group, the indan fragment ion with m/z 117.0698 (C_9H_9) was formed. Ring opening resulted in the formation of a tropylium ion with m/z 91.0542 (C_7H_7). NM-2-AI also formed diasteromers after hydroxylation (Figure 2B and 2C) with m/z 164.1069 ($C_{10}H_{14}ON$). For the (1S,2S)- and (1R,2R)-isomers, the more dominant fragmentation step was again the loss of the amino group instead of water, forming the corresponding fragment ion with m/z 133.0647 (C_9H_90). The fragment ion with m/z 146.0964 that resulted after elimination of the hydroxy group was also formed but with a much lower extent. Further fragmentation steps were similar to the corresponding diastereomer of 2-AI. For the (1S,2R)- and (1R,2S)-isomers, the elimination of the hydroxy group was preferred, forming the fragment ion with m/z 146.0964 ($C_{10}H_{12}N$). Further fragmentation led to the fragment ion with m/z 131.0729 (C_9H_9N) and the fragment ion with m/z 117.0698 (C_9H_9). Ring opening resulted in the fragment ion with m/z 105.0698 (C_8H_9). For the respective O-sulfated metabolites (Figure 2E and 2F) with m/z 244.0637 ($C_{10}H_{14}O_4NS$), the elimination of the sulfate group was preferred instead of the loss of the substituted amino group, forming the corresponding aglycon with m/z 164.1069 ($C_{10}H_{14}ON$). The fragment ion with m/z 213.0215 ($C_9H_9O_4S$) that resulted after cleavage of the methylated amino group was also formed, but with a much lower extent. Further fragmentation steps were similar that occurred for the corresponding phase I metabolite.

3.2 | Metabolites found in incubations using pHLM

As no metabolites of 2-AI were found, it can be assumed that it is only sparsely metabolized also in vivo. *N*-Hydroxylation (4 in Figure 4) and aliphatic hydroxylation (2 and 3 in Figure 4) were found for NM-2-AI.

3.3 | Metabolites found in incubations using pS9

2-AI was metabolized by *N*-acetylation (1Ac in Figure 3), a metabolic step that was not able to be catalyzed by pHLM since NAT is located in the cytosol of liver cells. NM-2-AI was *N*-hydroxylated forming a hydroxylamine (4 in Figure 4). Although identified after incubation with pHLM, the hydroxy metabolite formed after hydroxylation of the aliphatic ring system was not detected. This might be explained by the lower CYP activity of the pS9 fraction compared to pHLM.

3.4 | Activity studies using recombinant NAT isoenzymes

As the NAT2 is polymorphically expressed leading to different possible rates of acetylation in different individuals, activity studies are important.[12] NM-2-AI was not *N*-acetylated, most probably due to the steric restriction caused by the methyl substituent. *N*-Acetyl-2-AI was detected in incubations using pS9 and in incubations using the NAT2 isoenzyme. This is in accordance with the results of other studies that found a higher affinity of NAT2 toward alkylamines, such as the reversible dopamine-β-hydroxylase inhibitor etamicastat.[15] Since 2-AI is only metabolized by a few metabolic steps, the occurrence of polymorphically expressed enzymes being involved in its metabolism may lead to individually varying plasma concentrations and elimination half-lives.

3.5 | Identification of phase I and phase II metabolites in rat urine

2-AI was hydroxylated in beta position of the amine moiety leading to diasteromers (2 and 3 in Figure 3). In addition, the *N*-acetylated metabolite was detected (1Ac in Figure 3). NM-2-AI was also

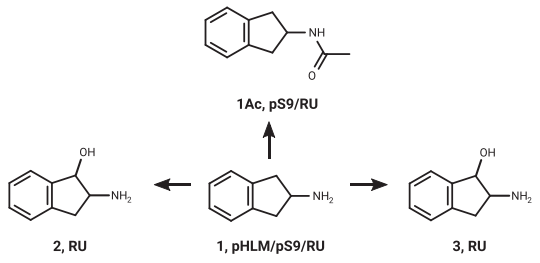

FIGURE 3 Metabolic pathways of 2-AI. RU = rat urine, pS9 = pooled human liver S9 fraction. Acetylation is indicated by a suffixed ac

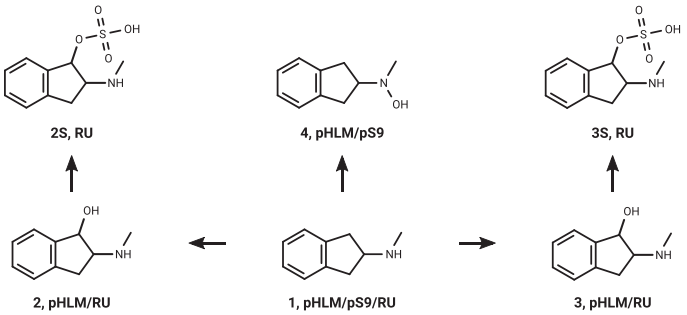

FIGURE 4 Metabolic pathways of NM-2-AI. RU = rat urine, pS9 = pooled human liver S9 fraction. Sulfates are indicated by a suffixed S

hydroxylated in beta position of the amine moiety forming corresponding (*E*)/(*Z*)-isomers (2 and 3 in Figure 4). In addition to in vitro incubations hydroxy NM-2-AI underwent sulfation leading to the corresponding sulfates of the diasteromers (2S and 3S in Figure 4).

3.6 | Proposed metabolic pathways

2-AI was sparsely metabolized. Hydroxylation (1 in Figure 3) in beta position of the amine moiety led to corresponding diasteromers (2 and 3 in Figure 3). *N*-acetylation was observed and led to metabolite 1Ac in Figure 3. Similar to 2-AI, the hydroxylation of NM-2-AI in beta position of the amine moiety led to the formation of corresponding diasteromers (2 and 3 Figure 4). Subsequent sulfation was also observed and led to the sulfated diasteromers (2S and 3S in Figure 4). Hydroxylation on the amino group led to the formation of a hydroxylamine metabolite (4 in Figure 4).

These findings are in line with previous studies. The metabolism of the related compound 5,6-methylenedioxy-2-aminoindane (MDAI) in rats was investigated by Zidkova et al by analyzing urine samples using HRMS amongst others.[16] The main metabolic pathways were found to be oxidative demethylenation followed by *O*-methylation and *N*-acetylation as well as formation of glucuronides and sulfates. However, the main portion of administered MDAI was excreted unchanged.

4 | CONCLUSIONS

The investigated aminoindanes were metabolized to a considerably low extend. 2-AI was merely hydroxylated and thereby formed diasteromers which were clearly distinguishable by their fragmentation. Acetylation of the amine moiety was the only metabolic reaction that was detectable in vitro while also occurring in vivo. Incubations using isolated isoforms of *N*-acetyl transferases revealed that this reaction was catalyzed by NAT2, a polymorphically expressed enzyme that might influence the hepatic elimination of 2-AI. The metabolism of NM-2-AI was quite similar to that of 2-AI. It also formed diasteromers after hydroxylation which additionally underwent sulfation. *N*-Acetylation was not detected for NM-2-AI but hydroxylation at the amine moiety led to the formation of a hydroxylamine.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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