Journal of Chromatography, 281 (1983) 135–149
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,196

REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF α-KETO ACID QUINOXALINOL DERIVATIVES

OPTIMIZATION OF TECHNIQUE AND APPLICATION TO NATURAL SAMPLES

DAVID J. KIEBER*,* and KENNETH MOPPER*
College of Marine Studies, University of Delaware, Lewes, DE 19958 (U.S.A.)
(Received July 28th, 1983)

SUMMARY

A rapid, sensitive and selective method to quantify α-keto acids in natural samples is described. The basis of this method is the reaction of α-keto acids with *o*-phenylenediamine to form highly fluorescent quinoxalinol derivatives. These derivatives are separated by reversed-phase high-performance liquid chromatography (RP-HPLC) and detected fluorometrically. Information is provided concerning optimal derivatization and chromatographic conditions. Sample cleanup steps that are usually required prior to RP-HPLC analysis are eliminated. The method is reproducible (± 1.90% at the 11 pmole level) and the fluorescent response is linearly related to α-keto acid concentration at both the μM and the nM level. Complete recoveries are obtained ($\geq$98%) at the μM level. The detection limit is in the pmole to fmole range per injected acid. Applications to physiological and environmental samples are illustrated.

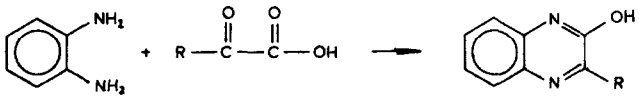
INTRODUCTION

α-Keto acids are key intermediates in a number of major biochemical pathways including glycolysis, photorespiration, and amino acid and carbohydrate metabolism[1]. Because of the biological importance of α-keto acids, numerous methods have been developed to quantify this class of compounds in biological samples.

Gas–liquid chromatography (GLC) has been widely used to quantify α-keto acids and has proven to be quite sensitive[2–6]. Unfortunately, GLC techniques require a number of steps both to clean up samples (isolate components of interest) and to produce volatile derivatives suitable for GLC analysis. High-performance liquid chromatographic methods of analysis (HPLC) using ultraviolet (UV) or fluorescence detection of derivatized α-keto acids are also quite sensitive[7–10]. However, like GLC

* Present address: Rosentiel School of Marine and Atmospheric Science, University of Miami, Miami, FL 33149, U.S.A.

0021-9673/83/$03.00



OPD α-KETO ACID 2-QUINOXALINOL

Fig. 1. Reaction between *o*-phenylenediamine (OPD) and an α-keto acid to form a substituted quinoxalinol derivative.

methods, most HPLC methods require numerous cleanup steps prior to HPLC analysis to remove highly fluorescent or UV-absorbing interferences that may co-elute with derivatized α-keto acids. Recently, a promising HPLC method to analyze α-keto acids in aqueous media has been developed[11] although no natural samples were tested. With this method, acids are separated by ion-exchange chromatography, then successively labeled with N-methyl nicotinamide chloride (NMN) and detected fluorometrically. This technique is simple to perform, rapid and sensitive. However, an anion-exchange column and post-column reaction system are needed, thereby necessitating an HPLC system dedicated for the analysis of this class of compounds. Furthermore, for seawater samples, a tedious desalting step is necessary prior to HPLC analysis.

Another HPLC method for the analysis of α-keto acids has been developed by Liao *et al.*[8]. With this method, α-keto acids were converted to their quinoxalinol derivatives by reaction with *o*-phenylenediamine (OPD) (Fig. 1) and subsequently separated by reversed-phase high-performance liquid chromatography (RP-HPLC) and detected by UV absorbance. A major drawback to their method is that a number of cleanup steps are needed before a sample can be analyzed. Also, these investigators did not present any information regarding optimal derivatization or chromatographic conditions. Recently, the quinoxalinol method has been modified[12,22] by using fluorescence instead of UV detection. However, these methods, like the method of Liao *et al.*[8], still require numerous sample cleanup steps prior to chromatographic injection.

In the present study, a detailed investigation of the advantages and limitations of the OPD technique is given; in particular, detailed information is provided concerning optimal derivatization and chromatographic conditions. This method has been modified to eliminate sample cleanup steps that are otherwise required prior to RP-HPLC analysis. Applications to biological and natural samples are illustrated.

MATERIALS AND METHODS

Apparatus

The high-performance liquid chromatograph consisted of the following equipment: a Model 421 system controller with two Model 100A pumps (Beckman Instrument Inc., Berkeley, CA), a Model UHP-7K automatic injector with a 30- or 500-μl sample loop (Valco Instruments Co., Houston, TX), a Model 650-10S scanning fluorescence spectrophotometer with a 20-μl quartz flow-cell (Perkin-Elmer, Norwalk, CT), and an SP4050 printer/plotter (Spectra-Physics, Santa Clara, CA). Chromatographic separations were carried out with a 100 × 4.6 mm stainless-steel Adsorbosphere reversed-phase (RP-18) column containing 3-μm packing (Applied

Science, State College, PA). A direct connect guard column containing RP-18 40-μm packing (Applied Science) was attached to the analytical column.

Chemicals

Distilled-in-glass organic solvents (Burdick and Jackson, Muskegon, MI) and buffer salts (Fisher Scientific, King of Prussia, PA) were HPLC grade. Reagent grade OPD was obtained from Fisher Scientific. α-Keto acid standards [sodium pyruvate (PA), sodium α-ketoisovalerate (KIVA), α-ketovaleric acid (KVA), sodium α-keto-isocaproate (KICA), sodium α-ketocaproate (KCA), sodium α-ketobutyrate (KBA), α-ketoglutaric acid (KGA), *p*-hydroxyphenylpyruvic acid (HPPA), sodium glyoxylate (GA), β-phenylpyruvate (PPA), oxaloacetic acid (OAA), sodium α-keto-β-methyl-*n*-valerate (MKVA), α-ketooctanoic acid (KOA), α-ketononanoic acid (KNA) and indole-3-pyruvic acid (IPPA)] were purchased from Sigma Chemical Company, St. Louis, MO. All organic solvents, α-keto acid standards, and buffer salts were used without further purification.

