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Liquid chromatographic determination of dicarboxylic acids based on intramolecular excimer-forming fluorescence derivatization

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Abstract

A highly sensitive and selective fluorimetric determination method for dicarboxylic acids (C_5–C_{12}) has been developed. This method is based on an intramolecular excimer-forming fluorescence derivatization with a pyrene reagent, 4-(1-pyrene)butyric acid hydrazide (PBH), followed by reversed-phase liquid chromatography (LC). The carboxylic acids were converted to the corresponding dipyrene-labeled derivatives by reaction with PBH in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. The derivatives afforded intramolecular excimer fluorescence (450–550 nm) which can clearly be discriminated from the normal fluorescence (370–420 nm) emitted from PBH and monopyrene-labeled derivatives of monocarboxylic acids. The structures of the derivatives and the emission of excimer fluorescence were studied by LC with mass spectrometry and with spectrofluorimetry, respectively. The PBH derivatives of the carboxylic acids could be separated by reversed-phase LC on an ODS column with isocratic elution. The detection limits (signal-to-noise ratio=3) were 1.3 fmol to undetectable for a 20-μl injection.

Keywords: Derivatization, LC; Excimer fluorescence; Carboxylic acids; Pyrenebutyric acid hydrazide

1. Introduction

Dicarboxylic acids (glutaric acid, adipic acid, suberic acid, sebacic acid, dodecanedioic acid, etc.) are minor metabolites of fatty acids, but their levels in body fluids are significantly increased in patients with inborn errors of fatty acid metabolism [1,2] including glutaric aciduria (acidemia) and other acyl CoA dehydrogenase deficiencies, and with diabetic ketoacidosis [3,4]. Therefore, their measurements take considerable roles in rapid diagnoses and accurate therapies of the metabolic disorders [5].

Many chromatographic methods have been proposed for the measurement of carboxylic acids; gas chromatography [1–5], liquid chromatography (LC) [6–14] and capillary electrophoresis [15–19] coupled with a wide variety of detection methods such as potentiometry [6,7,15], conductimetry [8,9], photometry [10,16], fluorimetry [11,12,17,18], and mass spectrometry (MS) [1–5,13,14,19].

In our previous research, we developed highly selective and sensitive determination methods for

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polyamines [20] and basic amino acids [21] in LC based on an intramolecular excimer-forming fluorescence derivatization. An excimer is a complex between a compound and its corresponding excited state. In these methods, all the primary and secondary amino moieties in polyamines and basic amino acids were labeled with a pyrene reagent, 4-(1-pyrene)butyric acid *N*-hydroxysuccinimide ester, and the derivatives afforded an intramolecular excimer fluorescence (450–520 nm) that is much longer than usual (monomer) fluorescence (370–420 nm) of monopyrene derivatives. By using these characteristics, the polyamino compounds were determined highly selectively, even though the sample was contaminated with monoamino compounds.

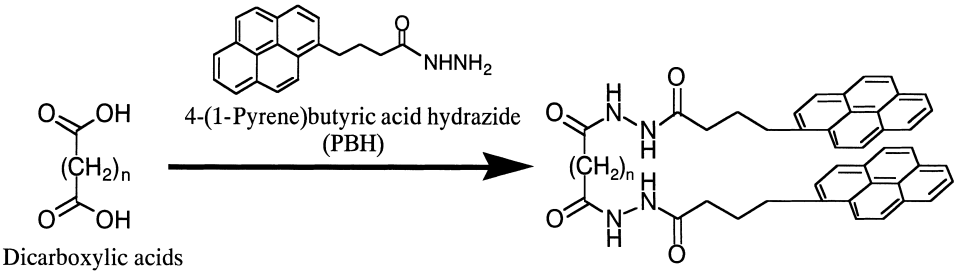
In the present study, we have examined the applicability of the above-mentioned intramolecular excimer-forming fluorescence derivatization to a wide variety of dicarboxylic acids (Fig. 1), and have developed highly sensitive determination method for dicarboxylic acids (C_5–C_{12}). The acids were converted to the corresponding dipyrene-labeled derivatives by 4-(1-pyrene)butyric acid hydrazide (PBH) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and the derivatives generated excimer fluorescence from the intramolecular dipyrene sites. The structures of dipyrene-labeled derivatives and the emission of intramolecular excimer fluorescence were studied by LC with MS and by fluorimetry.

