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FLUOROMETRIC DETERMINATION OF α-OXOMETHYLENE COMPOUNDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING N^1-METHYLNICOTINAMIDE CHLORIDE*

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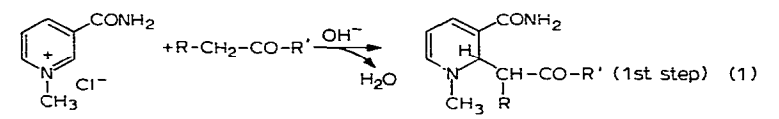
SUMMARY

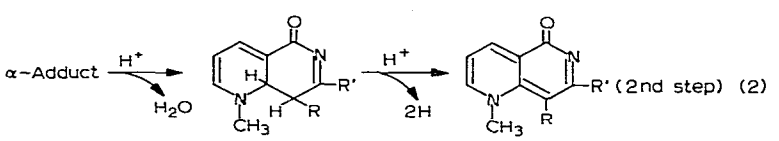
Before or after the high-performance liquid chromatographic separation, α-oxomethylene compounds are subjected to reaction with N^1-methylnicotinamide chloride in the presence of alkali followed by heating with acid to produce fluorophores, permitting their determination at the picomole level.

INTRODUCTION

We have recently developed a fluorometric assay of α-oxomethylene compounds ($R–CH_2–CO–R'$) at the picomole level by using N^1-methylnicotinamide chloride (NMN)[1]. The reaction probably proceeds as shown below. In the first step, α-oxomethylene compounds react with NMN in the presence of alkali to form α-adducts of NMN. In the second step, the resulting α-adducts are converted by brief heating with excess of acid into fluorophores via non-fluorescent cyclic α-adducts. The fluorophores are stable to acids and alkalis, and therefore the fluorogenic reaction seems to be promising as a method for pre- or post-column derivatization of α-oxomethylene compounds in their high-performance liquid chromatography (HPLC).

In this investigation, the applicability of NMN was examined.





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EXPERIMENTAL

Reagents and materials

N^1-Methylnicotinamide chloride (NMN) was purchased from Tokyo Kasei (Tokyo, Japan). The following α-oxomethylene compounds were purchased from commercial sources: methyl phenyl ketone (acetophenone), ethyl phenyl ketone (propiophenone), *n*-propyl phenyl ketone, cyclopentanone, cyclohexanone, cycloheptanone, sodium pyruvate and oxalacetic acid (*cis*-form) (guaranteed reagents), *n*-butyl phenyl ketone, cyclooctanone (extra-pure reagents) from Tokyo Kasei; acetone, methyl ethyl ketone (guaranteed reagents) from Kanto (Tokyo, Japan); and α-ketoglutaric acid monosodium salt from Sigma (St. Louis, Mo., U.S.A.). The α-oxomethylene compounds were dissolved in ethanol to make 10 m*M* stock solutions, except for the keto acids, which were dissolved in distilled water just before use. Other chemicals and solvents used were of analytical-reagent grade. The following chromatographic resins were used: Wako Gel LC-5K (5 μm) from Wako (Osaka, Japan), Iatrobeads 6CP-2020 (20 μm, styrene–divinylbenzene porous polymer) from Iatron Laboratories (Tokyo, Japan) and Co:Pell ODS (30 μm, C_{18} bonded pellicular vitreous beads) and Partisil-10 SAX (10 μm, microparticulate silica-bonded strong anion exchanger) from Whatman (Clifton, N.J., U.S.A.).

High-performance liquid chromatography

System for pre-column derivatization HPLC. The previous assay procedure[1] was modified as follows for microanalysis of α-oxomethylene compounds. A 10-μl volume of 6 *N* sodium hydroxide solution was transferred into a polyethylene microtest-tube (capacity 1.5 ml; Eppendorf, Hamburg, G.F.R.) and 10 μl each of the sample solution (containing 5 pmole–20 nmole of α-oxomethylene) and 50 m*M* NMN in 10^{-4} *M* hydrochloric acid were added in that order. The tube was allowed to stand for 10 min, 150 μl of 70.4% formic acid were added and the mixture was heated at 92° for 3 min. After cooling, an aliquot (0.5–20 μl) was injected into the chromatographic system. All of the procedures prior to the heating were performed in an ice-bath at 0°.