Organic clean water (Q water) was obtained by passing tap water through both a Milli RO and a Milli Q system containing an Organex Q attachment (Millipore, Bedford, MA).

o-Phenylenediamine purification

Since numerous reagent peaks were observed in chromatograms when samples were analyzed at high sensitivities, the OPD reagent was purified. The following purification procedure was employed: a mixture containing 0.25 g OPD, 5 ml Q water and 1.0 ml concentrated HCl solution (approximately 12 *M*) was reacted for 1 h at 45°C in a borosilicate glass test tube sealed with a PTFE-lined cap. After the reaction was completed, the mixture was cooled in an ice bath. A 1.5-ml aliquot of this mixture was passed through an RP-18 Sep-Pak column (Waters Assoc., Milford, MA) at a flow-rate approximately 1 ml/min. The eluate was collected and stored in an ice bath. Before a 1.5-ml aliquot was loaded onto the Sep-Pak, the Sep-Pak was conditioned with 25 ml methanol and 15 ml Q water. The eluate contained purified OPD that was used in the derivatization procedure. This reagent solution was stable for at least one week when stored frozen at −20°C.

Standard solutions

α-Keto acid standards (1 m*M*) were made in methanol–water (20:80) and stored at 4°C. All standards except KGA and OAA were stable for at least two weeks. The KGA and OAA standards decayed within a few days; therefore, these standards were made just prior to their use.

Derivatization procedure

The method of Liao *et al.*[8] was optimized; the following procedure was reproducible and quantitative for both natural samples and standards. Physiological fluids (*e.g.*, urine, blood serum) were deproteinated (sample diluted to 50% with methanol). These samples as well as environmental samples (*e.g.*, seawater) were filtered through a Whatman GF/F filter using a Gelman support system (Gelman Sciences, Ann Arbor, MI), made of Delrin and stainless-steel, attached to a 25-ml polypropylene syringe. To 2.5 ml of a filtered sample were added 0.5 ml purified OPD solution

(containing approximately 0.44 *M* OPD in 2 *M* hydrochloric acid) and 0.3 ml concentrated hydrochloric acid solution (approximately 12 *M*). This mixture was reacted in a PTFE-sealed test tube for 1 h at 45°C. After 1 h, the reacted mixture was immersed in an ice bath until analyzed. Just prior to HPLC analysis, samples were pH-adjusted with 1.6 ml of 6 *M* sodium acetate (pH 6). Between 20 and 500 μl were injected depending on the α-keto acid concentration expected in the sample.

HPLC conditions

All chromatographic separations were performed at room temperature and at a flow-rate of 1.0 ml/min. While chromatographic conditions were being optimized, analyses were performed isocratically. During optimization of the derivatization procedure and for the analysis of natural samples, a gradient elution was normally employed.

For gradient runs, the weaker component of the mobile phase (A) was 0.035 *M* ammonium acetate and the stronger component of the mobile phase (B) was acetonitrile. The gradient typically used was: 10–35% B in 22 min, isocratic at 35% B for 8 min and 35–10% B in 3 min. To elute strongly retained substances that degrade the HPLC column and produce ghost peaks, it was necessary to run the following gradient periodically (every sixth run): 35–100% B in 5 min, isocratic at 100% B for 10 min and 100–10% B in 8 min.

RESULTS

Optimization of chromatographic conditions

Organic phase. Methanol (MeOH), acetonitrile (ACN), ACN–tetrahydrofuran (THF) (98:2) and ACN–dimethylsulfoxide (DMSO) (98:2) were examined for their effect on the chromatographic separation of quinoxalinol derivatives. In all cases, the mobile phase consisted of 75% of the weaker eluent, water, and 25% of the stronger eluent, the organic phase. From Fig. 2 it is evident that there was a dramatic decrease in the capacity factor (k') for each α-keto acid derivative when switching the organic phase from MeOH to ACN due to a corresponding increase in solvent strength. This effect became more pronounced as the hydrophobic nature of the α-keto acid derivative increased. In contrast, a small addition of either DMSO or THF to ACN altered capacity factors only slightly, relative to ACN alone, suggesting that these solvents had similar solvent strengths. However, selectivities were somewhat different.

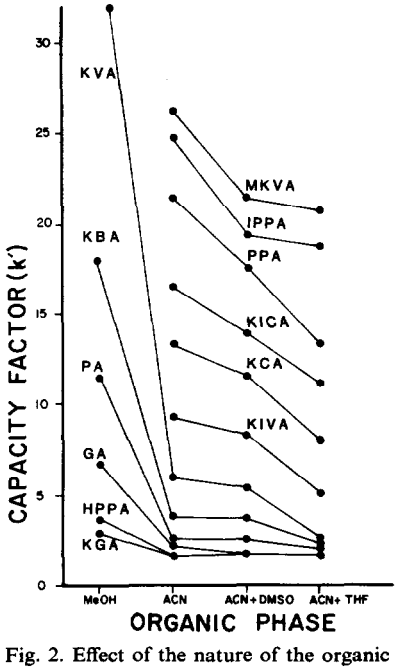
To further study the effect of THF on quinoxalinol separation, a dilution series of THF in ACN from 0 to 50% was examined. In all cases, the organic phase was held constant at 20% of the mobile phase. Results demonstrated that the capacity factor (k') only decreased slightly for each α-keto acid as the concentration of THF increased in the mobile phase which demonstrates that, for the compounds being separated, THF and ACN have similar solvent strengths. However, relative to ACN, THF affected separation selectivity by altering the retention of certain quinoxalinols preferentially. With an increase in THF, the resolution of MKVA and IPPA (initially eluted as one peak) gradually increased whereas the resolution of KVA and KBA decreased to the point where they were not resolved at 50% THF in ACN.

