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IDENTIFICATION OF CARCINOGENIC ACETATES OF FLUORENYLHYDROXAMIC ACIDS BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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SUMMARY

A method for the identification and quantitation of carcinogenic O-acetates of fluorenylhydroxamic acids by high-pressure liquid chromatography is described. The adsorbent is Corasil II and a mixture of ethyl acetate–*n*-hexane (1 : 1) is used as the solvent. N-Acetoxy-2-fluorenylacetamide or N-acetoxy-3-fluorenylacetamide are separable as single peaks from N-acetoxy-4-fluorenylacetamide and from N-acetoxy-2-fluorenylbenzamide. The peak height traced by the recorder is linearly proportional to the amount of compound in the effluent. The method can be utilized for the detection of 0.1–1.0 μg of compound. The method has been used to identify the products of the decomposition of N-acetoxy-2-fluorenylacetamide in aqueous media.

INTRODUCTION

It is generally accepted that the carcinogen N-2-fluorenylacetamide (2-FAA) acquires biological activity by sequential metabolic activation to a reactive form. In the first step which appears to be obligatory for carcinogenesis, the arylamide is converted by the hepatic mixed-function oxidase to the respective arylhydroxamic acid, N-hydroxy-2-FAA[1–3]. N-Hydroxy-2-FAA, although a highly active carcinogen for the rat by a variety of routes[4–6] and a transforming agent for rat-embryo fibroblasts in culture[7], may not be the final reactive form (ultimate carcinogen). There is indirect evidence that N-hydroxy-2-FAA is esterified to a sulfate, N-sulfoxy-2-FAA, by the hepatic sulfotransferase(s) of the rat[8–10]. The sulfate ester dissociates into an electrophilic arylamidonium ion which is thought to initiate hepatocarcinogenesis by interaction with cellular macromolecules[8]. However, formation of the sulfate ester of N-hydroxy-2-FAA appears to be restricted to the liver. The evidence indicates that sulfation does not occur in any of the extrahepatic tissues of the rat susceptible to the action of 2-FAA[6,11] or in rat-embryo cells in culture[7]. In these instances, it is theoretically possible that the acetate ester, N-acetoxy-2-FAA, is formed and may give rise to an electrophilic reactant in the same way as the sulfate ester. This supposition is based on the formation of N-acetoxy-2-FAA upon reaction of N-hydroxy-2-FAA with acetyl CoA[12] as well as on the demonstration that N-acetoxy-2-FAA is

a transforming agent for cultured embryonic cells of the rat and of the hamster[7,13]. Although N-acetoxy-2-FAA can be obtained by synthesis in pure form[14,15], isolation of the ester from biological systems and its subsequent quantitation is complicated by the instability of the compound upon thin-layer chromatography (TLC) on silica gel[16] and by its tendency to decompose in polar solvents[17]. In the present work, we have identified, by high-pressure liquid chromatography (HPLC), the heretofore unknown products of the decomposition of N-acetoxy-2-FAA in aqueous media. The identification of these products in a biological system containing N-hydroxy-2-FAA as a substrate would provide evidence, therefore, for the formation of N-acetoxy-2-FAA by acetylation of the hydroxamic acid. N-Acetoxy-3-FAA, N-acetoxy-4-FAA, and N-acetoxy-2-fluorenylbenzamide (N-acetoxy-2-FBA), which are reasonably stable in polar solvents, may be identified directly by HPLC as described below. N-Acetoxy-3-FAA and N-acetoxy-2-FBA as well as the respective arylhydroxamic acids, N-hydroxy-3-FAA and N-hydroxy-2-FBA, are potent carcinogens for the rat[5,18]; and N-acetoxy-3-FAA, N-hydroxy-3-FAA, and N-hydroxy-2-FBA are also transforming agents *in vitro*[7]. The transforming ability of N-acetoxy-2-FBA has not been investigated thus far. With the use of HPLC, we are now in a position to determine whether the carcinogenic fluorenylhydroxamic acids, N-hydroxy-2-FAA, N-hydroxy-3-FAA, and N-hydroxy-2-FBA are metabolized by tissue preparations and cells in culture to the carcinogenic acetate esters. If this were the case, the carcinogenicity of the hydroxamic acids, may, in fact, be due to the formation of the acetates. Experiments along these lines are in progress.