The jacketed column was maintained at 50° with a water-bath circulator (Type BT-35; Yamato Scientific, Tokyo, Japan). The eluent was delivered through stainless-steel tubing (0.25 mm I.D.) with a Mini-micro pump (Type KHD-16; Kyowa Seimitsu, Tokyo, Japan). On-column syringe injection of samples was performed through a loop injector (20 μl) using dual 4-way valves (Type KMM-4V-2, Kyowa Seimitsu) which operated up to 70 kg/cm². A 10-cm pulse-damping column filled with 40-μm glass beads (1 mm I.D.; Durrum, Palo Alto, Calif., U.S.A.) was placed between the pump and the injector. The outlet of the column was connected to a 14-μl quartz flow cell in a Shimadzu fluorescence detector (Type FLD-1; Shimadzu Seisakusho, Kyoto, Japan) equipped with a coated low-pressure mercury lamp emitting continuous light at 300–400 nm (maximum intensity at 360 nm) and an EM-3 secondary filter which cuts off light shorter than 405 nm. The outlet of the flow cell was connected to a 10-m back-pressure coil made of Teflon tubing (0.25 mm I.D.). The fluorescence intensity was recorded with a Shimadzu recorder (Type R-12; Shimadzu Seisakusho).

System for post-column derivatization HPLC. The chromatographic system described above was additionally modified as shown in Fig. 1. The column outlet was

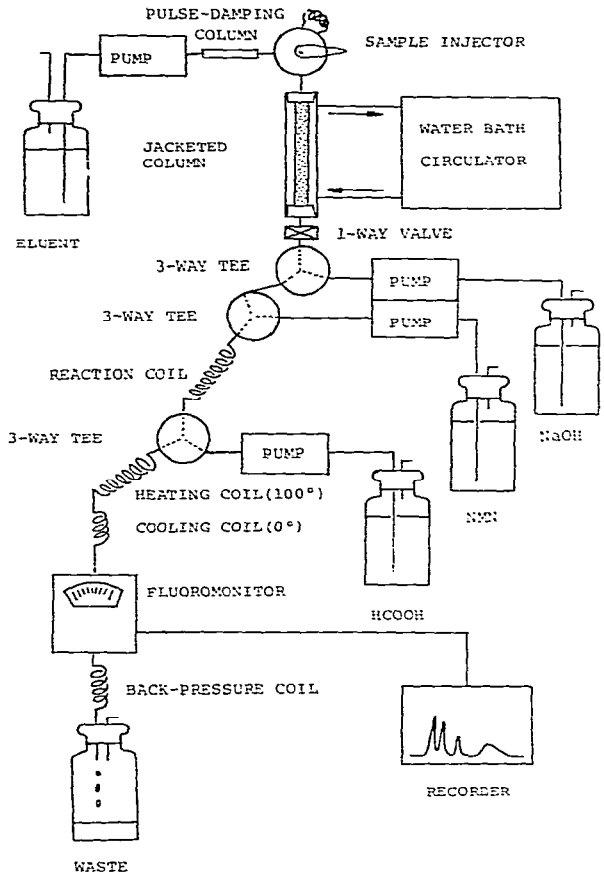

Fig. 1. Flow diagram for the HPLC post-column derivatization of α-oxomethylene compounds.

fitted with a one-way valve to prevent sodium hydroxide solution from entering the column and the eluate was mixed in a Teflon 3-way tee-piece (Kyowa Seimitsu) with 10 *N* sodium hydroxide solution, delivered through a 30-cm length of TFE tubing (0.5 mm I.D.) with a double plunger type Mini-micro pump (Type KHU-W-52; Kyowa Seimitsu). The outlet of the tee-piece was connected to a 3-cm length of Teflon tubing (0.5 mm I.D.), which was connected to the second Teflon 3-way tee-piece (Kyowa Seitmitsu). The alkaline eluate was mixed in the second tee-piece with 100 m*M* NMN in 10^{-4} *M* hydrochloric acid delivered with the other arm of the double plunger pump. The outlet of the second tee-piece was connected to a 10-m reaction coil made of Teflon tubing (0.25 mm I.D.). The latter was connected to the third 3-way tee-piece to which 88% formic acid was delivered with a Mini-micro pump (Type KSD-16). The outlet of the third tee-piece was connected to a 5-m heating coil made of stainless-steel tubing (0.5 mm I.D.), which was heated at 100° in a water-bath and was connected to a 30-cm cooling coil made of stainless-steel tubing (0.5 mm I.D.). The cooling coil was immersed in an ice-bath and its end was connected to the flow cell in the fluorescence detector.

