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Note

Analysis of 2-acetylaminofluorene and its metabolites by gas chromatography with electron-capture detection

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2-Acetylaminofluorene (2-AAF) is a well-studied model hepatocarcinogen. In the presence of the cytochrome P-450-dependent mixed function oxidase enzyme system it is metabolized to N- as well as ring-hydroxylated products. The N-hydroxy derivative is considered to be a more proximal carcinogen[1–3]. Ring hydroxylation, conjugation and elimination are considered to be detoxification processes[4].

Busk and Ahlborg[5] have studied the effect of retinol (vitamin A) as a modifier of the mutagenesis of 2-AAF and 2-aminofluorene (2-AF) in the Salmonella/microsome assay[6]. They have reported that low amounts of retinol increased the mutagenicity whereas at higher doses it remained unchanged. Formation of different proportions of active and inactive metabolites could be a factor in the modification of mutagenic activity, and hence quantitation of these metabolites is important.

Thin-layer chromatography[7] and liquid chromatography[8] are time-consuming or lack sensitivity. High-performance liquid chromatography (HPLC) has been used to analyse 2-AAF and metabolites in urine[9,10]. However, interferences can occur. Low amounts of the metabolites can also be measured using a scintillation counter when radioactive 2-AAF is used[11].

In our laboratory, 2-AAF and metabolites after incubation with rat liver homogenate are monitored by HPLC, with both UV and scintillation detection[15]. However, to provide further confirmation of the results it was decided to develop a sensitive and specific gas chromatographic (GC) assay. Razzouk and co-workers[12,13] and Batardy-Gregoire *et al.*[14] have reported sensitive methods to quantitate some of the 2-AAF metabolites using GC with electron-capture detection (ECD). We used a modification of their procedure to estimate 2-AAF and 1-OH, 3-OH, 5-OH, 7-OH,

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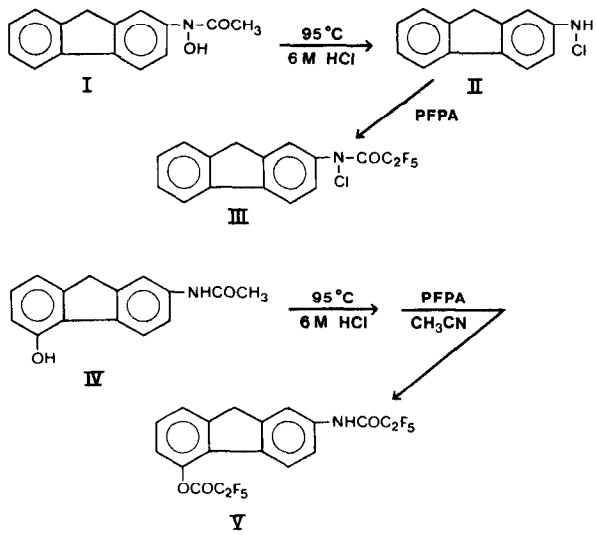
9-OH and N-OH-AAF as their pentafluoropropionyl derivatives from a mixture of these substances. The following reaction scheme was used.



EXPERIMENTAL

Reagents and chemicals

The unlabelled metabolites, 1-, 3-, 5-, 7-, 9- and N-hydroxy-2-acetylaminofluorene were generously provided by Dr. J. N. Keith (Illinois Institute of Technology, Chicago, IL, U.S.A.). 2-AAF (96% pure) was purchased from EGA Chemie (Steinheim, F.R.G.). N-[9-^{14}C]-2-acetylaminofluorene with a specific activity of 52.0 mCi/mmol was obtained from New England Nuclear (Boston, MA, U.S.A.). Pentafluoropropionic anhydride (Supelco), Aquasil (Pierce) and 2-aminobiphenyl were purchased from Novakemi (Stockholm, Sweden). Acetonitrile, benzene, methanol and other solvents (pesticide grade) and chemicals (reagent grade) were supplied by Labkemi (Kebo Grave, Stockholm, Sweden).

Columns and conditions for GC–ECD

A Varian 3700 GC was fitted with a 5% OV-225 glass column and a ^{63}Ni electron-capture detector. In this system, we found that a few picograms of the derivatives on the column could be detected. However, attempts to separate a mixture of the metabolites (1-OH, 3-OH, 5-OH, 7-OH, 9-OH, N-OH-2-AAF and 2-AAF) were not successful. Other systems using OV-17, SE-52, OV-1 and combinations or variation in the operating conditions did not provide adequate separation. The separation was, however, achieved by using a capillary column.

