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SEPARATION OF α-KETO ACIDS BY CAPILLARY SUPERCRITICAL FLUID CHROMATOGRAPHY AS THEIR QUINOXALINOL DERIVATIVES

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SUMMARY

α-Keto acids were derivatized with *o*-phenylenediamine to produce a nitrogen-containing quinoxalinol. The derivatives were analyzed by capillary supercritical fluid chromatography (SFC) with nitrogen thermionic detection. The quinoxalinols were analyzed without additional silylation as is required for efficient gas chromatographic analysis. α-Keto acids were also extracted from human urine and converted to the corresponding quinoxalinols. Simultaneous pressure and temperature programming was employed to optimize chromatographic efficiency. The nitrogen thermionic detector used demonstrated linearity over 3–4 orders of magnitude. A sensitivity of 1 pg N/s was obtained for 3-benzylquinoxalinol at a signal-to-noise ratio of 4:1.

INTRODUCTION

During the last several years, supercritical fluid chromatography (SFC) has been under extensive development as an effective method for the analysis of non-volatile and thermally labile compounds. For difficult separations, capillary SFC has been primarily applied to relatively non-polar mixtures originating from fossil fuels, plastics and fatty materials. Applications of SFC to polar substances, such as various biochemical compounds, natural products, or pharmaceuticals, remain uncommon due to the relatively poor solvating capacities of the conventional mobile phases (*e.g.*, carbon dioxide or nitrous oxide).

In order to extend the scope of SFC toward polar solutes, three general approaches appear feasible: (a) exploring the mobile phases with appreciable dipole moments (*e.g.*, ammonia or sulfur dioxide); (b) using a polar retention modifier; and (c) improving the solute's solubility in a mobile phase through chemical derivatization. Although all three approaches merit exploration, the sample derivatization is particularly attractive if the formed derivatives become sufficiently soluble in "comfortable" mobile phases such as carbon dioxide or nitrous oxide. This has recently been demonstrated with silylated oligosaccharides[1] and biological conjugates of steroid metabolites and bile acids[2].

The present communication deals with yet another example of a biologically important class of compounds, α-keto acids. The reaction of these compounds with *o*-phenylenediamine is known to proceed quantitatively, forming quinoxalinols:

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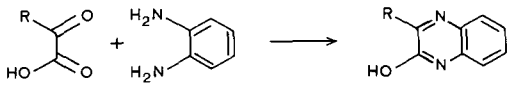

While this derivatization scheme was initially proposed for gas chromatographic (GC) studies[3], the restrictions of volatility are a problem. Such a difficulty is not experienced in SFC. An additional advantage of this solute derivatization approach is the incorporation of two nitrogen atoms into the structure, which facilitates highly sensitive detection by a nitrogen-sensitive thermionic detector.

EXPERIMENTAL

Apparatus

The supercritical fluid chromatograph was a home-made instrument as described previously[4]. A Brownlee Labs. micropump (Applied Biosystems, Santa Clara, CA, U.S.A.) with software version G, was used as the source of mobile phase. The mobile phase employed was SFC grade nitrous oxide (Scott Specialty Gases, Plumsteadville, PA, U.S.A.). The nitrous oxide cylinder also contained 1100 p.s.i. of helium head pressure to facilitate rapid filling of the pump without externally cooling the pump heads. Injection was accomplished via an electrically actuated high-pressure valve with an internal sample loop volume of 0.06 μl (Valco Instruments, Houston, TX, U.S.A.). The capillary columns were 10 m $\times$ 50 μm I.D. The fused-silica inner surface was deactivated prior to coating through treatment with polymethylhydrosiloxane (85 cSt) (Petrarch Systems, Bristol, MA, U.S.A.)[5]. The column was statically coated wth a 0.25-μm film of SE-30 at 50°C, and the stationary phase was crosslinked three times with azo-*tert.*-butane (Alfa Products, Danvers, MA, U.S.A.)[6].