The detailed chromatographic conditions are given in the figure legends.

RESULTS AND DISCUSSION

HPLC of fluorophores

The fluorescence of the NMN derivatives of α-oxomethylene compounds was generally most intense in acidic media, and the excitation and emission maxima were at 356–420 and at 420–472 nm, respectively[1]. Elution with alkaline eluents caused strong adsorption of fluorophores on the columns tested because of insolubility both in polar and non-polar solvents. Neutral eluents made the peaks rather broad. Acidic solvents in which the fluorophores were in the dicationic form gave the best separation with higher sensitivity. Fig. 2 illustrates an example of the separation of fluorescent NMN derivatives of some α-oxomethylene compounds on the column of Iatrobeads 6CP-2020 using acetic acid–methanol–water (6:1:21). Although unsymmetrical ketones ($R–CH_2–CO–CH_2–R'$) may give isomeric fluorophores in theory, methyl ethyl ketone and methyl isobutyl ketone gave single peaks on the styrene–divinylbenzene column and a silica column (Wako Gel LC-5K). The sensitivity of the fluorescence detection was markedly dependent on the compounds, as shown in Fig. 2. Generally, compounds having an α-oxomethylene group adjacent to an aromatic ring were detected with much higher sensitivity than aliphatic α-oxomethylenes. Acetophenone, one of the compounds giving the most intense fluorescence, could be determined in amounts as low as 0.5 pmole (Fig. 3).

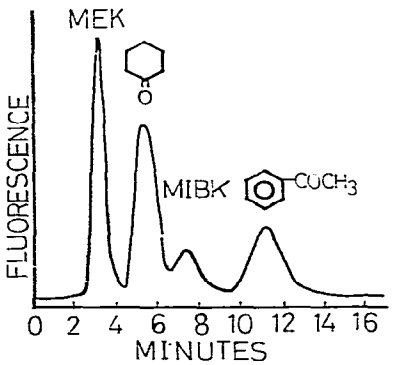

Fig. 2. HPLC separation of some α-oxomethylene compounds as their fluorescent NMN derivatives. Sample: mixture containing NMN derivatives of methyl ethyl ketone (400 pmole), cyclohexanone (500 pmole), methyl isobutyl ketone (2 nmole) and acetophenone (25 pmole). Column: Iatrobeads 6CP-2020 (20 μm; 30 cm × 3 mm I.D.) at 50°. Eluent: acetic acid–methanol–water (6:1:21). Eluent flow-rate: 0.5 ml/min.

HPLC of native α-oxomethylene compounds

The formation of α-adducts in alkaline media (the first step) is the rate-limiting step in the fluorogenic reaction, and the production of fluorophores increased with increasing concentrations of sodium hydroxide and NMN. The combination of 10 *N* sodium hydroxide solution and 50–100 m*M* NMN in 10^{-4} *M* hydrochloric acid gave satisfactory results in the first step. Fig. 4 shows the separation of C_5–C_8 cyclic ketones on a column of the pellicular reversed phase Co:Pell ODS by isocratic elution with 5% methanol.

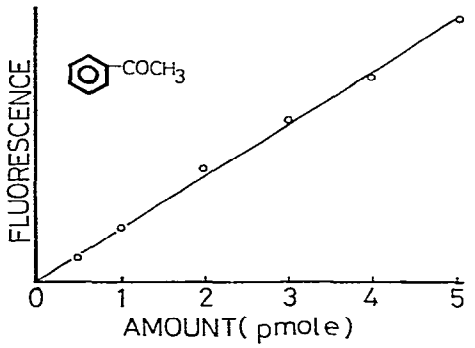

Fig. 3. Working curve for acetophenone. Acetophenone in various concentrations was converted into its fluorophore as described in the text. An aliquot (10 μl) of the reaction mixture was analysed under the same conditions as in Fig. 2. The peak height was plotted against the amount of acetophenone.