MATERIALS AND METHODS

Preparation of compounds

N-Acetoxy-2-FAA[15], m.p. 108–109°, N-acetoxy-3-FAA[19], m.p. 104–105°, N-acetoxy-4-FAA[18], m.p. 105–107°, N-acetoxy-2-FBA[5], m.p. 130–132°, N-(1-hydroxy)-2-FAA[20], m.p. 216–219°, and N-(3-hydroxy)-2-FAA[21], m.p. 247–249°, were prepared by the procedures described in the literature. N-(3-Acetoxy)-2-FAA, m.p. 233°, was obtained by a modification of the previously published procedure[22]. To a solution of the hydrochloride of N-(3-hydroxy)-2-fluorenamine (0.15 g, 0.65 mmole) in pyridine (5 ml) was added acetic anhydride (0.25 ml). The mixture stood at room temperature for 4 h and was then poured into cold water. The precipitate was collected, washed with water and recrystallized from 95% ethanol. TLC of the recrystallized material on silica gel GF_{254} (rapid-plate; Woelm, Eschwege, G.F.R.) with chloroform–methanol (95:5) as a solvent gave a single fluorescence-quenching spot, $R_F = 0.50$. The infrared spectra of the above compounds matched those of authentic samples. N-(1-Acetoxy)-2-FAA, whose preparation had not been described previously, was obtained by treating N-(1-hydroxy)-2-FAA[20] (0.15 g, 0.63 mmole) in pyridine (5 ml) with acetic anhydride (0.30 ml). After the reaction mixture had stood at room temperature for 4 h, the precipitate was collected and washed with water. The crude material was recrystallized successively from 95% ethanol and from benzene to yield the pure product (0.13 g, 73% yield), m.p. 197–198°; ν_{max}^{KBr} 3350 (N–H), 1740 (O–C=O), 1690 (N–H) cm^{-1}. TLC on silica gel GF_{254} (rapid-plate; Woelm) with ethyl acetate–benzene (7:3) as a solvent gave a single fluorescence-quenching spot, $R_F = 0.28$. Calculated for $C_{17}H_{15}O_3N$: C, 72.58; H, 5.37; N, 4.98. Found: C, 72.55; H, 5.45; N, 5.25.

Conditions of HPLC

The equipment used in the present experiments (column length, internal diameter of columns, solvent reservoir, pressure device, optical unit monitoring the column effluent) was that previously described[23]. The absorbance of the effluent relative to air was recorded with a strip chart recorder, Model 7101 BM (Hewlett-Packard, Palo Alto, Calif., U.S.A.). The adsorbent was Corasil II (particle size 37–50 μm) (Waters Ass., Milford, Mass., U.S.A.) and the solvent was ethyl acetate–*n*-hexane (1:1) throughout. The columns were packed mechanically with a dry-column packer (Chromatronix, Berkeley, Calif., U.S.A.) and operated at room temperature (25–26°) with an inlet pressure of 80 lbs./in.^{2}. The compounds (0.2–1.0 μg) in ethyl acetate or ethyl acetate–*n*-hexane (1:1) (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.).

Incubation of N-acetoxy-2-FAA in aqueous medium and isolation of the reaction products prior to HPLC

N-Acetoxy-2-FAA (0.1 μmole) in acetone (0.1 ml) was added to a 0.01 *M* phosphate buffer (10 ml), pH 7.4, containing inorganic salts in the following final concentrations: NaCl (137 m*M*), KCl (2.7 m*M*), $CaCl_2$ (0.9 m*M*) and $MgCl_2 \cdot 6\ H_2O$ (0.5 m*M*). Three separate mixtures were incubated for 2 h at 37°. The mixtures were then combined, cooled to 4° and extracted with diethyl ether (2 × 30 ml). The ether was washed once with cold water (60 ml), dried (anhydrous sodium sulfate) and evaporated under a stream of nitrogen. The residue was dissolved in ethyl acetate–*n*-hexane (1:1) (0.3 ml) and aliquots (10–20 μl) of this solution were injected on to the column.