Pressure restriction for the operation of a flame detector was accomplished by forming an integral restrictor[7] directly at the end of the coated column. The detector was a modified Perkin-Elmer Sigma 3 nitrogen–phosphorus detector. Polarization voltage, bead heating current and signal amplification were provided by a Perkin-Elmer stand-alone nitrogen–phosphorus detection electrometer (Perkin-Elmer, Norwalk, CT, U.S.A.). The rubidium silicate thermionic source was prepared according to the procedure of Lubkowitz *et al.*[8]. Beads containing 1.6% B_2O_3, 12.4% Na_2O, 74.0% SiO_2, 12.0% Rb_2O exhibited optimum sensitivity and lifetime. Typical detector operating conditions were: heating block temperature, 300°C; polarizing voltage, −250 V; bead heating current, 2.5 A; 1.0 ml/min hydrogen flow; 100 ml/min air flow.

Derivatization procedure

All α-keto acid standards were obtained in the free acid form (Sigma, St. Louis, MO, U.S.A.). Milligram amounts of the α-keto acid standards were dissolved in 2 ml of 4 *N* hydrochloric acid. To this solution 2 ml of a 1% solution of *o*-phenylenediamine in 2 *M* hydrochloric acid was added. The reaction was performed at 70°C for 1 h. The resultant quinoxalinols precipitated upon cooling and were collected by vacuum filtration. The filtrate was recrystallized from a 70% ethanol solution.

α-Keto acids were extracted from normal urine by a procedure similar to that reported by Langenbeck *et al.*[9]. After collection, 4 mg of sodium dithionite was added per ml of urine to stabilize aromatic α-keto acids[10]. A 20-ml aliquot of a 24-h urine

collection was acidified to pH 2 with 12 *M* hydrochloric acid. Urinary acids were extracted three times with 4 ml of ethyl acetate and twice with 4 ml of diethyl ether. The combined extracts were evaporated to dryness. The residue was dissolved in 0.5 ml of 4 *M* hydrochloric acid, and 1 ml of 1% *o*-phenylenediamine in 2 *M* hydrochloric acid was added to the solution. The reaction proceeded for 1 h at 70°C. After reaction was completed, the solution was saturated with 1.5 g of ammonium sulphate. The quinoxalinols were extracted three times with 5 ml of chloroform. The combined extracts were evaporated to dryness under a stream of dry nitrogen. The residue was reconstituted in 50 μl of acetone.