In relation to the organic phases tested, ACN was the solvent of choice. This

solvent was found to be a much stronger eluent than methanol. In addition, ACN had a similar solvent strength and is relatively inexpensive compared to either THF or DMSO.

A series of isocratic runs at 10, 20, 30, 40 and 50% ACN were performed to determine the effect of organic phase concentration on the separation of quinoxalinols. For all runs, the weak eluent was 0.035 *M* ammonium acetate (pH 7.0). In this series, *k'* decreased exponentially for each quinoxalinol derivative over the interval 0–50% ACN as expected[13]. For the best resolution in the shortest time, isocratic separations should be performed with 20–30% ACN. However, because of the large difference in *k'* between early and late eluting α-keto acid derivatives, gradient elution is preferred to isocratic elution.

Aqueous phase. The effect of buffer pH (at constant buffer concentration) on *k'* was examined using ammonium acetate. The pHs tested were: 4, 5, 6.2 and 8. All α-keto acids tested, except KGA, are expected to be non-ionic as their quinoxalinol derivatives within the pH range tested since the pK_a values of the nitrogen atoms (Fig. 1) are probably similar to their pK_a value in pyrazine, 0.6[14], and the pK_a of the hydroxyl group (Fig. 1) should be similar to the pK_a of the hydroxyl group in 2-hydroxyquinoline, 8.7[15]. As expected, results of the pH study indicated that the charge on quinoxalinols did not change significantly since *k'* for each α-keto acid derivative tested was constant over the pH range examined.



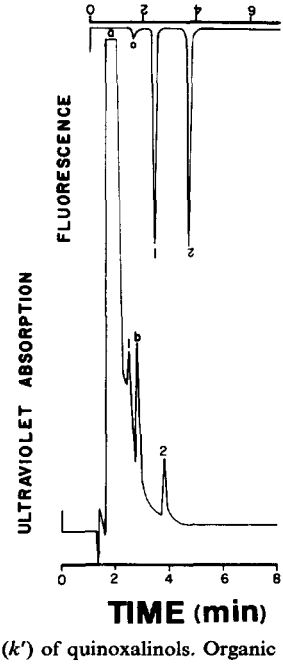


Fig. 2. Effect of the nature of the organic phase on the relative retention (*k'*) of quinoxalinols. Organic phases tested: 1, MeOH; 2, ACN; 3, ACN–DMSO, 98:2; and 4, ACN–THF, 98:2. See Materials and methods for a list of α-keto acid abbreviations and Results for organic phase abbreviations.

Fig. 3. Simultaneous detection of quinoxalinols (9 nmoles each) by fluorescence (excitation, 340 ± 5 nm; emission, 420 ± 5 nm) and UV absorption (245 ± 4 nm). Peaks: a and b, reagent peaks; 1, pyruvic acid; 2, α-ketobutyric acid.

The effect of buffer concentration on k' was also investigated (at constant buffer pH). The buffer concentrations examined were: 0, 0.01, 0.1, 0.2 and 0.35 *M* ammonium acetate. Buffer concentration, like buffer pH, did not affect k'. This result indicated that "salting-out" or "salting-in" effects[16,17] were not an important retention mechanism for quinoxalinols within the concentration range tested.

To determine the effect of changing the nature of the ionic medium of the aqueous phase on k' for each α-keto acid derivative, the following buffers were tested: sodium citrate, sodium borate, disodium hydrogen phosphate and ammonium acetate. In addition, water alone was tested. All chromatographic separations were performed isocratically at 25% ACN and 75% buffer. Buffer pH and concentration were always 7.0 and 0.5 *M* respectively. Results indicated that the nature of the ionic medium had a negligible effect on k' for all quinoxalinol derivatives suggesting that changes in retention due to relative differences in ionic strength or in the chemical nature of the buffer salt were not important at the pH tested.

Optimization of sensitivity

Fluorescence detection. The fluorescence of KBA and PA quinoxalinol derivatives at an excitation wavelength 340 ± 5 nm and an emission wavelength 420 ± 5 nm was compared to their UV absorption at 254 ± 4 nm to determine which method of detection is more sensitive. When these derivatives were detected by UV absorption, there was considerable interference in the detection of quinoxalinols because of the reagent. In contrast, the reagent and its by-products did not interfere with the detection of quinoxalinols when using fluorescence detection since these by-products have negligible fluorescence relative to quinoxalinols at the wavelengths chosen (Fig. 3). With proper wavelength selection, fluorescence detection dramatically increased the sensitivity of the method when compared with the sensitivity obtained using UV detection.

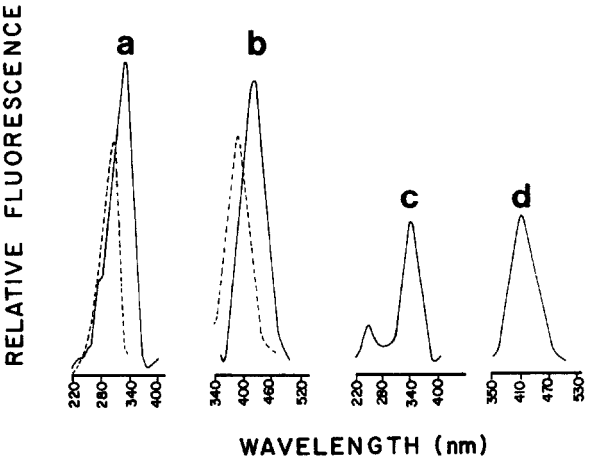


Fig. 4. Emission and excitation spectra (5-nm slits) for OPD, pyruvic acid (PA) quinoxalinol and α-ketobutyric (KBA) quinoxalinol in 0.35 *M* ammonium acetate–methanol (80:20): A, excitation spectra for PA (solid line, emission 410 nm) and OPD (dashed line, emission 360 nm); B, emission spectra for PA (solid line, excitation 340 nm) and OPD (dashed line, excitation 320 nm); C, excitation spectra for KBA (emission 410 nm); and D, emission spectra for KBA (excitation 340 nm).