RESULTS AND DISCUSSION

The mean retention times of N-acetoxy-fluorenylacetamides are listed in Table I and representative elution profiles of the compounds are shown in Fig. 1. All compounds were eluted reproducibly as single, sharp peaks. Although the method was not designed to resolve mixtures of N-acetoxy-fluorenylacetamides, we have readily separated N-acetoxy-2-FAA or N-acetoxy-3-FAA from N-acetoxy-4-FAA and N-acetoxy-2-FBA when a mixture of these compounds was applied to the column.

TABLE I

RETENTION TIMES OF THE ACETATES OF FLUORENYLHYDROXAMIC ACIDS CHROMATOGRAPHED ON CORASIL II

The HPLC conditions were those described in the text.

Compound	*Number of runs*	*Retention time (min)* *
N-Acetoxy-2-FAA	14	9.7
N-Acetoxy-3-FAA	12	8.9
N-Acetoxy-4-FAA	14	7.7
N-Acetoxy-2-FBA	16	6.8

* The values are the mean retention times of the number of runs listed. The average deviations from the means were $< \pm 3\%$.

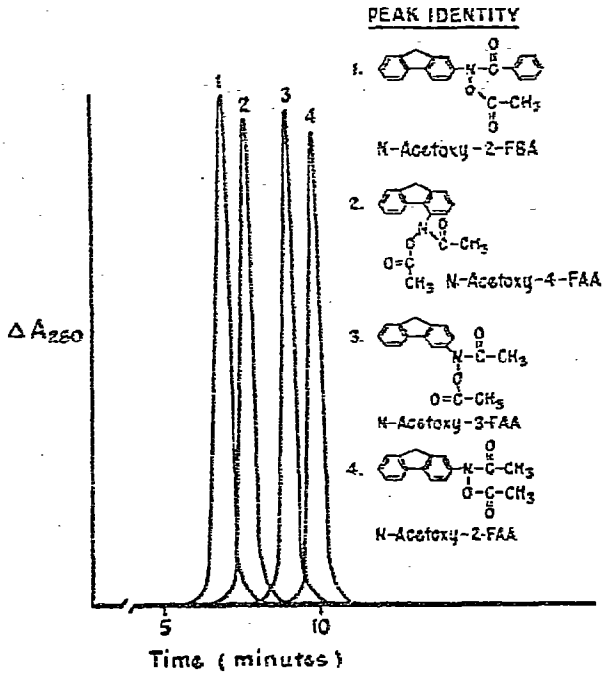

Fig. 1. Composite elution profile of N-acetoxy-fluorenylamides. Each compound was chromatographed on Corasil II as described in the text.

The separation of N-acetoxy-2-FAA from N-acetoxy-3-FAA is, however, not possible under the present experimental conditions. Fig. 2 shows that the peak height traced by the recorder is proportional to the amount of compound applied to the column within a range of 0.2–1.0 μg. Therefore, peak height is a convenient measure of the amount of compound in the effluent. Since as little as 0.1 μg of the acetate esters is readily detectable, the sensitivity of HPLC for the detection and quantitative estimation of these compounds is, in our experience, considerably greater than that of TLC. The ease of quantitation and the speed of the procedure are additional advantages that make HPLC the method of choice for the identification of these compounds.

HPLC is applicable to the identification of N-acetoxy-2-FAA isolated from non-polar media in which the ester is comparatively stable (Fig. 1). However, in polar solvents and especially in the aqueous media employed in biological experiments N-acetoxy-2-FAA decomposes and cannot be identified directly. Since the formation of N-acetoxy-2-FAA in biological systems is of major concern for the reasons stated above, the only approach open to us was the identification of the products resulting from the decomposition of N-acetoxy-2-FAA. Fig. 3A shows the typical elution profile of an ethereal solution that was obtained by extraction of an aqueous incubation system containing N-acetoxy-2-FAA and then subjected to HPLC. A compound with a retention time of 9.7 min corresponding to N-acetoxy-2-FAA was not seen. Instead, compounds A, B, C, D, E and F were noted. Compounds A and B were always seen when the medium was extracted with diethyl ether and when the