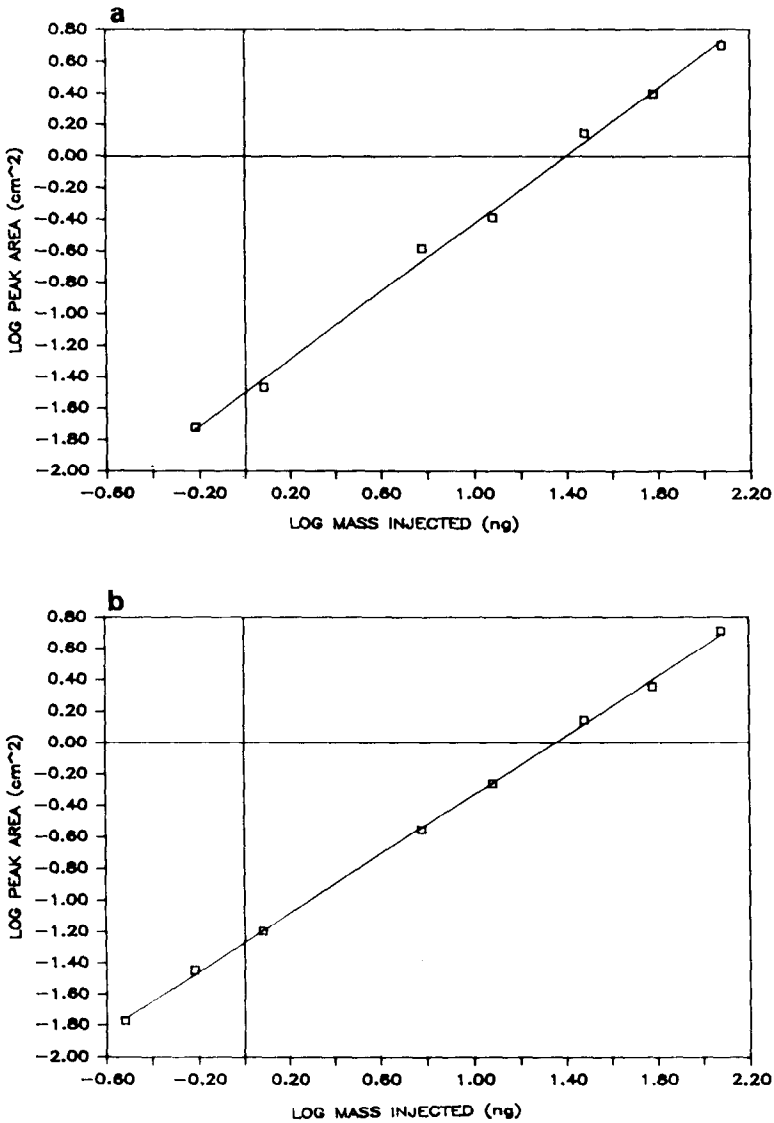

Fig. 1. Linearity of thermionic detector response. (a) *n*-Hexylquinoxalinol, (b) 3-benzylquinoxalinol.

RESULTS AND DISCUSSION

Calibration curves for both an aliphatic and aromatic α-keto acid quinoxalinol are shown in Fig. 1. Each point plotted was the average of five peak area measurements at each concentration. All peak area measurements had a relative standard deviation (R.S.D.) of 4% or less. Throughout the calibration study, a constant background bead current of 10 pA was maintained to improve the reproducibility of the detector response. Background current was controlled by making fine adjustments in the bead heating current.

Fig. 1a shows the calibration curve for *n*-hexylquinoxalinol from α-ketooctanoic acid. The response was linear over 3–4 orders of magnitude with a correlation coefficient of 0.9984. The slope of the log–log plot was 1.071. The sensitivity for *n*-hexylquinoxalinol was 2.0 pg N/s at a signal-to-noise ratio of 3:1.

Fig. 1b shows the calibration curve for 3-benzylquinoxalinol formed from phenylpyruvic acid. The detector response was linear over the same range as above, with a correlation coefficient of 0.9995. The slope of the log–log plot was 0.936. A sensitivity of 1.0 pg N/s was obtained for 3-benzylquinoxalinol at a signal-to-noise ratio of 4:1.

As shown in Fig. 2, quinoxalinol derivatives of aliphatic α-keto acids were

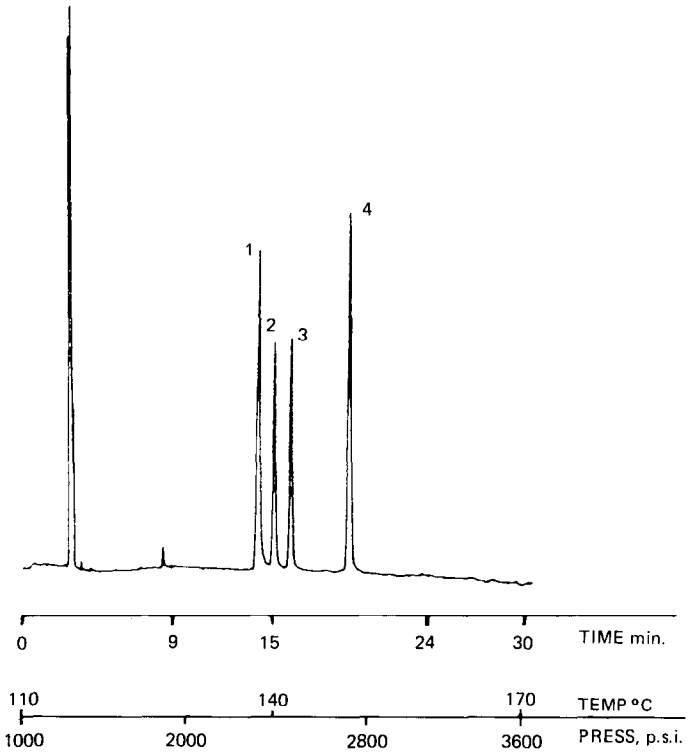

Fig. 2. Standard chromatogram of aliphatic quinoxalinols. Peak identification: (1) methylquinoxalinol from pyruvic acid; (2) ethylquinoxalinol from α-ketobutyric acid; (3) *n*-propylquinoxalinol from α-ketovaleric acid; (4) *n*-hexylquinoxalinol from α-ketooctanoic acid.

