Journal of Chromatography, 237 (1982) 447–456
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,475

SYSTEMS FOR THE SEPARATION OF METABOLITES OF THE CARCINOGEN, N-2-FLUORENYLACETAMIDE, BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

C. N. RYZEWSKI* and D. MALEJKA-GIGANTI
Department of Laboratory Medicine and Pathology, University of Minnesota, and Laboratory for Cancer Research, Veterans Administration Medical Center, Minneapolis, MN 55417 (U.S.A.)*
(Received October 22nd, 1981)

SUMMARY

Separation procedures for N-2-fluorenylacetamide (2-FAA) and its metabolites have been hampered by the unusually strong adsorption of N-hydroxy-N-2-fluorenylacetamide (N-OH-2-FAA), the proximate carcinogenic metabolite, to chromatographic packings. Results of investigations with C_2, C_3, C_8, and C_{18} column packings are presented showing that N-OH-2-FAA could be eluted and the separation of 2-FAA and eight other metabolites achieved with acidic mobile phases on C_2 and C_8 columns after a period of column conditioning. Elution of C_8 columns with mobile phases containing acetohydroxamic acid was less destructive to the columns while permitting the separation of 2-FAA and its metabolites with recovery yields of N-OH-2-FAA of 91.5%. Quantitation of these compounds was by integration of peaks detected by the spectroscopic method. Areas were linear for peaks representing from 5 to at least 50 ng of N-OH-2-FAA and of the eight other metabolites.

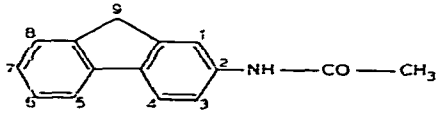
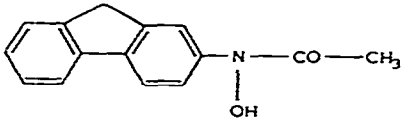
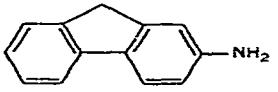
INTRODUCTION

The development of a separation procedure for a carcinogen, N-2-fluorenylacetamide (2-FAA), and its metabolites (structures and abbreviations of the compounds are listed in Table I) is warranted by the importance of 2-FAA in studies of N-arylamide carcinogenesis, the mechanism of xenobiotic biotransformations as well as the role of mixed-function oxidases. The primary metabolites of 2-FAA generated by the hepatic enzymes include phenols, N-OH-2-FAA, an alcohol, a ketone and an amine[1–4]. The development of an efficient separation procedure for these metabolites has been hampered by the unusually strong adsorption of N-OH-2-FAA to chromatographic column packings. Since N-OH-2-FAA is a proximate carcinogenic metabolite of 2-FAA[5], estimates of the amounts of the N-hydroxy compound formed in hepatic and extra-hepatic tumor targets are of particular importance in carcinogenesis. An additional difficulty with the separation procedures arises when conditions under which quantitative elution of N-OH-2-FAA is possible must be balanced with conditions allowing separation of the remaining metabolites.

0021-9673/82/0000–0000/$02.75

TABLE I

STRUCTURES OF 2-FAA AND ITS METABOLITES CORRESPONDING TO THE PEAK NUMBER IN THE HPLC ELUTION PROFILES

Structure	*Compound*	*Peak No.*
	N-2-Fluorenylacetamide [2-FAA]	10
Ring-oxidized derivatives:		
1-OH	N-(1-Hydroxy)-2-FAA [1-OH-2-FAA]	8
3-OH	N-(3-Hydroxy)-2-FAA [3-OH-2-FAA]	7
5-OH	N-(5-Hydroxy)-2-FAA [5-OH-2-FAA]	3
7-OH	N-(7-Hydroxy)-2-FAA [7-OH-2-FAA]	1
9-OH	N-(9-Hydroxy)-2-FAA [9-OH-2-FAA]	2
9=O	N-(9-Oxo)-2-FAA	6
	N-Hydroxy-2-FAA [N-OH-2-FAA]	9
	N-2-Fluorenamine [2-FA]	4
Derivative:		
9=O	N-(9-Oxo)-2-FA*	5

* Potential metabolite of 2-FA.