Emission and excitation wavelengths. The wavelengths used to detect quinoxalinols fluorometrically were determined on the basis of the emission and excitation spectra that were obtained (using 5-nm slits) for KBA and PA quinoxalinols in 0.35 *M* ammonium acetate–MeOH (80:20). The excitation maximum for each derivative was 340 nm while the emission maximum for each was 410 nm. However, the reagent fluoresced strongly at an excitation wavelength 320 ± 20 nm and an emission wavelength 380 ± 40 nm (Fig. 4). Therefore, to maximize the signal-to-noise ratio, the wavelengths used to detect α-keto acids during their analysis were excitation 340 ± 5 nm and emission 420 ± 5 nm.

Fluorescence intensity as a function of organic phase concentration. The fluorescence intensity of the KBA quinoxalinol was examined as a function of percent organic phase in 0.35 *M* ammonium acetate. Both MeOH and ACN were examined at six dilutions over the range 0–100%. Results indicated that as the concentration of organic phase increased in the mobile phase, the fluorescence of the KBA quinoxalinol decreased linearly (a four-fold decrease was observed over the range tested) suggesting that the organic phase quenched quinoxalinol fluorescence. Since ACN is a much stronger eluent than MeOH, less organic solvent is needed to elute quinoxalinols. Consequently, if ACN is used as the organic phase instead of MeOH, quinoxalinol fluorescence increases, thus improving the sensitivity of the method.

Optimization of derivatization procedure

Reaction time and temperature. The effect of reaction time on quinoxalinol formation was examined using a 1 m*M* standard mixture. Standard reaction conditions were employed except for reaction temperature (80°C instead of 45°C). Results indicated that the reaction is nearly complete after 10 min for all α-keto acids tested (Fig. 5).

The effect of reaction temperature on quinoxalinol formation was also examined with a 1 m*M* standard α-keto acid mixture, other reaction conditions were held constant. Results indicated that at temperatures higher than 40°C the reaction was nearly complete after 60 min for all α-keto acids tested (Fig. 6). However, reagent by-product peak areas increased exponentially with temperature from 1 to 10% of the total peak area (sum of all standard and by-product peak areas) over the temperature range 0–100°C. To minimize the formation of by-products, the derivatization reaction was normally run at 45°C; at this temperature, reagent peaks comprised 1.5% of the total peak area.

Reagent concentration. Employing standard reaction conditions, the OPD concentration was varied from 5 to 500 m*M*. The total α-keto acid concentration was held constant at 0.13 m*M*. The resulting molar ratio of reagent to total α-keto acid concentration ranged from 3800:1 to 38:1. Quinoxalinol formation was found to be independent of reagent concentration over the range 750:1 to 1900:1. This result is in agreement with those of Nielsen[18] and Mowbray and Ottaway[19] who found that a 100- to 1000-fold excess of OPD was needed to maximize quinoxalinol formation from aromatic α-keto acids and pyruvic acid. Use of greater than 1000-fold excess of OPD caused a decrease in quinoxalinol formation, especially for KBA and GA. Since OPD is a highly reactive compound that will readily react with numerous compounds normally found in natural samples (*e.g.*, carboxylic acids and monosaccharides), it is important that excess reagent is added to the sample to obtain quan-

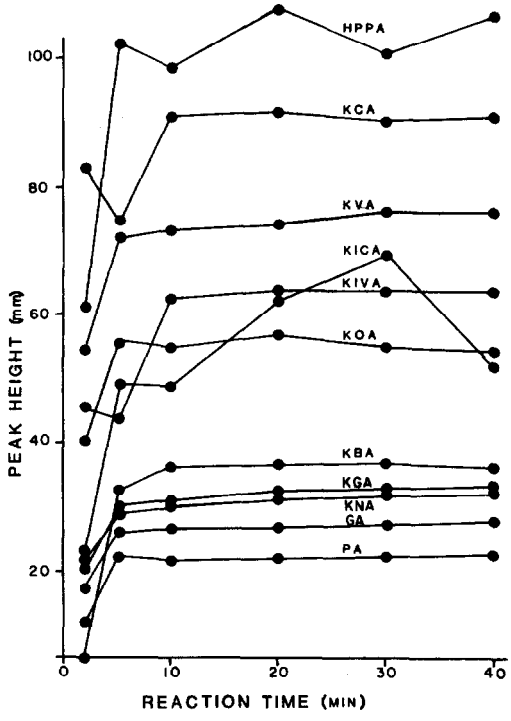


Fig. 5. Effect of reaction time on quinoxalinol formation. Reaction temperature held constant at 80°C. See Materials and methods for a list of α-keto acid abbreviations.

titative results. To assure that results are quantitative, the linearity of response for selected α-keto acids should be determined at concentrations observed in natural samples.

Reagent purification. At very high sensitivities needed to detect trace amounts of α-keto acids in some natural samples (*e.g.*, seawater), reagent and by-product interferences (*e.g.*, phenazines[19]) were important. Therefore, a study was performed to determine if reagent by-products that eluted at or near quinoxalinols during HPLC analysis could be selectively removed. To accomplish this, a number of blanks were examined.

