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Note

Separation and identification of metabolites of the arylamide, N-3-fluorenyl-acetamide, by high-pressure liquid chromatography

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The carcinogen, N-fluoren-2-yl-acetamide (N-2-FAA), interacts with cytochrome P_1-450 from hepatic microsomes of 3-methylcholanthrene (3-MC)-treated rats or guinea pigs to produce a type I binding spectrum[1]. The binding constants of N-2-FAA with cytochrome P_1-450 of the 3-MC-treated rat and guinea pig were $1.8 \cdot 10^{-6}$ *M* and $6.7 \cdot 10^{-6}$ *M*, respectively[2]. It has been reported that the isomer, N-fluoren-3-yl-acetamide (N-3-FAA), which is a weak carcinogen[3], also binds to cytochrome P_1-450 of hepatic microsomes of 3-MC-treated rats or guinea pigs to produce a type I binding spectrum[1]. The values of the binding constant for N-3-FAA were similar to those reported for N-2-FAA[2]. These data suggested that N-3-FAA may be metabolized in a similar fashion as N-2-FAA. Accordingly, we intend to extend our previous studies on the microsomal metabolism of N-FAA[1,4] to N-3-FAA. Previous work on the metabolism of N-3-FAA has shown that rats dosed with N-3-FAA excreted three phenolamides, N-(2-hydroxy)fluoren-3-yl-acetamide [N-(2-HO)3-FAA], N-(7-hydroxy)fluoren-3-yl-acetamide [N-(7-HO)3-FAA], and N-(2,7-dihydroxy)fluoren-3-yl-acetamide [N-(2,7-HO)3-FAA] in the urine[5,6]. In this report we present a method utilizing high-pressure liquid chromatography (HPLC) for the separation and identification of N-(2-HO)3-FAA and N-(7-HO)3-FAA which are the major urinary metabolites[6]. In addition, HPLC has been used in this study for the separation and identification of N-(3-acetamido)fluoren-9-ol [N-(9-HO)3-FAA] and N-(3-acetamido)fluoren-9-one [N-(9-oxo)3-FAA]. There is preliminary evidence that these compounds are metabolites of N-3-FAA[2]. It has also been reported recently that the isomeric N-2-FAA undergoes oxidation at C atom 9 *in vivo* and *in vitro* to N-(2-acetamido)fluoren-9-ol [N-(9-HO)2-FAA] and N-(2-acetamido)fluoren-9-one [N-9-oxo)2-FAA][7]. These oxidation products of N-2-FAA were isolated by thin-layer chromatography (TLC) and characterized by nuclear magnetic resonance. In the present study, we include a method for the separation and identification of N-2-FAA, N-(9-HO)2-FAA and N-(9-oxo)2-FAA by HPLC.

MATERIALS AND METHODS

Preparation of compounds

N-(9-HO)3-FAA was prepared by acetylation of 3-aminofluoren-9-ol[8] (0.25 g,

1.3 mmole) in benzene (30 ml) with acetic anhydride (0.16 ml, 1.7 mmole) in presence of triethylamine (0.005 ml) as a catalyst. The precipitate was collected and washed with *n*-hexane; m.p. 208–210°, 0.23 g, 75% yield. Recrystallization from ethanol–water (with Norit A) gave the pure compound, m.p. 210–211°. TLC on silica gel GF_{254} with chloroform–methanol (85:15) gave a single fluorescence-quenching spot, R_F 0.38; $\nu_{max.}^{KBr}$ 3280 (OH), 3090 (NH). 1660 (C=O), 1550 (C–NH) cm^{-1}; $m/e = 239$ (M^+). Calculated for $C_{15}H_{13}NO_2$:C, 75.29; H, 5.47; N, 5.86. Found: C, 75.59; H, 5.54; N, 6.04.

N-(9-oxo)3-FAA was prepared by treating 3-aminofluoren-9-one[8,9] (0.25 g, 13 mmoles) dissolved in a mixture of benzene (35 ml) and toluene (5 ml) with acetic anhydride (0.18 ml, 1.9 mmole) and triethylamine (0.025 ml). The compound was recrystallized from benzene: *n*-hexane; m.p. 219–221°, 0.15 g, 51% yield; $m/e = 237$ (M^+); $\nu_{max.}^{KBr}$ 3360 (sharp, NH), 1700 (C=O), 1550 (C–NH), cm^{-1}. Calculated for $C_{15}H_{11}NO_2$:C, 75.93; H, 4.67; N, 5.90. Found: C, 76.07; H, 4.90; N, 5.91.

N-(2-HO)3-FAA, m.p. 230–231°, was synthesized as described[5].

N-(7-HO)3-FAA, m.p. 268–272°, $m/e = 239$ (M^+), was obtained by a modification of the literature procedure[6]. Calculated for $C_{15}H_{11}NO_2$: C, 75.30; H, 5.48; N, 5.60. Found: C, 75.19; H, 5.57; N, 5.79.

