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4,5-Diaminophthalhydrazide as a highly sensitive chemiluminescence derivatization reagent for α-dicarbonyl compounds in high-performance liquid chromatography

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ABSTRACT

4,5-Diaminophthalhydrazide (DPH) was found to be a highly sensitive chemiluminescence derivatization reagent for α-dicarbonyl compounds in high-performance liquid chromatography. The reagent reacts with α-dicarbonyl compounds in dilute hydrochloric acid in the presence of β-mercaptoethanol to give highly chemiluminescent quinoxaline derivatives which produce chemiluminescence by reaction with hydrogen peroxide and potassium hexacyanoferrate(III). The DPH derivatives of five α-dicarbonyl compounds were separated on a reversed-phase column, TSKgel ODS-120T, with a mixture of acetonitrile and 10 m*M* ammonium acetate, followed by chemiluminescence detection. The detection limits are in the range 1.1–300 fmol for a 20-μl injection volume (signal-to-noise ratio 3).

INTRODUCTION

Several α-dicarbonyl compounds, such as methylglyoxal, diacetyl and 2,3-pentanedione, are present in physiological fluids [1,2] and foods [3,4]. However, despite various studies, the details of the roles of these compounds remain unknown. This might be partly due to the lack of a sensitive and specific method for the determination of α-dicarbonyl compounds. Many methods, including polarography [5], UV–visible spectrophotometry [6,7], fluorimetry [8], gas chromatography [9,10] and high-performance liquid chromatography (HPLC) with spectrophotometric [1,10] or fluorimetric detection [11,12] have been reported. Of these methods, the fluorimetric HPLC method [12] with 1,2-diamino-4,5-methylenedioxybenzene is the most sensitive.

On the other hand, chemiluminescence (CL) detection has been successfully introduced into HPLC analysis because of its high sensitivity [13–16] and several precolumn CL derivatization reagents have been reported [17,18]. No reagents, however, have been developed for α-dicarbonyl compounds.

In previous work, we developed 4,5-diaminophthalhydrazide dihydrochloride (DPH) [19] as a CL reagent for α-keto acids. Recently, it was found that α-dicarbonyl compounds also react with DPH under derivatization conditions different to those for α-keto acids to give quinoxaline derivatives (Fig.

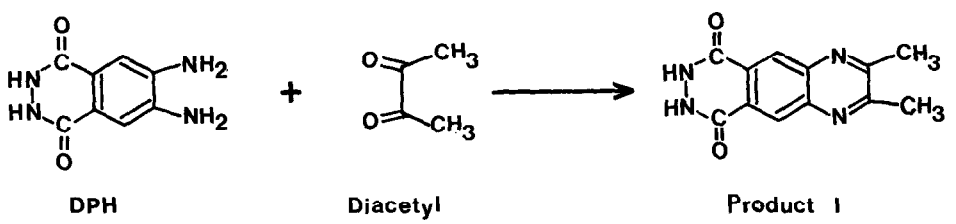

Fig. 1. Chemiluminescence derivatization reaction of diacetyl with 4,5-diaminophthalhydrazide (DPH).

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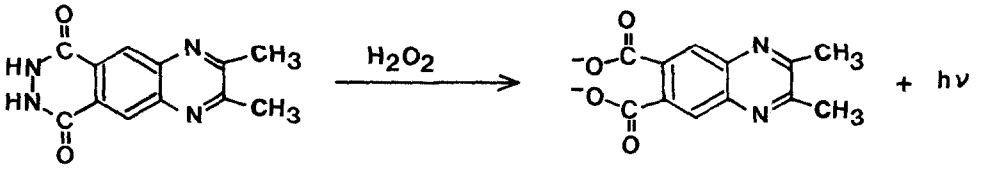

Fig. 2. Chemiluminescence reaction of the DPH derivative of diacetyl.

