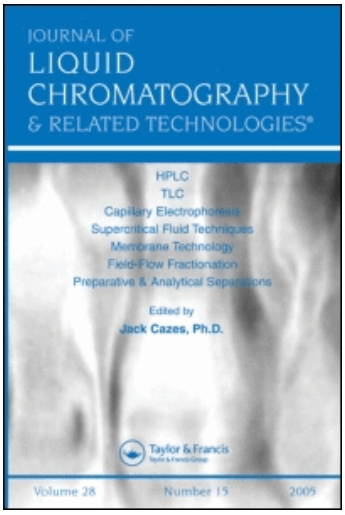

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A High Pressure Liquid Chromatography Procedure for the Separation of Metabolites of 2-Acetylaminofluorene from Cells in Culture

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A HIGH PRESSURE LIQUID CHROMATOGRAPHY PROCEDURE FOR THE SEPARATION OF METABOLITES OF 2-ACETYLAMINOFLUORENE FROM CELLS IN CULTURE

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ABSTRACT

A method is presented for the determination of pmole levels of the carcinogenic metabolite N-hydroxy-2-acetylaminofluorene (N-hydroxy-AAF) formed from N-2-acetylaminofluorene (AAF) by cells in culture. The extract from the cells and cell incubation medium was given a preliminary cleanup using thin layer chromatography. N-hydroxy-AAF was then isolated using high-pressure liquid chromatography (HPLC) with a Waters Bondapak C_{18}/Corasil column and an acetonitrile-water elution system. Ring hydroxylation products were eluted with 23% acetonitrile, and AAF and the deacetylation product 2-aminofluorene (AF) were eluted with 33% acetonitrile. Resolution of N-hydroxy-AAF was accomplished using a linear gradient of from 33% to 99% acetonitrile.

When cells in culture were incubated with 22 nmoles/ml of AAF for 6 h, 690 pmoles of N-hydroxy-AAF per 10^6 cells were formed by hamster hepatocytes compared with 13 and 8 pmoles/10^6 cells for human embryo and hamster embryo cells respectively. The rate of formation of N-hydroxy-AAF by hamster liver microsomes was 140 nmoles per h of incubation per mg of microsomal protein.

INTRODUCTION

The enzymatically catalyzed activation of AAF to metabolites carcinogenic to animals is believed to proceed through enzymatic oxidation of AAF to N-hydroxy-AAF (1,2) followed by the formation of the ultimate carcinogen, N-sulfate-AAF via catalysis by a

cytosol sulfotransferase (3-5). Variation in carcinogenicity between species, strain, and sex, as well as organospecificity, might be explained by differences in the activities of AAF metabolizing enzymes. Decreased capabilities for N-hydroxylation and N-sulfation of AAF, or the presence of competing nonactivating reactions such as deacetylation to AF, may account for differences in carcinogenicity (6). Malignant transformation of cells in culture by AAF may be under similar enzymatic controls. Hamster embryo cells are malignantly transformed by N-hydroxy-AAF but not by AAF (7). These cells may lack the ability to metabolize AAF to N-hydroxy-AAF.

The detection of pmole levels of radiolabeled metabolites of AAF from cells in culture requires the complete separation of such metabolites from the high radioactive background of AAF-[9-^{14}C]. Using HPLC, apparent separation of standards by absorbance measurements may not be sufficient because undetected small peak trailings may contain significant radioactivity. We report here an HPLC procedure which resolves N-hydroxy-AAF from high residual radioactive background and permits the determination of pmole amounts of this carcinogenic intermediate. Furthermore, this method was used to study the metabolism of AAF by human embryo cells, hamster embryo cells and hamster hepatocytes in culture.

EXPERIMENTAL

Apparatus

A Spectra-Physics Model 3500B Gradient Liquid Chromatograph equipped with a Model 770 Variable Wavelength Detector (Spectra-Physics, Santa Clara, CA) and a Waters Bondapak C_{18}/Corasil column (2' x 1/8") (Waters Associates, Inc., Framingham, MA) were used for all experiments.