N-(9-HO)2-FAA was prepared by acetylation of 2-aminofluoren-9-ol[8,10] (0.041 g, 0.21 mmole) in benzene (20 ml) with acetic anhydride (0.04 ml, 0.42 mmole) and triethylamine (0.05 ml). The precipitate was collected and washed with *n*-hexane, The compound, m.p. 249–250°, 0.035 g, 70% yield, gave a single fluorescence-quenching spot on TLC with chloroform–methanol (9:1); $m/e = 239$ (M^+).

N-(9-oxo)2-FAA was obtained by acetylation of 2-aminofluoren-9-one (Aldrich, Milwaukee, Wisc., U.S.A.) (0.20 g, 1.03 mmole) in benzene (9 ml) with acetic anhydride (0.20 ml, 2.1 mmole). The precipitate was collected and recrystallized from 95% ethanol and then from benzene–*n*-hexane; m.p. 233–236° (reported: 235–235.5°[10]). 0.17 g, 69% yield. TLC of the compound on silica gel GF_{254} with chloroform–methanol (95:5) gave a single, fluorescence-quenching spot, R_F 0.26.

HPLC conditions

The columns used in the present experiments were made of stainless-steel tubing (Supelco, Bellefonte, Pa., U.S.A.) and operated at room temperature (25–26°). The length of the column was 1.2 m and the I.D. was 2.0 mm. The columns were connected to a 500-ml solvent reservoir (Varian Aerograph, Walnut Creek, Calif., U.S.A.). Pressure (70 lbs./in.^{2}, unless otherwise stated) was applied by a gas tank filled with helium and regulated by a pressure-reducing valve. The adsorbent was Corasil II (particle size 37–50 μm) obtained from Waters Assoc. (Milford, Mass., U.S.A.). The columns were packed with a dry-column packer (Chromatronics, Berkeley, Calif., U.S.A.). The eluting solvent was diethyl ether–*n*-hexane (9:1) which was degassed by boiling prior to use. The adsorbance of the effluent relative to air was monitored at 280 nm by means of a double-beam monitoring unit with an optical path-length of 10 mm (Pharmacia, Piscataway, N.J., U.S.A.). The change in absorbance was recorded with a strip-chart recorder (Model SR 204; Heath-Schlumberger, Benton Harbor, Mich., U.S.A.). Prior to use the columns were washed with the eluting solvent for 0.5 h. The compounds (0.1–1.0 μg) dissolved in ethyl acetate (5–

25 μl) were injected into the column by means of a Pressure-Lok liquid syringe C-160 (Precision Sampling, Baton Rouge, La., U.S.A.).