The reagent blank initially tested consisted of 0.25 g OPD, 5 ml Q water and 1 ml concentrated hydrochloric acid. This blank was reacted for 1 h at 45°C, then an aliquot was pH-adjusted (pH 6) and injected into the liquid chromatograph. Results of this analysis (Fig. 7) indicated that numerous compounds were present. In an attempt to remove these compounds, an aliquot (1.5 ml) of the original blank was passed through an RP-18 Sep-Pak column at an approximate flow-rate of 1 ml/min. Part of the eluent was then pH-adjusted (pH 6) and subsequently analyzed by HPLC. Results indicated that a significant fraction of the by-products were removed by sorption onto the Sep-Pak column; only hydrophilic interferences were not removed. Another fraction of the eluent was re-reacted for 1 h at 45°C, pH-adjusted (pH 6), then analyzed. In contrast to the original blank, no fluorescent interferences developed in the re-reacted blank (Fig. 8); results from this experiment also indicated that

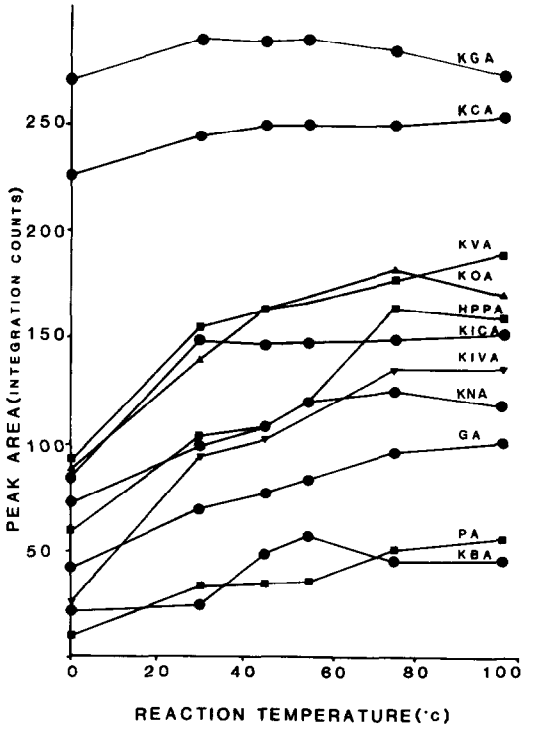


Fig. 6. Effect of reaction temperature on quinoxalinol formation. Reaction time 60 min. See Materials and methods for a list of α-keto acid abbreviations.

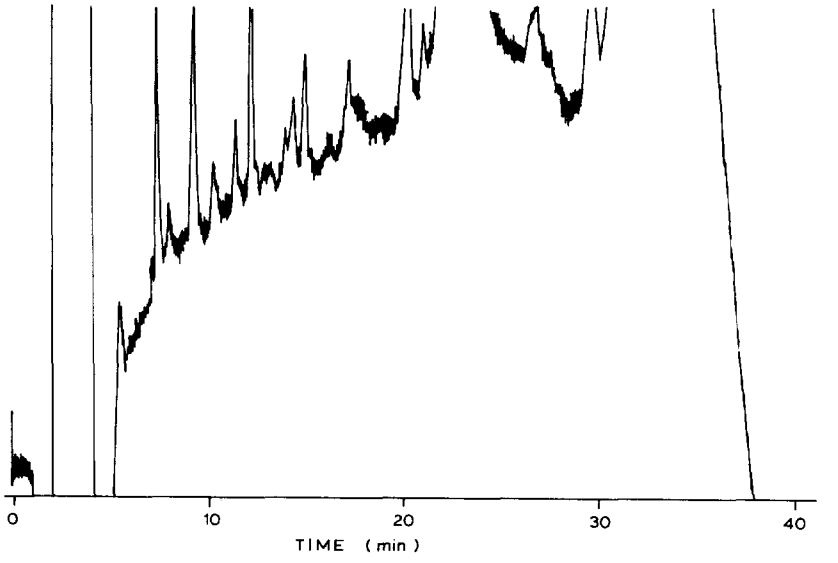


Fig. 7. Chromatogram of a reagent blank before it was passed through an RP-18 Sep-Pak column.

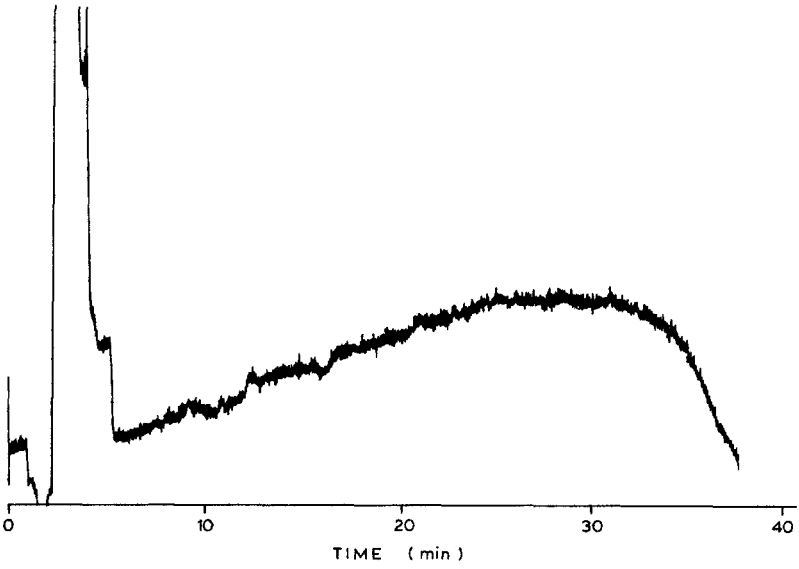


Fig. 8. Chromatogram of a reagent blank that was passed through an RP-18 Sep-Pak column.

these interferences originated from the reagent and not from the Q water. This result was further demonstrated by re-reacting another fraction of the eluent with 0.1 g OPD. The reactivity of the Sep-Pak-cleaned blank (containing purified reagent) to a 10-μM standard α-keto acid mixture was found to be identical to the reactivity obtained when this standard mixture was added to 0.25 g OPD. This indicated that a significant amount of OPD was not lost when the reagent solution was passed through a Sep-Pak.