2. Experimental

2.1. Reagents and solutions

PBH was purchased from Molecular Probes (Eugene, OR, USA), and was used without further purification. Dicarboxylic acids and other chemicals were of the highest purity available, and were used as received. Distilled water, further purified with a Milli-QII system (Millipore, Milford, MA, USA), was used for all aqueous solutions.

Stock solutions (1.0 m*M*) of dicarboxylic acids were prepared in dimethyl sulfoxide (DMSO) and stored at 4 °C. These solutions were stable for at least 1 month, and were diluted further with DMSO to the required concentrations before use. The solu-



Dicarboxylic acid	n	Abbreviation
Malonic acid	1	C_3
Succinic acid	2	C_4
Glutaric acid	3	C_5
Adipic acid	4	C_6
Pimeric acid	5	C_7
Suberic acid	6	C_8
Sebacic acid	8	C_{10}
Dodecanedioic acid	10	C_{12}

Fig. 1. Intramolecular excimer-forming fluorescence derivatization of dicarboxylic acids with 4-(1-pyrene)butyric acid hydrazide.

tions of PBH (5 m*M* in DMSO), EDC (2 *M* in water) and pyridine [20% (v/v) in DMSO] were prepared before use and used up within a day.

2.2. Apparatus and operation conditions

2.2.1. LC detection systems

An isocratic LC system consisted of a Jasco (Tokyo, Japan) PU-980 chromatograph pump, a Rheodyne (Cotati, CA, USA) Model 7125 syringe-loading sample injector equipped with a 20-μl sample loop, a Jasco DG-980-50 on-line degasser, a reversed-phase column, YMC-Pack ODS-AM (250× 4.6 mm I.D., particle size 5 μm; YMC, Kyoto, Japan), and a Hitachi (Tokyo, Japan) L-7480 spectrofluorimeter fitted with a 12-μl flow-cell. Aqueous 65% (v/v) acetonitrile was used as a mobile phase. The flow-rate was set at 1.0 ml/min and the column temperature was ambient (23±3 °C). The fluorescence detector was operated at the excitation and emission wavelengths of 345 and 480 nm, respectively, and the slit-widths of both the monochromators were set at 15 nm. For comparative studies, monomer fluorescence was monitored at the excitation and emission wavelengths of 345 and 378 nm, respectively.

2.2.2. LC–MS system

A Finnigan (San Jose, CA, USA) LCQ, ion-trap mass spectrometer equipped with an electrospray ionization (ESI) interface, was used in place of the fluorescence detector. Other separation conditions were the same as described in the previous section. The effluent from the LC column was directly introduced to the LC–MS interface without splitting. The ion source voltage and temperature of the heated capillary were set at +4.5 kV and 275 °C, respectively. Nitrogen gas was used as both sheath gas (85 p.s.i.; 1 p.s.i.=6894.76 Pa) and auxiliary gas (20 p.s.i.). The scan range was set at m/z 100–1000.

2.2.3. Fluorimetry

Uncorrected fluorescence spectral measurements were performed with a Hitachi F-2500 fluorescence spectrophotometer in 10×10 mm quartz cells; spectral bandwidths of 5 nm were used for both the excitation and emission monochromators.

2.3. Derivatization procedure

To 200 μl of a dicarboxylic acid solution placed in a 1.5-ml Reacti-vial (Pierce, Rockford, IL, USA), 100-μl aliquots of 20% (v/v) pyridine and 2 *M* EDC solutions, and 200 μl of 5 m*M* PBH solution were successively added. The vial was tightly sealed and heated at 40 °C for 60 min in a block heater, Pierce Reacti-Therm (Model 18970). After cooling in ice-water, the reaction mixture was directly injected into the chromatograph. To prepare the reagent blank, a 200-μl volume of DMSO in place of the standard solution was subjected to the same procedure.

