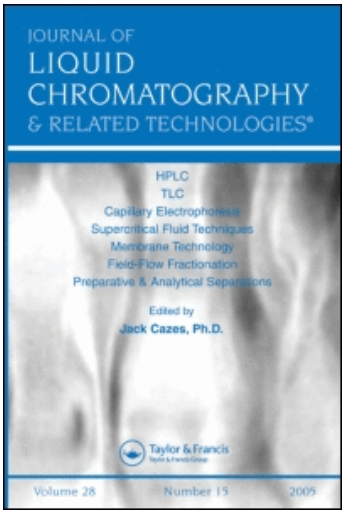

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Glenn D. Newport[a]; Constance C. Weis[a]; Robert W. West[a]; James W. Stanley[b]
[a] National Center for Toxicological Research Jefferson, Arkansas [b] Pepsico, Inc., Purchase, New York

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AN ANALYTICAL APPROACH TO METABOLIC PROFILING OF AROMATIC COMPOUNDS USING LIQUID CHROMATOGRAPHY

James W. Stanley (Present Address: Pepsico, Inc., Purchase, New York 10577), Glenn D. Newport, Constance C. Weis and Robert W. West, National Center for Toxicological Research Jefferson, Arkansas 72079

ABSTRACT

An analytical approach to metabolic profiling of aromatic compounds is described for both conjugated and "free" metabolites in biological systems. Initially, an ethyl acetate extraction removes the less polar metabolites. A salting-out procedure using Sephadex G-10 is combined with reverse-phase high-performance liquid chromatography (HPLC) to analyze the water-soluble conjugates directly using sequential uv and fluorescence detection. The "free" metabolites are also derived from individual conjugate peaks by enzymatic or hydrolytic procedures and then re-chromatographed by HPLC. Metabolites in the ethyl acetate fraction are similarly analyzed by reverse-phase HPLC. The utility of this method is demonstrated for 2-acetylaminofluorene (2-AAF and 4-ethylsulfonyl-naphthalene-1-sulfonamide (ENS), two compounds of interest in the study of murine bladder carcinogenesis.

INTRODUCTION

A practical problem in metabolic studies is the analysis of water-soluble conjugates and metabolites of the parent compound. This is true both *in vivo* (where urine or blood are often the biological matrix) and *in vitro* (where tissue slices, isolated liver-cell preparations or perhaps tissue culture are the media for analysis). In the past many water-soluble, non-extractable

metabolites have been analyzed indirectly using enzymatic cleavage or acid-base hydrolysis, neither of which often account for a high percentage of the components. Although incomplete profiles may be satisfactory in some cases, current interest is directed toward obtaining more complete profiles including water-soluble components. Often water-soluble conjugates are labile and/or of such high molecular weight that they are not amenable to gas chromatographic (GC) analysis. Our interest in isolating and determining the biological properties of these conjugated metabolites prompted development of this procedure. The aromatic amines (2-AAF and ENS) selected for study are of interest in carcinogenesis, and present two problems typically found in drug metabolism studies: (a) the parent compound is extensively metabolized to water-soluble components, and (b) the spectrum of components includes "free" metabolites that present difficulties in performing chromatographic analysis.

For example, N-hydroxylation of aromatic amines is a key metabolic step in activation of these compounds to proximate carcinogens (1). These are converted further to water-soluble conjugates which, in turn, may serve as vehicle for transport to target tissues. In the case of ^{14}C-AAF, >95% of the dose administered appears in urine as water-soluble material (conjugates and/or adducts). To understand and properly define the mechanism of action, direct analysis of these constituents is highly desirable. Since some of the "free" metabolites are significantly more difficult to analyze by chromatography than the conjugates, direct analysis offers a higher probability of a complete profile of this compound.

The other compound, ENS, is a sulfonamide reported to be a murine bladder carcinogen about which little was known metabolically (2). Although ENS and 2-AAF are aromatics, they represent distinct chemical classes and serve as diverse representative examples for this approach.

MATERIALS AND METHODS

Chemicals. [9-^{14}C]-2-Acetylaminofluorene (2-AAF)(20 mCi/mmol), purchased from New England Nuclear (Boston, MA), was shown to be

>99% pure by thin-layer (TLC) and high-performance liquid chromatography (HPLC) using both the adsorption (silica gel) and reverse-phase (silanized silica-TLC, Vydac Reverse Phase-HPLC) mode. Unlabelled 2-AAF was purchased from Aldrich Chemical Company (Milwaukee, WI); its purity checked by the above method was >99%. Unlabelled standards of 2-aminofluorene (2-AF), the 7,5,3, or 1-ring hydroxy-2-acetylaminofluorene and analogues (7-OH-2-AAF, 5-OH-2-AAF, 3-OH-2-AAF, 1-OH-2-AAF), and N-hydroxy-2-acetylaminofluorene (N-OH-2-AAF) were obtained from Dr. Charles Irving (V. A. Hospital, Memphis, TN). The N-glucuronide of N-OH-2-AAF was prepared biosynthetically by Dr. Charles King (Michigan Cancer Foundation, Detroit, MI) and its purity determined by HPLC.

4-[2'-^{14}C]-ethylsulfonylnaphthalene-1-sulfonamide and its unlabelled forms were obtained by custom synthesis from Mallinckrodt Chemical Co., (St. Louis MO); they were found to be >99% pure by TLC and HPLC by using uv and fluorescent detection. Both 4-[2'-hydroxy]-ethylsulfonylnaphthalene-1-sulfonamide and 4-ethylsulfonylnapthalene-1-sulfonic acid [Na^+ salt] were obtained by custom synthesis from Los Alamos Research Laboratories (New Mexico), and their purity checked by TLC and HPLC.

n-Propylsulfonylnaphthalene-1-sulfonamide (PNS) was synthesized in-house by a published method (3); high resolution mass spectral analysis was performed, and the purity checked by TLC and HPLC.