Materials

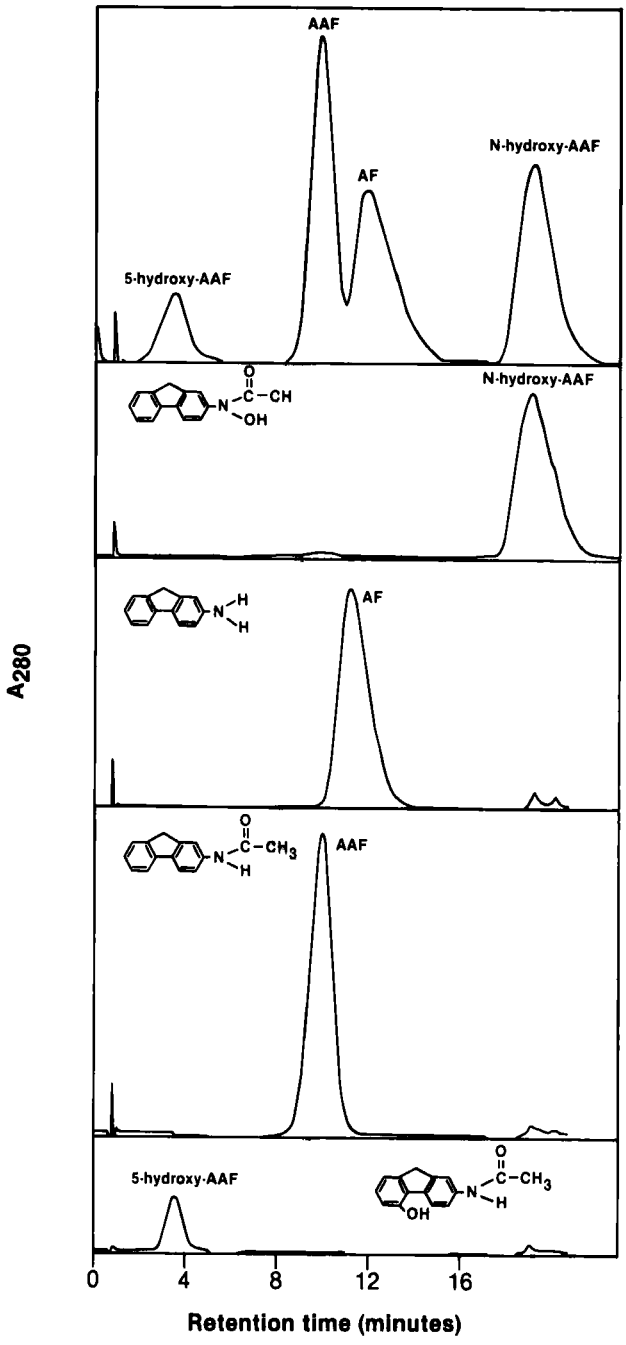
AAF-[9-^{14}C] (46 mCi/mmole) was purchased from New England Nuclear Corporation, Boston, MA. Syrian golden hamsters (Ela/ENG)

were purchased from Engle Laboratory Animals, Farmersburg, IN, and human embryo cells were obtained from HEM Research, Rockville, MD. Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Grand Island Biological Corporation, Grand Island, NY, and fetal calf serum was from Flow Laboratories, Rockville, MD. TLC silica gel GF plates (250 micron) were obtained from Analtech, Newark, DE.

Preparation and Treatment of Cells and Microsomes

Hamster embryo cells, hepatocytes and liver microsomes were prepared from Syrian golden hamsters. Human embryo cells were obtained as a frozen pool at generation 0 and were subcultured to make pools of cells which were frozen at generations 10 and 14. Primary hamster embryo cells and human embryo cells at generation 10 or 14 were reconstituted 24 h before use. All cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2 mM L-glutamine and 10% (v/v) heat-inactivated fetal calf serum. For AAF-^{14}C metabolism studies, duplicate groups of human embryo and hamster embryo cells were treated in monolayer with 5 ml of medium containing 4.8 µg/ml (0.22 µmoles/ml) of AAF-[9-^{14}C]. Cells were treated simultaneously in suspension and in monolayer. Duplicate groups of all cells were disaggregated and the volumes adjusted so that the medium contained 1 x 10^7 cells per ml. To harvest the cells, the monolayer cultures were scraped into the medium and centrifuged at 500 x g for 10 min.

Hamster liver microsomes were prepared from 7-8 week old animals. Following decapitation, the livers from six animals were pooled and homogenized in three volumes of ice-cold 150 mM KCl using a Sorval Omni-Mixer. The homogenate was centrifuged at 9,000 x g for 20 min. The supernatant was then centrifuged at 105,000 x g for 30 min and the pellet was washed twice with 150 mM KCl and stored at -90°C. Protein concentrations were determined by the method of Lowry, et al (8). Microsomal assay mixtures (1 ml) contained 100 µmoles of potassium phosphate buffer (pH 7.5), 8 µmoles of $MgCl_2$, 33 µmoles of KCl, 1.5 µmoles of NADPH, 25 µg of



AAF-[9-^{14}C] and 0.2 to 0.8 mg of microsomal protein to initiate the reaction. Incubation was for 30 min at 37°.

