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Metabolism of the Tobacco Carcinogen 2-Amino-9H-pyrido[2,3-b]indole (AαC) in Primary Human Hepatocytes

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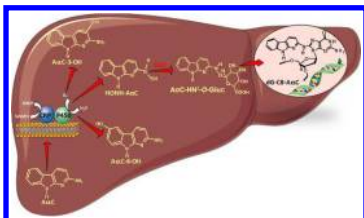
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KEYWORDS: 2-amino-9H-pyrido[2,3-b]indole (AαC), primary human hepatocytes, metabolism, P450 1A2, DNA adduct, UDP-glucuronosyltransferase.

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ABSTRACT

2-Amino-9*H*-pyrido[2,3-*b*]indole (AαC) is the most abundant carcinogenic heterocyclic aromatic amine (HAA) formed in mainstream tobacco smoke. AαC is a liver carcinogen in rodents, but its carcinogenic potential in humans is not known. To obtain a better understanding of the genotoxicity of AαC in humans, we have investigated its metabolism and its ability to form DNA adducts in human hepatocytes. Primary human hepatocytes were treated with AαC at doses ranging from 0.1 to 50 μM and the metabolites were characterized by UPLC/ ion trap multistage mass spectrometry (UPLC/MSⁿ). Six major metabolites were identified: a ring-oxidized doubly conjugated metabolite, *N*²-acetyl-2-amino-9*H*-pyrido[2,3-*b*]indole-6-yl-oxo-(β-D-glucuronic acid) (*N*²-acetyl-AαC-6-*O*-Gluc); two ring-oxidized glucuronide (Gluc) conjugates: 2-amino-9*H*-pyrido[2,3-*b*]indol-3-yl-oxo-(β-D-glucuronic acid) (AαC-3-*O*-Gluc) and 2-amino-9*H*-pyrido[2,3-*b*]indol-6-yl-oxo-(β-D-glucuronic acid) (AαC-6-*O*-Gluc); two sulfate conjugates, 2-amino-9*H*-pyrido[2,3-*b*]indol-3-yl sulfate (AαC-3-*O*-SO₃H) and 2-amino-9*H*-pyrido[2,3-*b*]indol-6-yl sulfate (AαC-6-*O*-SO₃H); and the Gluc conjugate, *N*²-(β-D-glucosiduronyl)-2-amino-9*H*-pyrido[2,3-*b*]indole (AαC-*N*²-Gluc). In addition, four minor metabolites were identified: *N*²-acetyl-9*H*-pyrido[2,3-*b*]indol-3-yl sulfate (*N*²-acetyl-AαC-3-*O*-SO₃H); *N*²-acetyl-9*H*-pyrido[2,3-*b*]indol-6-yl sulfate (*N*²-acetyl-AαC-6-*O*-SO₃H), *N*²-acetyl-2-amino-9*H*-pyrido[2,3-*b*]indol-3-yl-oxo-(β-D-glucuronic acid) (*N*²-acetyl-AαC-3-*O*-Gluc), and *O*-(β-D-glucosiduronyl)-2-hydroxyamino-9*H*-pyrido[2,3-*b*]indole (AαC-HN²-*O*-Gluc). The latter metabolite, AαC-HN²-*O*-Gluc is a reactive intermediate which binds to DNA to form the covalent adduct *N*-(2'-deoxyguanosin-8-yl)-2-amino-9*H*-pyrido[2,3-*b*]indole (dG-C8-AαC). Pre-incubation of hepatocytes with furafylline, a selective mechanism-based inhibitor of P450 1A2, resulted in a strong decrease in the formation of AαC-HN²-*O*-Gluc and a concomitant decrease in DNA adduct formation. Our findings describe the major pathways of metabolism of AαC in primary human hepatocytes

and reveal the importance of N-acetylation and glucuronidation in metabolism of A α C. P450

1A2 is a major isoform involved in the bioactivation of A α C to form the reactive A α C-HN²-

O-Gluc conjugate and A α C-DNA adducts.

INTRODUCTION

Epidemiologic studies conducted over the past two decades have consistently shown that smoking is a risk factor for liver and gastrointestinal tract cancer.¹⁻³ Cigarette smoking is a prominent source of exposure to a number of genotoxicants including nitrosamines, aromatic amines, polycyclic aromatic hydrocarbons, and heterocyclic aromatic amines (HAA).⁴ HAA are also formed in well-done cooked meat, poultry and fish,⁵ and some occur in diesel gas exhaust.^{6,7} 2-Amino-9*H*-pyrido[2,3-*b*]indole (AαC) is by far the most abundant HAA formed in tobacco smoke with a level range between 25 to 260 ng per cigarette.⁸⁻¹¹ These amounts are comparable to those of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), and 25-100 times higher than those of 4-aminobiphenyl (4-ABP) and benzo[*a*]pyrene (B[*a*]P),^{9,12,13} these three chemicals are recognized as human carcinogens.^{3,14}

AαC has been detected in urine of smokers of the Shanghai Cohort study.¹⁵ The urinary levels were positively correlated to the number of cigarettes smoked per day. A similar finding was reported in Zengshu, China, where AαC was present at higher levels in the urine of smokers than nonsmokers.¹⁶ Recently, a tobacco smoking cessation study conducted in the United States revealed that AαC was present in urine during the smoking phase in greater than 90% of the subjects, and the geometric mean urinary level of AαC decreased by 87% six weeks after cessation of tobacco usage.¹⁷ These urinary biomarker data demonstrate that tobacco smoking is a significant source of AαC exposure. The data reported in the literature on urinary level of AαC in human are restricted to two reports where the mean level of AαC in urine of subjects who smoked greater than 20 cigarette per day range between 12 and 35 pg/mg creatinine.¹⁵⁻¹⁷

AαC induces liver and blood vessel tumors in CDF1 mice,^{5,18} it induces *lacI* transgene mutations in the colon of C57BL/6 mice,¹⁹ and aberrant crypt foci, an early biomarker of colon neoplasia, in Big Blue mice.²⁰ However, the genotoxicity of AαC remains unknown in humans and only few reports have been reported on the genotoxicity of AαC *in vitro* in human cells. AαC is genotoxic in human lymphoblastoid cells (MLC-5)²¹ and in peripheral blood lymphocytes cells, when assessed the by the comet and micronucleus assays.²² We also showed that AαC forms high and persistent levels of DNA adducts in primary human hepatocytes.^{23,24} The major DNA adduct formed by AαC is *N*-(deoxyguanosin-8-yl)-2-amino-9*H*-pyrido[2,3-*b*]indole (dG-C8-AαC)^{25,26} which is regarded as a mutagenic lesion.²⁷ The levels of dG-C8-AαC formed in primary human hepatocytes were greater than those dG-C8 adducts formed with other HAA, including 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) or the structurally related arylamine 4-aminobiphenyl (4-ABP).²⁴

AαC requires metabolism to exert its genotoxic effects. AαC undergoes metabolic activation, by P450 1A2-catalyzed *N*-oxidation of the exocyclic amine group to form 2-hydroxyamino-9*H*-pyrido[2,3-*b*]indole (HONH-AαC).^{28,29} Conjugation enzymes such as *N*-acetyltransferases (NAT) or sulfotransferases (SULT), catalyze the conversion of HONH-AαC to unstable esters, which undergo heterolytic cleavage to form the presumed short-lived nitrenium ion, which covalently adducts to DNA.³⁰ In contrast to the NAT and SULT enzymatic pathways of bioactivation, the UDP-Glucuronosyltransferases (UGT) are largely viewed as conjugation pathways that lead to detoxication.³¹ UGT-mediated conjugation of glucuronic acid (Gluc) to the exocyclic amino groups of HAA or *N*-hydroxylated HAA is a detoxification pathway for HAA.³¹⁻³³ However, we discovered that the UGT also catalyze the

O-glucuronidation of HONH-A α C to form the A α C-HN²-*O*-Gluc conjugate, a reactive metabolite that contributes to the genotoxicity of A α C.³⁴

The abundance of A α C in tobacco smoke and its propensity to undergo bioactivation by hepatic enzymes provides a plausible biochemical mechanism that may explain how A α C can induce DNA damage and play a role in the development of liver and digestive tract cancers in smokers.^{1,35,36} However stable biomarkers must be developed and implemented in molecular epidemiological studies designed to assess the role of chemical exposures to A α C in cancer risk. For this purpose, a better understanding of the metabolism of A α C is required.