High-Performance Liquid Chromatography. Waters Associates (Milford, MA) Model 6000 pumps and Model 660 programmer were used along with a U6K injector system. Detection of individual peaks was performed with a Perkin-Elmer (Norwalk, CT) LC-55 uv/visible detector coupled on-line with an Aminco-Bowman (Silver Springs, MD) spectrophotofluorometer (SPF with ratio photometer) equipped 100 µl flow cell. The uv wavelengths used were either 254 nm or 280 nm, and are shown on the figures. For the fluorescence detection of ENS and 2-AAF products, the excitation and emission wavelengths were Ex 310 nm, Em 390 nm and Ex 297 nm, Em 366 nm, respectively. Collection of fractions was made with a Gilson (Middleton, WI) Model FC-80H Micro Fractionator. Normal flow rate was 0.5 ml/min. Eighty 1-min

samples were transferred to scintillation vials for counting radioactivity. In all cases, linear gradients or isocratic conditions were used.

The HPLC columns used were: Vydac Reverse Phase, (10 μ)(The Separations Group, Hesperia, CA): μBondapak$_{18}$ (10 μ) Waters Associates, Milford, MA), or Zorbax ODS (5-7 μ) (DuPont, Wilmington, DE) for reverse-phase work. Zorbax was found to be suitable for all but the N-OH-2-AAF separations.

Chromatography-Packings and Solvents. The XAD-2 material was obtained as washed and sized beads from Bio-Rad Laboratories (Richmond, CA)(Bio-Beads SM-2). Three additional methanol (Burdick and Jackson-LC grade, Muskegon, MI) washes were performed (10 ml/g) before use, followed by extensive washing with water (Millipore Milli-Q-System purified) to hydrate the beads.

The Bio-Gel P-2 (Bio-Rad Laboratories) was used as received, although several void volumes of mobile phase were passed through columns before use.

The Sephadex G-10 (Pharmacia, Inc., Piscataway, NJ) was used as received. A standard hydration procedure consisted of heating the G-10 (four volumes of water/g) to 90°C in a water bath for 2 hr. When solutions of 0.2 M ammonium acetate (NH_4AC) were used as the eluate, the column was washed with four void volumes before use.

The Sephadex LH-20 (Pharmacia) was hydrated in a similar manner to G-10 except 10 volumes of water/g LH-20 was used.

The solvents used for this study included: (a) methanol (LC grade from Burdick and Jackson), (b) 2-propanol (Certified grade from Fisher Scientific, St. Louis, MO); (c) acetic acid (Reagent grade from Fisher Scientific) which was re-distilled before use in an all glass system; (d) water which was purified in a Millipore Milli-Q-System fed by a deionized water source or distilled water fed by a deionized water source.

Urine Collections. The BALB/cStCrlfC3H/Nctr strain of mouse was used in the metabolism work. Mice were stomach intubated with a total of 1 mg of 2-AAF or ENS, including varying amounts of ^{14}C-labelled material. Some urine samples were collected directly by

bladder palpation, normally yielding 50-200 µl per mouse. Other collections were made for 24 hours over ice in a small glass metabolism cage designed in the laboratory (4). Normal 24 hr urine volume was about one ml.

Sephadex G-10 Clean-Up. After low speed centrifugation to pellet particulate matter, the urine (1 ml) was made 0.2 M using NH_4Ac, and a 1-ml aliquot was carefully layered on top of a (1 cm diameter x 20 cm length) column of Sephadex G-10 containing the hydrated equivalent of 2 g dry packing, previously conditioned with NH_4Ac. Twelve (12) ml of 0.2 M NH_4Ac were then passed through the column (designated: NH_4Ac fraction) followed by 10 ml (ENS) or 20 ml (2-AAF) of water (designated H_2O fraction). The H_2O fraction was then either lyophilized or analyzed directly by HPLC. The whole procedure, while taking several hours, can be performed with minimal technician time and in the dark and/or cold room with a large number of samples run simultaneously. For animals treated with ENS, urine was extracted three times with two volumes of ethyl acetate, before G-10 clean-up. This was necessary for removal of unmetabolized parent compound and conjugated metabolites, where turnover to water-soluble conjugates is <95%.

In Vitro Liver Slice Incubations Work-Up (ENS). Liver slices were obtained as follows: 8-wk old BALB/c female mice were sacrificed by cervical dislocation, and the livers immediately removed and placed in ice-cold Krebs Ringer Bicarbonate buffer. The livers were placed on top of double-thickness buffer-soaked filter paper, transferred to a Brinkman Mickle Tissue Chopper, and sliced into 0.1 mm sections. The slices were weighed (≈100 mg) and transferred into a 25 ml Erylenmeyer flask containing 3 ml Krebs-Ringer Bicarbonate buffer. The flasks were gassed for 2 min with 95/5:O_2/CO_2, fitted with rubber stoppers and vents, and incubated with shaking for time periods up to 2 hrs at 37°C in a water bath. The substrate (radiolabelled ENS) was added to the Krebs-Ringer buffer before slices were added, usually 10 µl of DMSO/3.0 ml of incubation mixture.

After incubation, the mixture and slices were transferred to centrifuge tubes. Three centrifugations were made at low speeds

(1,000 rpm), each followed by decanting the supernatant, adding additional buffer (3.0 ml) and vortexing. Combined washes were pooled, centrifuged at 7,500 rpm for 20 min at 5°C, and the clear supernatant removed by Pasteur pipette.

The procedure from this point on was identical to that performed on urine samples.

RESULTS AND DISCUSSION

In developing a clean-up procedure with wide applicability, several factors were considered, i.e., the clean-up procedure should be: (a) amenable to the objectives of profiling a large percentage of the metabolites, therefore, not too selective, and (b) compatible with the analytical methods of choice.

Generally, extractable metabolites (i.e. those readily partitioned into ethyl acetate or ethyl ether) can be cleaned up by familiar procedures; however, exceptions have been observed with aromatic amines and are discussed later.

Clean-up of "water-soluble" metabolites is more difficult since they usually are associated with rather complex biological matrices, e.g., urine. Most endogenous urinary components are polar, and advantage should be taken of this property during clean-up. Our approach exploited the common functionality of a non-polar moiety (aromatic rings) in both the ENS and 2-AAF to perform group separation. Other objectives were to de-proteinize and remove inorganics from the water-soluble fraction.