Initially, applications of high-performance liquid chromatography (HPLC) to the separation of the metabolites of 2-FAA involved a combination of normal-phase and reversed-phase HPLC[6,7]. A separation procedure employing only reversed-phase HPLC was developed by Thorgeirsson and Nelson[8]. Procedures based on reversed-phase HPLC developed subsequently[9–11] were modifications of the original procedure[8] and employed a mobile phase buffered to a pH of 8.5 or 9.0, at which dissolution of the silica matrix occurs. Moreover, elution of N-OH-2-FAA was slow, its peak broad and its quantitation unreliable. The nature of the secondary interactions contributing to the adsorption of N-OH-2-FAA has not been elucidated. It is suspected that the interactions may be due to metal impurities found in silica[12]. It has been shown, that N-OH-2-FAA and certain other hydroxamic acids can be selectively eluted from reversed-phase columns after complexation with ferric ions during chromatography[13]. However, this method was not applicable to the separation of other metabolites of 2-FAA. More recently, 2-FAA and eight of its metabolites were separated by reversed-phase HPLC with a mobile phase containing desferal mesylate[14]; this agent presumably chelates the sites of adsorption for the hydroxamic acids[12]. However, the addition of desferal mesylate by itself was not sufficient to block the chemisorptive sites, since the procedure required pre-saturation of the column with a solution of N-OH-2-FAA for several hours[14].

We present a procedure for separation of 2-FAA and eight of its metabolites,

which involves isocratic elution from a reversed-phase column with a mobile phase containing acetohydroxamic acid. Employing the labelled compound, we show that N-OH-2-FAA is recovered quantitatively, peak detection is by the spectroscopic method and quantitation by peak integration.

MATERIALS AND METHODS

Preparation of compounds

2-FAA (Aldrich, Milwaukee, WI, U.S.A.) was recrystallized from ethanol–water (7:3) and had a m.p. of 196–198°C. N-OH-2-FAA, m.p. 150–151°C (ref. 15); 1-OH-2-FAA, m.p. 210–212°C (ref. 16); 3-OH-2-FAA, m.p. 247–249°C (ref. 17); 5-OH-2-FAA, m.p. 214–216°C (ref. 18); 7-OH-2-FAA, m.p. 230–232°C (ref. 19); 9-OH-2-FAA, m.p. 249–250°C and N-(9-oxo)-2-FAA, m.p. 233–236°C (ref. 20); 2-FA, m.p. 127–129°C (ref. 21) and N-(9-oxo)-2-FA, m.p. 154–156°C (ref. 22) were prepared by the published procedures. The IR and UV spectra of the compounds matched those of the authentic samples. The compounds were found to be pure by thin-layer chromatography on silica gel GF_{254} with chloroform–methanol (95:5) or ethyl acetate–benzene (7:3) as mobile phase. The compounds for HPLC analyses were dissolved in methanol, and a stock solution containing 0.5 or 1.0 mg/ml of each compound was prepared.

Solvents and chemicals for HPLC

Sodium heptanesulfonate, oxalic acid and all solvents used for chromatography were glass-distilled/HPLC grade from Matheson, Coleman and Bell (Cincinnati, OH, U.S.A.). The methanesulfonic acid and trimethylamine hydrochloride were from Aldrich. The chloroacetic acid was from Mallinckrodt (Paris, KE, U.S.A.). Acetic acid was spectral grade, called Photorex reagent grade, from J. T. Baker (Phillipsburg, NJ, U.S.A.). Acetohydroxamic acid was purchased from Sigma (St. Louis, MO, U.S.A.). All solvents were pre-mixed to minimize exothermic mixing effects.