In another experiment, the effect of adjusting the pH of the reaction mixture on by-product formation was studied. To examine this effect, a Sep-Pak-cleaned blank was pH-adjusted (pH 6) and subsequently analyzed by HPLC at 0, 2 and 3 h. This blank was compared to a blank that was only pH-adjusted just prior to its chromatographic analysis at times: 0, 1.5, 3, 4 and 6 h. Results demonstrated that fluorescent by-products formed in the blank that was pH-adjusted at $T = 0$ and subsequently analyzed 3 h later. However, no by-products developed in the blank that remained acidic for up to 6 h prior to HPLC analysis. Therefore, it is recommended that the pH of the reaction mixture be adjusted just prior to injection.

Hydrochloric acid concentration. The effect of acid concentration in the reaction mixture on quinoxalinol formation was examined over the range 0–4 M hydrochloric acid. All reaction parameters were held constant in this experiment except for acid concentration. Quinoxalinol formation increased substantially for all α-keto acids (except HPPA which decreased slightly) over the range 0–2 M hydrochloric acid indicating that this reaction is acid-catalyzed as previously observed[20]. With a further increase in acid concentration from 2 to 4 M, quinoxalinol formation was nearly constant for all α-keto acids tested.

Final pH of reaction mixture. The effect of the final pH of the reaction mixture on quinoxalinol formation was examined. After the derivatization reaction was com-

plete, the pH of the reaction mixture was adjusted with 6 *M* sodium hydroxide to one of the following pH values: 4.92, 6.12, 7.82, 8.70 or 11.20. Results indicated that quinoxalinol formation was independent of pH except at pH 11.2 where a slight increase in formation was observed for KGA and KNA, while a small decrease was observed for KBA.

Temporal stability of quinoxalinols. A standard mixture of 12 α-keto acids was reacted with OPD employing the standard derivatization procedure, then pH-adjusted to one of the following pH values with 6 *M* sodium hydroxide: 4.16, 5.25, 6.26, 8.68, 9.78 or 11.30. All pH-adjusted samples were stored frozen at −20°C. The stability of each quinoxalinol in the standard mixture was examined at each pH. Samples were analyzed (see Materials and methods for details of the gradient employed) at the following times: 1, 2, 3, 4, 5, 7, 9, 23 and 35 days. On each day that samples were analyzed, the fluorometer was calibrated with quinine sulfate. In general, quinoxalinol derivatives were quite stable with time at all pH values tested. However, for PA there was an increase then a decrease (usually to its original value found on day 1) in peak area for all pH values tested except 11.30. α-Ketobutyric acid showed an increase in peak area at pH 5.25 between 5 and 23 days then a decrease between days 23 and 35; KGA decreased dramatically in peak area between days 23 and 35 at pH 4.16 even though it was quite stable between 1 and 23 days. Since KBA and PA were not completely resolved from one another or from a third unknown peak, it is suspected that changes in their concentration with time were, in part, due to an artifact resulting from improper integration of these partially resolved peaks.

Precision

To determine the overall precision of the method, five separate standard mixtures each containing 10 different α-keto acids (1 μ*M* each) were derivatized, then analyzed in a series of five identical gradient runs. For this derivatization and analysis procedure, the average coefficient of variation (%CV) in peak area for all α-keto acids at the 11 pmole level (amount injected) was 1.90%; the average %CV in retention time was 0.33%.

Sensitivity

In its present form, the sensitivity of the quinoxalinol method is approximately 10 fmole per injected acid for most α-keto acids tested. However, because of blank interferences, the sensitivity of this method for KGA, HPPA and PA is approximately 1 pmole. This detection limit can be improved by either increasing the amount of sample injected (normally 30–500 μl are injected) or by reacting more sample (normally 2.5 ml are reacted), then concentrating the quinoxalinols in the sample using a Sep-Pak column containing RP-18 material or by employing a column-switching technique[13]; precolumn concentration procedures are only feasible for α-keto acids that do not have blank interferences (*e.g.*, KBA).

Linearity

Linearity of the fluorescent response was determined for KGA, GA, PA and KVA over the concentration range 9–70 n*M*. Nine dilutions were tested. Linear regression analysis indicated that the fluorescent response was linearly related to α-