Clean-Up Material Selection. Several popular materials (XAD-2, Bio-Gel P-2, Sephadex LH-20 and Sephadex G-10) used for clean-up were investigated as to their ability to provide the desired properties; of these, Sephadex G-10 was preferable. The strengths and weaknesses of each packing are discussed subsequently. Amberlite XAD-2, a nonionic, styrene-divinylbenzene copolymer, has a surface area of approximately 300 m^2/g, and a rigid, porous (approximately 900-Å pore diam.) structure (5). It is a very useful adsorbant for clean-up of drugs from urine and other aqueous systems. Primary use has been limited to parent drugs in an attempt to eliminate the extraction step in analysis (6,7). Some limited column chromatography has

also been reported (8,9). Several workers (10) have reported problems with recovery of certain drugs; however, the nature of the material suggests a possible utility in the clean-up of water-soluble metabolites of aromatics. Fig. 1-A shows an attempt to selectively elute metabolites of ENS from this material in an open column using a linear gradient of methanol in water from 0-100%. Several points are evident; the material has a high affinity for

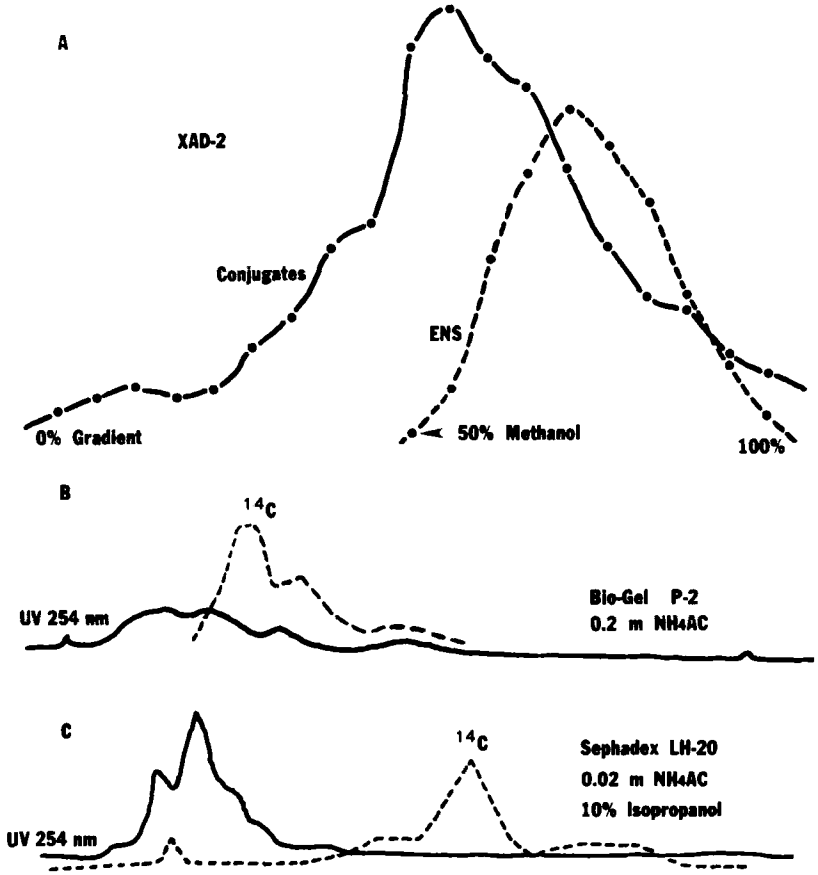

FIGURE 1.

Elution profiles of ENS metabolites from various adsorbant materials.

aromatic ring systems, as nearly 50% methanol is required to elute even the water-soluble metabolites of this compound. Unfortunately, selective elution is difficult to achieve since most of the uv-absorbing compounds in urine, as well as the parent compound, eluted in the same region of the gradient. In addition, recovery of extractable metabolites required excessive volumes of solvent/g of adsorbant.

Bio-Gel P-2, a copolymer of acrylamide and N-N-methylene-bis-acrylamide, is essentially a gel-permeation material for low molecular weight compounds (1000 m.w.). By virtue of its chemical makeup, hydrophobic interactions play a minor role in its chromatography in aqueous media (11). Even at high ionic strength (see Fig. 1-B), the water-soluble metabolites of ENS are only slightly retained relative to the uv-adsorbing constituents in urine.

Sephadex LH-20, a hydroxypropyl derivative of G-24, is hydrophobic in nature, and although developed for gel-permeation work in organic solvents, LH-20 is increasingly used for adsorption chromatography (11). The higher affinity of LH-20 for aromatic compounds over G-10 is clearly evident from a comparison of Fig. 1-C and 2-A&B. At relatively low ionic strength, 10% isopropanol in an isocratic mode is required to elute the ENS water-soluble metabolites. Thus, LH-20 is, in general, a useful clean-up material with higher affinity for aromatics and lypophilic substances.

Sephadex G-10 is a beaded cross-linked matrix formed by cross-linking dextran B-512 with epichlorohydrin (11,12). The G-10 gel is the most highly cross-linked member and is used for low molecular-weight gel-filtration chromatography of aqueous compounds. In addition, Sephadex displays some subtle, but useful, adsorption properties with aromatic systems (13). These interactions are most certainly hydrophobic in nature as an increase in ionic strength greatly alters the elution profile of ENS water-soluble metabolites (Fig. 2-A&B). By manipulating this factor, a simple, gentle, yet effective clean-up of these water-soluble metabolites is achieved. Aromatic water-soluble metabolites, which are retained on G-10 at