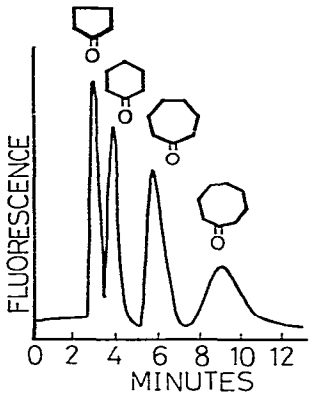

Fig. 4. HPLC separation of some native cyclic ketones. Sample: mixture containing cyclopentanone (200 pmole), cyclohexanone (200 pmole), cycloheptanone (2 nmole) and cyclooctanone (4 nmole). Column: Co:Pell ODS (30 μm; 50 cm × 3 mm I.D.). Eluent: 5% methanol. Flow-rates: eluent, 1.36 ml/min; 10 *N* NaOH, 0.17 ml/min; 100 m*M* NMN in 10^{-4} *M* HCl, 0.68 ml/min; 88% formic acid, 0.68 ml/min. Temperatures: column, 50°; reaction coil, 40°; heating coil, 100°; cooling coil, 0°.

In general, aliphatic α-oxomethylene compounds required heating of the reaction coil in order to accelerate the addition reaction, although the heating also accelerated the alkaline hydrolysis of amide groups of α-adducts and NMN. By heating the reaction coil at 40°, as little as 5 pmole of cyclopentanone and cyclohexanone were determined (Fig. 5). However, cycloheptanone and cyclooctanone were much less fluorescent in the system.

Some phenones were also analysed on the same column with 25% methanol as the eluent (Fig. 6). When the reaction coil was maintained at 0°, acetophenone and propiophenone gave intense fluorescence. Working curves for acetophenone and propiophenone are linear in the range 25–250 pmole of compound (Fig. 7). Heating the reaction coil at 40–50° increased the sensitivities of *n*-propyl phenyl ketone and *n*-butyl phenyl ketone, whereas it markedly decreased the sensitivities of acetophenone and propiophenone. Pyruvate, α-ketoglutarate and oxalacetate were separated on a Partisil-10 SAX column with isocratic elution of 0.1 *M* potassium phosphate solution

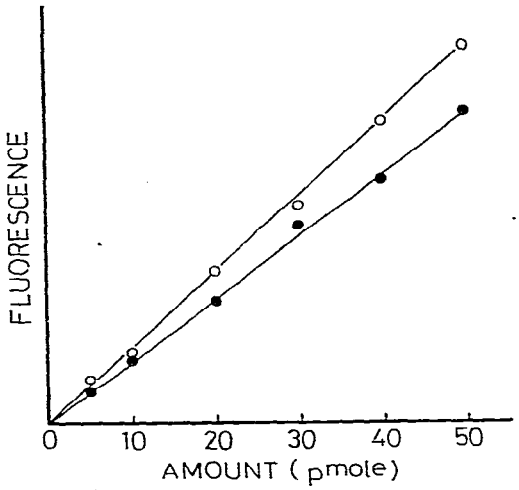

Fig. 5. Working curves for cyclopentanone (○) and cyclohexanone (●). Conditions as in Fig. 4. Peak-height ratios of these compounds to 500 pmole of cycloheptanone (internal standard) were plotted on the ordinate.

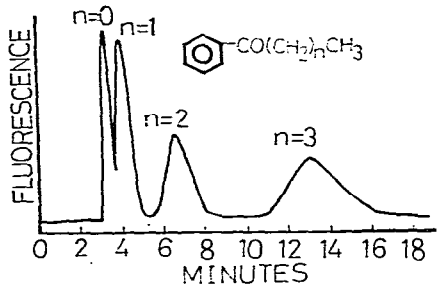

Fig. 6. HPLC separation of some native phenones. Sample: mixture containing acetophenone (200 pmole), propiophenone (200 pmole), *n*-propyl phenyl ketone (25 nmole) and *n*-butyl phenyl ketone (50 nmole). Eluent: 25% methanol. Temperature of reaction coil: 0°. Other conditions as in Fig. 4.