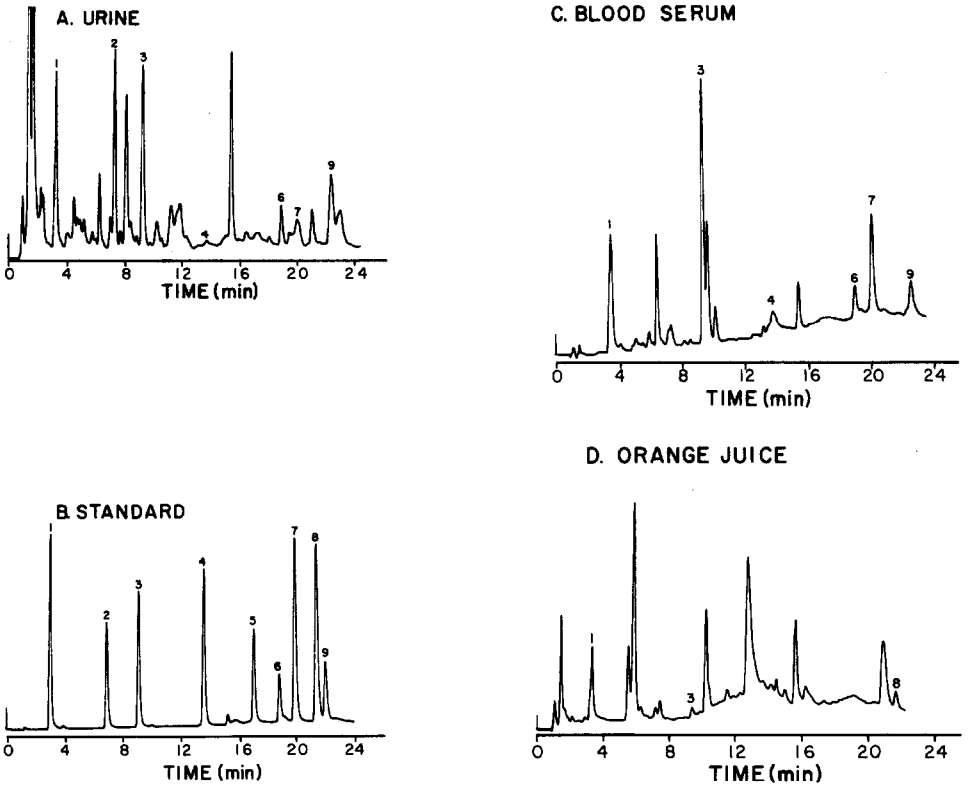


Fig. 9. Typical chromatograms of deproteinated physiological samples and standard. Derivatization procedure and gradient as in Materials and methods. Column: Adsorbosphere RP-18, 100 × 4.6 mm, 3-μm packing. Flow-rate, 1 ml/min. Wavelengths: excitation, 340 ± 5 nm; emission, 420 ± 5 nm. 30 μl injected. Chromatograms: A, urine; B, standard, each peak 11 pmol; C, blood serum; and D, fresh orange juice. Peaks: 1 = α-ketoglutaric acid; 2 = glyoxylic acid; 3 = pyruvic acid; 4 = α-ketobutyric acid; 5 = α-ketovaleric acid; 6 = α-ketoisovaleric acid; 7 = α-ketocaproic acid; 8 = α-ketoisocaproic acid; and 9 = β-phenylpyruvic acid.

keto acid concentration over the range tested ($r > 0.995$ for all α-keto acids tested). The result is in agreement with results obtained at the μM level[2,8,19,21].

Recovery

The recovery of the OPD technique was determined for 10 α-keto acids (1 μM each) added to a seawater sample from the mid-Atlantic continental shelf in relation to a standard aqueous mixture of these acids. Results of three identical runs for both the seawater sample and standard indicate that there was complete recovery (≥98%) for all α-keto acids tested. In contrast, when the OPD method was used in conjunction with numerous sample cleanup steps[12] to quantify α-keto acids in seawater, low recoveries were obtained for the α-keto acids tested; 50% recoveries were obtained for PA, KBA and KIVA, and for GA the recovery was 13%.

DISCUSSION

Relative to other chromatographic techniques, HPLC separation and on-line fluorometric detection of quinoxalinols is a rapid and sensitive method to quantify α-keto acids in physiological and environmental samples. The method is precise as well as fairly insensitive to changes in chromatographic or derivatization conditions. In addition, quinoxalinol formation is linearly related to α-keto acid concentration at levels found in natural samples so that quantitative results are easily and rapidly obtainable.

If narrow bandpass or interference filters (< 10 nm) are used during the fluorometric detection of quinoxalinols, then this method, besides being rapid and sensitive, is also quite selective for α-keto acids even in complex organic mixtures such as physiological fluids. This eliminates sample cleanup steps that increase the likelihood of sample contamination and reduce sample recoveries. Sample cleanup steps are normally required when employing a less selective method of detection[8]. For example, Steinberg and Bada[12] used wide-bandpass filters (excitation, 330–400 nm;

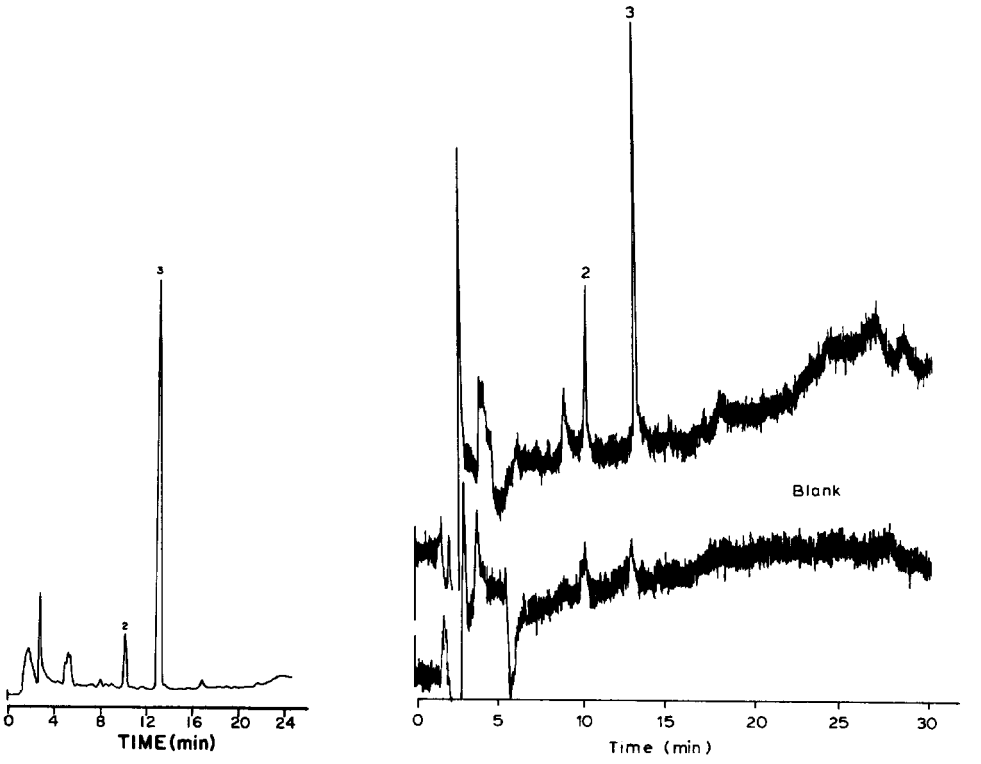


Fig. 10. Chromatogram of filtered, salt marsh, sediment pore water. Derivatization procedure and gradient as in Materials and methods; peaks as in Fig. 9; 30 μl injected.