Studies have been devoted to the metabolism of A α C *in vitro* with hepatic human liver microsomes or recombinant human P450s^{29,30,37-39} and *in vivo* mainly in rats^{26,37,40} and mice.⁴¹ However, knowledge about the major metabolic pathways of A α C and the key enzymes involved in bioactivation of this carcinogen in liver and extrahepatic tissues of humans are limited. There is only one study reported the metabolism of A α C in human hepatocarcinoma cell line HepG2.⁴⁰ This cell line is not fully metabolically competent since it does not express some important metabolism enzymes such as UGT.⁴⁰ In contrast, primary human hepatocytes are the gold standard to investigate different pathways of carcinogen metabolism, since this cell model retains liver function for at least seven days and expresses phase I and phase II enzymes as well as cofactors at physiological concentrations.⁴²

The aim of our study was to characterize the major metabolites of A α C formed in human liver using primary cultured human hepatocytes. The metabolites were characterized by UPLC/MSⁿ in conjunction with HPLC and ultraviolet detection, and by treatment with the deconjugation enzymes arylsulfatase and β -glucuronidase. Our findings show that A α C undergoes multiple pathways of metabolism that include *N*²-acetylation, *N*²-glucuronidation,

1
2
3 as well as ring-oxidation at the C-3 and C-6 atoms of the heterocyclic ring of A α C. The
4
5 metabolic activation of A α C through *N*-oxidation was shown to occur by formation of A α C-
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7 HN²-*O*-Gluc, a genotoxic metabolite that reacts with DNA.³⁴ Preincubation of hepatocytes
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9 with furafylline, a selective inhibitor of P450 1A2⁴³ resulted in a strong decrease in the
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11 formation of A α C-HN²-*O*-Gluc with a concomitant decrease in DNA adducts. Our data
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13 describes for the first time, the metabolism of A α C in human liver , and the importance of
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15 P450 1A2 in formation of A α C-HN²-*O*-Gluc and DNA adducts.
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MATERIALS AND METHODS

Caution. *A α C and its derivatives are potential human carcinogens. These chemicals must be handled in a well-ventilated fume hood with proper use of gloves and protective clothing.*

Chemicals. A α C was purchased from the Toronto Research Chemicals (Toronto, ON, Canada). [4b,5,6,7,8,8a-¹³C₆]A α C was a gift from Dr. Daniel Doerge, National Center for Toxicological Research (Jefferson, AR). Human liver microsomes were obtained from the Tennessee Donor Services, Nashville, TN, and kindly provided by Prof. F. P. Guengerich, Vanderbilt University. DMSO, ethoxyresorufin, methoxyresorufin, methanol, acetonitrile, ascorbic acid, hydrochloric acid, ammonium acetate, sulfatase from *Helix pomatia* ($\geq 10,000$ units/g solid) and β -Glucuronidase from *Helix pomatia* ($\geq 30,000$ units/g solid) were purchased from Sigma Aldrich (St. Louis, MO, USA). A α C-3-OH and A α C-6-OH were prepared with human liver microsomes or rat liver microsomes and spectroscopically characterized as previously reported.^{17,34} *N*-(deoxyguanosin-8-yl)-2-amino-9*H*-pyrido[2,3-*b*]indole (dG-C8-A α C) and [¹³C₁₀]-dG-C8-A α C were prepared as previously described.²⁷

Cell Isolation, Culture, and Treatment. Human liver samples were obtained from patients undergoing liver resection for primary or secondary hepatomas through the Centre de Ressources Biologiques (CRB)-Santé of Rennes (<http://www.crbsante-rennes.com>). The demographics information is provided in supporting information (Table S1). The research protocol was conducted under French legal guidelines and fulfilled the requirements of the local institutional ethics committee. Hepatocytes were isolated by a two-step collagenase perfusion procedure and seeded in petri dishes at a density of 3×10^6 viable cells/19.5 cm² dish, in William's medium (Gibco, Life technologies, Carlsbad, CA, USA), supplemented by bovine serum albumin (1 g/L) (Life technologies), glutamine (2 mM) (Life technologies), bovine insulin (5 μ g/mL) (Life technologies), penicillin (10 U/mL) (Life technologies),

streptomycin (10 $\mu\text{g/mL}$) (Life technologies) and 10% fetal calf serum (v/v) (Life technologies). After 18 h of cell seeding, the media was replaced with cell media lacking fetal calf serum and containing hydrocortisone hemisuccinate (54 μM) (Laboratoire SERB, Paris, France). After 36 h of culture, the differentiated cells were incubated with A α C in DMSO (0.1% v/v) for 24 h. At the end of treatment, the supernatants were collected and the cells were washed with PBS. Cell pellets were collected by centrifugation at 3500 g for 10 min at 4 $^{\circ}\text{C}$. Both supernatants and cell pellets were stored at -80 $^{\circ}\text{C}$ until further use. To assess the role of P450 1A2 in the metabolism of A α C, the cells were pre-treated with furafylline (5 μM) or 0.1% DMSO (v/v) for 24 h. The media was then renewed with furafylline (5 μM) or 0.1% DMSO (v/v), and the cells were incubated with A α C (0.1, 1, 10 or 50 μM) for an additional 24 h. At the end of each time point, the cell media was collected for metabolite analysis and the cellular pellets were collected after PBS washing for DNA adducts measurements.

EROD/MROD Activity. Ethoxyresorufin O-deethylase (EROD) and methoxyresorufin O-demethylase (MROD) activities associated with P450 1A1/2⁴⁴ and P450 1A2,⁴⁵ respectively, were measured in all primary cultured hepatocytes used in this study as described previously.²⁴ The reaction rates were linear over the reaction time and proportional to protein concentration.⁴⁶

Samples preparation and HPLC analysis of the major metabolites of A α C. Cell media was treated by 3 volumes of cold methanol and incubated on ice for 30 min, followed by a centrifugation at 20 000 g for 10 min at 4 $^{\circ}\text{C}$. The supernatant was collected and evaporated to dryness, and the extracts were dissolved in HPLC grade water. The metabolites were separated and collected with an Agilent model 1100 HPLC Chemstation (Palo Alto, CA) equipped with UV/Vis detector. The metabolites were separated with Aquasil C18 column

(4.6 x 250 mm, 5 μ m particle size) from Thermo Scientific (Bellefonte, PA). The chromatography commenced isocratically at 95% A solvent (20 mM ammonium acetate and 5% acetonitrile) for 10 min, followed by a linear gradient over 20 min to 50% B (acetonitrile) at a flow rate of 1 mL/min. The column was then washed with 100% acetonitrile and re-equilibrated at starting solvent conditions. Once collected, the metabolites were evaporated to dryness and stored at -80 °C until LC/MS analysis.

UV Spectral Characterization of A α C metabolites by treatment with deconjugating enzymes or acid hydrolysis. The proposed ring oxidized sulfate conjugates (A α C-3-*O*-SO₃H and A α C-6-*O*-SO₃H) and the ring oxidized Gluc conjugates (A α C-3-*O*-Gluc and A α C-6-*O*-Gluc) were hydrolyzed, respectively with sulfatase or β -glucuronidase. The metabolites purified by HPLC (~25 ng) were concentrated to dryness by vacuum centrifugation, resuspended in 25 μ L of 50 mM potassium phosphate buffer (pH 7.0) and incubated with 25 μ L of sulfatase (200 units/mL) or 25 μ L of β -glucuronidase (300 units/mL) for 1 h at 37 °C. The enzymes solutions and buffers were purged with argon before use, and the incubations were conducted in tightly closed Eppendorf tubes to minimize oxidation of the deconjugated metabolites. The hydrolysis was terminated by the addition of 1 volume of ice-cold methanol. The precipitated proteins were removed by centrifugation and the supernatants were analyzed by HPLC as described above. The proposed *N*-acetylated ring-oxidized A α C Gluc conjugate (*N*²-acetyl-A α C-6-*O*-Gluc) was subjected to enzymatic hydrolysis by β -glucuronidase under the same conditions as described above, followed by acid hydrolysis (0.1 M HCl) for 3 h at 60 °C. After addition of 1 volume of ice-cold methanol and incubation for 5 min on ice, the precipitated protein was eliminated by centrifugation. The supernatant was dried and re-suspended in HPLC grade water. The hydrolysis products were analyzed by HPLC as described above. The UV spectra of each hydrolysis products was compared to those spectra

of A α C-6-OH and A α C-3-OH, which were produced with human liver microsome as previously reported.^{34,41}

Solid-phase extraction (SPE) of hepatocyte metabolites for UPLC-ESI-MSⁿ. The cell media containing A α C metabolites were processed by SPE using Oasis HLB 1 cc (30 mg) cartridge (Waters, Milford, MA, USA), prior to UPLC-ESI-MSⁿ analysis. After the conditioning the cartridge with 1 mL of methanol, followed by 1 mL of LC/MS grade water, the cell media (100-500 μ L) diluted in 2 mM ammonium acetate (added up to 1 mL) were loaded in the cartridge. The cartridge was washed with 1 mL of 2 mM ammonium acetate, and metabolites were eluted with 1 mL of methanol. The eluates were evaporated to dryness by vacuum centrifugation at 42 °C. The residues were dissolved in mobile phase buffer (2 mM ammonium acetate).