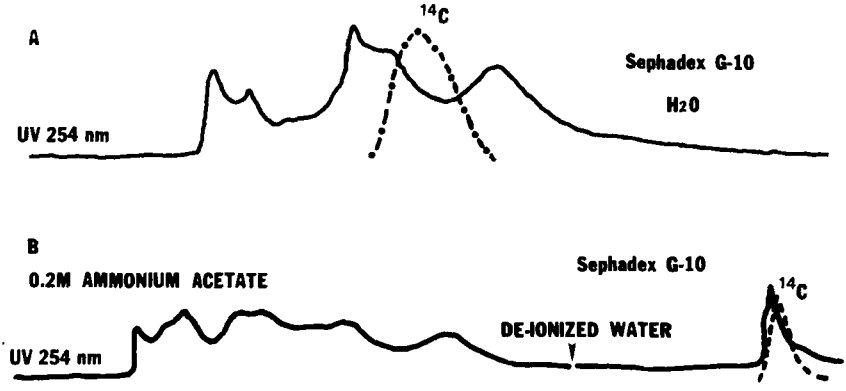

FIGURE 2

Elution profiles of ENS urinary metabolites on Sephadex G-10 at low and high ionic strengths.

high ionic strength, can be easily eluted near the void volume of the column by switching to distilled water as the mobile phase. The eluted metabolites are free from protein, most uv-absorbing components in urine, and inorganic salts. In addition, this separation can be performed in the cold for maximum stability of labile metabolites. The water fraction is then analyzed using the HPLC system for separation of individual metabolites.

Two additional uses of Sephadex G-10 in clean-up of aromatic amine ring systems are shown in Fig. 3. For 2-AAF and benzidine, both the fluorene ring (Fig. 3-A) and the biphenyl ring (Fig. 3-B) demonstrate similar chromatographic behavior on this gel.

The Analysis of Individual Metabolite-Conjugates on HPLC. Reverse-phase chromatogaphy was found to be useful for fractionating conjugates which are labile and not extractable. The approach was similar to that used in the clean-up step, that of using ionic strength to enhance the affinity of the conjugates for the C-18 bonded-phase packings, and alcohol gradients to separate individual components. Chromatograms of the metabolites of ENS, isolated from the liver-slice system, are shown in Fig. 4. The chromatograms

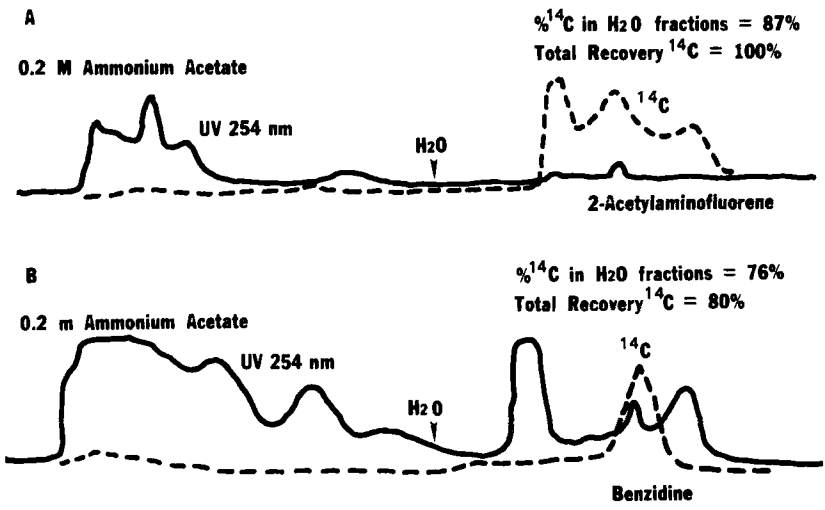

FIGURE 3

Urinary metabolites of 2-acetylaminofluorene and benzidine. Elution profiles of 0.2 M ammonium acetate fractions on Sephadex G-10 using a combination of high and low ionic strength to selectively elute conjugated metabolites.

show how a complete analysis was obtained using a combination of initial ethyl acetate extraction and HPLC (ENS and B-OH ENS) as in Fig. 4-A., followed by Sephadex G-10 clean-up and HPLC of the water-soluble components (Fig. 4-B). The major radioactive uv peak (Peak 1, Fig. 4-B) in the water-soluble fraction is unstable and breaks down under acidic pH, elevated temperatures, or on acidic absorbants (silica) to the major fluorescent peak (Peak 2, Fig. 4-B), which is the deaminated sulfonic acid, 4-ethylsulfonylnaphthalene-1-sulfonic acid. This latter compound is actually an artifact of sample manipulation, due to the inherent instability of the parent conjugate, and not a true metabolite. Presence of this unstable conjugate (Peak 1) probably would not have been detected using conventional procedures since: (a) it is not cleaved to extractable products by enzymatic treatments such as B-glucuronidase or sulfatase; and (b) it quantitatively breaks down to the sulfonic acid