Columns

The C_{18} (MCH-10) and the end-capped C_{18} (N-MCH-10) columns were from Varian Instruments (Sunnyvale, CA, U.S.A.). The C_8 columns included Zorbax C_8 from DuPont (Wilmington, DE, U.S.A.), Ultrasphere Octyl from Altex (Berkeley, CA, U.S.A.), IBM Octyl from IBM Instruments (Danbury, CT, U.S.A.), LiChrosorb RP-8 from E. Merck (Darmstadt, G.F.R.). The 5- and 10-μm LiChrosorb RP-2 columns were from E. Merck, and the Zorbax ODS and Zorbax TMS were from DuPont.

Chromatographic equipment

All chromatographic analyses were carried out on the Varian Instruments Model 5060 liquid chromatograph. Spectroscopic peak detection was done with a Varian Instruments UV50 variable-wavelength detector whose flow cell was maintained at 31°C with a circulating water bath (type K2/R, MGW Lauda, G.F.R.). Peak areas and peak heights were measured by an integrating recorder from Hewlett-Packard (Model 3390A). Samples were injected by means of a pneumatically actuated injector from Valco Instruments (Houston, TX, U.S.A.) equipped with a 10-μl loop.

Preparation and determination of the recovery of N-OH-2-[9-^{14}C]FAA

N-OH-2-[9-^{14}C]FAA was prepared from 2-nitro[9-^{14}C]fluorene (California Bionuclear Corp. (Sun Valley, CA, U.S.A.), Lot No. 2529, specific radioactivity 14.7 mCi/mmole) that had been diluted with the unlabelled compound to a specific radioactivity of 5.8 mCi/mmole. 2-Nitro-[9-^{14}C]fluorene was reduced and subsequently acetylated as described previously[21]. N-OH-2-[9-^{14}C]FAA was recrystallized from ethanol–water (8:2) and had a m.p. of 148–150°C. The labelled compound was purified by HPLC employing an Altex Ultrasphere Octyl column (150 × 4.6 mm, 5 μm particle diameter) and 10% (v/v) dimethyl sulfoxide (DMSO), 16% (v/v) isopropanol, 10% (v/v) 0.5 *M* sodium chloroacetate, pH 3.0, as mobile phase. The specific radioactivity of the pure compound was 5.4 mCi/mmole. From 15,000 to 40,000 dpm of radioactivity in 10 μl of methanol was used per injection. The amount of radioactivity collected under the N-OH-2-FAA peak was compared to the amount of radioactivity collected directly from the 10-μl injector loop. All collected samples were diluted with mobile phase to the same final volume (15 ml), frozen in a dry ice–ethanol bath, and lyophilized overnight in 40-ml conical tubes. A 10-ml volume of scintillation liquid[21] was then added to each tube, which contained a transparent yellowish residue, and the tubes were shaken to insure that the residue became suspended in the liquid. The precipitate which formed was removed by centrifugation in an International Equipment Company Centrifuge (Model PR-2) at 600 *g* for 20 min. The amount of radioactivity in the supernatant was determined using a Packard Liquid Scintillation Spectrometer (Model 3255). The counts were corrected for quenching by means of an external standard. The counting efficiency was 70%.

RESULTS AND DISCUSSION

Adsorption of N-OH-2-FAA to reversed-phase columns

N-OH-2-FAA was eluted with methanol at a flow-rate of 2 ml/min as a broad peak for 30 to 50 min from C_{18} (MicroPak MCH-10, MicroPak N-MCH-10, Zorbax ODS), C_8 (Zorbax C_8), and C_3 (Zorbax TMS) columns. The use of acetonitrile, isopropanol or tetrahydrofuran (THF) and the addition of sodium heptanesulfonate or tetramethylammonium chloride to these solvents did not improve the elution of this hydroxamic acid.