The instrumentation consisted of a Carlo Erba Fractovap (Series 4160), LT Programmer (Model 400), ECD control (Model 251) and a ^{63}Ni electron-capture detector (Model HT 25). A Vitatron strip chart recorder was operated at 1 mV for

full scale deflection. A fused-silica column (20 m × 0.31 mm I.D.) coated with SE-52 (cross-linked, df = 0.37 μm) was installed to operate in the "on column injection" mode. Hydrogen (1.5 ml/min) was used as carrier gas and 5% argon–methane (20 ml/min) as the make-up gas. The instrument was operated in the constant-current mode, and the operation was programmed from 70 to 210°C with the detector temperature at 250°C.

Derivatization and relative calibration

Pyrex culture tubes (10 ml) were silanized with a 1% aqueous solution of Aquasil. A standard methanol solution of 2-AAF and metabolites was prepared containing: 1-OH, 2.1 μg; 3-OH, 2.28 μg; N-OH, 1.2 μg; 5-OH, 2.6 μg; 7-OH, 2.0 μg; 9-OH-AAF, 2.74 μg and 2-AAf, 2.0 μg per ml, respectively.

A solution of 2-aminobiphenyl (9 μg/ml) was used as the internal standard. From 50 to 500 μl of the metabolite standard solution and 100 μl of the marker solution were placed in the culture tubes. The methanol was evaporated to dryness with a current of nitrogen. Hydrochloric acid (6 *M*, 0.5 ml) was added and the mixture was heated at 95°C, for 1 h. The tubes were cooled and centrifuged, and the aqueous solution was evaporated to dryness at 40°C under vacuum. Acetonitrile (0.5 ml), 1% triethylamine in benzene (200 μl) and pentafluoropropionic anhydride (50 μl) were now added, and the tubes were allowed to stand at room temperature for 1 h. The solvents were removed at 40°C under vacuum. Benzene (5 ml) was added, and the tubes were vortexed for 1 min. The benzene solution was washed twice with 2 ml of phosphate buffer (pH 6), and 1 μl was injected into the column. The operation was programmed from 70 to 210°C.

The peaks were recorded on a Vitatron chart recorder (1mV full scale) (Fig. 1). Peak height ratios were plotted against weight ratios to obtain calibration curves. The standard curves for N-OH-2-AAF and 5-OH-AAF are given as examples (Fig. 2).

Five replicate analyses with 100 μl of the standard mixture and 50 μl of the internal standard were performed, and the coefficient of variation (C.V.) was calculated from the peak height ratios. The following C.V. was obtained for the various metabolites: 9-OH-AAF, 0.073; 2-AAF, 0.066; N-OH-AAF, 0.124; 5-OH-AAF, 0.153; 7-OH-AAF, 0.094 and 3-OH-AAF, 0.125.

Each of the metabolites, 2-AAF and 2-aminobiphenyl were individually derivatized and injected into the column under the same conditions to determine their retention times. The various derivatives were also examined by GC–MS to establish their structure. The results will be published elsewhere.

To check the efficiency of the derivatization procedure the following scheme was employed. [^{14}C]-2-AAF was incubated with rat liver homogenate, and the mixture of 2-AAF and metabolites was isolated and recovered as a methanol solution[15]. A small amount of this solution (200 μl containing *ca.* 2 μg and radioactivity *ca.* $1.2 \cdot 10^6$ dpm) was derivatized as before. (The aqueous and organic phases were saved.) When the sample was injected, the chromatogram showed peaks corresponding to 2-AAF, N-OH-2-AAF, 9-OH-2-AAF, 5-OH-2-AAF and 7-OH-2-AAF. There were hardly any other peaks.

The pooled aqueous and organic phases were evaporated to dryness. The residues were added to 5 ml of PCS xylene cocktail, sonicated for 5 min and centrifuged,