analyzed by capillary SFC without silylation of the remaining hydroxyl group as is required for efficient GC analysis[9,10]. Each peak corresponds to approximately 3 ng of quinoxalinol injected on column. Resolution was optimized through a simultaneous temperature/pressure program. At high mobile phase pressure (density) the solute diffusion coefficient (D_m) decreases, leading to a corresponding loss of efficiency. Fields and Lee[11] have shown that chromatographic efficiency in SFC decreases by nearly 75% during a density programmed run. To partially offset the efficiency loss at higher mobile phase densities, higher column temperatures can be used. The increase in

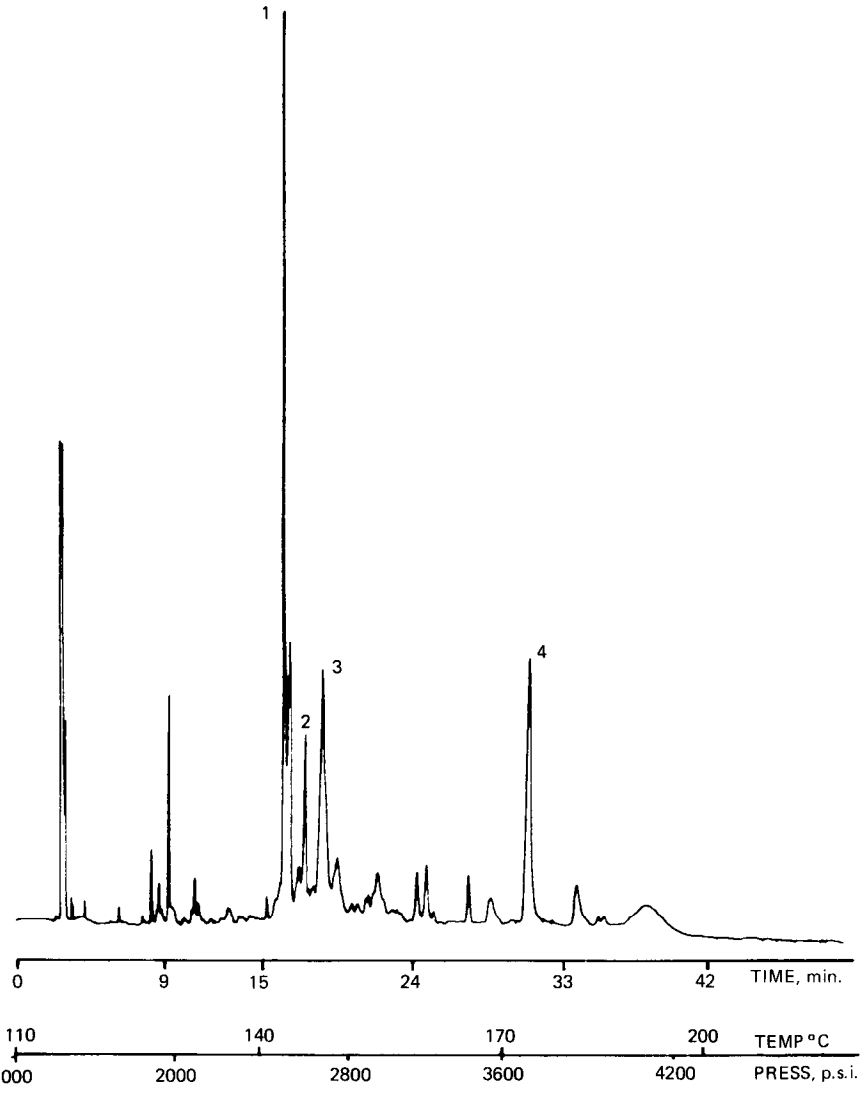

Fig. 3. Chromatogram of quinoxalinol derivatives of human urinary α-keto acids. Peaks tentatively: (1) methylquinoxalinol; (2) ethylquinoxalinol; (3) *p*-propylquinoxalinol; (4) 3-benzylquinoxalinol.

operating temperature enhances separation through two effects: (1) an increase in the mobile phase diffusion coefficient; and (2) an increase in solute volatility which corresponds to a decrease in capacity factor (k'). In both the standard and urinary quinoxalinol chromatograms (Figs. 2 and 3) a slow temperature ramp of 2°C/min was initiated at injection to enhance efficiency.