Elution of N-OH-2-FAA as a sharp, unretained peak from C_{18} columns was achieved by the use of DMSO, dimethylformamide (DMF) or dimethylacetamide (DMA). Dilution of these solvents to increase the retention time of N-OH-2-FAA resulted in large decreases in the recovery of this compound and in excessive peak broadening. Acidification of the mobile phase with acetic acid, formic acid or methanesulfonic acid improved recovery of the compound and peak shape, while addition of ion-pairing agents such as sodium heptanesulfonate or tetramethylammonium chloride did not. However, even acidified mobile phases containing DMSO, DMF or DMA could not be diluted to the extent needed to simultaneously increase the retention time of N-OH-2-FAA, to permit its quantitative recovery and to separate the other 2-FAA metabolites on C_{18} columns.

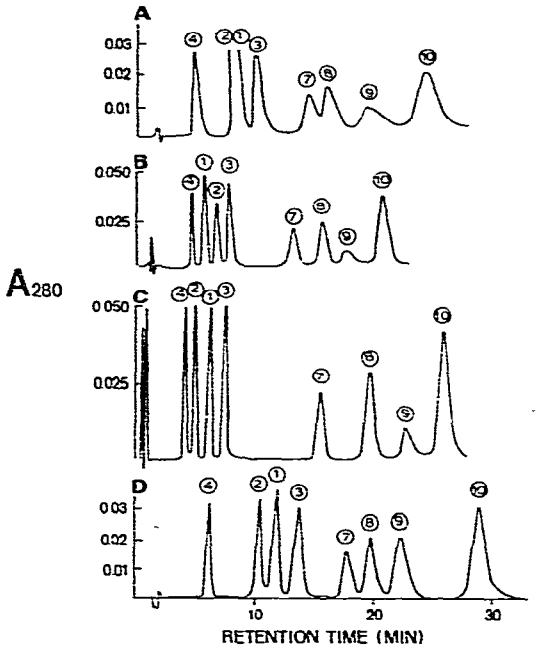


Fig. 1. Separation of 2-FAA and its metabolites on C_2 and C_8 columns. A, LiChrosorb RP-2, 10-μm particles, 250 × 4.6 mm (I.D.); flow-rate 2 ml/min; mobile phase, DMSO (10%, v/v)–formic acid (18%, w/v). B, Zorbax C_8, 250 × 4.6 mm I.D.; flow-rate 2 ml/min; mobile phase, DMSO (30%, v/v)–formic acid (17.6%, w/v)–phosphoric acid (0.85%, w/v). C, Ultrasphere Octyl, 5-μm particles, 150 × 4.6 mm I.D.; flow-rate 2 ml/min; mobile phase, DMSO (20%, v/v)–isopropanol (10%, v/v)–oxalic acid (1%, w/v). D, LiChrosorb RP-2, 5-μm particles, 250 × 4.6 mm I.D.; flow-rate 1.8 ml/min; mobile phase, methanol (25%, v/v–)acetic acid (5%, v/v)–oxalic acid (1%, w/v)–Na_2HPO_4 (50 m*M* final concentration). Compounds are identified in Table I and were injected each at a concentration of 50 μg/ml in 10 μl.

Separation of 2-FAA and its metabolites on C_2 and C_8 columns

The C_2 and C_8 columns provided more suitable stationary phases for separation of 2-FAA and its metabolites. Four separation procedures were developed for these compounds on such columns by adjusting the relative proportions of DMSO, isopropanol, methanol, formic acid, acetic acid and oxalic acid in the mobile phase (Fig. 1). A feature common to all four procedures is that mobile phases are strongly acidic. Although it appeared that DMSO or DMF was necessary for elution of N-OH-2-FAA as a result of work with C_{18} columns, given the proper conditions (Fig. ID), such solvents could be eliminated from the mobile phaxe. DMSO was used in preference to DMF because 1-OH-2-FAA and N-OH-2-FAA tend to co-elute when DMF is added as the organic modifier.