UPLC-ESI-MSⁿ analysis. Ultraformance liquid chromatography electrospray ionization mass spectrometry (UPLC-ESI-MS) analyses was performed with NanoAcquity UPLC system (Waters Corp., New Milford, MA) equipped with a Waters Symmetry C18 Trap Column (180 μ m x 20 mm, 5 μ m particle size) (Waters Corp., New Milford, MA) and an Advance CaptiveSpray ion source (Michrom Bioresources, Auburn, CA) interfaced with a linear quadrupole ion trap mass spectrometer (LTQ Velos, Thermo Fisher, San Jose, CA). Solvent A was 2 mM ammonium acetate and solvent B was 95% acetonitrile and 5% H₂O. After 3 min at 5% of B, a linear gradient was employed, starting at 5% B, arriving at 50% B in 20 min and ended at 99% B at 25 min at a flow rate of 5 μ L/min. The transitions were as follow: A α C-*O*-SO₃H ([M+H]⁺ at m/z 280.1 > 200.1 >); A α C-*O*-Gluc ([M+H]⁺ at m/z 376.1 > 200.1 >); *N*²-acetyl-A α C-*O*-SO₃H ([M+H]⁺ at m/z 322.1 > 241.1 >); *N*²-acetyl-A α C-*O*-Gluc ([M+H]⁺ at m/z 418.1 > 242.1 >); A α C-*N*²-Gluc ([M+H]⁺ at m/z 360.1 > 183.1

>); A α C-HN²-O-Gluc ([M+H]⁺ at m/z 376.1 > 200.1 >); A α C ([M+H]⁺ at m/z 184.1 > 167.1 >).

Isolation and Digestion of DNA for Adduct Measurements. Cell pellets containing 3 $\times$ 10⁶ primary human hepatocytes were homogenized in 400 μ L of TE buffer at pH 8.0 (50 mM Tris-HCl and 10 mM EDTA) and incubated with RNase T1 (319 U) and RNase A (20 μ g) for 30 min at 37 °C. Then proteinase K (200 μ g) and SDS (0.05% final concentration) were added and the mixture was incubated for 1 h at 37 °C. The DNA was purified by the phenol/chloroform extraction, followed by precipitation with ethanol.⁴⁷ DNA was resuspended in 200 μ L of sterile water and quantified with a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). Each DNA sample was spiked with isotopically labeled internal standards at a level of 1 adduct per 10⁷ bases. DNA digestion was performed in 5 mM Bis-Tris-HCl buffer (pH 7.1). DNA was incubated for 3.5 h with DNase I and Nuclease P1 at 37 °C followed by 18 h incubation with alkaline phosphatase and phosphodiesterase at 37 °C.⁴⁸ After vacuum centrifugation to the dryness, digested DNA was resuspended in 30 μ L of 1:1 water/DMSO (v/v) and sonicated for 5 min. Samples were then centrifuge for 5 min at 21 000 g and the supernatant was transferred to LC vials.

UPLC/MS³ measurement of DNA adducts. DNA adduct measurements were performed with the NanoAcquity UPLC system (Waters Corp, New Milford, MA) equipped with a Waters Symmetry trap column (180 μ m $\times$ 20 mm, 5 μ m particle size), a Michrom C18 AQ column (0.3 mm $\times$ 150 mm, 3 μ m particle size), and a Michrom Captive Spray source interfaced with LTQ Velos. Chromatographic conditions were described previously.²⁴ The ions were monitored at the MS³ scan stage as follows: dG-C8-A α C (m/z 449.1 > 333.1 > 209.2, 291.4, 316.4); [¹³C₁₀]-dG-C8-A α C (m/z 459.1 > 338.1 > 210.2, 295.5, 321.5).

RESULTS

Identification of the major metabolites of A α C formed in human hepatocytes. Six major metabolites were produced by incubating primary human hepatocytes with of A α C (50 μ M) and arbitrarily named M1 to M6 as function of the order of their elution time by HPLC (Figure 1). All of the metabolites retained the characteristic UV chromophore of A α C with slight changes in the spectral properties. The metabolites were collected and infused into the LTQ Velos ion trap MS for characterization.

ESI-MS product ion and UV spectra of A α C metabolites. The product ion spectrum of A α C-*N*²-Gluc ([M+H]⁺ at *m/z* 360.1) (M5) at MS² stage, shown in Figure 2A, displays fragment ions at *m/z* 280.1 ([M+H-2H₂O-CO₂]⁺), *m/z* 226.1 ([M+H-C₄H₆O₅]⁺) and at *m/z* 184.1 ([M+H-C₆H₈O₆]⁺). The latter two fragment ions are attributed to the positively charged acetyl derivative of A α C and the protonated A α C. These results are consistent with a previous study.⁴⁹

The two A α C-*O*-Gluc isomers (M2 and M3) are readily distinguished by their product ion spectra at MS³ scan stage. The product ion spectrum of A α C-6-*O*-Gluc ([M+H]⁺ at *m/z* 376.1) (M2) at MS³ scan stage displays two fragments ions: one ion at *m/z* 200.1 ([M+H-C₆H₈O₆]⁺) attributed to the positively charged A α C-6-OH, which formed by the cleavage of the Gluc moiety, and the second ion at *m/z* 183.0 ([M+H-C₆H₈O₆-NH₃]⁺) is attributed to the loss of NH₃ from A α C-6-OH (Figure 2B). In the case of A α C-3-*O*-Gluc, ([M+H]⁺ at *m/z* 376.1) (M3) the fragment ions at *m/z* 200.1 and *m/z* 183.0 are also observed, in addition to a prominent fragment ion at *m/z* 155.1 ([M+H-C₆H₈O₆-NH₃-CO]⁺), which is proposed to occur by the successive loss of NH₃ and CO from A α C-3-OH (Figure 2C). The ion at *m/z* 155.1 is a prominent product ion of A α C-3-OH, but not for A α C-6-OH, when these hydroxylated A α C derivatives undergo CID with a triple quadrupole MS, or by ion trap at the MS³ scan stage

(unpublished results, R. Turesky). The differences in the pattern of fragmentation of A α C-*O*-Gluc isomers lead us to tentatively assign M3 as A α C-3-*O*-Gluc and M2 as A α C-6-*O*-Gluc.

The hydrolysis products of M2 and M3 obtained by treatment with β -glucuronidase showed that M2 co-eluted with A α C-6-OH and displayed an UV spectrum identical to that of A α C-6-OH, whereas the hydrolysis product of M3 co-eluted with A α C-3-OH and exhibited the same UV spectrum as A α C-3-OH (Figure S1). The A α C-6-OH and A α C-3-OH reference compounds produced by human liver microsomes were characterized by $^1\text{H-NMR}$, which unambiguously identified the sites of ring-oxidation of A α C.^{17,34} Taken together these results lead us to identify M2 as A α C-6-*O*-Gluc and M3 as A α C-3-*O*-Gluc.

As was observed for the *O*-Gluc conjugates, the isomeric sulfate conjugates can be distinguished by their product ion spectra. The product ion spectrum of A α C-6-*O*-SO₃H (M6) ($[\text{M}+\text{H}]^+$ at m/z 280.0) at MS³ stage displays two fragments ions at m/z 200.0 ($[\text{M}+\text{H}-\text{SO}_3]^+$) and m/z 183.0 ($[\text{M}+\text{H}-\text{SO}_3-\text{NH}_3]^+$) (Figure 2D). The product ion spectrum of A α C-3-*O*-SO₃H (M4) ($[\text{M}+\text{H}]^+$ at m/z 280.0) at MS³ stage also displays the fragment ions at m/z 200.1 and 183.0, and a major fragment ion at m/z 155.0 ($[\text{M}+\text{H}-\text{SO}_3-\text{NH}_3-\text{CO}]^+$) attributed to the loss of NH₃ and CO from A α C-3-OH (Figure 2E). These results lead to the tentative identification of M4 as A α C-6-*O*-SO₃H and M6 as A α C-3-*O*-SO₃H. The sulfatase hydrolysis product of M4 and M6 co-eluted by HPLC and exhibited the characteristic UV spectra of A α C-3-OH and A α C-6-OH, respectively support the assignment of M4 as A α C-6-*O*-SO₃H and M6 as A α C-3-*O*-SO₃H (Figure S1).

The product ion spectrum of *N*²-acetyl-A α C-6-*O*-Gluc (M1) ($[\text{M}+\text{H}]^+$ at m/z 418.1) at MS³ scan stage is shown in Figure 3A. A major fragment ion is observed at m/z 200.1 ($[\text{M}+\text{H}-\text{C}_6\text{H}_8\text{O}_6-\text{C}_2\text{H}_3\text{O}]^+$) attributed to the cleavage of the Gluc and the acetyl linkages. Fragment ions at m/z 183.1 ($[\text{M}+\text{H}-\text{C}_6\text{H}_8\text{O}_6-\text{C}_2\text{H}_3\text{O}-\text{NH}_3]^+$) and m/z 172.1 ($[\text{M}+\text{H}-\text{C}_6\text{H}_8\text{O}_6-$

$\text{C}_2\text{H}_3\text{O-CO}]^+$) are also observed. A fragment ion at m/z 155.1 attributed to the successive loss of NH_3 and CO from protonated $\text{A}\alpha\text{C-6-OH}$ was also observed. However, this ion is relatively minor in abundance. The position of oxidation and identification of the metabolite as N^2 -acetyl- $\text{A}\alpha\text{C-6-O-Gluc}$ was determined by comparing the UV spectrum of hydrolysis product to those UV spectra of $\text{A}\alpha\text{C-6-OH}$ and $\text{A}\alpha\text{C-3-OH}$. The N^2 -acetyl- $\text{A}\alpha\text{C-6-O-Gluc}$ hydrolysis product (enzymatic hydrolysis using β -glucuronidase followed by acid hydrolysis) co-eluted and exhibited an UV spectrum identical to that of $\text{A}\alpha\text{C-6-OH}$, whereas the UV spectrum and retention time of $\text{A}\alpha\text{C-3-OH}$ were different. These data confirm the identification of M1 as N^2 -acetyl- $\text{A}\alpha\text{C-6-O-Gluc}$ (Figure 3B).