3. Results and discussion

3.1. LC separation conditions

Reversed-phase LC was most suitable for the separation of PBH derivatives of dicarboxylic acids. Four reversed-phase columns [ODS (YMC-Pack C_{18}), octyl (YMC-Pack C_8), butyl (YMC-Pack C_4), and phenyl (YMC-Pack Ph) columns; 250×4.6 mm I.D., particle size 5 μm each] were tested. Among them, ODS column resulted in most satisfactory separation; resolution values between adipic acid and pimelic acid were 1.5–3.0 (ODS), 0.8–1.7 (octyl), 0.1–0.6 (butyl) and 0–0.2 (phenyl) using various mobile phases. Typical chromatograms obtained with standard mixtures of seven dicarboxylic acids and two monocarboxylic acids (*n*-caprylic and *n*-capric acids) are shown in Fig. 2. All the dicarboxylic acids gave the respective single peaks, and they were separated from each other and also from the peaks of early eluting blank components (Fig. 2A). Any peak for the dicarboxylic acids could not be observed when the monomer fluorescence was monitored at 378 nm (Fig. 2B). On the other hand, monocarboxylic acids afforded the respective monomer fluorescence peaks (Fig. 2D), but they did not give any peak in the excimer fluorescence detection (Fig. 2C). Furthermore, all of the tested monocarboxylic compounds described later (Section 3.5) did not give any peak in the excimer fluorescence detection. Thus, this method permits highly selective determination of dicarboxylic acids in the samples containing monocarboxylic acids.