The formation of AF was determined by TLC. The band migrating with an AF standard was quantified using a Packard radiochromatographic scanner. Samples, together with AF, AAF and N-hydroxy-AAF standards, were spotted under yellow lights on 250 micron silica gel plates as rapidly as possible and were placed in chromatography tanks before the solvent had evaporated. The plates were developed in chloroform:methanol (99:1) in the presence of ammonia fumes. As much as 61% of AF was lost when samples were allowed to dry before chromatography, but when plates were placed in the chromatography tank before the samples had dried, 99% was recovered. For identification of AF, the appropriate band (Rf = 0.75) was extracted with chloroform and its structure verified by gas chromatography and mass spectrometry.

RESULTS

Resolution of N-Hydroxy-AAF By High Pressure Liquid Chromatography

The resolution of N-hydroxy-AAF from ring hydroxylation products and AAF is shown in Figure 1. Excellent separation of N-hydroxy-AAF-[9-^{14}C] was achieved without cross-contamination, and this permitted accurate determination of pmole levels of this metabolite which originated from the incubation of relatively

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FIGURE 1

Separation of N-hydroxy-AAF from AAF and other metabolites of AAF. Conditions: Column, Waters Bondapak C_{18}/Corasil (2' x 1/8"); elution solvent system, acetonitrile:water; gradient conditions, a) 23% acetonitrile for 4 minutes b) gradient of 30%/minute to 33% acetonitrite c) 33% acetonitrite for 12 minutes d) gradient of 33%/minute to 99% acetonitrile; flow rate, 2 ml/min; temperature, ambient.

large amounts of AAF. Total ring hydroxylation products were eluted with 23% acetonitrile in water for 4 min followed by a 30%/min gradient to 33% acetonitrile. Following the elution of AAF and AF, N-hydroxy-AAF was resolved by initiating a rapid linear gradient (2 min) from 33% to 99% acetonitrile. Gradients longer than 2 min resulted in poorer resolution of N-hydroxy-AAF. The partial resolution of 5-hydroxy-AAF and 7-hydroxy-AAF was achieved by elution with 15% acetonitrile in H_2O (Figure 2).

Determination of N-Hydroxy-AAF Produced by Cells in Culture and by Liver Microsomes

The cells homogenized in 150 mM KCl, media (containing 7 μg/ml of unlabeled carrier N-hydroxy-AAF) and liver microsome incubation mixtures previously incubated with AAF-[9-^{14}C] were all extracted three times with two volumes of chloroform and the extract dried over anhydrous sodium sulfate. The chloroform volume was con-

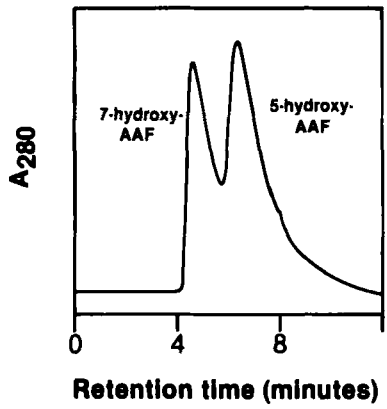

FIGURE 2

Chromatogram of mixture of 5-Hydroxy-AAF and 7-Hydroxy-AAF. Conditions: Column, Waters Bondapak C_{18}/Corasil (2' x 1/2"); elution solvent system, 15% acetonitrile in water; flow rate, 2.4 ml/min; temperature, ambient.

densed with a Rotoevaporator and then further reduced under a stream of nitrogen. A preliminary resolution of the metabolites was made using TLC by streaking the sample on a 250 micron silica gel plate and developing in chloroform:methanol (98:2) in the presence of ammonia fumes. Rf values for standards were 0.21 for N-hydroxy-AAF, 0.43 for AAF, and 0.75 for AF. An area from, but not including, the origin up to the AAF peak was scraped and eluted twice with methanol. This area included N-hydroxy-AAF and ring-hydroxylated products as determined by TLC and HPLC using authentic standards of N-hydroxy-AAF, 7-hydroxy-AAF and 5-hydroxy-AAF. The combined methanol eluates were reduced under a stream of nitrogen and the eluted metabolites further separated using the HPLC method described above.

By extraction with chloroform, 82% of added unlabeled N-hydroxy-AAF (7 μg/ml) was recovered from the media. On elution from silica gel with methanol following TLC, 58% of the N-hydroxy-AAF was recovered, and following HPLC 44% was recovered. Total recovery of N-hydroxy-AAF during the entire isolation procedure (extraction from tissue culture medium, condensation of extract for TLC, elution from silica gel, condensation of eluate and HPLC) was 11%.

Metabolism of AAF by Cultured Cells and Liver Microsomes

When cells were incubated for 6 h in monolayer with 4.8 μg/ml of AAF-[9-^{14}C], hamster hepatocytes metabolized AAF to N-hydroxy-AAF at a rate greater than 50 times that by human embryo and hamster embryo cells (Table 1). Metabolism of AAF to AF was 7-fold greater by human embryo cells than by hamster embryo cells. AF formation with hamster hepatocytes was 500 times greater than with hamster embryo cells.