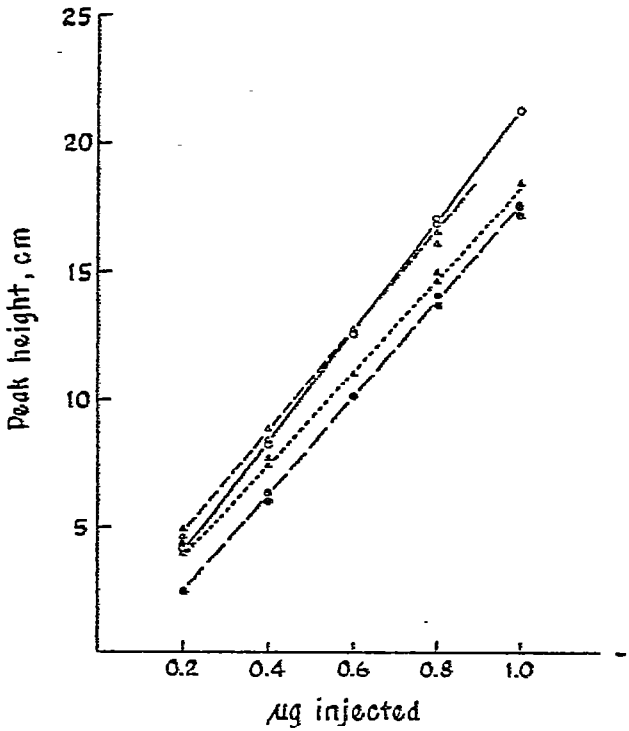

Fig. 2. Relation between peak height and amount of N-acetoxy-fluorenylamide subjected to HPLC. The HPLC conditions are described in the text. ●, N-Acetoxy-2-FAA; ○, N-acetoxy-3-FAA; ▲, N-acetoxy-4-FAA; △, N-acetoxy-2-FBA.

extracts were subjected to HPLC. These compounds are not derived, therefore, from N-acetoxy-2-FAA. Compound C has not been identified as yet. Compounds D, E and F had the same retention times as N-(1-hydroxy)-2-FAA, N-(3-hydroxy)-2-FAA and N-(1-acetoxy)-2-FAA, respectively (Fig. 3B, Table II). The identity of compounds D, E and F with the *o*-amidofluorenols and N-(1-acetoxy)-2-FAA, respectively, was confirmed by co-chromatography of the ether extract with the authentic compounds (Fig. 3C). Liquid chromatography of an aliquot of the ethereal extract on Carbowax 400/Porasil C, a method previously described for the detection and identification of fluorenylhydroxamic acids[23], gave no evidence for N-hydroxy-2-FAA. This excluded decomposition of N-acetoxy-2-FAA to the hydroxamic acid. The data summarized in Table III lead us to conclude that N-acetoxy-2-FAA in aqueous media undergoes a rearrangement in which the acetoxy group migrates to carbon-atom 1 of the fluorene moiety to yield N-(1-acetoxy)-2-FAA. The latter compound is then partially hydrolyzed to N-(1-hydroxy)-2-FAA. N-(3-Hydroxy)-2-FAA arises from the electrophilic attack of hydroxyl groups from the medium on the positively charged carbon-atom 3 of the resonance form of the arylamidonium ion that results from the dissociation of N-acetoxy-2-FAA in polar media[17]. This follows from the observation that N-(3-hydroxy)-2-FAA was also identified by HPLC in incubation mixtures that were extracted immediately after the addition of N-acetoxy-2-FAA to the buffer (Table III). Under these conditions, the elution profile of the extract showed no trace