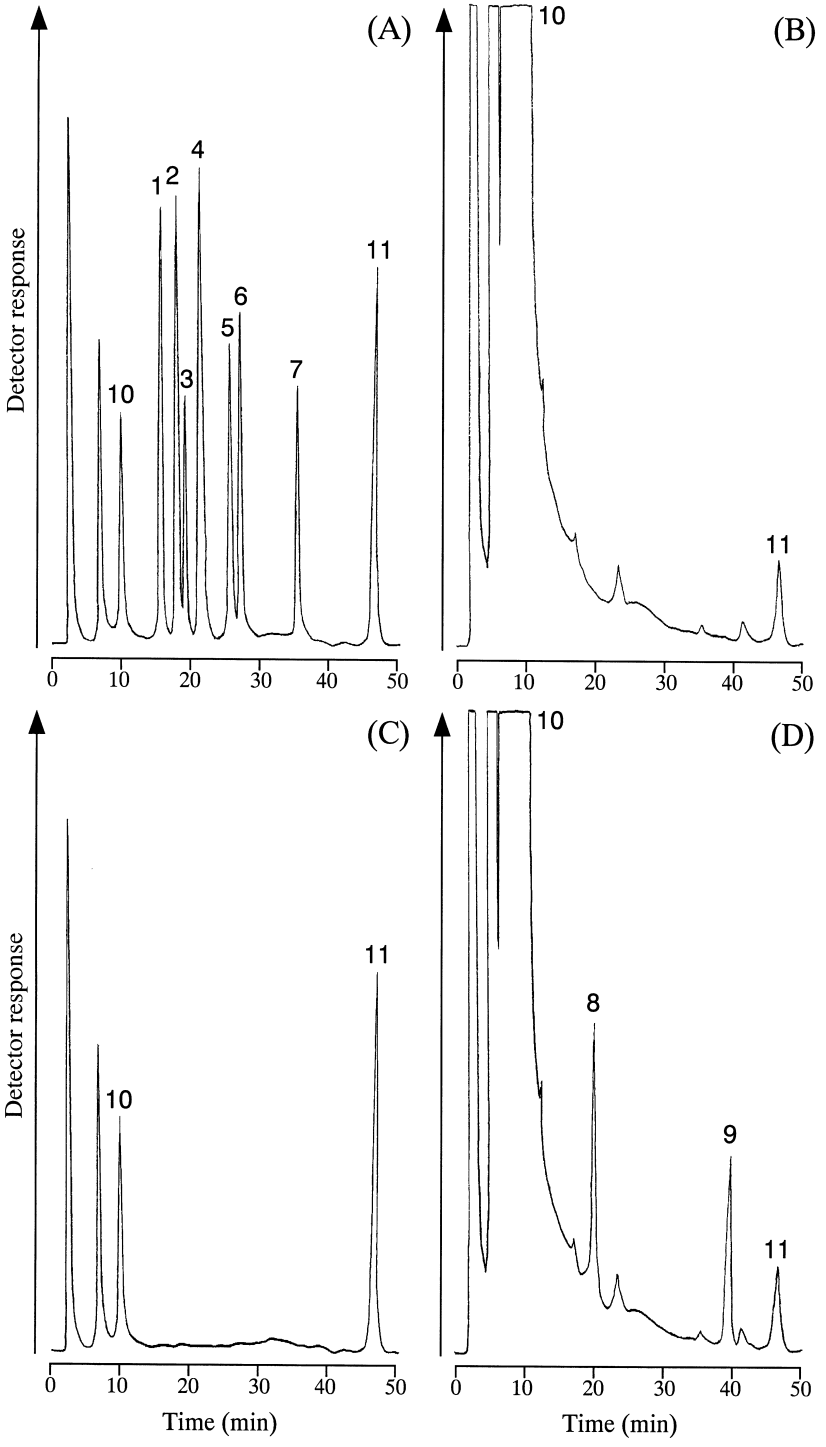


Fig. 2. Chromatograms obtained with the pyrene-labeled dicarboxylic acids (A and B) and monocarboxylic acids (C and D) with the excitation wavelength at 345 nm. Detections: (A) and (C), excimer fluorescence region (emission 480 nm); (B) and (D), monomer fluorescence region (emission 378 nm). Peaks: 1, C_6; 2, C_7; 3, C_3; 4, C_8; 5, C_5; 6, C_{10}; 7, C_{12}; 8, *n*-caprylic acid; 9, *n*-capric acid; 10, PBH; 11, reagent dimer; others, reagent blanks and/or unknown. Amounts (pmol on column): C_3=83, other dicarboxylic acids=17.

As for the retention times of the PBH derivatives of dicarboxylic acids, they became reasonably longer with increasing the carbon numbers except for malonic acid and glutaric acid. This may be explained by their structure. PBH derivatives of the longer-chain acids are compactly folded mainly due to the intramolecular pyrene to pyrene attraction, but the shorter ones cannot form these structures by the chain strain.

3.2. Structural analysis by LC–MS

The structures of PBH derivatives of seven dicarboxylic acids were studied by LC–MS with ESI interface. The selected ion chromatograms [monitoring ion ranges (m/z); 673.5±0.5 (malonic acid), 701.5±0.5 (glutaric acid), 715.5±0.5 (adipic acid), 729.5±0.5 (pimelic acid), 743.5±0.5 (suberic acid), 771.5±0.5 (sebacic acid), and 799±0.5 (dodecanedioic acid)] suggested that the components of the fluorescence peaks are ascribable to dipyrene-labeled derivatives of dicarboxylic acids as shown in Fig. 1. Mass spectra for the peaks also provided the following quasi-molecular ions ($[M+H]^+$, m/z) as base peaks; 673.5 (malonic acid), 701.4 (glutaric acid), 715.4 (adipic acid), 729.4 (pimelic acid), 743.5 (suberic acid), 771.6 (sebacic acid), and 799.6 (dodecanedioic acid). When detected at m/z ($[M+H]^+$) corresponding to the monopyrene-labeled derivatives, no significant peak was observed in the respective selected ion chromatograms. These LC–MS data strongly supported that both the carboxyl moieties were derivatized with PBH under the present derivatization conditions (Fig. 1).