A common drawback to the four procedures (Fig. 1) is that separation can be achieved only after the columns are conditioned by washing with the acidic mobile phase for an extended period of time. Hence, these mobile phases will not work immediately when applied to new columns. Column packings were found to be unstable when such mobile phases were used. A gradual decline in retention capacity of columns occurred initially and became rapid after 70 to 80 sample injections. The loss

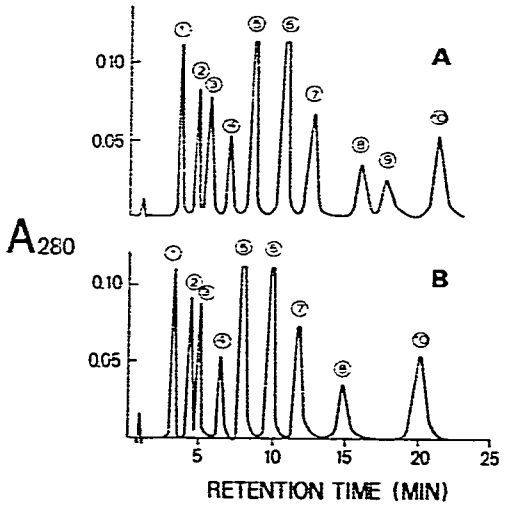


Fig. 2. Effect of the presence in (A) or absence from (B) the mobile phase of acetohydroxamic acid on the separation of 2-FAA and its metabolites on an Ultrasphere Octyl column, 150 × 4.6 mm I.D. operated at 30°C and a flow-rate of 2 ml/min with an operating pressure of 4750 p.s.i. The mobile phase contained DMSO (10%, v/v), isopropanol (16%, v/v), THF (1%, v/v), 0.5 *M* sodium chloroacetate, pH 3.0 (10%, v/v) and acetohydroxamic acid (1%, w/v), when added. Compounds are identified in Table I and were injected each at a concentration of 50 μg/ml in 10 μl.

of retention capacity was due presumably to the hydrolysis of the organic matrix from silica. Column voiding became evident after 80 to 100 sample injections.

The difficulties associated with the use of such separation procedures might be due to a limitation of derivatized silica gel packing material. Nevertheless, the procedures were used to separate 2-FAA and its metabolites for 25 to 50 sample injections. Should developments in the technology of packing materials result in more stable column packings, these solvent systems would be of greater value.

Elution of N-OH-2-FAA with a mobile phase containing acetohydroxamic acid

Elution of N-OH-2-FAA and separation of the other metabolites of 2-FAA with less damage to columns became possible with mobile phases containing acetohydroxamic acid. The addition of acetohydroxamic acid to the mobile phase is required for elution of N-OH-2-FAA, but has little effect on the elution of the other compounds (Fig. 2). With the use of acetohydroxamic acid, the strongly acidic conditions used previously to achieve the separation (Fig. 1) were found unnecessary. The pH of the aqueous portion of the mobile phase was 5.65 if the chloroacetate buffer was not added. To achieve the separation, the mobile phase was buffered to pH 3.0 to control the retention time of 2-FA, which increased with increasing pH and at pH 4.68 became identical with that for 1-OH-2-FAA.

Extensive washing of the column with mobile phase containing acetohydroxamic acid for several weeks did not condition the column to permit elution of N-OH-2-FAA with a mobile phase devoid of this modifier (Fig. 2B). Elution of N-OH-2-FAA with acetohydroxamic acid, therefore, was achieved by reversible competition for adsorption sites rather than by an irreversible conditioning process which elim-

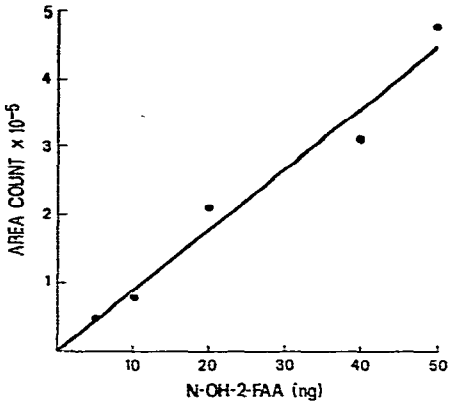


Fig. 3. Linearity of integrator response with the amount of N-OH-2-FAA injected in 10 μl of methanol and eluted as described in Fig. 2A.

inates such sites. In three experiments, an average of 91.5% of injected radioactivity of N-OH-2-[9-^{14}C]FAA was recovered under its peak.