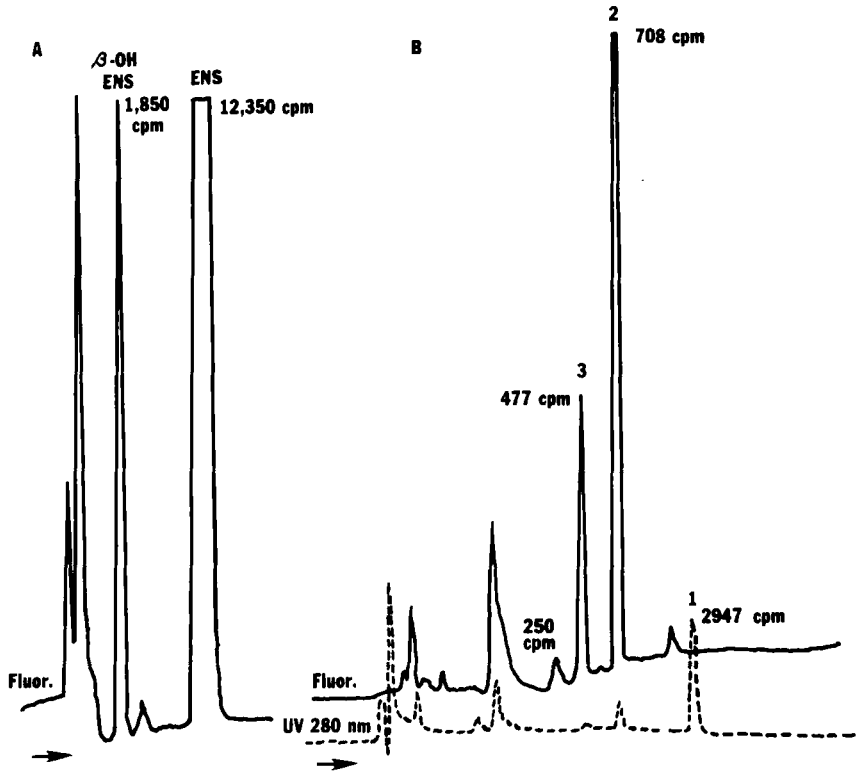

FIGURE 4

HPLC profiles of ENS metabolites from a liver-slice system. (A) Ethyl acetate extractable metabolites. Chromatographic conditions: column-Vydac Reverse Phase; solvent-43% MeOH in 0.02 M NH_4Ac; flow rate-0.5 ml/min. (B) Water soluble metabolites which were eluted selectively from Sephadex G-10. Chromatographic conditions: column-Vydac Reverse Phae; solvent-linear gradient from 1 to 10% isopropyl alcohol in 0.02 M NH_4Ac, 30 min; flow rate-0.5 ml/min.

under acidic conditions, pH 2.0. The sulfonic acid (Peak 2) was previously reported as a metabolite (2) using a more classical approach. Indirect evidence indicates that this uv conjugate (Peak 1) is an N-glucuronide or the parent sulfonamide, based upon its slow release of parent compound when treated with B-glucuronidase, disruption of fluorescence associated with substitution on the

sulfonamide nitrogen, and acid-hydrolysis products (14,15). The other major fluorescent peak (Peak 3, Fig. 4-B) is the O-glucuronide of the major extractable metabolite 4-2'-hydroxy-sulfonylnaphthalene-1-sulfonamide (B-OH-ENS)(Fig. 4-A). Figure 5 demonstrates the utility of this approach when comparing the metabolism of close analogs. The propyl analog (Fig. 5-C&D) was of interest as a reportedly inactive hyperplastic agent in regard to inducing pathology in the urinary tract of mice. Since the compound demonstrates similar in vitro properties to ENS (e.g. carbonic anhydrase inhibition), this compound was of interest. Initial work with the extractable metabolites on TLC using fluorescent scanning (Fig. 5 inserts) revealed that indeed there were some major differences in metabolism. ENS was converted in vivo as in vitro (Fig. 4) to a more polar metabolite (B-OH ENS), but it was also excreted in significant amounts unchanged (Fig. 4-E&F inserts), while the propyl analog (PNS) did not appear in the urine unchanged (see Fig. 5-C&D inserts-broken line shows where the standard of PNS would be expected). Instead, two more polar metabolites were observed. Incubation of the urine with B-glucuronidase (16 hr at 37°C) after initial extraction with ethyl acetate confirmed these differences and revealed an additional finding; in contrast to the propyl analog, small amounts of ENS were released during prolonged incubation. Both compounds revealed the expected presence of glucuronide conjugates of the principal polar metabolite observed in the initial ethyl acetate extracts (Fig. 5-C-F). The ability to investigate the water-soluble metabolites intact (Fig. 5) helped to explain the differences observed on TLC for these two analogs.

While ENS is conjugated to an N-glucuronide (tentative) which can be cleaved either back to the parent compound or to the sulfonic acid, the propyl analog exhibited no evidence of this behavior. The differences in the distribution co-efficients for these analogs (ENS, 9.2, PNS 20.3, octanol/H_2O), indicates that a significant percentage of ENS found in the urine may represent a simple excretion difference based on lipophilicity rather than breakdown of its