To determine if treatment of cells in suspension (vs. monolayer) would be more effective for metabolism and transformation studies, human embryo and hamster embryo cells in suspension were treated with 4.8 μg/ml of AAF-[9-^{14}C] for 6 h. The metabolism of

TABLE 1

Metabolism of AAF to N-Hydroxy-AAF and AF by Cultures of Human Embryo and Hamster Embryo Cells, and Hamster Hepatocytes[a]

	pMoles Formed/10^6 Cells		pMoles AAF Metabolized/10^6 Cells
	N-Hydroxy-AAF	AF	
Monolayer culture[b]			
Human embryo cells	13 ± 0.7	2240 ± 90	2240 ± 40
Hamster embryo cells	7.7 ± 0.1	310 ± 30	340 ± 3
Hamster hepatocytes			
1 day old	691	153,000	161,000
2 day old	472	110,000	114,000
Suspension culture[c]			
Human embryo cells	1.0 ± 0.7	345 ± 4	380 ± 1
Hamster embryo cells	7.8 ± 2.1	230 ± 8	300 ± 40

[a] Cells were treated with 4.8 μg/ml of AAF-(9-^{14}C) and incubated for 6 h at 37°.

[b] Values in duplicate are means ± avg dev from 2 groups containing 3 cell incubations per group. Where single values are shown, groups contained 10 cultures.

[c] Values and means ± avg dev from duplicate incubations.

AAF by human embryo cells was significantly lower than metabolism by these cells in monolayer. Metabolism by hamster embryo cells was similar to metabolism in monolayer.

Table 2 shows the formation of N-hydroxy-AAF and AF following the metabolism of AAF by hamster liver microsomes in the presence of NADPH. N-hydroxy-AAF formation was linear between 0.2 mg/ml and 0.4 mg/ml of microsomal protein. Due to competition by

TABLE 2

Metabolism of AAF to N-Hydroxy-AAF and AF by Hamster Liver Microsomes in the Presence of NADPH[a]

Microsomes mg/ml	pMoles Formed/hr[b]	
	N-Hydroxy-AAF	AF
0	0.3 ± 0.1	0 ± 0
0.2	14 ± 2	2260 ± 280
0.4	30 ± 2	2170 ± 240
0.8	42 ± 3	2440 ± 240

[a] Incubation mixtures contained 25 μg/ml of AAF-(9-^{14}C) and 1.5 mM NADPH.

[b] Values are means ± avg dev from triplicate samples.

oxidase and deacetylase enzymes for AAF, AF formation was not linear at these protein concentrations. In the absence of NADPH (and without the competing N-hydroxylation reaction) AF formation was linear between 0.2 mg/ml and 0.4 mg/ml of microsomal protein (Table 3).

DISCUSSION

While the malignant transformation of cells by AAF in the absence of exogenous enzyme systems has not been reported, N-hydroxy-AAF is capable of malignantly transforming hamster embryo cells (7). The inability of AAF to transform cells in culture may be linked to a low rate of hydroxylation of AAF to N-hydroxy-AAF. In animals, the rate of conversion of AAF to N-hydroxy-AAF is related to its carcinogenicity. AAF, a carcinogen in the rat, mouse, and hamster is converted to N-hydroxy-AAF

TABLE 3

Metabolism of AAF to AF by Hamster Liver Microsomes in the Absence of NADPH

Microsomes mg/ml	nMoles AF Formed/Hr[b]
0	0
0.05	4 ± 0.5
0.1	12 ± 1.6
0.2	22 ± 1.3
0.4	33 ± 3.3

[a] Incubation mixtures contained 25 mg/ml of AAF-(9-^{14}C).

[b] Values are means ± avg dev from triplicate samples.

in these species. In the guinea pig, however, AAF is not metabolized to N-hydroxy-AAF and is not carcinogenic (2, 9, 10).

Our studies show that N-hydroxylation of AAF by hamster embryo and human embryo cells in culture occurs at a very low rate while the rate of N-hydroxylation by hepatocytes is 90-fold and 50-fold greater, respectively. This low rate of N-hydroxylation by hamster embryo cells may be a reason why AAF does not malignantly transform these cells.

The determination of pmole levels of N-hydroxy-AAF was made possible by combining a cleanup with TLC and elution of hydroxylated AAF metabolites followed by HPLC. Using a Waters Corasil - C_{18} column and an acetonitrile water elution system, we were able to retain N-hydroxy-AAF on the column while eluting the ring-hydroxylated products, AAF and AF. This system allowed the elution of all significant background radioactivity by delaying the start of the gradient and subsequent elution of N-hydroxy-AAF while still retaining excellent peak conformation.

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