

IN VITRO METABOLISM OF ARYLAMINE HAIR DYES IN HUMAN HEPATIC MICROSOMES AND HUMAN HEPATOCYTES

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RESULTS

ABSTRACT

Metabolism is a key consideration in the safety assessment of primary arylamines used in oxidative hair dyes, especially for the assessment of potential carcinogenicity, since N-hydroxylation formation by CYPs is regarded as a key step in the activation of certain arylamines that have been identified as human bladder carcinogens (4-aminobiphenyl, 2-naphthylamine, benzidine). Previous studies have shown that *p*-phenylenediamine and 2,5-diaminotoluene, two widely used oxidative hair dye ingredients, do not form mono-oxygenated metabolites in cryopreserved human hepatocytes, hepatic microsomes or recombinant CYPs. Only the mono- and diacetylated metabolites were observed in human hepatocytes. The purpose of the present work was to screen additional arylamine hair dyes for oxidative metabolism in human hepatic microsomes and to conduct more in depth metabolism studies with any compounds exhibiting evidence of oxidative metabolism. Five primary arylamines that are widely used in oxidative hair dyes were screened and showed no evidence of oxidative metabolism. One compound, 4-amino-2-hydroxytoluene (AHT), showed evidence of formation of two hydroxylated metabolites by human, rat, and mouse hepatic microsomes. However, there was no evidence of covalent binding of ¹⁴C-radiolabeled AHT to human microsomal protein, suggesting that the monohydroxylated metabolites formed were not biologically reactive. One of these metabolites was confirmed to be hydroxylated on the methyl group and the other is likely to be ring hydroxylated. Further in vitro studies in human, rat, and mouse hepatocytes indicated that phase II reactions (sulfation, acetylation, glucuronidation) predominate in the intact hepatocyte and that mono-oxygenated metabolites are not detected. Our results indicate that none of the hair dyes tested show evidence of hepatic metabolism to potentially biologically reactive oxidative metabolites. As such, the metabolism of these compounds appears distinct from that exhibited by known human bladder carcinogens.

INTRODUCTION

In a case-control epidemiology study conducted in Los Angeles an association was found between the use of permanent (i.e., oxidative) hair dyes and bladder cancer risk (1). In a subsequent publication from the same study the increase in bladder cancer risk was reported to be associated with N-acetyltransferase-2 (NAT2) slow acetylator phenotype (2). These investigators concluded that modification of the risk by NAT2 genotype/phenotype implicates arylamines used in permanent hair dyes as playing a role in bladder carcinogenesis. More recent epidemiology studies have not shown an association between permanent hair dye use and bladder cancer (3,4), and NAT2 polymorphisms did not significantly modify the bladder cancer risk estimates (3). Nevertheless, the Los Angeles study had raised questions about permanent hair dyes (which contain arylamines as ingredients) because some arylamines such as benzidine, 4-aminobiphenyl, and 2-naphthylamine, are known human bladder carcinogens, and N-acetyltransferase enzymes are involved in the metabolism of these carcinogens. At the time the Los Angeles study was published there were few data in the literature on the metabolism of hair dye arylamines. In our previous work, *p*-phenylenediamine and 2,5-diaminotoluene have shown no evidence for formation of hydroxylated metabolites by human hepatic microsomes or by specific human CYP enzymes (5,6). The experiments presented here extend the evaluation of hepatic metabolism to additional arylamine hair dyes. The arylamines selected for study in these experiments and in the previously published work (5,6) are those used at the highest volume in permanent hair dyes.

METHODS

Pooled Human Liver Microsomes

Pooled human liver microsomes were obtained from Gentest, Woburn, MA. The microsomes were pooled from microsome preparations from 26 female and 20 male subjects. Characterization of the enzymatic activity of the pooled microsomes was provided by the supplier.

Microsomal Incubations

Test chemicals (10 and 100 μM) and the positive control (100 μM 2-aminofluorene) were incubated with pooled liver microsomes (1 mg protein/ml) in the presence or absence of an NADPH regenerating system. The reactions were initiated by the addition of microsomes, incubated at 37°C for one hour, and terminated by addition of an equal volume of acetonitrile. Samples were centrifuged to remove protein and the supernatant was flash frozen and stored at -70°C until analysis.

Cryopreserved Human Hepatocytes

Cryopreserved human hepatocytes were obtained from In Vitro Technologies. Suspended hepatocytes (1x10⁶ cells/ml) were incubated with substrate (10 and 100 μM) for up to four hours. 500 μl aliquots were removed at selected time points and the reaction stopped by the addition of an equal volume of acetonitrile. Samples were centrifuged to remove protein and the supernatant was flash frozen and stored at -70°C until analysis.

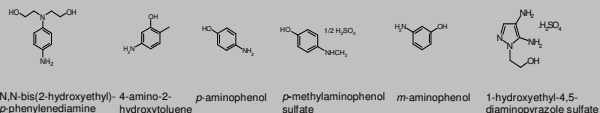
Sample Analysis

Analyses for metabolites of test chemicals was carried out using reverse phase HPLC with MS or radiochemical detection. For MS detection, a Waters Quattro Micro MS was used in scanning mode (+ve and -ve ion) for selected ion detection of the parent ion and potential metabolite ions. Under the conditions used the LC/MS detection method did not permit quantification. For radiochemical detection, a Berthold Radioflow LB509 detector was used. The limit of detection was approximately 5% of total [¹⁴C]labeled AHT injected on column. Total injected on column was 30 nanocuries and limit of detection was approximately 1.5 nanocuries.