3.3. Excimer fluorescence from PBH derivatives of dicarboxylic acids

Fig. 3 shows normalized fluorescent emission spectra of the fluorescence peak eluates for the dicarboxylic acids, *n*-caprylic acid, and PBH in LC. As in the cases with polypyrene-labeled derivatives of polyamines and basic amino acids [20,21], and with dipyrenylalkanes [22], the PBH derivatives of dicarboxylic acids afforded excimer fluorescence characterized by structureless and broad peak around 480 nm. On the other hand, *n*-caprylic acid and the reagent blank gave only monomer fluorescence from monopyrene-labeled *n*-caprylic acid and/or PBH. The ratios of fluorescence intensity in the excimer region to that in the monomer region (378 nm) differed among the dicarboxylic acids and were

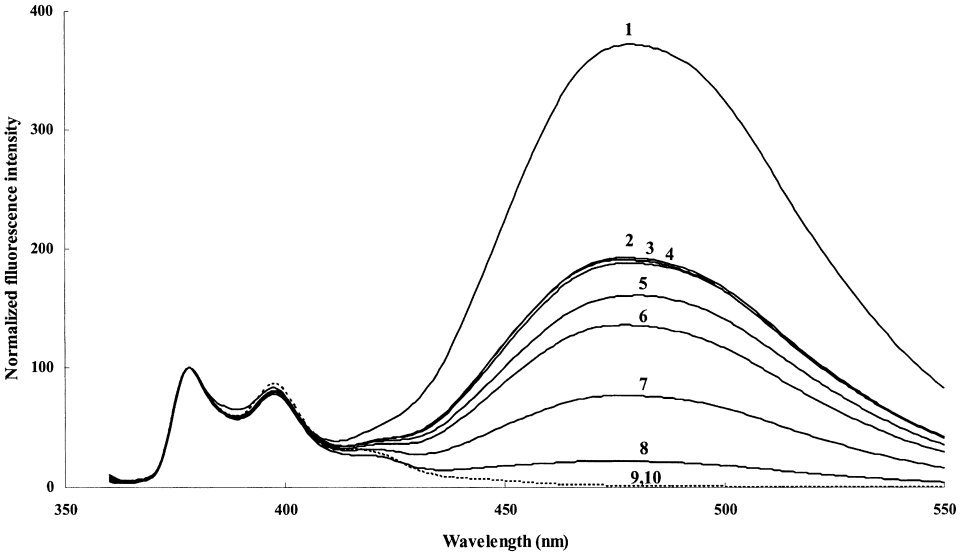


Fig. 3. Fluorescence emission spectra (excitation 345 nm) of the eluates for pyrene-labeled dicarboxylic acids and *n*-caprylic acid, and for PBH from the LC column. Each spectrum was normalized to the first peak at 378 nm. Curves: 1, C_5; 2, C_6; 3, C_4; 4, C_8; 5, C_{10}; 6, C_7; 7, C_{12}; 8, C_3; 9, *n*-caprylic acid; 10, PBH.

basically independent of their chain lengths. Malonic acid and dodecanedioic acid gave relatively weak excimer fluorescence probably due to the steric hindrance to the excimer formation. The effect of solvent on the emission of excimer fluorescence was quite similar to that in the polypyrene derivatives of polyamines [20]. All the derivatives showed intense excimer fluorescence in aqueous 50–90% (v/v) solutions of water-soluble organic solvents (tetrahydrofuran, acetonitrile, methanol, and ethanol).

From these observations, we can conclude that the present method is highly selective for dicarboxylic acids by measuring the intramolecular excimer fluorescence from their dipyrene derivatives.