Acetohydroxamic acid of high purity has no appreciable absorbance at 280 nm, which permits elution profiles for the 2-FAA metabolites to be monitored by spectroscopic methods. Minimum detectable quantitites of N-OH-2-FAA are limited by detector noise levels. When the detector noise level was stabilized to 10^{-4} absorbance unit, peak areas could be accurately determined for quantities of N-OH-2-FAA as low as 5 ng (Fig. 3). Integrator response was found to be linear for peaks representing 5 to 50 ng of N-OH-2-FAA and of the other metabolites. The upper limit for linear integrator response was not determined, but appeared to be much greater than 50 ng. Peaks representing <5 ng of N-OH-2-FAA were visible in recorder tracings, but could not be integrated at this noise level. The use of fixed-wavelength detectors, with noise specifications nearly an order of magnitude lower than those of variable-wavelength detectors, should permit quantitative detection of N-OH-2-FAA at <5 ng.

The separation of 9-OH-2-FAA from 5-OH-2-FAA (peaks 2 and 3, respectively), and the separation of 1-OH-2-FAA, N-OH-2-FAA and 2-FAA (peaks 8, 9 and 10, respectively), required fine adjustments in mobile-phase composition. Applications in which the quantities of 2-FAA are large compared to those of the metabolites, conditions encountered during *in vitro* metabolic studies, necessitated that the peak for 2-FAA be well separated from the other peaks. The position of the peak for N-OH-2-FAA was shifted away from the 2-FAA peak toward the 1-OH-2-FAA peak by the addition of THF. Simultaneously, the separation between 9-OH-2-FAA and 5-OH-2-FAA was improved by the presence of THF in the mobile phase. The amount of THF which could be added to the mobile phase was limited by the tendency of the 1-OH-2-FAA and N-OH-2-FAA peaks to superimpose with increasing THF content.

Acetohydroxamic acid and stainless steel

With mobile phases containing acetohydroxamic acid, the liquid which sometimes leaked around high-pressure fittings was orange to red in color. When nuts made of 316 stainless steel were placed in solutions containing 1% (w/v) of ac-

TABLE II

RETENTION TIMES FOR 2-FAA AND ITS METABOLITES AND MOBILE PHASE COMPOSITIONS IN SEPARATIONS ON SEVERAL C_8 COLUMNS

Column dimensions, 150 mm × 4.6 mm I.D.; flow-rate, 2 ml/min, except for III, for which it was 3 ml/min; column temperature, 30°C; a guard column of Perisorb RP-2, 35 × 4 mm I.D. was used for all separations.

Columns		*Retention time (min)*					
		7-OH-2-FAA	*9-OH-2-FAA*	*5-OH-2-FAA*	*2-FA*	*9-Oxo-2-FA*	*9-Oxo-2-FAA*
I	Ultrasphere Octyl (No. 1)***	3.42	4.60	5.34	6.75	8.27	10.40
II	Ultrasphere Octyl (No. 2)	3.39	4.91	5.68	7.28	8.79	10.93
III	Zorbax C_8	2.95	3.90	4.78	5.51	7.81	10.17
IIIa	Zorbax C_8 ‡	3.07	4.09	4.85	6.07	7.80	9.02
IV	IBM C_8	3.57	4.54	5.22	8.72	—	—

* Added as percentage w/v
** 0.5 *M* Sodium chloroacetate, pH 3.0.
*** See Fig. 2A for chromatogram.
‡ See Fig. 4 for chromatogram.
‡‡ Methanesulfonic acid added to a final concentration of 20 m*M*.

etohydroxamic acid, the solution became yellow within 1 h at room temperature. If, after 2 days of contact with the solution of acetohydroxamic acid, the nuts were washed with isopropanol and transferred to a fresh solution of acetohydroxamic acid, the solutions became weakly discolored only after more than 1 week. A surface impurity in the steel which becomes complexed by acetohydroxamic acid could account for the initial discoloration.