1). Further, the quinoxaline derivatives generated intense CL by reaction with hydrogen peroxide in the presence of potassium hexacyanoferrate(III) in alkaline media (Fig. 2), conditions which are different to those for α-keto acids. In this work, we examined the optimum derivatization and CL reaction conditions and developed a sensitive and selective method for the determination of α-dicarbonyl compounds using DPH, based on HPLC with CL detection.

EXPERIMENTAL

Chemicals and solutions

All chemicals and solvents were of analytical-reagent grade, unless stated otherwise. Distilled water, purified with a Milli-Q II system (Millipore), was used for all aquous solutions. Hydrogen peroxide (31%, v/v) was purchased from Mitsubishi Gas Kagaku (Tokyo, Japan). The α-dicarbonyl compounds listed Table I were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). DPH dihydrochloride was prepared as described previously [19,20]. DPH solution (8.0 m*M*) was prepared in 0.2 *M* hydrochloric acid containing 0.2 *M* β-mercaptoethanol. This solution was used within 4 h. Hydrogen peroxide (40 m*M*) and potassium hexacyanoferrate (III) (25 m*M*) solutions were prepared in water and 2.75 *M* sodium hydroxide, respectively.

Apparatus and HPLC conditions

The time course of the CL reaction was measured with a Laboscience TD-4000 lumiphotometer. Uncorrected fluorescence spectra were measured with a Hitachi 650-60 spectrofluorimeter in a 10 × 10 mm quartz cell; a spectral bandwidth of 5 nm was used in both excitation and emission monochromators. ^{1}H nuclear magnetic resonance spectra were obtained with a Hitachi R-90H spectrometer at 90 MHz using a *ca.* 1% (w/v) solution of [$^2H_6$]dimethyl sulfoxide containing tetramethylsilane as an internal standard. Field desorption mass spectra were taken with a JEOL HX-110 spectrometer. Uncorrected melting points were measured with a Gallenkamp melting point apparatus.

Fig. 3. shows a schematic diagram of the HPLC–CL system. Chromatography was performed with a Model 655A-11 high-performance liquid chromatograph (Hitachi, Tokyo, Japan) equipped with a Rheodyne Model 7125 syringe-loading sample injector valve (20-μl loop). Chromatograms were recorded with a SIC Chromatocorder 11 (System Instruments, Tokyo, Japan). The DPH derivatives of α-dicarbonyl compounds were separated on a TSKgel ODS-120T reversed-phase column (250 × 4.6 mm I.D.; particle size 5 μm) (Tosoh, Tokyo, Japan) by isocratic elution with acetonitrile–10 m*M* ammonium acetate (27:73, v/v) as eluent. The flow-rate of the mobile phase was 1.0 ml/min. The column temperature was ambient (18–25°C).

The eluate from the HPLC column was mixed with the hydrogen peroxide and the potassium hexacyanoferrate(III) solutions delivered by two Hitachi L-6000 pumps using T-type mixing devices. The flow-rates of the hydrogen peroxide and potassium hexacyanoferrate(III) solutions were 1.0 and 2.0 ml/min, respectively. The CL generated was monitored by a Model 825-CL intelligent CL detector (Jasco, Tokyo, Japan) equipped with a 90-μl flow cell. Stainless-steel tubing (0.5 mm I.D.) was used for the HPLC system.

Preparation of chemiluminescent compound from diacetyl

DPH (0.2 g, 1.0 mmol) and diacetyl (0.1 g, 1.2 mmol) were dissolved in 15 ml of 0.2 *M* hydrochloric acid containing 0.2 *M* β-mercaptoethanol. The mixture was heated in a boiling water-bath for 1 h. The precipitate that was produced during the heating, was filtered off and then recrystallized from di-