Characterization of minor metabolites of $\text{A}\alpha\text{C}$ in human hepatocytes. We assessed the metabolite formation in primary human hepatocytes treated with various concentrations of $\text{A}\alpha\text{C}$ (0.1, 1, 10 and 50 μM) over 24 h followed by UPLC/ MS^n analysis. In addition to the previously described metabolites, several minor metabolites were identified. There were two N -acetylated ring-oxidized $\text{A}\alpha\text{C}$ sulphate conjugates (N^2 -acetyl- $\text{A}\alpha\text{C-6-O-SO}_3\text{H}$, N^2 -acetyl- $\text{A}\alpha\text{C-3-O-SO}_3\text{H}$), one N -acetylated ring-oxidized $\text{A}\alpha\text{C}$ Gluc conjugate (N^2 -acetyl- $\text{A}\alpha\text{C-3-O-Gluc}$) and the genotoxic metabolite, $\text{A}\alpha\text{C-HN}^2\text{-O-Gluc}$ (Figure 4).

The product ion spectra of the two N^2 -acetyl- $\text{A}\alpha\text{C-O-SO}_3\text{H}$ conjugates ($[\text{M}+\text{H}]^+$ at m/z 322.1) at the MS^3 scan stage revealed a prominent fragment ion at m/z 224.1 ($[\text{M}+\text{H}-\text{H}_2\text{SO}_4]^+$) which occurs by cleavage of the sulfate linkage. In addition, fragment ions were observed at m/z 200.1 ($[\text{M}+\text{H}-\text{SO}_3-\text{C}_2\text{H}_3\text{O}]^+$) attributed to the loss of SO_3 and the acetyl linkage to form a protonated $\text{A}\alpha\text{C-OH}$ species (Figure 4A and 4B). We were unable to distinguish the identities of these two isomers based on mass spectral data; however, based on their polarity and the fact that the retention time for the $\text{A}\alpha\text{C-6-O-SO}_3\text{H}$ metabolite was characteristically shorter than that of $\text{A}\alpha\text{C-3-O-SO}_3\text{H}$, the metabolite observed at t_R 15.2 min

was tentatively assigned as *N*²-acetyl-AαC-6-*O*-SO₃H and the metabolite observed at *t*_R 16.9 min assigned as *N*²-acetyl-AαC-3-*O*-SO₃H (Figure 5).

In the case of *N*²-acetyl-AαC-3-*O*-Gluc ([M+H]⁺ at *m/z* 418.1), The product ion spectra at MS³ stage shows a fragment ion at *m/z* 224.1 ([M+H-C₆H₈O₆-H₂O]⁺), which is attributed to the cleavage of the Gluc linkage and the loss of water. In addition, we observed a fragment ion at *m/z* 200.1 attributed to the protonated AαC-3-OH resulting from the concomitant cleavages of the Gluc and the acetyl moieties (Figure 4D). Based on its polarity and its longer retention time in comparison to its *N*²-acetyl-AαC-6-*O*-Gluc isomer described above, we tentatively identified this metabolite as *N*²-acetyl-AαC-3-*O*-Gluc (Figure 5).

The product ion spectrum of the previously reported AαC-HN²-*O*-Gluc ([M+H]⁺ at *m/z* 376.0) at MS³ stage shown in Figure 4C displays two fragment ions: the ion at *m/z* 184.0 ([M+H-C₆H₈O₇]⁺) is attributed to the positively charged AαC, which is formed by the cleavage of the Gluc linkage. The ion at *m/z* 167.0 ([M+H-C₆H₈O₇-NH₃]⁺) is attributed to the loss of NH₃ from the protonated AαC. The online LC-ESI-MSⁿ consecutive reaction product ion spectra acquired in the negative mode of AαC-HN²-*O*-Gluc [M-H]⁻ at *m/z* 374.1 at the MS² and MS³ scan stages support the proposed structure with an *O* linkage formed between the HONH-AαC and Gluc. The base peak ion in the product ion spectrum at the MS² scan stage was observed at *m/z* 193.0 [M-H-AαC]⁻. The second generation product ion spectrum of AαC-HN²-*O*-Gluc acquired on *m/z* 193.0 shows the typical CID fragmentation pattern previously reported for the glucuronate,⁵⁰ and proved that the linkage formed between the glucuronic acid and HONH-AαC occurred through the oxygen atom of HONH-AαC (Figure S2). These spectral data are consistent with the published data on the metabolite produced by recombinant UGTs.³⁴

In addition to these fully characterized metabolites, we observed the formation of several other minor metabolites, which are isomers of those characterized above and include two N^2 -acetyl-A α C-*O*-Gluc, one N^2 -acetyl-A α C-*O*-SO₃H, and one A α C-*N*-Gluc.

A α C metabolism at low concentrations in human hepatocytes. The biotransformation of A α C was examined as a function of concentration in primary human hepatocytes treated with 0.1, 1 and 10 μ M of A α C. As shown in Figure 6, the concentration of A α C did not greatly impact its metabolism; indeed the patterns of metabolites formed were comparable at all of the concentrations studied. Based on the total ion counts of each metabolite in positive ion mode, the N^2 -acetyl-A α C-6-*O*-Gluc is the most prominent metabolite, followed by the two ring-oxidized A α C glucuronide conjugates (A α C-3-*O*-Gluc and A α C-6-*O*-Gluc) and the two ring-oxidized A α C sulfate conjugates (A α C-3-*O*-SO₃H and A α C-6-*O*-SO₃H). However, these estimates are approximate as the ionization efficiencies, particularly those of the sulfate conjugates in the positive ion mode, may result in underestimation of product formation. The findings show that the ring oxidation of C3 and C6 position constitutes a major pathway of metabolism of A α C. It is noteworthy that the formation of the genotoxic metabolite A α C-HN²-*O*-Gluc occurred at all the concentrations studied.

Role of P450 1A2 in A α C metabolism and DNA adduct formation in human hepatocytes. Human P450 1A2 is well recognized as the major isoform responsible for bioactivation of a number of HAA.^{29,51-53} The role of P450 1A2 in the formation of A α C metabolites in hepatocytes was assessed with furafylline, a selective, mechanism-based P450 1A2 inhibitor.⁴³ In our experimental conditions, we observe that 24 h of treatment with 5 μ M of furafylline leads to more than 80% decrease of P450 1A2 activity (date not shown). As shown in Figure 7, the pre-incubation of human hepatocytes with furafylline resulted in a strong decrease in the levels of A α C-HN²-*O*-Gluc with a concomitant increase in the amount

of unmetabolized A α C and A α C-*N*²-Gluc. These results demonstrate that P450 1A2 is the major isoform involved in the metabolism of A α C and formation of A α C-HN²-*O*-Gluc. In addition, we observed that P450 1A2 inhibition lead to a decrease in the levels of the two ring-oxidized A α C sulfate conjugates (A α C-3-*O*-SO₃H and A α C-6-*O*-SO₃H), the two ring-oxidized A α C glucuronide conjugates (A α C-3-*O*-Gluc and A α C-6-*O*-Gluc) and the two *N*-acetylated A α C-6-OH conjugates (*N*²-acetyl-A α C-6-*O*-Gluc and *N*²-acetyl-A α C-6-*O*-SO₃H). In contrast, the relative abundance of the *N*²-acetyl-A α C-3-*O*-SO₃H and *N*²-acetyl-A α C-3-*O*-Gluc were increased by the inhibition of P450 1A2. Thus, other P450s may have catalysed the C-3 oxidation *N*²-acetyl-A α C. The potency of furafylline P450 1A2 mediated inhibition was most pronounced when A α C was incubated at low substrate concentrations, indicating that other P450s²⁹ or oxidases¹⁷ contributed to the metabolism of A α C at higher substrate concentrations.

The levels of dG-C8-A α C were measured in conjunction with the amounts of A α C-HN²-*O*-Gluc formed in human hepatocytes treated with several doses of A α C (0.1, 1 and 10 μ M) in the presence or absence of furafylline. As shown in Figure 8, A α C-HN²-*O*-Gluc and dG-C8-A α C were formed in a dose-dependent manner. The inhibition of P450 1A2 lead to a 70 - 80% decrease in the amount of A α C-HN²-*O*-Gluc and dG-C8-A α C adduct levels. These results signify that P450 1A2 is the major isoform involved in the bioactivation of A α C in human hepatocytes, to form genotoxic metabolites, including A α C-HN²-*O*-Gluc, and dG-C8-A α C adduct formation. A representative UPLC-ESI/MS³ chromatogram and product ion spectra of DNA adduct are shown in supplementary data (Figure S3). The product ion spectrum is in excellent agreement to our previously published data²⁷.