New Ultrasphere Octyl columns

When the mobile phase used to achieve the separation in Fig. 2A was applied to new Ultrasphere Octyl columns, resolution between the 9-OH-2-FAA and the 5-OH-2-FAA peaks was diminished and the N-OH-2-FAA peak was broader, tailing into the 2-FAA peak. Increasing the concentration of acetohydroxamic acid from 1 to 2% (w/v) restored the separation between the N-OH-2-FAA and the 2-FAA peaks. Minor adjustments in isopropanol, DMSO and THF (Table II) restored the remainder of the separation to that shown in Fig. 2A.

Although losses in retention capacity were not observed with buffered mobile phases containing acetohydroxamic acid, column voiding appeared to be a persistent problem with the Ultrasphere Octyl columns. Voiding was evident as a broadening of the peaks near their bases, obscuring the separation. Column function could be restored by running the column in the direction opposite to that prescribed by the manufacturer. With the three Ultrasphere Octyl columns tested, column voiding was apparent within 1 week of daily use. However, after being turned upside down, the columns could be used daily for at least 2 months.

Pre-equilibration of the mobile phase with silica by interposing a silica precolumn between the pump and the injector did not alleviate the voiding problem, nor did the use of a guard column between the injector and the analytical column. The

				Percent of mobile phase (v/w)				
3-OH-2-FAA	*1-OH-2-FAA*	*N-OH-2-FAA*	*2-FAA*	*Isopropanol*	*DMSO*	*THF*	*Acetohydroxamic acid**	*Chloroacetate***
12.37	15.68	17.37	20.97	16	10	1	1	10
14.11	18.17	20.03	23.78	15.5	9	1.5	2	10
11.94	15.25	17.59	21.37	16	10	1	1	10
11.54	14.57	16.25	19.54	18	9.5	1.5	1.25	10
12.92	16.21	18.54	22.77	16	10	1	1	0§§

reason for the voiding is not understood. However, the operating pressure of 4750 p.s.i. needed to maintain a flow-rate of 2 ml/min in a 150 × 4.6 mm column packed with 5-μm Ultrasphere Octyl may be a factor.

Zorbax C_8 column

With the mobile phase used to generate the chromatogram in Fig. 2A, 2-FAA and its metabolites were separated on a Zorbax C_8 column (150 × 4.6 mm). The Zorbax C_8 column was found to have a greater retention capacity than the Ultrasphere Octyl column, hence a flow-rate of 3 ml/min was required to complete the separation in less than 25 min (Table II). Increasing the strength of the mobile phase and making adjustments in its composition allowed the separation to be achieved in the same amount of time at a flow-rate of 2 ml/min (Fig. 4). The use of the Zorbax column had two important advantages over the use of Ultrasphere Octyl columns. Less acetohydroxamic acid (1.25%, w/v) was required to elute N-OH-2-FAA with the Zorbax column as compared to the new Ultrasphere columns (2.0%, w/v). The operating pressure required to maintain a flow-rate of 2 ml/min with the Zorbax C_8 columns (Fig. 4) was 2700 p.s.i. or 2000 p.s.i. less than that required with the Ultrasphere Octyl columns.

Coverage of the silica matrix

As a final note, during the work to achieve elution of N-OH-2-FAA and a separation of the 2-FAA metabolites, it was apparent that column packings with more extensive coverage of the silica matrix by the organic matrix were better suited to this purpose. Both the Zorbax C_8 and the Ultrasphere Octyl packings were end-capped for maximum coverage of surface silanols. Packings which are not end-capped, such as LiChrosorb RP-8 (data not shown) and the IBM C_8 (Table II) pose a