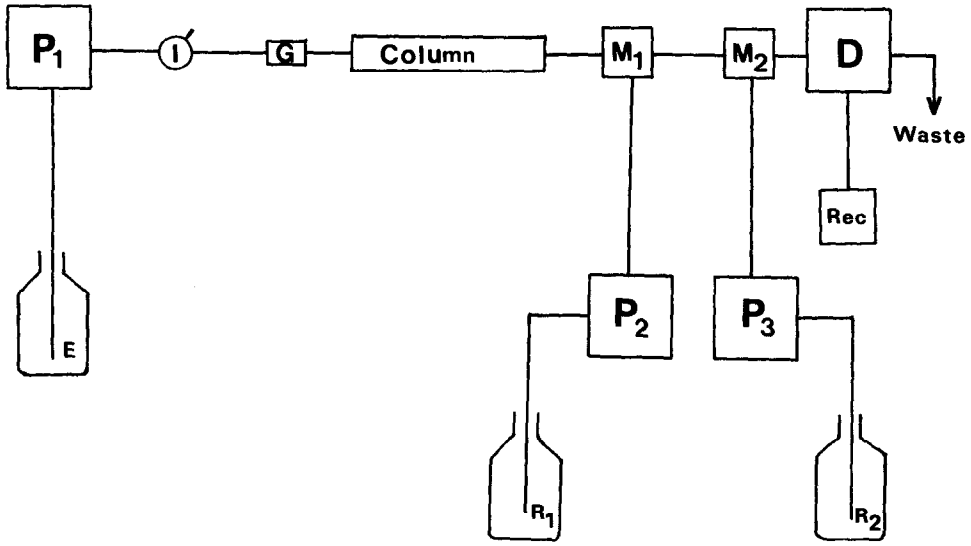

Fig. 3. Schematic flow diagram of the HPLC–CL system. P_1–P_3 = HPLC pumps; I = injector valve (20 μl); D = detector; G = guard column (TSK gel ODS-120T); Column = TSK gel ODS-120T (250 × 4.6 mm I.D.); M_1 and M_2 = mixing devices; Rec = recorder; E = mobile phase; R_1 = hydrogen peroxide solution; R_2 = potassium hexacyanoferrate(III) solution. Flow-rates: E = 1.0, R_1 = 1.0, R_2 = 2.0 ml/min.

methyl sulphoxide–water (9:1, v/v) to give product I (Fig. 1) (80 mg, 0.33 mmol) as brownish yellow needless, m.p. 330°C (decomposition). ^{1}H NMR, δ (ppm) 2.76 (6H, s, C-CH_3), 8.55 (2H, s, aromatic protons), 11.38 (2H, s, NH). Mass spectrum *m/z* 242 (M^+, base peak). Analysis: calculated for $C_{12}H_{10}N_4O_2$, C 59.50, H 4.16, N 23.13; found, C 59.73, H 4.16, N 23.00%.

Procedure

To a 100-μl aliquot of a test solution of α-dicarbonyl compounds, placed in a screw-capped tube (100 × 15 mm I.D.) were added 100 μl of the DPH solution. The tube was tightly closed and heated at 100°C for 45 min. A 20-μl aliquot of the final reaction mixture was injected into the chromatograph.

RESULTS AND DISCUSSION

HPLC conditions

A good separation of the DPH derivatives of five α-dicarbonyl compounds listed in Table I was achieved on a TSK gel ODS-120T column by isocratic elution with acetonitrile–10 m*M* ammonium acetate (27:73, v/v). A typical chromatogram obtained with a standard mixture is shown in Fig. 4. The individual α-dicarbonyl compounds gave single peaks in the chromatogram.