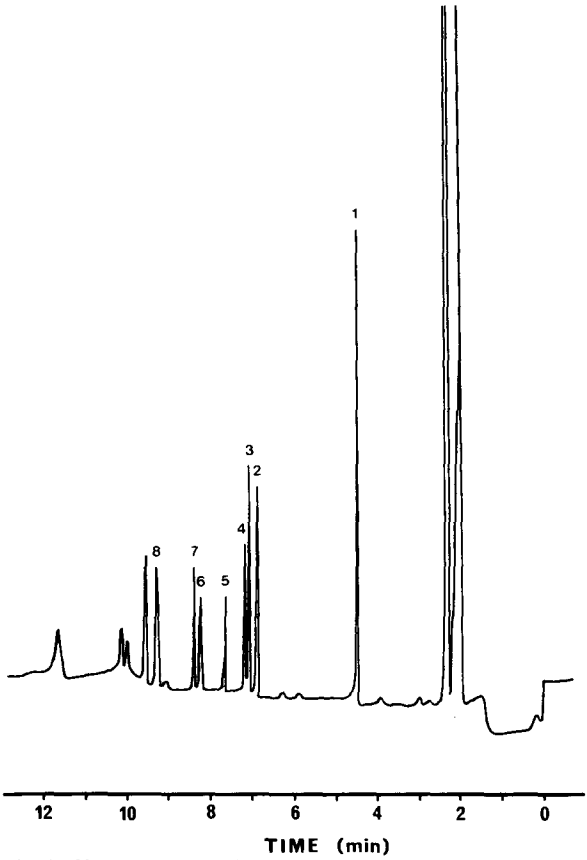


Fig. 1. Chromatogram (electron-capture detection) of a mixture of 2-AAF and its metabolites (PFP derivatives) separated on an SE-52 fused-silica capillary column. Initial temperature, 70°C; programmed to 190°C at 40°C/min and then to 210°C/min for 12 min. Peaks: 1 = 2-ABP; 2 = 1-OH-AAF; 3 = 9-OH-AAF; 4 = 3-OH-AAF; 5 = 2-AAF; 6 = N-OH-AAF; 7 = 5-OH-AAF; 8 = 7-OH-AAF. The peaks correspond to *ca.* 20–40 pg of the various PFP derivatives injected into the column at an attenuation of 256 (on-column injection mode).

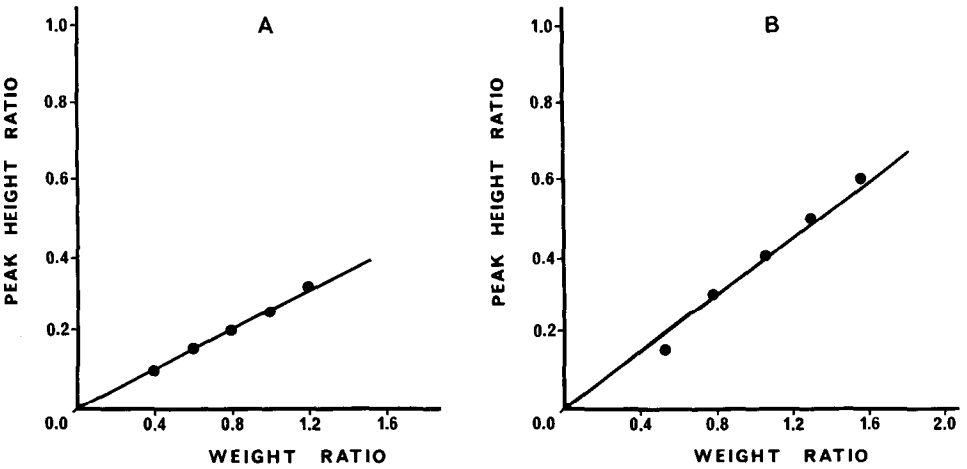


Fig. 2. Relative calibration curves of (A) N-OH-AAF *vs.* 2-ABP and (B) 5-OH-AAF *vs.* 2-ABP, with electron-capture detection.

and the clear solutions were used for scintillation. The radioactivity in the aqueous and benzene phases was calculated from a quenching curve. It was noticed that only *ca.* 3% of the total radioactivity remained in the aqueous phase. This clearly demonstrates that the derivatization procedure is very satisfactory.

RESULTS AND DISCUSSION

The pentafluoropropionyl (PFP) derivatives of 2-AAF and its metabolites are very sensitive to electron-capture detection. Picogram amounts injected on the column can be quantitated. It was noticed in our studies that the PFP derivative from N-OH-2-AAF (III) was *ca.* 5 times more sensitive than the corresponding trifluoroacetyl derivative. In our preliminary studies, with a fused-silica column (20 m × 0.21 mm I.D.) coated with SE-52 used in the "split-splitless" mode, we had a better separation of the 9-OH and 3-OH metabolites than in the present study. However, too much baseline drift made it unfeasible for our work.

The derivatization procedure is quite effective, as shown from the studies using [^{14}C]-2-AAF. The standard curves are linear for at least 15–1000 pg injected on the column, and there is very little scatter except for the 1-OH-metabolite. In dilute solution, this compound decomposes rapidly on standing for a few hours, at room temperature. Even the 3-OH derivative decomposes on standing, though less rapidly. Since this phenomenon is time-dependent, adsorption on the glass surface might not be the reason. It is more likely that inherent instability or hydrolysis by the alkaline glass surface causes this problem. The stability of perfluoroacyl esters of phenols in the presence of water has been studied by Ehrsson *et al.*[16] and Lamparsky and Westrick[17]. Such esters are stable in the presence of water if the pH of the aqueous phase is less than 6. Batardy-Gregoire *et al.*[14] also found that the HFB derivatives of ring-hydroxylated metabolites of 2-AAF readily decompose on standing. The molecular structures of these derivatives have been established by GC–MS and will be reported elsewhere.

In our preliminary studies, metabolite formation on incubation with rat liver homogenate shows a minimum when 10 μg of vitamin A was added to the incubation mixture. The methodology reported here does not enable one to differentiate between 2-AAF and 2-AF. Further work is in progress to solve this problem.

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