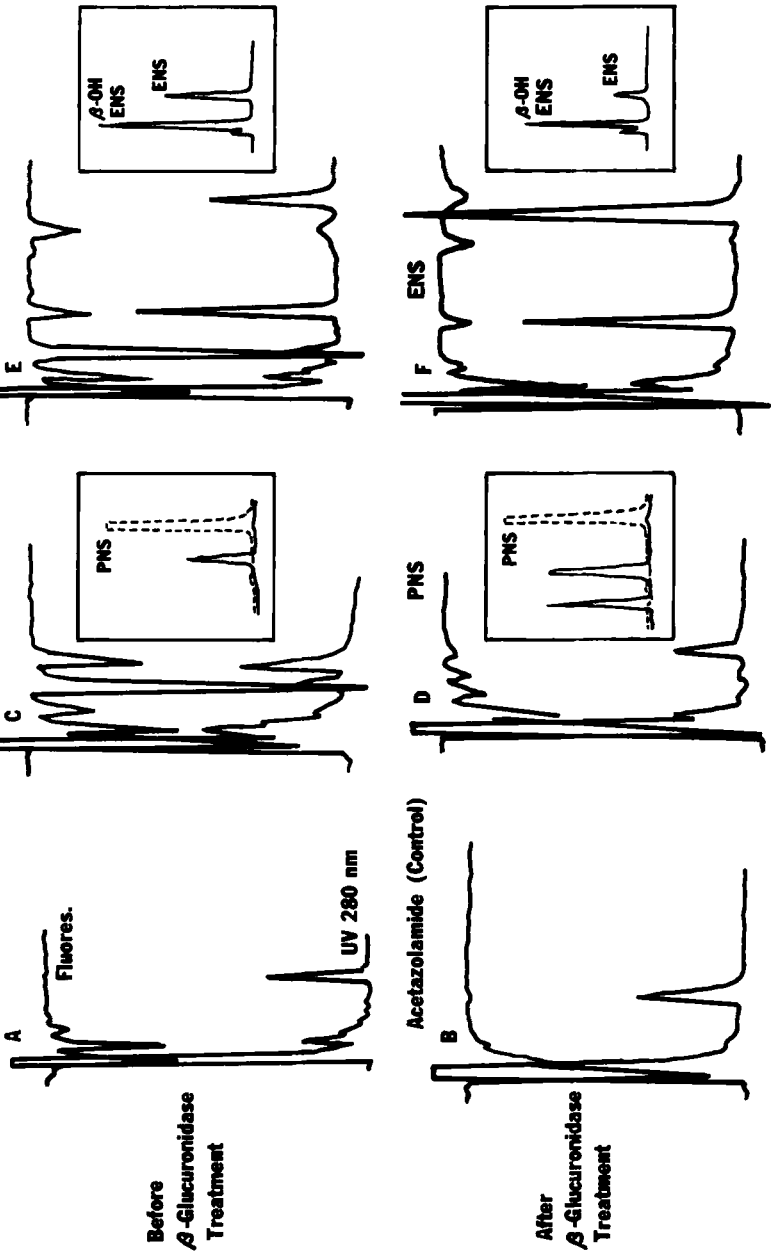

FIGURE 5. HPLC profiles of water-soluble urinary metabolites before and after treatment with B-glucuronidase. (Compare upper and lower figures, E.G., C and D). Control compound, Acetazolamide (A&B), is compared to metabolites of PNS, 4-n-propylsulfonylnaphthalene-1-sulfonamide (C&D), and 4-ethylsulfonylnaphthalene-1-sulfonamide (E&F). Inserts represent profiles of extracted metabolites on TLC using fluoresence scanning.

conjugate. Thus, the origin of the observed metabolites on TLC was easily traced (Fig. 5) by a comparison of the water-soluble metabolite profile before and after B-glucuronidase treatment. The acetazolamide-treated animal was used as a control under similar diuresis (Fig. 5).

Another example of the utility of LC is represented by the analysis of the 2-AAF metabolites. For this compound, one of the difficulties was to find a chromatographic system to analyze all the major metabolites simultaneously. These included 2-AAF, 2-AF, 7-OH, 5-OH, 3-OH, 1-OH and N-OH AAF. Several papers (16,17,18) addressing this problem have appeared recently. The principal difficulty which had been encountered in previous work (16,17,18) was the reactivity or extreme affinity of hydroxamic acids for active adsorbants, and thus, systems which were satisfactory for ring hydroxy metabolites (19) and 2-AAF were not for N-OH AAF (a key metabolite) (1). Since methods are needed for both in vitro and in vivo work with this compound, two alternative approaches are presented. One involved direct analysis of the "free" metabolites which may be of interest to those involved with non-conjugating systems such as microsomal work. When streaking of N-OH-2-AAF was observed in TLC on either silica gel (E. Merck) or silanized silica (E. Merck), it was not surprising to find that this compound did not elute from a bonded reverse-phase column under solvent conditions that adequately separated the remaining metabolites (Fig. 6-A). Since this problem was a result of residual underivatized Si-OH groups present on any bonded-phase column with silica gel support, the minimization of such interaction was attempted. Based on published work (20,21), acetic acid was incorporated into the mobile phase. Acetic acid is a powerful solvent in normal-phase chromatography but very similar to methanol in its characteristics as a mobile phase in reverse-phase work (20). Thus, as is shown in Fig. 6-B, the incorporation of acetic acid did result in the elution of N-OH-2-AAF at a relative retention consistent with its expected polarity in a true reverse-phase separation. The fine tuning of

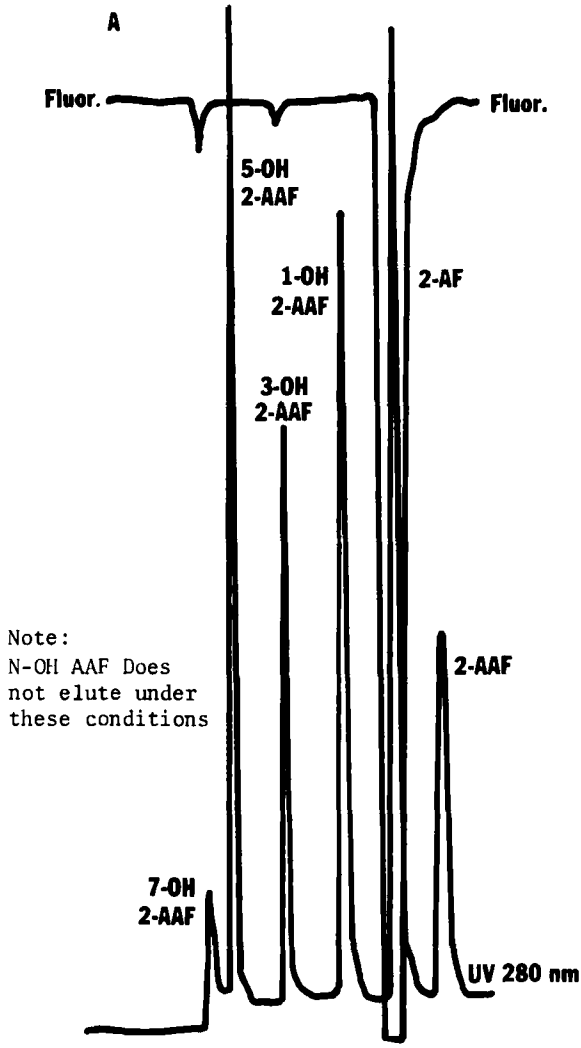

FIGURE 6-A

HPLC profile of standards of 2-AAF and ring-hydroxy metabolites using reverse-phase mode and methanol/water mobile phase. Chromatographic conditions: column-Vydac Reverse Phase; solvent-43% CH_3OH in 0.02 M NH_4Ac; flow rate-0.5 ml/min.