3.4. Optimum derivatization conditions

Optimization studies were carried out to maximize the excimer fluorescence peak area using glutaric acid, pimelic acid, and dodecanedioic acid as model short-chain, middle-chain, and long-chain dicarboxylic acids, respectively. Some pyrene-label reagents for carboxylic acids are commercially available: PBH, 1-pyrenemethylamine, 1-pyrenepropylamine, 1-pyrenyldiazomethane (PDAM), and 1-(bromoacetyl)pyrene. Of the reagents, PBH gave the highest excimer fluorescence peaks for all the dicarboxylic acids under the common derivatization procedures [11,12]. The optimum concentrations of PBH and EDC were 2–10 m*M* and 1–4 *M* in the respective reagent solutions; 5 and 2 *M* were selected, respectively, as the optimum. The derivatization reactions of carboxyl group with hydrazide-type reagents efficiently proceed in the presence of EDC (condensation reagent), base and water-miscible organic solvent. Of the tested bases (pyridine, triethylamine, *N*,*N*,*N*′,*N*′-tetraethylethylenediamine, quinuclidine and potassium carbonate), pyridine afforded maximum peak areas in the concentration ranges 10–50% (v/v); 20% (v/v) was selected and this did not cause any damage of the LC column. As water miscible organic solvents, methanol, ethanol, acetonitrile, *N*,*N*-dimethylformamide, and DMSO were investigated; the maximum peak areas for all the acids were obtained when DMSO was used for the preparations of test sample, PBH, and pyridine solutions. The derivatization reaction proceeded more rapidly with increasing reaction temperature in the ranges 0–40 °C, and the peak areas for the acids reached maxima after reactions of 120, 90, and 60 min at 0, 20, and 40 °C, respectively. Higher temperature than 50 °C causes the decrease in the peak areas. The reaction at 40 °C for 60 min was selected for obtaining high sensitivity and reproducible results. There are not significant differences in the above optimum conditions among the tested carboxylic acids.

The PBH derivatives of the acids in the final reaction mixture were stable, and still gave the constant fluorescence intensities after standing for at least 24 h in the dark at 4–25 °C.

3.5. Method validation (calibration graph, precision, detection limits, and specificity)

The relationships between the amounts of dicarboxylic acids (glutaric acid, pimelic acid, and dodecanedioic acid) and the peak heights were linear over the concentration range of 10 pmol/min–10 nmol/ml in a sample solution, which corresponded to 0.067–67 pmol per 20 μl injection volume. The linear correlation coefficients ($n=5$) were 0.999, 1.000 and 0.999, and the *y*-intercepts of the linear regression lines were corresponded to −0.13, 0.09 and 0.08 nmol/ml in a sample solution, respectively, in the above range. The between-day precision values throughout the entire process were established by repeated determinations ($n=8$) using the mixtures of the acids (50 and 5000 pmol/ml each in a sample solution, 0.33 and 33 pmol each per 20 μl injection volume); the relative standard deviations were within 5.1 and 2.3%, respectively.

Fig. 4 shows the chromatogram obtained with a standard mixture of dicarboxylic acids at an extremely low concentration. The detection limits (signal-to-noise ratio=3) for dicarboxylic acids (C_5–C_{12}) were 2.3–4.3 fmol per 20 μl injection volume (Table 1), which corresponded to 0.35–0.65 pmol/ml in a sample solution, respectively. This intramolecular excimer-forming derivatization method is as sensitive as other highly sensitive derivatization reagents [11,12]. Table 1 shows detection limits and retention times for dicarboxylic acids including the aliphatic dicarboxylic acids, unsaturated carboxylic acids, and aromatic carboxylic acids. Most of the dicarboxylic acids and citric acid (tricarboxylic acid) reacted with

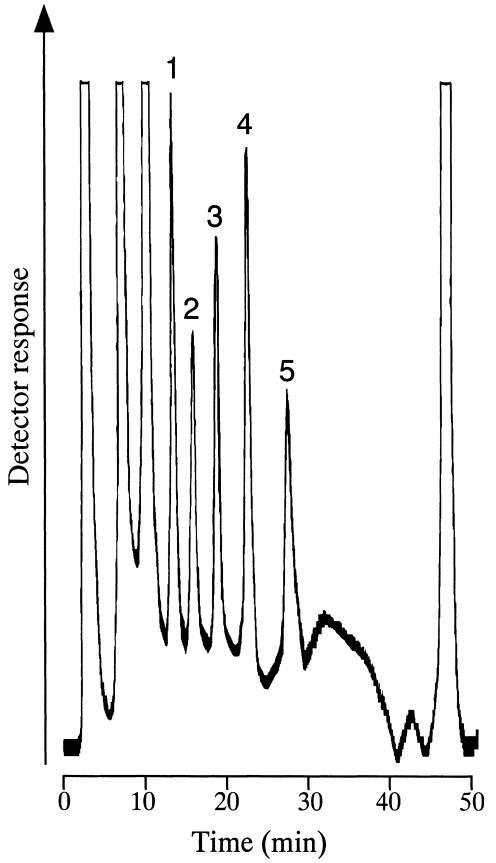


Fig. 4. Chromatogram of the PBH derivatives of di- and tricarboxylic acids by excimer fluorescence detection. Peaks: 1, L-malic acid; 2, C_6; 3, methylsuccinic acid; 4, citric acid; 5, C_{10}. Amounts: 17 fmol each on column.

