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Note

Rapid analysis of small molecules in the presence of DNA by high-performance liquid chromatography using internal surface reversed-phase silica

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"Internal surface reversed-phase" (ISRP) chromatography was first developed by Pinkerton and co-workers and utilized glycine-phenylalanine-phenylalanine (GFF) [1] or Diol-Gly-Phe-Phe peptide [2] bonded phases The characteristic feature of these ISRP supports is the presence of two surfaces, an outer porous hydrophilic layer and an inner hydrophobic surface. ISRP chromatography combines the properties of size-exclusion chromatography (SEC) and reversed-phase chromatography with, for example, large biopolymers being excluded from the hydrophobic inner surface wherein small bioactive molecules (drugs and their metabolites) are resolved from one another and from the more rapidly migrating polymers.

There are several reports on the use of ISRP columns for the analysis of drugs and their metabolites by direct injection of plasma [3,4], serum [5–7] and protein samples [8,9], without pre-clean up and extraction procedures. However, we are unaware of any such use of this support for the analysis of mixtures containing small organic molecules and nucleic acids. The analysis of such mixtures is important, for example, in studies of carcinogenic polycyclic aromatic hydrocarbons and their metabolites [10]. Our own requirement was for a methodology which would permit the rapid analysis of mixtures of DNA and small molecules which photolytically bind to the nucleic acid. We herein report the first application of an ISRP column for the analysis of small molecules in the presence of DNA.

EXPERIMENTAL

Reagents and materials

E-Urocanic acid (*E*-UA), *E*-indoleacrylic acid (*E*-IA), calf thymus DNA (Type I, sodium salt, "highly polymerized") and Sephadex G-100–120 (bead size 40–120

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μm, excludes molecular masses > 100 000) were purchased from Sigma (St. Louis, MO, U.S.A.). *E-p*-Methoxycinnamic acid (*E-p*MCA) was from Aldrich (Milwaukee, WI, U.S.A.). The corresponding *Z*-isomers were obtained by photolysing the *E*-isomers in 0.1 *M* sodium phosphate (pH 7.0). HPLC-grade methanol was from Burdick and Jackson Labs. (McGaw Park, IL, U.S.A.). Deionized water was distilled from glass using a Corning MP-1 water still.

Photolysis

An authentic DNA–IA photoadduct was prepared by photolysing a 6.0-ml degassed 0.1 *M* sodium phosphate (pH 7.0) solution of *E*-IA (1.4 m*M*, 0.26 mg/ml) and calf thymus DNA (2.8 m*M*, 0 91 mg/ml) in a turntable at 17°C with a Pyrex-filtered (λ > 294 nm) medium-pressure mercury lamp (Canrad-Hanovia, Model 679A-36, Newark, NJ, U.S.A.) for 15 h. The DNA was purified by several dialyses and precipitations and finally subjected to Sephadex chromatography. A comparable study of DNA and radiolabeled IA has been shown to lead to incorporation of the label to a level of 295 nmol IA per mg DNA (0.097 IA per base) [11]. Release of the IA from the DNA was achieved by photolysis with a Canrad-Hanovia (Model 688A-45) low-pressure mercury resonance lamp (λ = 254 nm).

Chromatography

DNA which had been reacted with the acrylic acids was further purified by passage through a Sephadex G-100–120 column (430 mm × 11 mm I.D.). It was then subjected to high-performance liquid chromatography (HPLC) using a Varian 5000 Series programmable solvent delivery liquid chromatograph fitted with a Rheodyne 7125 (Cotati, CA, U.S.A.) injection port with either a 0.2-ml or a 2.0-ml injection loop, a Varian 2050 variable-wavelength detector and a 250 mm × 4 6 mm I.D., 5-μm ISRP (GFF-S5-80) column (Regis, Morton Grove, IL, U.S.A.). Unless otherwise specified, a 50-μl sample was injected and the data were collected and processed on a Perkin-Elmer LCI-100 computing integrator. The aqueous mobile phase consisted of sodium phosphate (pH 7 0) freshly prepared and filtered through a 0.45-μm Nylon filter. The organic modifier was mixed by the ternary solvent delivery capability system. The detector was set at 254 nm for monitoring UA and at 280 nm for monitoring IA and *p*MCA.