DISCUSSION

The aim of this study was to characterize the major metabolites of A α C formed in primary human hepatocytes using UPLC/MSⁿ. Our data demonstrate that A α C is subjected to direct conjugation reactions such as *N*-glucuronidation, but also double and triple biotransformation reactions by the combination of *N*-oxidation or ring-oxidation followed by *O*-sulfonation, *O*-glucuronidation, as well as *N*-acetylation. The amounts of *N*-acetyl-A α C were extremely low in hepatocytes, indicating that this metabolite undergoes further metabolism (unpublished data, R. Turesky). We detected 10 stable metabolites including six major metabolites: A α C-3-*O*-SO₃H, A α C-6-*O*-SO₃H, A α C-3-*O*-Gluc, A α C-6-*O*-Gluc, *N*²-acetyl-A α C-6-*O*-Gluc and A α C-*N*²-Gluc, and four minor products: *N*²-acetyl-A α C-6-*O*-Gluc, *N*²-acetyl-A α C-6-*O*-SO₃H, *N*²-acetyl-A α C-3-*O*-SO₃H and A α C-HN²-*O*-Gluc.

Ring oxidation of A α C at the C-3 and C-6 atoms of the heterocyclic ring are the major pathways of A α C metabolism in human hepatocytes. These oxidation pathways are similar to the data reported by Raza et al. using human liver microsomes, where three oxidized products of A α C were identified: one *N*-oxidized product (HONH-A α C) and two ring-oxidized products (A α C-3-OH and A α C-6-OH).²⁹ The ratio of ring-oxidized products to *N*-oxidized product was estimated to be 85:15 and was relatively constant across the several human liver microsomal preparations.²⁹ Since A α C-3-OH and A α C-6-OH possess no mutagenic activities,^{28,29} the predominant routes for A α C metabolism in human hepatocytes appear as detoxication pathways. However, A α C-3-*O*-SO₃H also can be produced by the rearrangement of the genotoxic *N*-sulfate ester of HONH-A α C.¹⁷ Moreover, A α C undergoes metabolism to produce higher levels of DNA adducts than 4-ABP, PhIP or MeIQx in human hepatocytes.^{23,24} Therefore, DNA adducts are important biomarkers to measure when characterizing procarcinogen metabolism in cell systems. Although A α C undergoes extensive

metabolism by N^2 -acetylation and oxidation, we have not yet detected DNA adducts of A α C that possesses an *N*-acetyl group (unpublished observations, R. Turesky) as was reported for the structurally related aromatic amine, 2-aminofluorene.^{54,55}

There are large interspecies differences in metabolism of A α C in human hepatocytes compared to those previously reported *in vivo* in rats.⁴⁰ The four major metabolites of A α C identified in the bile and urine of adult male Sprague-Dawley rats treated intravenously with A α C were: A α C-3-*O*-SO₃H, N^2 -acetyl-A α C-3-*O*-SO₃H, N^2 -acetyl-A α C-6-*O*-SO₃H, N^2 -acetyl-A α C-3-*O*-Gluc.⁴⁰ The latter three metabolites were produced in relatively minor amounts in primary human hepatocytes. This discrepancy may be explained, in part by the different catalytic activities and regioselectivity of human and rat enzymes orthologues toward this procarcinogen. We previously reported interspecies differences in metabolism of two other HAA, PhIP and MeIQx in rodent and human hepatocytes.^{52,53} Indeed, the catalytic activity of human P450 1A2 was 9 to 11 fold higher than rat P450 1A2 in the *N*-oxidation of MeIQx and PhIP,^{53,56} and human P450 1A2 catalyzed the *N*-oxidation as the major pathway of metabolism whereas ring oxidation of these HAA was negligible.^{52,53,57,58} The biotransformation of A α C was also previously investigated in the human liver HepG2 cell line, where four major metabolites were identified: A α C-3-*O*-SO₃H, A α C-6-*O*-SO₃H, N^2 -acetyl-A α C-6-*O*-SO₃H and N^2 -acetyl-A α C.⁴⁰ The absence of Gluc metabolites is not surprising since this cell line is devoid of UGT.^{40,59} Taken together, these metabolic data show that rats and HepG2 cell lines do not fully capture the metabolism of A α C that occurs in human hepatocytes.

The genotoxicity of A α C is dependent upon *N*-oxidation of A α C. *In vitro* studies conducted with human liver microsomes or recombinant human P450s reveal that P450 1A2 is the primary isoform involved in *N*-oxidation and bioactivation of A α C.^{26,29} We previously

reported that P450 1A2 is the primary isoform involved in *N*-oxidation of A α C in primary human hepatocytes and that the catalytic activity of P450 1A2 is critical for A α C derived DNA adduct formation.²³ In this current study, we demonstrate that P4501A2 is also the major isoform involved in metabolism of A α C in human hepatocytes, by use of furafylline, a selective inhibitor of P450 1A2.⁴³ There is a strong correlation among P450 1A2 activity, A α C-HN²-*O*-Gluc and dG-C8-A α C formation in human hepatocytes. A α C-HN²-*O*-Gluc is a biologically reactive metabolite that binds to DNA,^{34,60} however, other reactive intermediates such as the *N*-sulfooxy- or *N*-acetoxy esters of A α C also bind to DNA.^{30,61,62} In contrast to the *N*-sulfooxy- or *N*-acetoxy esters of A α C, which are labile, A α C-HN²-*O*-Gluc is sufficiently stable and can be isolated and measured.^{61,62} NAT1 and NAT2 and SULT1A2 catalyse the DNA binding of HONH-A α C to DNA *in vitro*.³⁰ The relative contributions of UGT, NAT, SULT or other conjugating enzymes in the metabolic activation of A α C in humans remain to be determined. Pretreatment of hepatocytes with furafylline also strongly decreased the levels of sulfate and Gluc conjugates of A α C-3-OH and A α C-6-OH, but the *N*-acetylated derivatives of these metabolites increased when cells were pretreated with furafylline. These findings suggest that *N*-acetyl-A α C undergoes C-3 and C-6 oxidation by P450s other than P4501A2.

Recently, some oxidative products of A α C, including A α C-3-*O*-SO₃H, were detected in plasma of liver specific P-450 reductase null mice, suggesting that a non-hepatic P450 enzymes catalysed oxidation of A α C in the mouse model.⁴¹ The level of DNA adduct formation of A α C in liver was also unaffected in the liver specific P-450 reductase null mice treated with high doses of A α C.⁴³ Our data show that the contribution of non-P450 oxidases to the metabolism of A α C and bioactivation in human hepatocytes is minor.

In summary, human hepatocytes extensively metabolize A α C into more than ten products and also produce high levels of DNA adducts. Some of the metabolites of A α C may be used as biomarkers of exposure to A α C in molecular epidemiological studies since A α C is extensively metabolized in humans.^{17,63} A α C-3-OSO₃H has already been employed as a urinary biomarker and frequently detected in the urine of smokers.¹⁷ The development of A α C-HN²-O-Gluc as a urinary biomarker could provide valuable information about the extent of bioactivation of A α C in humans. Given the high levels of A α C in mainstream tobacco smoke,⁸⁻¹¹ and the relatively high levels of A α C DNA adducts formed in human hepatocytes compared to other HAA or aromatic amines,^{23,24} further studies on the potential role of A α C in tobacco-associated liver and gastrointestinal cancers are warranted.

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SUPPORTING INFORMATION

Regioisomer characterization of A α C-6-O-Gluc, A α C-3-O-Gluc, A α C-6-O-SO₃H and A α C-3-O-SO₃H. LC/MS² product ion mass spectra of A α C-HN²-O-Gluc in negative ion mode.

Second generation product ion spectrum of A α C-HN²-O-Gluc acquired on *m/z* 193 in negative ion mode. UPLC-ESI/MS³ chromatograms of dG-C8-A α C formed in primary human hepatocytes with or without inhibition of P4501A2 with furafylline. Primary human hepatocytes were pre-treated for 24 h with 0.1 % DMSO, or furafylline (5 μ M) followed by 24 h of treatment with 0.1 % DMSO (Ctrl), or A α C (0.1, 1 or 10 μ M). Donor information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