RESULTS AND DISCUSSION

Fig. 1A shows the separation of a mixture of N-3-FAA, N-(9-oxo)3-FAA and

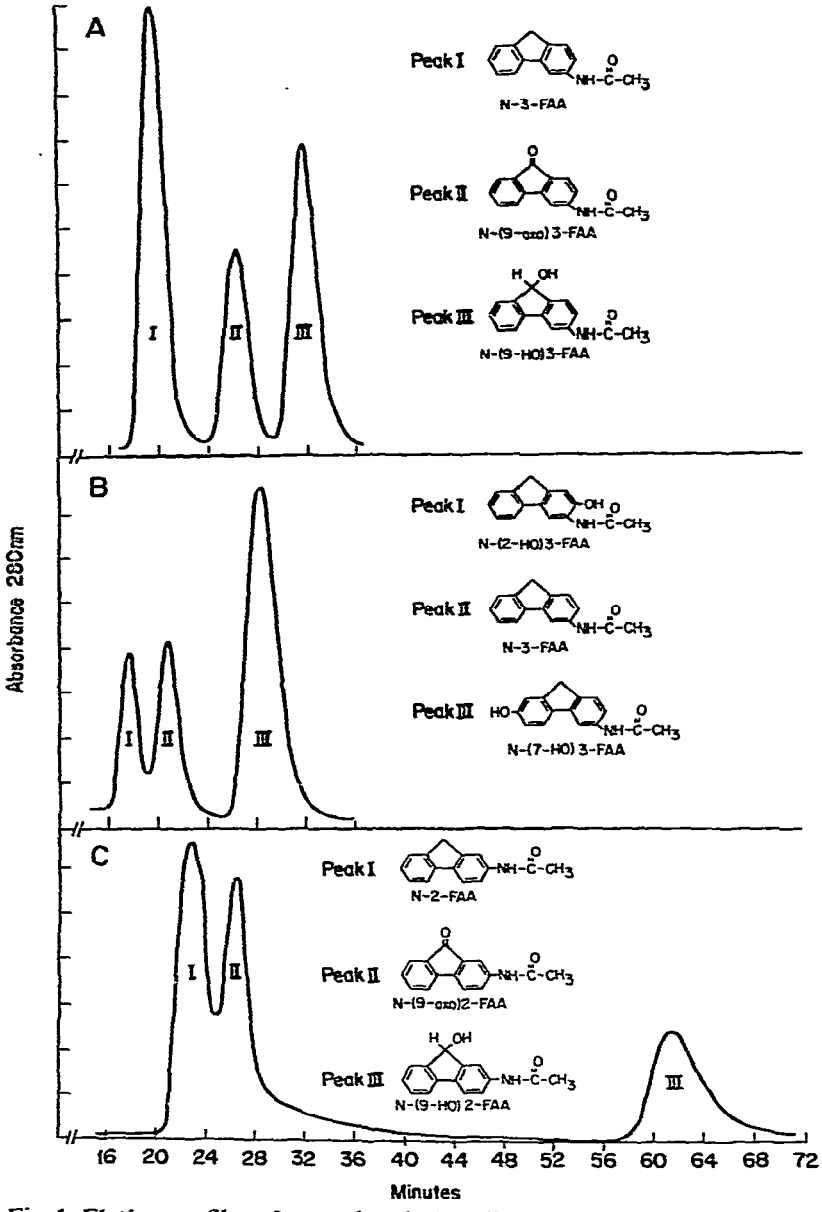

Fig. 1. Elution profiles of neutral and phenolic derivatives of N-3-FAA and N-2-FAA. The HPLC conditions are described in the text. A, Neutral derivatives of N-3-FAA; B, phenolic derivatives of N-3-FAA; C, neutral derivatives of N-2-FAA.

TABLE I

RETENTION TIMES OF N-3-FAA AND N-2-FAA AND THEIR DERIVATIVES

The HPLC conditions were those described in the text. The values are the mean retention times of the number of runs listed. The standard deviation from the means were 1% or less.

Compound	*Number of runs*	*Retention time (min)*
N-3-FAA	11	20
N-(9-oxo)3-FAA	7	24
N-(9-HO)3-FAA	7	31
N-(2-HO)3-FAA	6	16
N-(7-HO)3-FAA	4	26
N-2-FAA	6	23
N-(9-oxo)2-FAA	6	27
N-(9-HO)2-FAA	6	62

N-(9-HO)3-FAA on Corasil II with diethyl ether–*n*-hexane (9:1) as eluent. The average retention times of these three compounds are given in Table I. It may be seen from Fig. 1 and Table I that N-3-FAA is clearly separated from its oxidation products, N-(9-HO)3-FAA and N-(9-oxo)3-FAA. Fig. 1B shows the elution profile of N-3-FAA and its phenolic metabolites, N-(2-HO)3-FAA and N-(7-HO)3-FAA. The data indicate that the retention times of the N-(7-HO)3-FAA and of the C-9 oxidation products of N-3-FAA overlap. However, this will present no problem in the projected metabolic studies since phenolic and neutral derivatives of the substrate, N-3-FAA, are separated by solvent partition prior to HPLC[4]. Fig. 1C depicts the separation of N-2-FAA from its neutral oxidation products, N-(9-HO)2-FAA and N-(9-oxo)2-FAA. These compounds appear to be minor metabolites of N-2-FAA[7]. We have found that the retention time of N-(9-HO)2-FAA (62 min, Table I) can be shortened to 40 min with the use of diethyl ether at an inlet pressure of 80 lbs./in.^{2} instead of a mixture of diethyl ether–*n*-hexane. Under these conditions, these paration of the two oxidation products from the substrate, N-2-FAA, is still excellent. All of the compounds subjected to HPLC in this investigation showed a linear relationship between peak height and amount applied to the column (Fig. 2). Similar linearity for

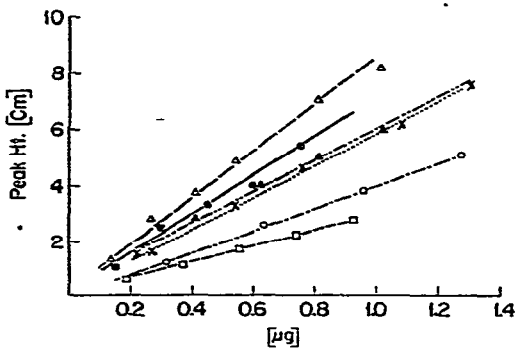

Fig. 2. Relation between peak height and amount of compound subjected to HPLC. The HPLC conditions are described in the text. Each value represents the mean of 2–3 determinations. □- -□, N-(9-HO)3-FAA; ○-·-○, N-(7-HO)3-FAA; ×···×, N-(9-HO)2-FAA; ▲··—··▲, N-(9-oxo)2-FAA; ●—●, N-(2-HO)-3-FAA; △--△, N-(9-oxo)3-FAA.

other derivatives of N-2-FAA and N-3-FAA on HPLC has been demonstrated[11,12]. Due to its sensitivity, power of separation and relatively rapid elution of the applied compounds, the method described here appears superior to paper chromatography and TLC which have been used previously for the separation and identification of compounds of this type[5-7]. Preliminary data with microsomal preparations of the liver of the rat and guinea pig indicate that N-(9-HO)3-FAA and N-(9-oxo)3-FAA are major metabolites of N-3-FAA[2].

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