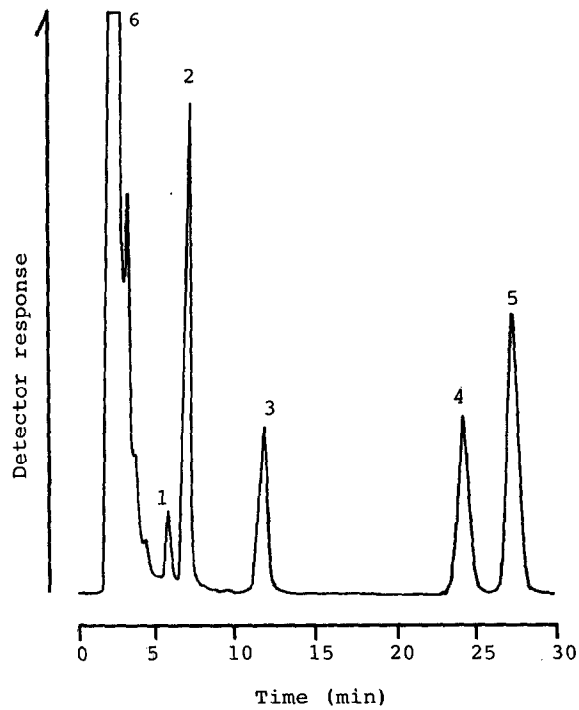

Fig. 4. Chromatogram of the DPH derivatives of five α-dicarbonyl compounds. A portion (100 μl) of a standard mixture of α-dicarbonyl compounds listed in Table I (1 mmol for 3,4-hexanedione and 10 pmol each for the other compounds per injection volume) was treated according to the procedure. Peaks: 1 = phenylglyoxal; 2 = diacetyl; 3 = 2,3-pentanedione; 4 = 3,4-hexanedione; 5 = 2,3-hexanedione; 6 = reagent blank.

Derivatization conditions

Diacetyl and 2,3-pentanedione were selected as model compounds to establish reaction conditions suitable for a more general method. α-Dicarbonyl compounds reacted with DPH in dilute hydrochloric acid, but not in neutral or alkaline solution. Hydrochloric acid at 0.18–0.30 *M* in the DPH solution gave maximum peak heights; 0.20 *M* was adopted for the preparation of the DPH solution. β-Mercaptoethanol was used to facilitate the derivatization of α-dicarbonyl compounds with DPH. The peak heights for the compounds were maximum at 0.20 *M* β-mercaptoethanol in the DPH solution. Other reductants such as sodium sulphite, sodium hydrogensulphite and sodium dithionite showed low reactivities, less than 50% of that obtained with β-mercaptoethanol.

The DPH solution gave the most intense and constant peaks at concentrations >6.0 m*M*; 8.0 m*M* was adopted. The derivatization reaction proceeded more rapidly as the reaction temperature was increased (Fig. 5). The peak heights became maximum and constant after heating at 100°C for 30 min. Therefore, heating at 100°C for 45 min was adopted in the recommended procedure.

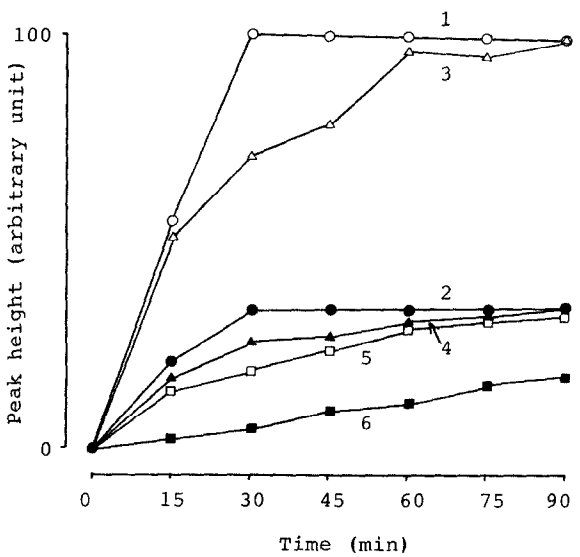

Fig. 5. Effect of reaction time and temperature on the derivatization reaction of diacetyl and 2,3-pentanedione with DPH. Temperatures: 1 and 2 = 100°C; 3 and 4 = 80°C; 5 and 6 = 50°C. Compounds: 1, 3 and 5 = diacetyl; 2, 4 and 6 = 2,3-pentanedione.

The DPH derivatives in the final solution were stable for at least 72 h in daylight at ambient temperature.