ABBREVIATIONS

A α C, 2-Amino-9*H*-pyrido[2,3-*b*]indole; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; 4-ABP, 4-aminobiphenyl; B[a]P, benzo[a]pyrene; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinolone; dG-C8-A α C, *N*-(deoxyguanosin-8-yl)-2-amino-9*H*-pyrido[2,3-*b*]indole; NAT, *N*-acetyltransferases; SULT, Sulfotransferases; UGT, UDP-Glucuronosyltransferases; UPLC/MSⁿ, UPLC/ion trap multistage mass spectrometry; HONH-A α C, 2-hydroxyamino-9*H*-pyrido[2,3-*b*]indole; A α C-3-OH, 2-amino-3-hydroxy-9*H*-pyrido[2,3-*b*]indole; A α C-6-OH, 2-amino-6-hydroxy-9*H*-pyrido[2,3-*b*]indole; A α C-3-*O*-SO₃H, 2-amino-9*H*-pyrido[2,3-*b*]indol-3-yl-sulfate; A α C-6-*O*-SO₃H, 2-amino-9*H*-pyrido[2,3-*b*]indol-6-yl-sulfate; A α C-3-*O*-Gluc, 2-amino-9*H*-pyrido[2,3-*b*]indol-3-yl-oxo-(β -D-glucuronic acid); A α C-6-*O*-Gluc, 2-amino-9*H*-pyrido[2,3-*b*]indol-6-yl-oxo-(β -D-glucuronic acid); *N*²-acetyl-A α C-3-*O*-SO₃H, *N*²-acetyl-9*H*-pyrido[2,3-*b*]indol-3-yl sulfate; *N*²-acetyl-A α C-6-*O*-SO₃H, *N*²-acetyl-9*H*-pyrido[2,3-*b*]indol-6-yl sulfate; *N*²-acetyl-A α C-3-*O*-Gluc; *N*²-acetyl-2-amino-9*H*-pyrido[2,3-*b*]indol-3-yl-oxo-(β -D-glucuronic acid); *N*²-acetyl-A α C-6-*O*-Gluc, *N*²-acetyl-2-amino-9*H*-pyrido[2,3-*b*]indol-6-yl-oxo-(β -D-glucuronic acid); A α C-*N*²-Gluc, *N*²-(β -D-glucosiduronyl)-2-amino-9*H*-pyrido[2,3-*b*]indole; A α C-HN²-

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O-Gluc, *O*-(β-D-glucosiduronyl)-2-hydroxyamino-9*H*-pyrido[2,3-*b*]indole; SPE, Solid-phase
extraction

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Figure Legends

Figure 1. HPLC-UV profile (A) and chemical structures (B) of A α C metabolites formed in primary human hepatocytes incubated with 50 μ M of A α C over 24 h.

Figure 2. LC/MS² product ion mass spectra of A α C-*N*²-Gluc (*m/z* 360.1 >) (A) and LC/MS³ product ion mass spectra of A α C-6-*O*-Gluc (*m/z* 376.1 > 200.1 >) (B), A α C-3-*O*-Gluc (*m/z* 376.1 > 200.1 >) (C), A α C-6-*O*-SO₃H (*m/z* 280.1 > 200.1 >) (D), A α C-3-*O*-SO₃H (*m/z* 280.1 > 200.1 >) (E) in positive ionization mode. The proposed mechanism of formation of the prominent fragment ion of A α C-3-OH at *m/z* 155.0 is presented.

Figure 3. Identification and characterization of *N*²-Acetyl-A α C-6-*O*-Gluc. (A) LC/MS³ product ion mass spectra of *N*²-Acetyl-A α C-6-*O*-Gluc (*m/z* 418.1 > 242.1 >) formed in primary human hepatocytes. *N*²-Acetyl-A α C-6-*O*-Gluc was purified by HPLC and characterized by mass spectrometry. (B) UV spectra of A α C-6-OH, A α C-3-OH, *N*²-acetyl-A α C-*O*-Gluc and its hydrolysis product obtained after β -glucuronidase followed by acid treatment of *N*²-acetyl-A α C-*O*-Gluc as described in material and method.

Figure 4. LC/MS³ product ion mass spectra of *N*²-acetyl-A α C-6-*O*-SO₃H (*m/z* 322.1 > 241.1 >) (A), *N*²-acetyl-A α C-3-*O*-SO₃H (*m/z* 322.1 > 241.1 >) (B), A α C-HN²-*O*-Gluc (*m/z* 376.1 > 200.1 >) (C) and *N*²-acetyl-A α C-3-*O*-Gluc (*m/z* 418.1 > 242.1 >) (D). Primary human hepatocytes were treated with 0.1, 1 and 10 μ M of A α C and the metabolites derived from A α C were characterized by mass spectrometry.

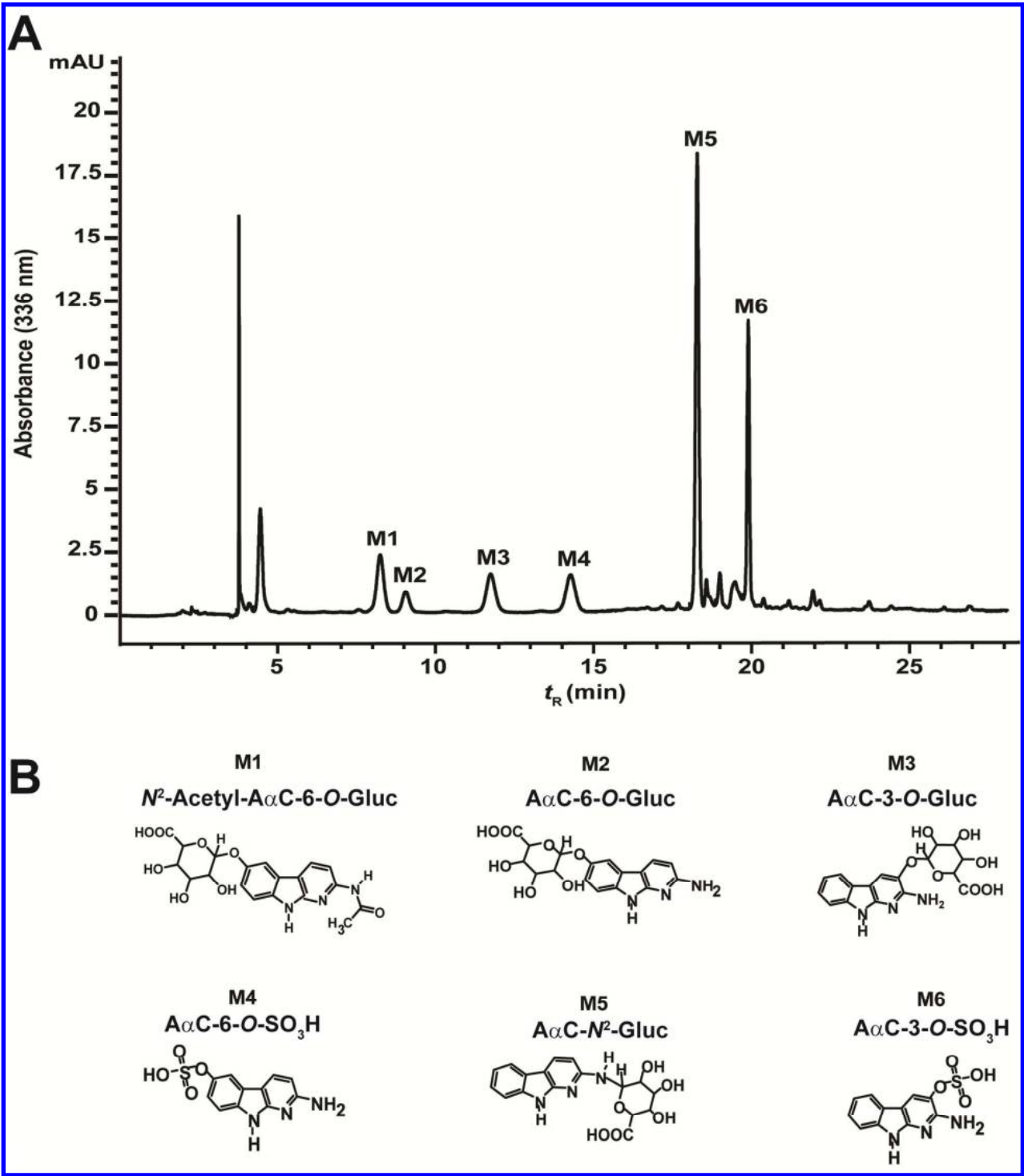
Figure 5. Mass chromatograms of A α C metabolites formed during 24h in primary human hepatocytes incubated 0.1, 1 and 10 μ M of A α C.

Figure 6. Distribution of A α C metabolites formed as a function of dose in two human primary hepatocyte preparations after 24 h of incubation. Primary human hepatocytes were incubated with various concentrations of A α C (0.1, 1 and 10 μ M) during 24 h and the relative ion abundance of each metabolites derived from A α C formed were determined by mass spectrometry.

Figure 7. Role of P450 1A2 in A α C metabolism in human hepatocytes. Cells were pre-treated with furafylline (5 μ M) for 24h prior to treatment with A α C (0.1, 1 and 10 μ M) during 24h. The relative abundance of each A α C derived metabolites was determined by mass spectrometry. (Student's *t*-test, * $P < 0.05$; ** $P < 0.01$, *** $P < 0.005$ versus control).

Figure 8. Correlation between DNA adducts levels derived from A α C and the relative abundance of A α C-HN²-O-Gluc formed in human hepatocytes. Relative abundance of A α C-HN²-O-Gluc (**A**) and dG-C8-A α C (**B**) formed in human hepatocytes. Cells were pre-treated with furafylline (5 μ M) for 24h prior to incubation with 0.1, 1, 10 and 50 μ M of A α C for 24h and the relative abundance of A α C-HN²-O-Gluc and dG-C8-A α C levels derived from A α C formed were estimated by mass spectrometry. (Student's *t*-test, * $P < 0.05$; ** $P < 0.01$, *** $P < 0.005$ versus control).