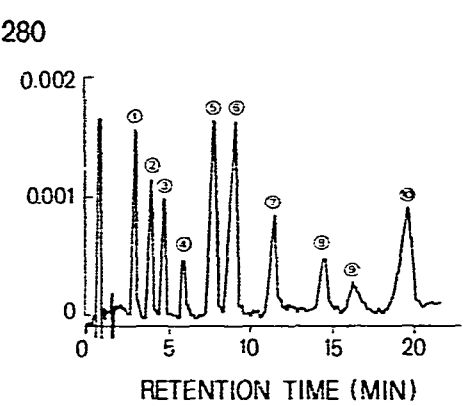


Fig. 4. Separation of 2-FAA and its metabolites on a Zorbax C_8 column 150 × 4.6 mm I.D. operated at 30°C and a flow-rate of 2 ml/min with an operating pressure of 2700 p.s.i. The mobile phase contained DMSO (9.5%, v/v), isopropanol (18%, v/v), 0.5 *M* sodium chloroacetate, pH 3.0 (10%, v/v), and acetohydroxamic acid (1.25%, w/v). Compounds are identified in Table I and were injected each at a concentration of 0.5 μg/ml in 10 μl.

problem to the elution of 2-FA in addition to the other problems associated with separations of the 2-FAA metabolites. Perhaps it is the more extensive coverage of the silica found with C_2 packings that permitted the elution of N-OH-2-FAA from such packings more readily.

ACKNOWLEDGEMENTS

This investigation was supported by the PHS Grant No. CA-28000 awarded by the National Cancer Institute, DHHS, and by U.S. Veterans Administration. The authors thank Mr. Milo Polovina for technical assistance.

REFERENCES

1 U. S. Seal and H. R. Gutman, *J. Biol. Chem.*, 234 (1959) 648.
2 C. C. Irving, *J. Biol. Chem.*, 239 (1964) 1589.
3 P. D. Lotlikar, M. Enomoto, J. A. Miller and E. C. Miller, *Proc. Soc. Exp. Biol. Med.*, 125 (1967) 341.
4 E. Kaplan, H. R. Gutmann and T. H. Emory, *Biochem. Pharmacol.*, 27 (1978) 1581.
5 J. A. Miller, *Cancer Res.*, 30 (1970) 559.
6 H. R. Gutmann, *Anal. Biochem.*, 58 (1974) 469.
7 F. R. Fullerton and C. D. Jackson, *Biochem. Med.*, 16 (1976) 95.
8 S. S. Thorgeirsson and W. L. Nelson, *Anal. Biochem.*, 75 (1976) 122.
9 R. Raineri, J. A. Poiley, M. K. Ernst, T. Hillesund and R. J. Pienta, *J. Liquid Chromatogr.*, 1 (1978) 457.
10 J. H. N. Meerman, A. B. D. van Doorn and G. J. Mulder, *Cancer Res.*, 40 (1980) 3772.
11 E. Hara, K. Kawajiri, O. Gotoh and Y. Tagashira, *Cancer Res.*, 41 (1981) 253.
12 M. D. Corbett and B. R. Chipko, *Anal. Biochem.*, 98 (1979) 169.
13 J. A. Hinson, L. R. Pohl and J. R. Gillette, *Anal. Biochem.*, 101 (1980) 462.
14 C. L. Smith and S. S. Thorgeirsson, *Anal. Biochem.*, 113 (1981) 62.
15 E. C. Miller, J. A. Miller and H. A. Hartmann, *Cancer Res.*, 21 (1961) 815.
16 M. A. Morgan and H. R. Gutmann, *J. Org. Chem.*, 24 (1959) 1163.
17 E. K. Weisburger and J. H. Weisburger, *J. Org. Chem.*, 19 (1954) 964.
18 E. K. Weisburger and J. H. Weisburger, *J. Org. Chem.*, 20 (1955) 1396.
19 J. H. Weisburger and E. K. Weisburger, *J. Chem. Soc., Part I*, (1954) 758.
20 H. R. Gutmann and E. Kaplan, *J. Chromatogr.*, 144 (1977) 136.
21 E. J. Barry, D. Malejka-Giganti and H. R. Gutmann, *Chem. Biol. Interact.*, 1 (1969/70) 139.
22 O. Diels, *Chem. Ber.*, 34 (1901) 1758.