Chemiluminescence reaction

The optimum CL reaction conditions were examined by setting the flow-rates of the hydrogen peroxide and potassium hexacyanoferrate(III) solutions at 1.0 and 2.0 ml/min, respectively. The CL intensities were affected by the concentrations of hydrogen peroxide, potassium hexacyanoferrate (III) and sodium hydroxide. The concentrations of these reagents were varied one at a time to establish the maximum intensity obtainable. Based on these experiments (Fig. 6), concentrations of 40 m*M* hydrogen peroxide, 25 m*M* potassium hexacyanoferrate(III) and 2.75 *M* sodium hydroxide were selected.

The length of tubing between the second device (M_2 in Fig. 2) and the detector affected the CL response. The peak heights increased with decreasing length of the tubing; 5 cm was tentatively selected.

The extra-column dispersion caused by the post-column reaction was examined by monitoring with a UV detector set between the column and the first mixing device. The half-widths of the peaks after the postcolumn reaction were broadened about 1.2-fold.

Calibration graph, precision and detection limits

The relationships between the peak heights and the amounts of the individual α-dicarbonyl compounds were linear up to at least 5 nmol per 20-μl injection volume.

The precision was established by repeated determinations ($n = 10$) using a mixture of five α-dicarbonyl compounds (400 pmol for 3,4-hexanedione and 4 pmol each for the other compounds per 20-μl injection). The relative standard deviations did not exceed 3% for all the compounds.

The detection limits are listed in Table I; these values were determined by derivatization of trace amounts of analytes. All the compounds tested except 3,4-hexanedione can be determined at sub-femtomole levels. The sensitivities are *ca.* 20–70 times higher than those of the most sensitive fluorimetric HPLC method so far using 1,2-diamino-4,5-methylenedioxybenzene [12]. The reason for the low sensitivity with 3,4-hexanedione is unknown.

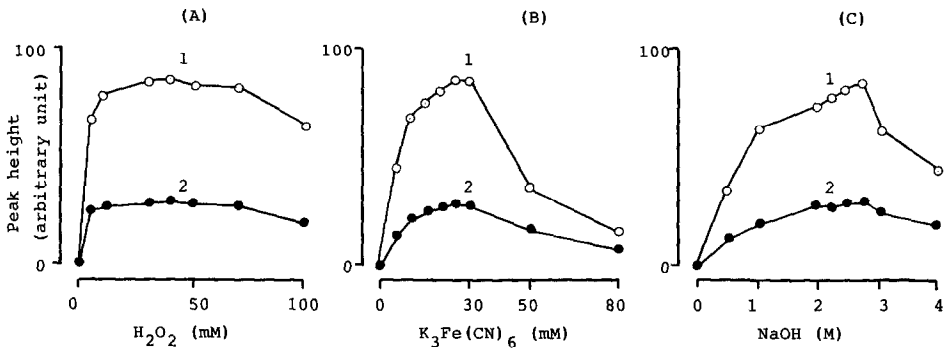

Fig. 6. Effects of (A) hydrogen peroxide, (B) potassium hexacyanoferrate(III) and (C) sodium hydroxide concentrations on the CL peak heights. Curves: 1 = diacetyl; 2 = 2,3-pentanedione.

Reaction of other substances with DPH

Glyoxal and methylglyoxal also gave single peaks at a retention time of 3.0 min for both compounds. However, the peaks were overlapped with reagents peaks under the selected HPLC conditions. α-Keto acids [19] and sialic acids [21] also react with DPH to give CL. Under the present derivatization conditions, however, α-keto acids (α-ketobutyric and phenylpyruvic acids) and sialic acids (N-acetyl- and N-glycolylneuraminic acids) gave *ca.* 1/10–1/15 of the CL intensity of α-dicarbonyl compounds (phenylglyoxal and diacetyl). None of the other physiologically important substances examined generated CL under the recommended conditions at a concentration of 10 mmol/ml. The compounds tested were seventeen L-amino acids, inositol, D-glucose, D-fructose, D-galactose, D-mannose, D-maltose, D-xylose, D-lactose, histamine, tyramine, tryptamine, 2-phenylethylamine, urea, citrulline, bilirubin, glutathione, uric acid, lactic acid, acetoacetic acid, malic acid, palmitic acid, nicotinamide, vitamin D_3, guanosine, cytosine, thymidine, adenosine, cholesterol, cortisone, epiandrosterone, aldosterone and epinephrine. The results suggest that the present derivatization method is usefully selective for α-dicarbonyl compounds.