RESULTS AND DISCUSSION

We have recently been interested in the photolytically induced covalent binding of a series of acrylic acid derivatives to DNA. The compounds we have studied (Fig. 1) include 3-(1H-imidazol-4-yl)-2-propenoic acid (urocanic acid, UA, a metabolite of histidine found in the skin), 3-(1H-indol-3-yl)-2-propenoic acid (indoleacrylic acid, IA, a tryptophan metabolite found in animals and plants) and 3-(4-methoxyphenyl)-2-propenoic acid (*p*-methoxycinnamic acid, *p*MCA, com-

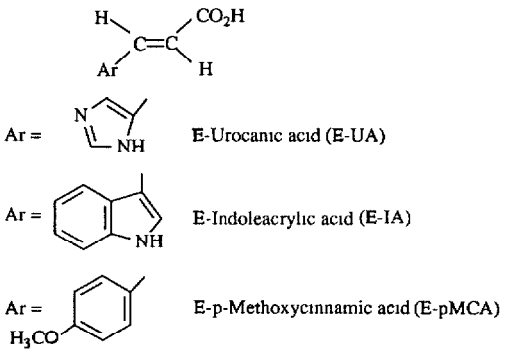

Fig 1. Structures of acrylic acid derivatives

monly used as its 2-ethylhexyl ester as a sunscreen). We have demonstrated that *E*-UA [12], *E*-IA [11] and *E*-*p*MCA [13] can each be photoactivated in the presence of DNA and bind irreversibly to the biopolymer. One of the binding mechanisms identified is a 2 + 2 cycloadditon of the acrylic acid ethylenic bond with the 5,6 double bond of thymidine in DNA to form cyclobutane adducts [11,14].

A diagnostic test for this mechanism is the re-irradiation of the photomodified DNA with higher-frequency (*i.e.* 254 nm) light, whereby the cyclobutane adducts are typically cleaved to give back the intact acrylic acid derivatives and thymidine. The procedure usually requires pre-digestion of the DNA with enzymes or acid, but there would be not necessity to do so if the photomodified DNA could be irradiated and the mixture analyzed directly for the release of *e.g.* acrylic acid derivatives. In addition to the obvious advantages of speed and convenience, a direct analysis of the DNA mixtures would permit the assay for certain cyclobutane adducts, such as those formed between psoralens and DNA, which are sensitive to enzymatic hydrolysis and thus do not survive DNA degradation [15]. We have thus tested the ISRP column's ability to resolve and quantitate small molecules in the presence fo DNA by chromatographing authentic mixtures of DNA and the *E*- and *Z*-isomers of the acrylic acid derivatives, and have applied this technique to the analysis of *E*-IA and *Z*-IA photolytically released from modified DNA.

Fig. 2 displays the elution profiles for mixtures of DNA and each of the acrylic acid derivatives. Note that the ISRP column successfully resolves the *E*/*Z* (*i.e.*, *trans*/*cis*) isomers, the capacity factors for which are presented in Table I.

The separation of the isomers of *p*MCA was studied as a function of the methanol content in the mobile phase, with the results shown in Fig. 3. Decreasing concentrations of methanol increase both the absolute retention times and the isomer resolution, while giving rise to a small diminution in back-presure. Using 0.1 *M* sodium phosphate without an organic modifier (flow-rate 1.0 ml/min), the *E*- and *Z*-isomers of IA eluted at 11.5 and 8.1 min, respectively, while the corre-

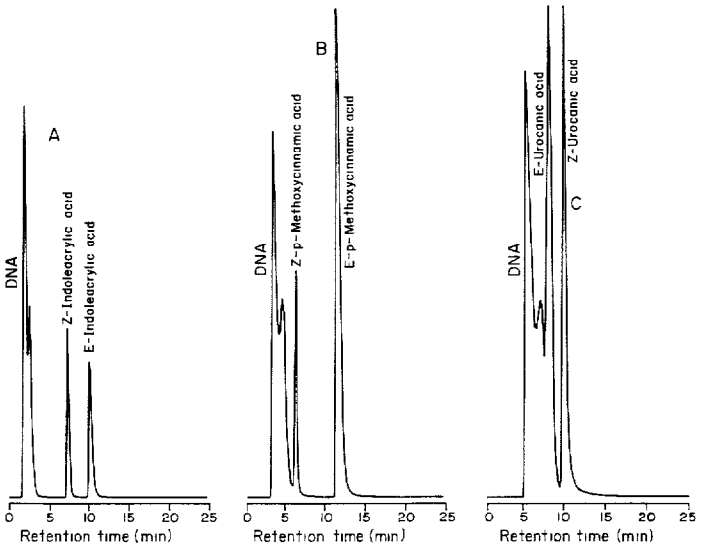

Fig 2 Chromatograms of DNA (1 7 m*M*, 0 56 mg/ml) spiked with acrylic acid derivatives (0 3 m*M*, 0 04–0.06 mg/ml) Mobile phase 0 1 *M* sodium phosphate (pH 7 0) (A) IA, flow-rate 1.0 ml/min, (B) *p*MCA, flow-rate 0 5 ml/min; (C) UA, flow-rate 0 3 ml/min, injection volume 50 μl