Fig. 11. Upper chromatogram: filtered seawater; peaks approximately 1 pmol. Lower chromatogram: reagent blank. Derivatization procedure and gradient as in Materials and methods; peaks as in Fig. 9; 500 μl injected.

emission, 410–520 nm) for the fluorometric detection of quinoxalinols. With this wavelength selection, their method was not selective for α-keto acids. Consequently, numerous steps, 8, were required to isolate components of interest from fluorescent interferences.

One drawback to the method is that OAA forms two fluorescent products upon condensation with OPD[19], carboxymethyl quinoxalinol (OAA derivative) and its decarboxylated counterpart methyl quinoxalinol (PA derivative). This result was confirmed in the present study, although it was not examined in detail. Once formed, the carboxymethyl quinoxalinol is known to slowly decompose (3–4 weeks) to methyl quinoxalinol[19]. Consequently, it may be difficult to quantify PA in the presence of OAA. However, if the ratio of formation of OAA quinoxalinol to PA quinoxalinol is determined to be constant over a short-term basis (24 h), then it will be possible to correct for the OAA interference knowing the peak-height ratio OAA–PA. If this ratio changes too rapidly and is not constant even over a short time-frame then it will be necessary to correct results for potential OAA interference either by eliminating OAA from the sample or by measuring it by an ancillary technique. It may be possible to selectively eliminate OAA in a sample by addition of malate dehydrogenase and the reduced form of nicotinamide adenine dinucleotide (NADH)[19]. An alternative approach would be to use affinity chromatography employing an immobilized enzyme system to remove OAA[13]. In relation to the NADH–malate dehydrogenase method, there is less chance for sample contamination using an immobilized enzyme system. Also, the enzyme in the latter method can be recycled making it cost-effective. In contrast to OAA, KGA and all other α-keto acids tested, except IPPA, only formed one fluorescent product, when reacted with OPD. Furthermore, HPPA could be detected in our method; whereas, in another recently published fluorometric method[22], it was not possible to detect this acid.

Even though it may be difficult to analyze OAA by the OPD technique, this method, in general, is ideal for the analysis of trace levels (nM–μM) of α-keto acids in chemically complex samples. Derivatization conditions are relatively mild (<2 M hydrochloric acid, 45°C) and the reaction is complete for most α-keto acids after 60 min. In addition, once OPD condenses with α-keto acids, the resultant quinoxalinol products are quite stable, except for OAA, so that HPLC analysis does not have to be performed immediately after derivatization is complete.

With this technique, numerous physiological and environmental samples were analyzed (Fig. 9–11); for all samples, no sample cleanup or concentration steps were necessary, except for deproteination and/or filtration.

ACKNOWLEDGEMENTS

This work is part of an M.Sc. thesis (D.J.K.) at the University of Delaware, College of Marine Studies, which was partially supported by a Delaware River and Bay Authority (DRBA) grant to J.H.S.; and a student research grant from the Explorers Club, Philadelphia, PA; and a University of Delaware Research Foundation Grant (UDRF) to K.M.

REFERENCES

1 A. L. Lehninger, *Biochemistry*, Worth Publishers, New York, 1975, 2nd ed., p. 1104.
2 U. Langenbeck, H. U. Möhring and K.-P. Dieckmann, *J. Chromatogr.*, 115 (1975) 65.
3 T. C. Cree, S. M. Hutson and A. E. Harper, *Anal. Biochem.*, 92 (1978) 156.
4 H. P. Schwarz, I. E. Karl and D. M. Bier, *Anal. Biochem.*, 108 (1980) 360.
5 F. Rocchiccioli, J. P. Leroux and P. Cartier, *J. Chromatogr.*, 226 (1981) 325.
6 T. S. Viswanathan, C. E. Hignite and H. F. Fisher, *Anal. Biochem.*, 123 (1982) 295.
7 T. Hayashi, T. Sugiura, H. Terada, S. Kawai and T. Ohno, *J. Chromatogr.*, 118 (1976) 403.
8 J. C. Liao, N. E. Hoffman, J. J. Bardoriak and D. A. Roth, *Clinical Chem.*, 23 (1977) 802.
9 T. Hirata, M. Kai, K. Kohashi and Y. Ohkura, *J. Chromatogr.*, 226 (1981) 25.
10 T. Hayashi, H. Tsuchiya, H. Todoriki and H. Naruse, *Anal. Biochem.*, 122 (1982) 173.
11 H. Nakamura and Z. Tamura, *Anal. Chem.*, 51 (1979) 1679.
12 S. M. Steinberg and J. L. Bada, *Mar. Chem.*, 11 (1982) 299.
13 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley, New York, 1979, p. 863.
14 R. F. Brown, *Organic Chemistry*, Wadsworth Publishing, Belmont, CA, 1975, p. 1008.
15 M. Windholz, S. Budavari, L. Y. Stroumtsos and M. N. Fertig (Editors), *The Merck Index*, 9th ed., Merck and Co., Rahway, NJ, 1976.
16 S. Glasstone, *Physical Chemistry*, Van Nostrand, New York, 1946, 2nd ed., p. 1320.
17 C. Horvath and W. Melander, *J. Chromatogr. Sci.*, 15 (1977) 393.
18 K. H. Nielsen, *J. Chromatogr.*, 10 (1963) 463.
19 J. Mowbray and J. H. Ottaway, *J. Biochem.*, 120 (1970) 171.
20 D. H. Cram and G. S. Hammond, *Organic Chemistry*, McGraw-Hill, New York, 1959, p. 316.
21 U. Langenbeck, A. Hoinowski, K. Mantel and H.-U. Möhring, *J. Chromatogr.*, 143 (1977) 39.
22 T. Hayashi, H. Tsuchiya and N. Naruse, *J. Chromatogr.*, 273 (1983) 245.