Microsomal Protein Binding

[¹⁴C]-labeled AHT was incubated in the presence or absence of an NADPH-regenerating system and liquid scintillation counting was used to quantify the amount of [¹⁴C]-AHT that remained bound to microsomal protein following repeated washes with acetonitrile.

Test Chemicals



Positive Control Chemical

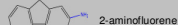


Fig. 1 2-Aminofluorene Positive Control

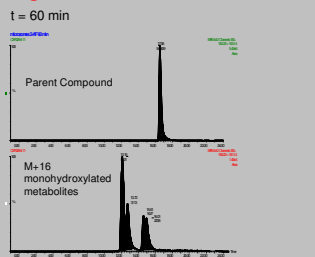
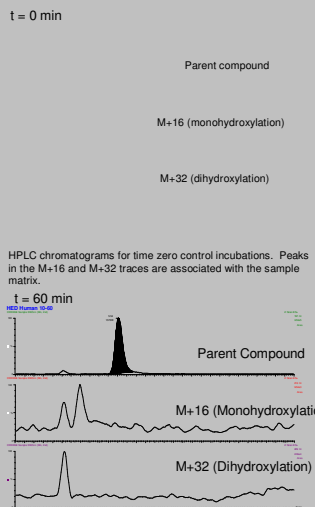


Fig. 2 N,N-bis(2-hydroxyethyl)-p-phenylenediamine



HPLC chromatograms obtained after incubation of pooled human liver microsomes with 10 μM N,N-bis(2-hydroxyethyl)-p-phenylenediamine for 60 minutes. The y-axis of each plot is adjusted such that the tallest peak detected is allocated a value of 100%. Only sample matrix peaks were observed in the M+16 and M+32 traces. There was no evidence of the production of oxidative metabolites at t = 60 min.

HPLC chromatograms obtained after incubations with p-aminophenol, p-methylaminophenol sulfate, m-aminophenol, and 1-hydroxyethyl-4,5-diaminopyrazole sulfate were similar to those obtained with N,N-bis(2-hydroxyethyl)-p-phenylenediamine, i.e., showing no evidence for the production of oxidative metabolites (data not shown).

Fig. 3 4-Amino-2-hydroxytoluene (AHT)

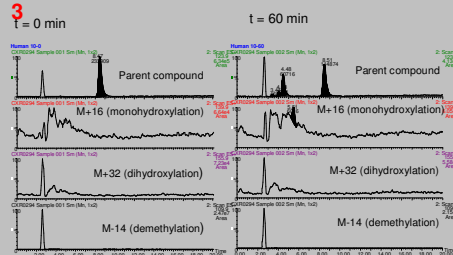


Fig. 4 Hydroxymethyl Metabolite of AHT – Synthetic Standard

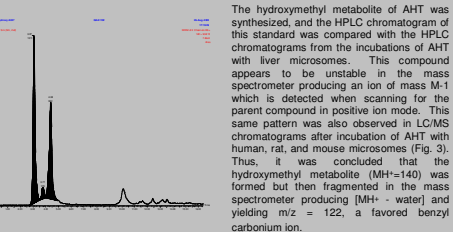


Table 1 Assay for Covalent Binding of ¹⁴C-AHT to Human Microsomal Protein

[¹⁴ C]-AHT	+/- M NADPH regenerating system	Amount of [¹⁴ C]-AHT bound to microsomal protein (pmol bound/mg protein/h)	
		Experiment 1	Experiment 2
40 μM	+	271	273
	-	343	413
130 μM	+	845	779
	-	1077	1165

Human liver microsomes were incubated with [¹⁴C]-AHT in the presence or absence of an NADPH regenerating system. There was no evidence for enzymatically-mediated covalent binding of AHT to human microsomal proteins. Therefore, the hydroxylated metabolites of AHT were concluded to be not biologically reactive. The second hydroxylated metabolite was concluded to likely be a ring hydroxylated metabolite (see Fig. 5).

Fig. 5 Metabolism of AHT by Human, Rat, and Mouse Liver Microsomes

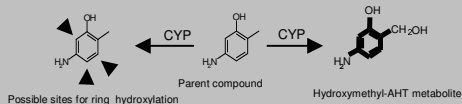


Table 2 Metabolism of AHT in Rat, Mouse and Human Hepatocytes – LC/MS Detection

	10 μM AHT				100 μM AHT			
	Acetyl	Sulfate	Glucuronide	Hydroxyl	Acetyl	Sulfate	Glucuronide	Hydroxyl
Human	+++	+++	-	-	+++	+++	+	-
Rat	++	+++	-	-	+++	+++	+	-
Mouse	-	+++	-	-	+++	+++	+	-

- Not Detected + Possible Peak Detected but at Limit of Detection (-2X background)
 ++ Small Peak Detected +++ Large Peak Detected

Human, rat, and human hepatocytes were incubated with 10 μM or 100 μM AHT, and samples were analyzed by LC/MS. In all three species the major metabolites present at both concentrations were the acetylated and sulfated metabolites. Glucuronidation was detectable only at the higher concentration of AHT and only with LC/MS and not with the less sensitive radiochemical detection method. The mono-hydroxylated metabolites observed previously in microsomal incubations were not detectable.

CONCLUSIONS

There is no evidence that the hair dye arylamines investigated in this study undergo hepatic metabolic activation (N-hydroxylation) that is characteristic of the known arylamine human bladder carcinogens. One compound that did show evidence of some metabolism by hepatic microsomes (AHT) was studied further in order to characterize the oxidative metabolites. These were concluded to be non-biologically reactive.

When metabolism of AHT was studied in intact human hepatocytes, N-acetylation and sulfation were determined to be the primary routes of metabolism, and glucuronidation represented a minor pathway. AHT was preferentially metabolized via these pathways and no oxidative metabolism could be detected.

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