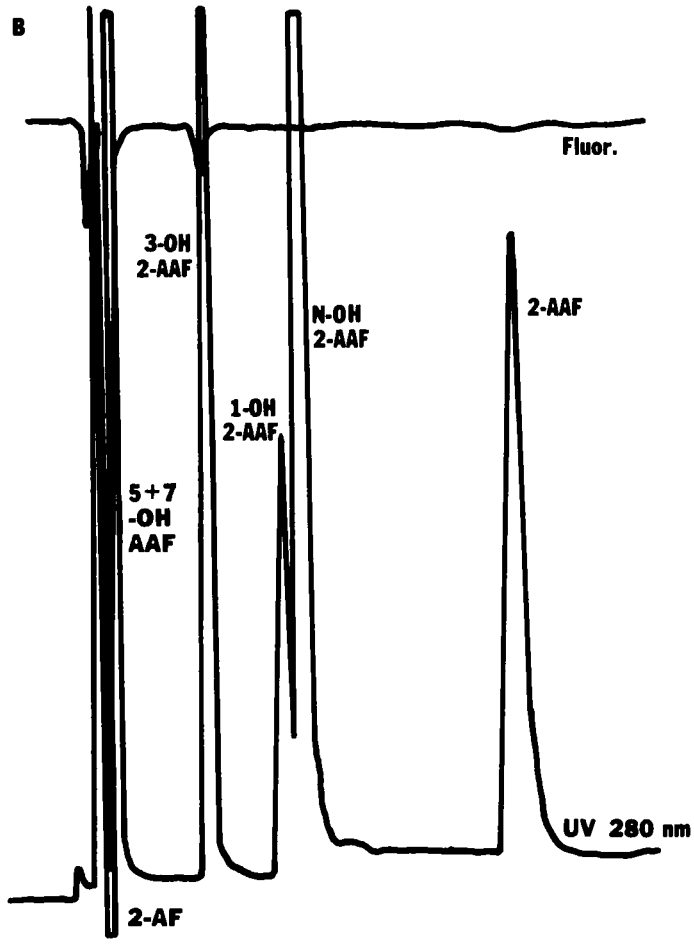

FIGURE 6-B

Reverse-phase separation of 2-AAF metabolites in acetic acid. Chromatographic conditions: column-Vydac Reverse Phase; solvent-20% CH_3COOH, 15% CH_3OH, 65% 0.02 M NH_4Ac; flow rate-0.5 ml/min.

this separation was achieved by incorporation of an ion-pairing counter ion for the now protonated 2-aminofluorene (2-AF)(Fig. 6-C), thus increasing the retention time for this species (22).

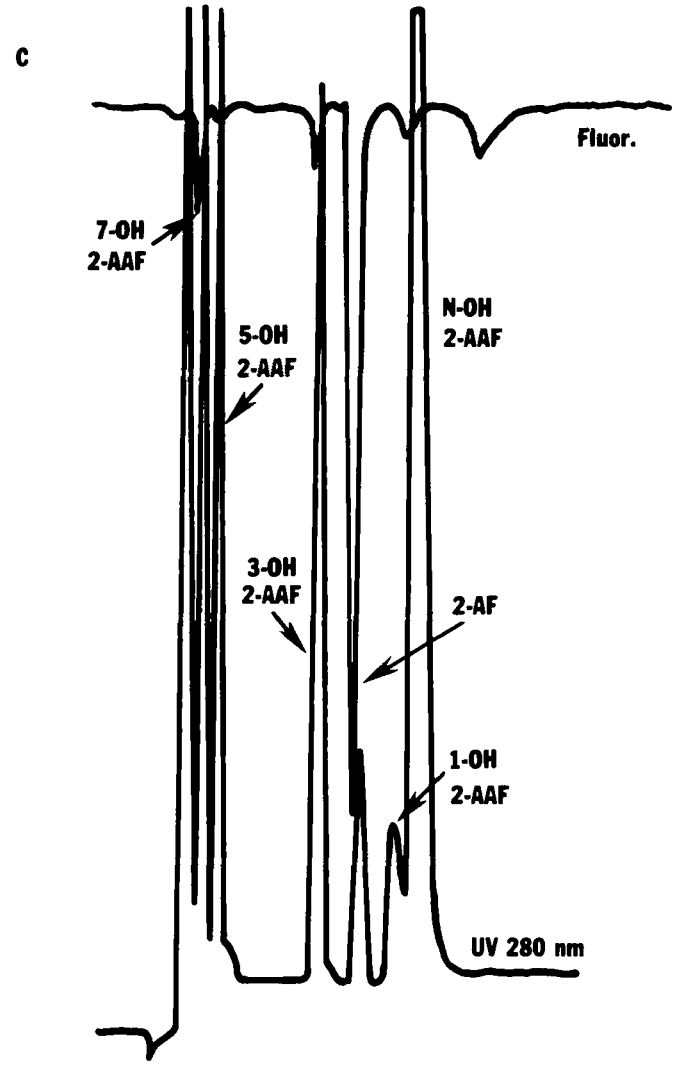

FIGURE 6-C

Separation of 2-AAF metabolites in acetic acid mobile phase with ion-pairing conditions. Chromatographic conditions: same as Figure B except add 0.005 M heptane sulfonic acid to mobile phase.

For in vivo work with urine a different approach was taken since the primary metabolites discussed above appear as conjugates. These conjugated metabolites were analyzed directly using the Sepahdex G-10 HPLC approach. An HPLC chromatogram of the water-soluble metabolites of 9-^{14}C-2-AAF isolated from urine of a mouse which had been stomach intubated with 0.5 mg of 9-^{14}C-2-AAF, and the urine collected for 16 hr, is shown in Fig. 7. As indicated in Fig 7, there are 5 major components, one of which is a doublet (Peak 2) observed in BALB/c female mice. The insert in Fig. 7 is an HPLC chromatogram of N-OH-2-AAF-glucuronide run under identical conditions showing that Peak 4 has the same retention time. Thus, this method provides a direct approach for the separation and identification of the amounts of N-OH-AAF which are excreted in the urine. Treatment with B-glucuronidase does release a compound with identical chromatographic properties as N-OH-2-AAF. Fig. 8-A&B demonstrates how individual conjugate peaks can be identified by collecting and treating with B-glucuronidase, then re-extracting and confirming identity with authentic standards. In this case, 7-OH and 5-OH-2-AAF were released upon enzymatic treatment, along with an unknown metabolite whose glucronide had a retention time very similar to the O-glucuronide of 5-OH-2-AAF (see doublet Peak 2, Fig. 7). Although there are several unidentified peaks (e.g. Peak 3, Fig. 7) which were observed in mouse urine upon treatment with 2-AAF, the utility of the approach as a separation method is the purpose of this discussion.