TABLE I

CAPACITY FACTORS OF ACRYLIC ACID DERIVATIVES ON AN ISRP COLUMN

The analyses were done in duplicate with reproducible chromatograms

Compound	Capacity factor	
	E-Isomer	*Z*-Isomer
Indoleacrylic acid	5 38	3 56
p-Methoxycinnamic acid	2 64	0 99
Urocanic acid	0 57	0 90

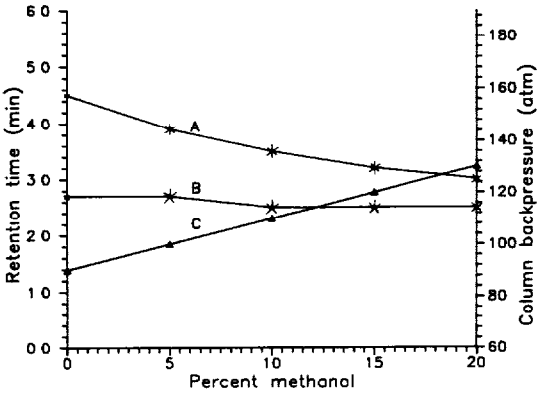

Fig 3. Column backpressure (line C), and *E*- and *Z*-*p*MCA chromatographic retention times (lines A and B, respectively) as a function of percent methanol Mobile phase, 0.05 *M* sodium phosphate (pH 7.0), flow-rate, 1 0 ml/min, injection volume, 50 μl of a 1.0 m*M* (0.18 mg/ml) solution of the isomeric mixture

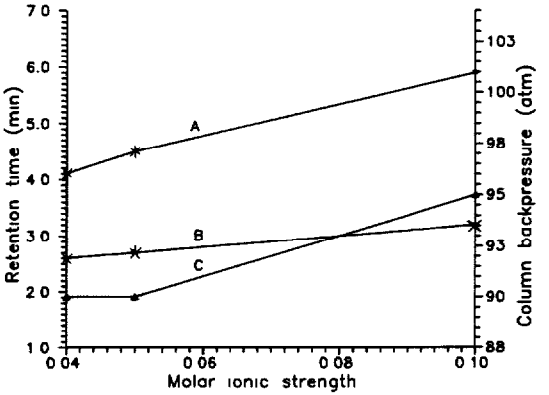

Fig 4 Chromatographic retention times of *E*- and *Z*-*p*MCA (lines A and B, respectively) and column backpressure (line C) as a function of eluent ionic strength Mobile phase, sodium phosphate (pH 7 0), flow-rate, 1 0 ml/min; injection volume, 50 μl of a 1 0 m*M* (0 18 mg/ml) solution of the isomeric mixture

sponding retention times of *E*- and *Z*-UA were 8.1 and 9.7 min (flow-rate 0.3 ml/min). The effect of the mobile phase ionic strength on the retention times of *p*MCA is presented in Fig. 4, where it is evident that the isomer resolution increases at higher ionic strength, primarily due to a large increase in the *E*-isomer retention time; there is no significant effect on the back-pressure.

To test the ability of ISRP chromatography to detect and resolve small organics released from DNA, a 1.0-ml solution of purified DNA (0.36 m*M*, 0.12 mg) which had been reacted with IA was re-irradiated at 254 nm and the photolysate (50 μl) injected directy onto the column. The chromatogram (Fig. 5) showed two new peaks at 8.1 and 11 5 min, corresponding to *Z*-IA and *E*-IA, respectively (confirmed by co-injection with authentic samples).

In a separate experiment, the quantum efficiencies (ϕ's) for disappearance of

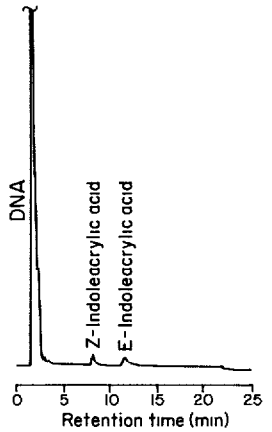

Fig 5 Chromatogram of DNA–indoleacrylic acid photoadduct after re-irradiation at λ = 254 nm for 20 min. Mobile phase, 0 1 *M* sodium phosphate (pH 7 0), flow-rate, 1 0 ml/min, injection volume, 50 μl

the acrylic acid derivatives upon co-photolysis with DNA using 308-nm light (XeCl laser) were determined by direct injection of the photolysates onto the column and quantitating the unreacted compounds (data not shown).

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