Figure 1.



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Figure 2.

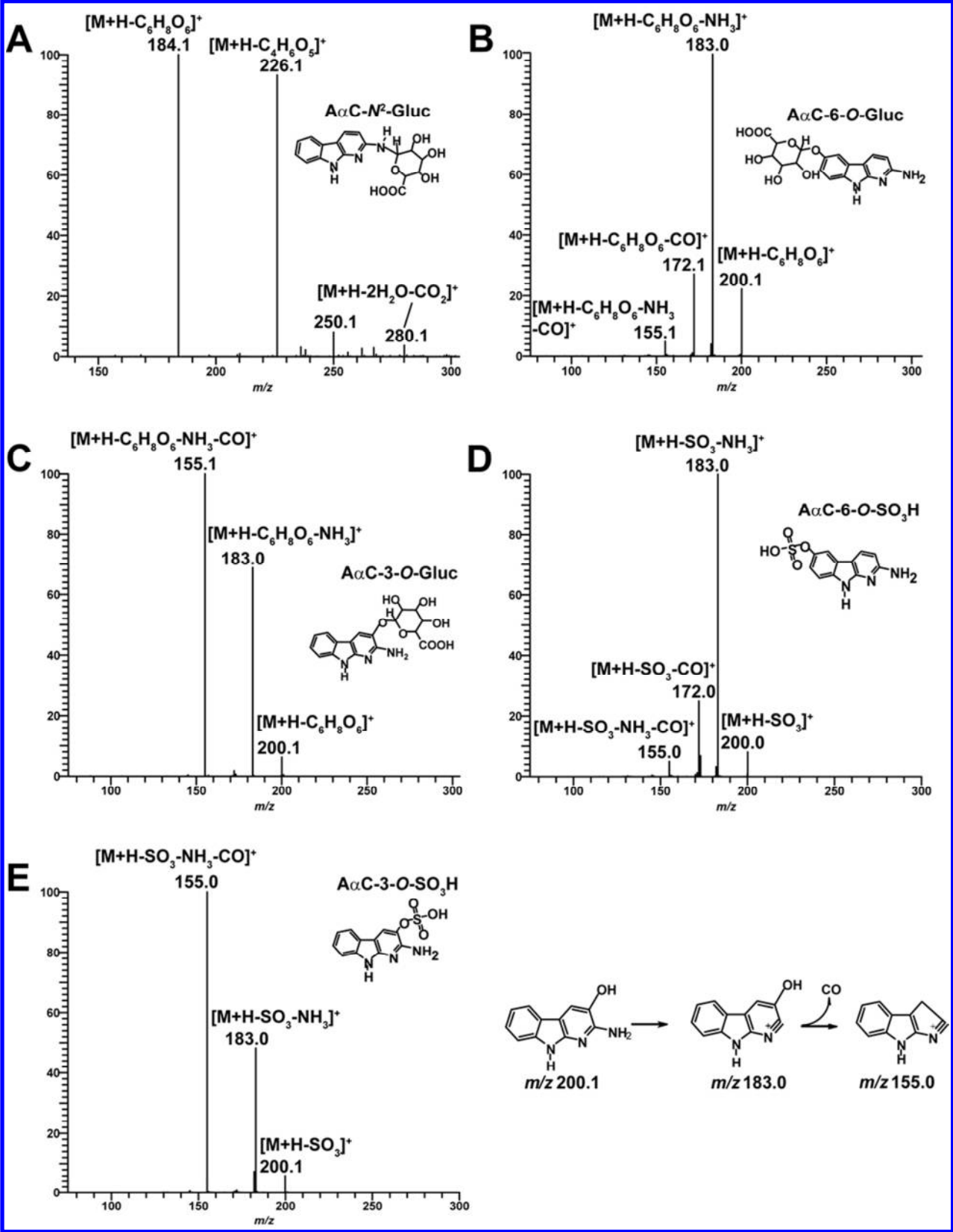


Figure 3.

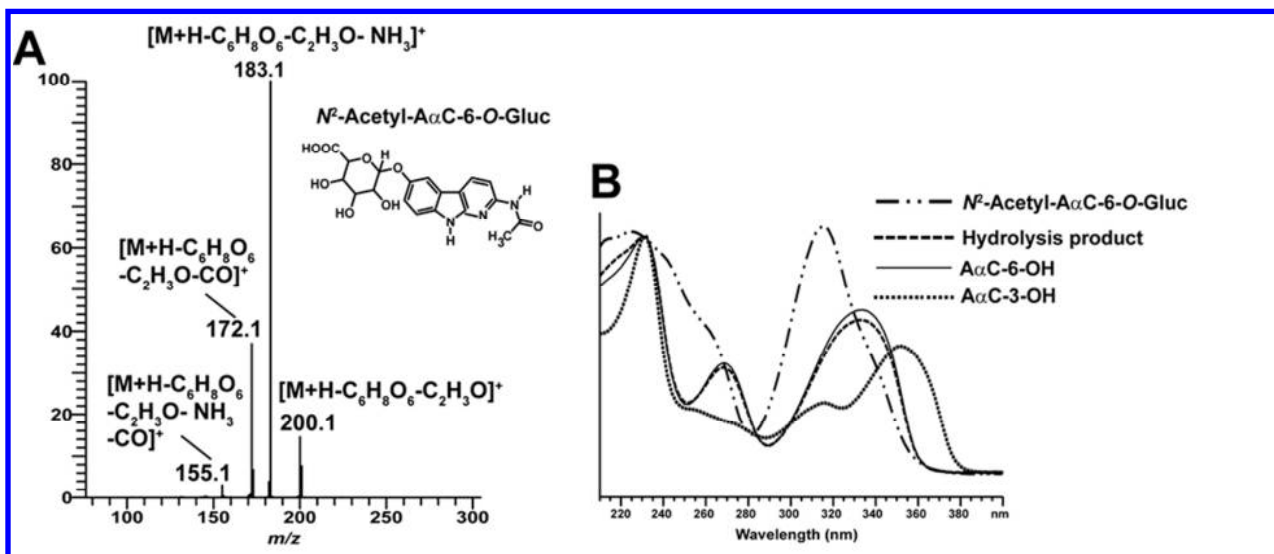


Figure 4.

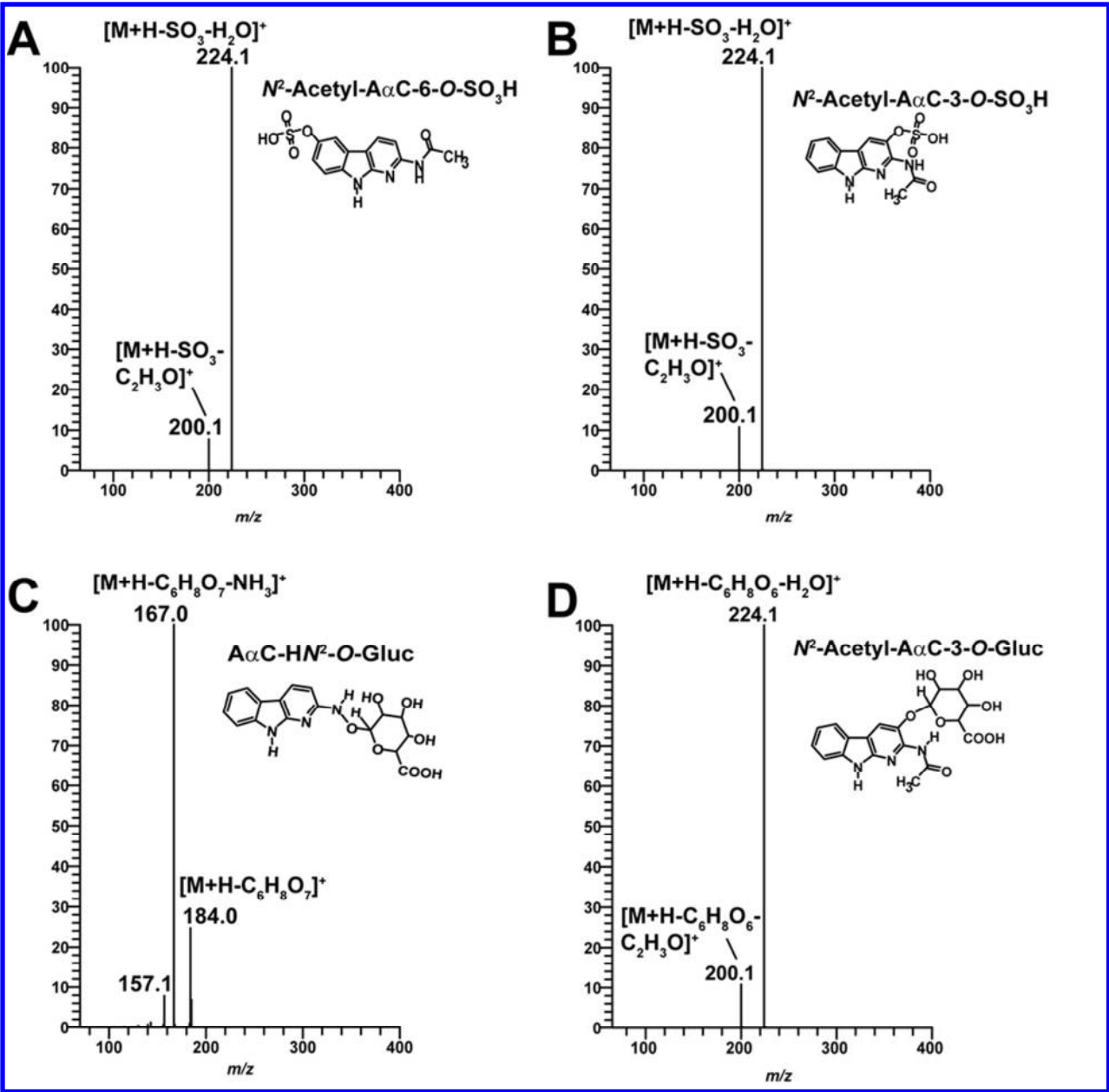


Figure 5.