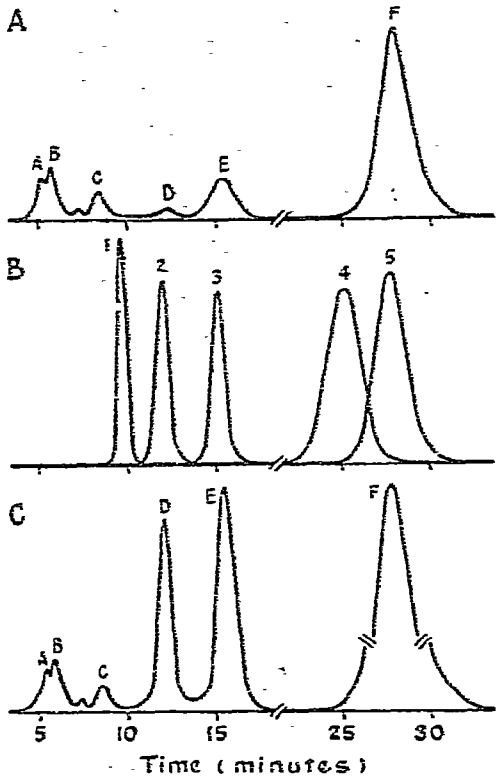

Fig. 3. Identification of the products of the decomposition of N-acetoxy-2-FAA in phosphate buffer. A, Elution profile of an ethereal extract obtained after incubation of N-acetoxy-2-FAA in phosphate buffer for 2 h at 37°. The conditions of incubation, extraction, and HPLC are described in the text. B, Elution profile of N-acetoxy-2-FAA (1), N-(1-hydroxy)-2-FAA (2), N-(3-hydroxy)-2-FAA (3), N-(3-acetoxy)-2-FAA (4) and N-(1-acetoxy)-2-FAA (5). C, Co-chromatography of an ethereal extract obtained after incubation of N-acetoxy-2-FAA in phosphate buffer as described above and a mixture of compounds 2, 3, and 5 listed under B. The increase in the height of peaks D, E, and F was accounted for by the amounts of compounds 2, 3, and 5 added to the extract.

TABLE II

RETENTION TIMES OF N-*o*-ACETOXY FLUORENYLACETAMIDES AND OF THE CORRESPONDING *o*-HYDROXYFLUORENYLACETAMIDES CHROMATOGRAPHED ON CORASIL II

The HPLC conditions were those described in the text.

Compound	*Number of runs*	*Retention time (min)**
N-(1-Acetoxy)-2-FAA	3	27.5
N-(3-Acetoxy)-2-FAA	3	25.4
N-(1-Hydroxy)-2-FAA	3	12.1
N-(3-Hydroxy)-2-FAA	3	15.3

* The values are the mean retention times of the number of runs listed. The average deviations from the means were $< \pm 3\%$.

TABLE III

CHROMATOGRAPHIC EVIDENCE FOR THE REARRANGEMENT OF N-ACETOXY-2-FAA AND FOR THE STABILITY OF N-(1-ACETOXY)- AND N-(3-ACETOXY)-2-FAA IN PHOSPHATE BUFFER

*Compound incubated**	*Incubation time (h)*	*Compounds identified by HPLC***	*Yield (%)****
N-Acetoxy-2-FAA	0	N-Acetoxy-2-FAA	70
		N-(3-Hydroxy)-2-FAA	10
	2	N-(1-Acetoxy)-2-FAA	60
		N-(3-Hydroxy)-2-FAA	5
		N-(1-Hydroxy)-2-FAA	<5
N-(1-Acetoxy)-2-FAA	0	N-(1-Acetoxy)-2-FAA	70
	2	N-(1-Acetoxy)-2-FAA	65
		N-(1-Hydroxy)-2-FAA	10
N-(3-Acetoxy)-2-FAA	0	N-(3-Acetoxy)-2-FAA	65
		N-(3-Hydroxy)-2-FAA	<5
	2	N-(3-Acetoxy)-2-FAA	60
		N-(3-Hydroxy)-2-FAA	5

* Equimolar amounts of each compound dissolved in acetone were incubated in a phosphate buffer as described in the text.
** The conditions of chromatography are described in the text.
*** Estimated by reference to calibration curves obtained as described in the text.

of N-(3-acetoxy)-2-FAA. Moreover, N-(3-acetoxy)-2-FAA that had been incubated in phosphate buffer at 37° for 2 h was recovered in good yield by ether extraction of the incubation system and was readily identified by HPLC (Table III). An *ortho* rearrangement of N-acetoxy-2-FAA has been reported upon TLC of the ester on silica gel[16]. This acid-catalyzed rearrangement differs from that observed here in that the major product of the reaction was N-(3-acetoxy)-2-FAA rather than N-(1-acetoxy)-2-FAA, the principal product of the rearrangement in aqueous media at neutral pH. For practical purposes, the simultaneous identification of N-(1-acetoxy)-2-FAA and of N-(1-hydroxy)- and N-(3-hydroxy)-2-FAA in the elution profile of an ethereal extract of a biological system containing a potential precursor of N-acetoxy-2-FAA would be strong evidence for the formation and transient existence of this carcinogen.

ACKNOWLEDGEMENT

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