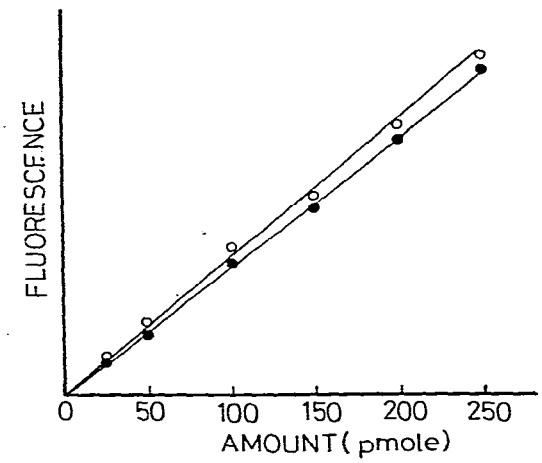

Fig. 7. Working curves for acetophenone (○) and propiophenone (●). Conditions as in Fig. 6. Peak-height ratios of these compounds to 25 nmole of *n*-propyl phenyl ketone (internal standard) were plotted on the ordinate.

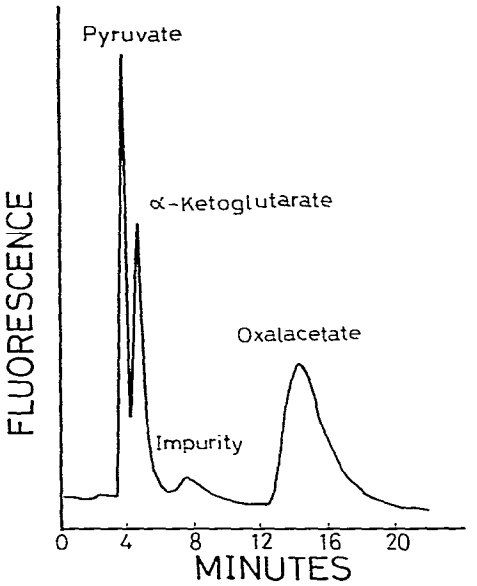

Fig. 8. HPLC separation of native α-keto acids. Sample: mixture containing sodium pyruvate (200 pmole), α-ketoglutaric acid monosodium salt (2 nmole) and oxalacetic acid (2 nmole). Column: Partisil-10 SAX (10 μm; 15 cm × 3 mm I.D.) at ambient temperature. Eluent: 0.1 *M* potassium phosphate (pH 5.5). Other conditions as in Fig. 4.

(pH 5.5), as shown in Fig. 8. Approximately 25 pmole of pyruvate was determined. The ion-exchanger method was demonstrated to be directly applicable to the analysis of urinary α-keto acids without any pre-treatment of the samples (10 μl).

In this investigation, NMN has been successfully applied to the fluorometric analysis of α-oxomethylene compounds by HPLC in both pre-column and post-column derivatization methods. The fluorogenic reaction is specific for the α-oxomethylene group as both the carbonyl and the α-methylene groups are simultaneously required for the formation of fluorophores. The sensitivity of the reaction permits the determination of some reactive α-oxomethylene compounds at the picomole level under mild conditions. The insensitive response of cycloheptanone, cyclooctanone, *n*-propyl phenyl ketone and *n*-butyl phenyl ketone is due primarily to steric hindrance of the α-oxomethylene groups in the first and/or second step of the reaction. At present, the pre-column derivatization method surpasses the post-column derivatization method in sensitivity. This is obviously due to the difference in the period of the addition reaction of α-oxomethylenes with NMN in alkaline media (10 min in the former and less than 2 min in the latter). Therefore, by using a longer reaction coil the sensitivity of the post-column derivatization method would be increased, with some loss of separation.

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REFERENCE

1 H. Nakamura and Z. Tamura, *Anal. Chem.*, 50 (1978) in press.