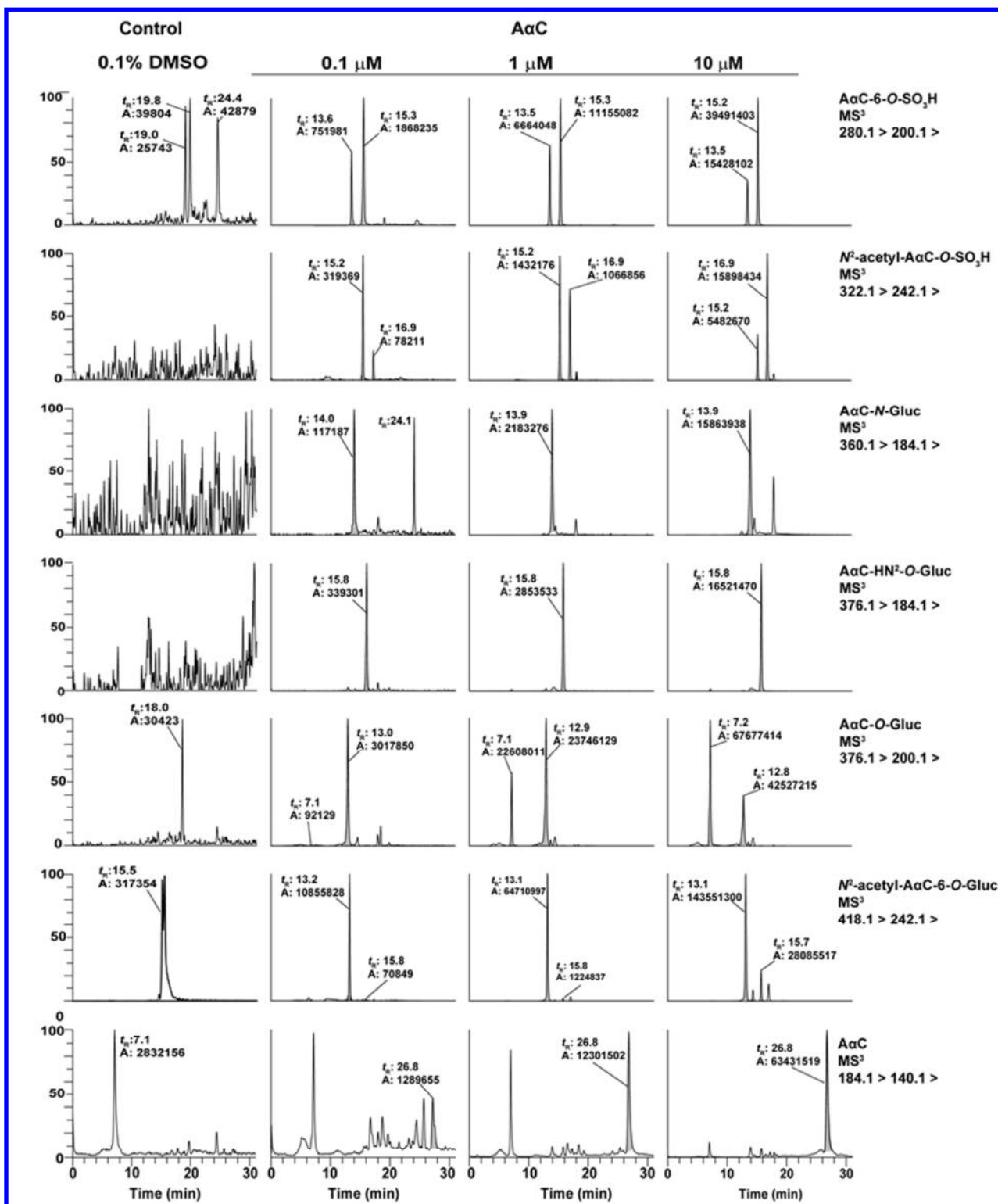


Figure 6.

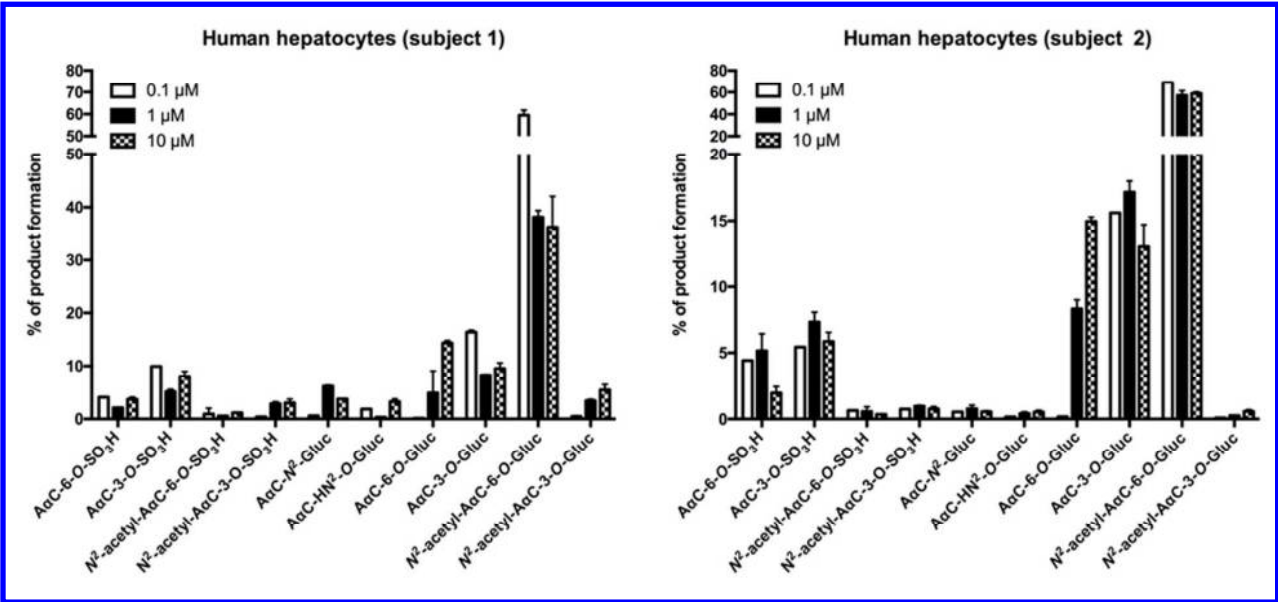


Figure 7.

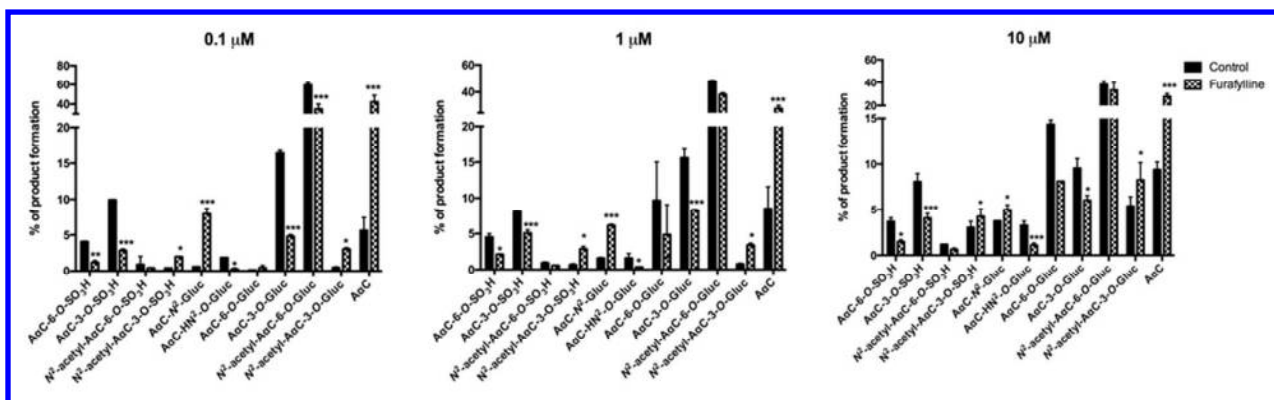


Figure 8.