The chromatogram of urinary α-keto acids is shown in Fig. 3. Peaks were tentatively identified by retention time comparison with standard quinoxalinols. Peaks corresponding to aliphatic, aromatic and dicarboxylic α-keto acid derivatives are evident in the chromatogram. The aliphatic region of the chromatographic profile occurs at 15–25 min, while the aromatic quinoxalinols elute approximately 30 min after injection. The two broad chromatographic zones eluting after 33 min have tentatively been assigned to the carboxyquinoxalinols of α-ketoglutaric and α-keto-adipic acids. These derivatives contain a free carboxylic acid moiety which increases their polarity and consequently decreases their solubility in supercritical nitrous oxide. Therefore, these compounds are more susceptible to irreversible interaction with the residual surface silanols which have not been deactivated in the column coating process. The calibration curve for the quinoxalinol from α-ketoglutaric acid exhibited non-linearity for the lower mass range. This result is indicative of irreversible interaction occuring in the analysis of the carboxyquinoxalinol. These interactions can result in a broadened chromatographic peak profile. Two possibilities exist to improve chromatographic efficiency for the carboxyquinoxalinols: (1) the use of a more inert capillary column with a greater stationary film thickness (0.5–1 μm), or (2) the addition of a polar mobile phase modifier to enhance the solvent strength of supercritical nitrous oxide. Our preliminary results indicate that the nitrogen thermionic detector shows neglible loss of sensitivity or selectivity with mixed supercritical mobile phases containing up to 10 mol% of an organic solvent such as methanol or isopropanol.

The consequence of increased supercritical mobile phase solvent strength is a decreased solute retention. To maintain resolution of the early eluting peaks in the urine chromatogram, mobile phase modification must occur only at the end of the analysis. Therefore, the composition of the mobile phase must be programmed in a manner analogous to gradient elution in high-performance liquid chromatography[12].

The efficiency of capillary SFC combined with sensitive and selective thermionic detection is ideal for the analysis of biologically important compounds existing in complex physiological matrices. Additional derivatization reactions which incorporate heteroatom-containing moieties into the compounds of biochemical interest are under investigation. Minor detector modifications allow selective thermionic response to other heteroatoms besides nitrogen. The sensitivity and selectivity of the thermionic detector response can thus be exploited in the analysis of other biologically important compounds.

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REFERENCES

1 T. L. Chester and D. P. Innis, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 9 (1986) 209–212.
2 C. Borra, F. Andreolini and M. Novotny, *Anal. Chem.*, in press.
3 U. Langenbeck, H.-U. Möhring and K.-P. Dieckmann, *J. Chromatogr.*, 115 (1975) 65–70.
4 M. Novotny and P. David, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 9 (1986) 647–651.
5 C. L. Woolley, R. C. Kong, B. E. Richter and M. L. Lee, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 7 (1984) 329–332.
6 B. W. Wright, P. A. Peaden, M. L. Lee and T. J. Stark, *J. Chromatogr.*, 248 (1982) 17–34.
7 E. J. Guthrie and H. E. Schwartz, *J. Chromatogr. Sci.*, 24 (1986) 236–241.
8 J. A. Lubkowitz, B. P. Semonian, J. Galobardes and L. B. Rogers, *Anal. Chem.*, 50 (1978) 672–676.
9 U. Langenbeck, A. Hoinowski, K. Mantel and H.-U. Möhring, *J. Chromatogr.*, 143 (1977) 39–50.
10 U. Langenbeck, A. Mench-Hoinowski, K.-P. Dieckmann, H.-U. Möhring and M. Petersen, *J. Chromatogr.*, 145 (1978) 185–193.
11 S. M. Fields and M. L. Lee, *J. Chromatogr.*, 349 (1985) 305–316.
12 C. R. Yonker and R. D. Smith, *Anal. Chem.*, 59 (1987) 727–731.