Classical approaches to metabolite analysis, either because of their inability to detect certain metabolites of conjugates, or their inherent potential of introducing artifacts throughout the procedure, have limited applicability to many in vivo studies. The advantage of this LC analytical approach lies in its ability to directly assess water-soluble components that are not amenable to classical methods. For animals maintained on long-term dietary studies, it is feasible to monitor metabolite profiles at normal

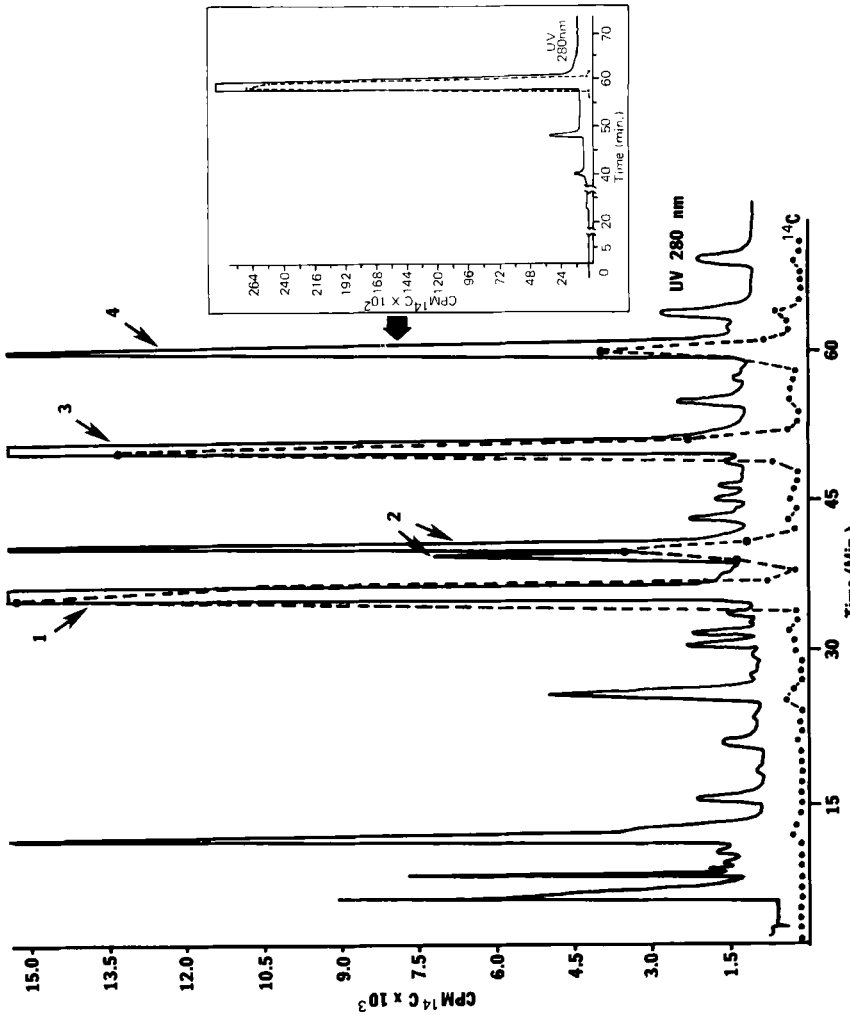

FIGURE 7. Water soluble metabolites of ^{14}C-2-AAF from urine of a BALB/c mouse. Insert is a chromatogram of N-OH-2-AAF- glucuronide standard.

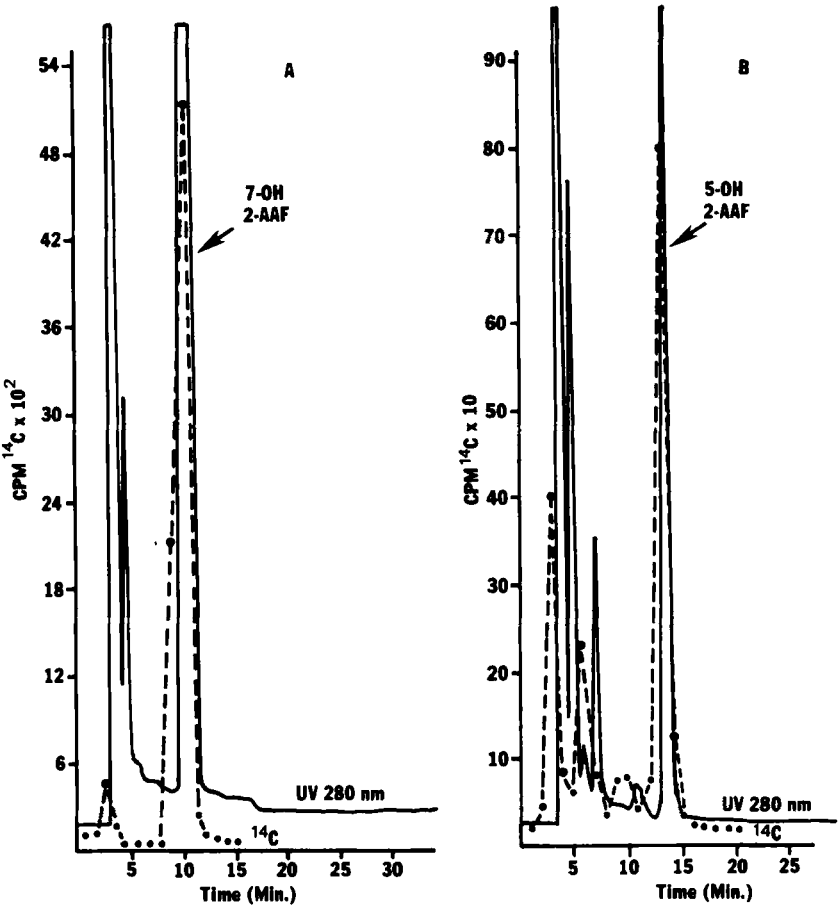

FIGURE 8

HPLC chromatograms of two peaks from Figure 7 after collection and treatment with B-glucuronidase.

levels. Future development in this regard may well be found in the analysis of water-soluble substances of broad interest to researchers in the life science area.

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