PBH to afford corresponding single excimer fluorescence peaks under the proposed derivatization and LC conditions (Table 1). However, there were undetectable or less sensitively detectable dicarboxylic acids probably due to the following reason; (1) steric hindrance of the first derivatized carboxyl moiety against the other carboxylic group, causes poor yield of the dipyrene derivative as shown in the PDAM derivatization of short-chain dicarboxylic acids [23], (2) low reactivity of the carboxyl group(s) also decreases the yield, and (3) steric hindrance to the formation of the excimer in the dipyrene derivatives causes weaker emission of the excimer fluorescence as in the case with malonic acid in Fig. 2.

The following biological compounds having only one carboxyl moiety or none in a molecule, at a concentration of 10 nmol/ml, did not afford any peak under the present conditions; the compounds tested were monocarboxylic acids (formic acid, acetic acid, *n*-butyric acid, *n*-caprylic acid, *n*-capric acid, palmitic acid, stearic acid, oleic acid, homovanillic acid, 5-hydroxyindole-3-acetic acid, and phenylpyruvic acid), L-ascorbic acid, neutral and basic amino acids, ammonia, acetylcholine, serotonin, catecholamines (epinephrine, norepinephrine, and dopamine), sugars (D-glucose, D-fructose, D-galactose, D-ribose, *N*-acetyl-D-glucosamine, maltose, and sucrose), nucleic acid bases (adenine, guanine, thymine, cytosine, and uracil), and other compounds (methanol, acetone, phenol, cholesterol, creatine, creatinine, and urea).

These observations suggest that the present derivatization method has enough precision, sensitivity, and selectivity for the biological and biomedical applications.

4. Conclusions

Unique derivatization technique, an intramolecular excimer-forming derivatization, has successfully applied to the highly selective determination method for dicarboxylic acids. The acids afforded intramolecular excimer fluorescence (450–550 nm), which can clearly be discriminated from normal fluorescence (370–420 nm) from PBH derivatives of monocarboxylic acids and PBH itself. This property as well as prolonged Stokes shift also allows highly sensitive detection of dicarboxylic acids. Therefore, the present method should be applicable to biological and biomedical investigations of the metabolic disorders accompanied by the changes of the dicarboxylic acids levels in biological samples.

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Table 1
Detection limits and retention times for dicarboxylic acids and citric acid

Dicarboxylic acid	Detection limit[a] (fmol)	Retention time (min)
Malonic acid (C_3)	22	18.6
Succinic acid (C_4)	6.9	15.2
Glutaric acid (C_5)	3.6	25.2
Adipic acid (C_6)	2.5	15.8
Pimeric acid (C_7)	2.4	17.3
Suberic acid (C_8)	2.3	20.7
Sebacic acid (C_{10})	3.3	26.4
Dodecanedioic acid (C_{12})	4.3	35.3
Methylsuccinic acid	1.9	17.5
L-Malic acid	1.3	12.6
α-Ketoglutaric acid	25	21.7
Citric acid	1.4	21.6
Fumaric acid	N.D.[b]	–
Maleic acid	N.D.[b]	–
Aspartic acid	6.7	16.6
Glutamic acid	N.D.[b]	–
o-Phthalic acid	16	25.1
3,4-Pyridinedicarboxylic acid	89	18.3
2,3-Pyridinedicarboxylic acid	N.D.[b]	–
6-Methyl-2,3-pyridinedicarboxylic acid	12	27.2

[a] $S/N=3$.
[b] Not detected.

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