TABLE I

RETENTION TIMES AND DETECTION LIMITS FOR DPH DERIVATIVES OF α-DICARBONYL COMPOUNDS

Compound	Retention time (min)	Detection limit (fmol)[a]
Phenylglyoxal	5.7	8.7
Diacetyl	6.8	1.1
2,3-Pentanedione	11.9	3.2
2,3-Hexanedione	27.3	1.9
3,4-Hexanedione	24.5	300.0

[a] The amount in the injection volume (20 μl) giving a signal-to-noise ratio of 3.

Chemiluminescent product in the determination of diacetyl

In order to investigate the structure of the CL products, diacetyl was employed as a model compound. The reaction products of α-dicarbonyl compounds with 1,2-diaminobezene [11] and 1,2-diamino-4,5-methylenedioxybenzene [12] have been characterized as quinoxaline derivatives. Thus the reaction product of diacetyl with DPH should be 2,3-dimethyl-7,8-dihydropyridazino[4,5-g]quinoxaline-6,9-dione; these identifications were based on the analytical and spectral data (see Experimental).

The time course of the CL reaction of the DPH derivative of diacetyl is shown in Fig. 7. The CL reached maximum intensity *ca.* 2 s after the reaction started, and then quenched rapidly.

With luminol, the aminophthalate ion, which was produced during the CL reaction, was proved to be a light-emitting species [22]. Therefore, the fluorescent spectra were measured after the reaction was performed using the DPH derivative. The excitation and emission maxima of the fluorescence were 370 and 433 nm, respectively. Consequently, 2,3-dimethylquinoxaline-6,7-dicarboxylic acid (Fig. 2),

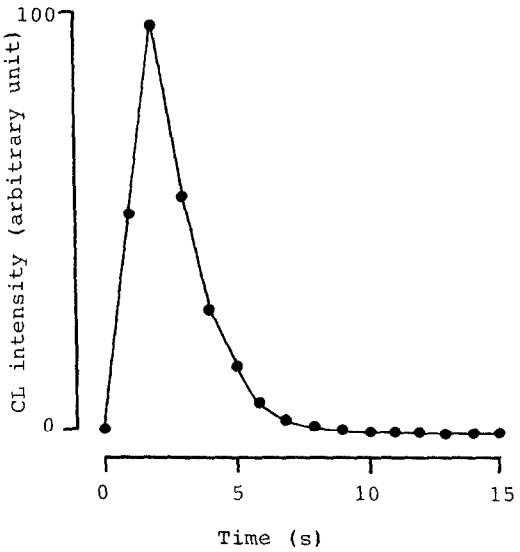

Fig. 7. Time course of the CL reaction of the DPH derivative of diacetyl. A 100-μl portion of the derivative solution ($1 \cdot 10^{-6}$ *M*) was mixed with 100 μl of the hydrogen peroxide solution in a polystyrene tube (65 × 8 mm I.D.). The CL reaction was initiated by automatic injection of 100 μl of the potassium hexacyanoferrate(III) solution into the tube. Each point corresponds to the integrated CL intensity at 1-s intervals.

which was expected to occur in the reaction was estimated to emit light with a maximum at 433 nm.

Because this chemiluminescence reaction is similar to that of luminol, similar factors such as other oxidants and cations may affect the reaction [23,24]. Therefore, in determinations on real samples, the effects of interferents unseparated from the analyte should be considered.

The method permits the highly sensitive and selective determination of α-dicarbonyl compounds, and can be applied to the sensitive determination of biogenic α-dicarbonyl compounds in body fluids and diacetyl in foodstuffs. Further studies are in progress.

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