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1,2-Diarylethylenediamines as sensitive pre-column derivatizing reagents for chemiluminescence detection of catecholamines in HPLC*

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Abstract: Chemiluminescence detection in high-performance liquid chromatography for derivatives of catecholamines (norepinephrine, epinephrine and dopamine) and isoproterenol was studied on the basis of the peroxyoxalate chemiluminescence reaction. The amines and isoproterenol, derivatized with 1,2-diarylethylenediamines, were separated on a reversed-phase HPLC column (TSK gel ODS-120T) with isocratic elution using a mixture of imidazole buffer (pH 5.8, 120 mM)–methanol–acetonitrile (6:2:9, v/v/v). The eluate was detected by a post-column chemiluminescence reaction system, using bis[4-nitro-2-(3,6,9-trioxadecyloxycarbonyl)phenyl]oxalate and hydrogen peroxide. Of the 14 1,2-diarylethylenediamines investigated, it was found that 1,2-bis(3-chlorophenyl)ethylenediamine, 1,2-bis(3,4-dichlorophenyl)-ethylenediamine and 1,2-bis(4-chlorophyenyl)ethylenediamine were the most sensitive derivatives for all catecholamines. The derivatization and peroxyoxalate chemiluminescence reaction conditions were optimized for 1,2-bis(3-chlorophenyl)-ethylenediamine. The chromatographic detection limits for catecholamines were approximately 40–120 amol for an injection volume of 100 μl (signal-to-noise ratio of 3).

Keywords: *Catecholamines; peroxyoxalate chemiluminescence; norepinephrine; epinephrine; dopamine; isoproterenol; diarylethylenediamine; Bis[4-nitro-2-(3,6,9-trioxadecyloxycarbonyl)phenyl]oxalate; reversed-phase liquid chromatograpy.*

Introduction

Catecholamines (CAs) such as epinephrine (E), norepinephrine (NE) and dopamine (DA) are important markers for the diagnosis of several diseases [1]. Of the many methods reported for the determination of CAs [2], high-performance liquid chromatography (HPLC) is the most prevalent technique for simultaneous quantification of CAs and has gained increasing use in the clinical laboratories. HPLC methods involving electrochemical detection [3–5], fluorometric detection [6–12] and chemiluminescence (CL) detection [13–15] have been developed. Post-column fluorescence derivatization of CAs in HPLC using peroxyoxalate CL detection has also been reported [15]. In a previous study the authors have investigated the use of 1,2-diphenylethylenediamine (DPE) as a pre-column derivatizing reagent for the sensitive and selective determination of CAs via peroxyoxalate CL detection; this method permitted the determination of CAs in 50 μl human plasma and 0.25 μl urine [16].

Diarylethylenediamines have also been investigated as fluorogenic reagents for CAs [17]. The present study explores the use of further highly sensitive fluorogenic reagents for the CL detection of CAs; 14 different species of diarylethylenediamine have been synthesized and screened as pre-column derivatizing reagents for CAs in HPLC with peroxyoxalate CL detection. The derivatization and CL reaction conditions have been studied and optimized for the HPLC analysis of CAs, using norepinephrine, epinephrine and dopamine as model systems.

Experimental

Reagents and solutions

NE bitartrate and DA hydrochloride were purchased from Wako Pure Chemicals (Osaka, Japan), and E bitartrate was from the Sigma Chemical Company (St Louis, MO, USA) and

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Table 1
Chemical structures and abbreviations for the diarylethylenediamines screened

1,2-Bis(aryl)ethylenediamine:aryl group	Abbreviation
3-Chlorophenyl	m-CED
3,4-Dichlorophenyl	DCED
4-Chlorophenyl	p-CED
4-Fluorophenyl	p-FED
1-Chlorophenyl	o-CED
4-Biphenylyl	BPED
1-Naphthyl	NED
4-Methoxyphenyl	p-MOED
2-Hydroxyphenyl	o-HED
3,4-Dimethoxyphenyl	DMOED
4-Methylphenyl	p-MED
3-Pyridyl	PED
4-Carboxyphenyl	p-CRED
3,4-Methylenedioxyphenyl	MDED

isoproterenol (IP) hydrochloride from Nakalai Chemicals (Kyoto, Japan). Diarylethylenediamines (DAEs) (abbreviations Table 1) were synthesized as previously reported [17]. DAE solutions (pH 6.5; 10 mM for DCED, BPED, NED and DMOED; 50 mM for the others) were prepared by dissolving 0.1 (or 0.5) mmol in 8 ml aqueous 60% acetonitrile, adjusting the pH* with 0.1 M hydrochloric acid to pH 6–7 and completing to volume (10.0 ml) with 60% aqueous acetonitrile. Bis[4-nitro-2-(3,6,9-trioxadecyloxycarbonyl)phenyl]oxalate (TDPO), bis[2-(3,6-dioxaoctyloxycarbonyl)-phenyl]oxalate (DOPO), bis(2,4,6-trichlorophenyl)oxalate (TCPO) and bis(2,4-dinitrophenyl) oxalate (DNPO) (all from Wako Pure Chemicals) were used after first dissolving them in ethyl acetate. Imidazole was recrystallized twice from toluene and 120 mM imidazole buffer was prepared in water; the pH was adjusted with nitric acid. Hydrogen peroxide in aqueous solution (31%, w/w) (Mitsubishi Gas Kagaku Co., Tokyo, Japan) was diluted with a mixture of ethyl acetate–acetonitrile (9:1, v/v) to appropriate concentrations. Bis(2-hydroxyethyl)iminotris-(hydroxymethyl)methane buffer (Bis–Tris; Dojindo Laboratories, Kumamoto, Japan) was prepared (0.1 M) by dissolving 209 mg Bis–Tris in 8.0 ml of 0.5 M potassium hydroxide; the pH was adjusted with hydrochloric acid (0.1 M) to pH 6.5, and completed to volume (10.0 ml) with 0.5 M potassium chloride solution. All other chemicals were of reagent grade. Deionized water was used.

Fluorescence derivatization

To 10 μl of an aqueous CA solution in a test tube were added successively: 600 μl acetonitrile, 400 μl Bis–Tris buffer (pH 6.5, 0.1 M) containing potassium chloride (0.5 M), 10 μl potassium hexacyanoferrate(III) (100 mM) and 200 μl DAE (50 mM). The mixture was incubated at 37°C for 25 min. A portion of the mixture was subjected to HPLC.

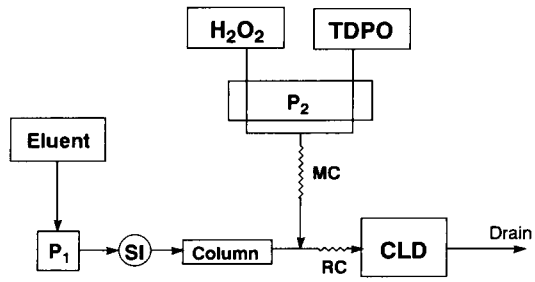

Figure 1
Block diagram of the LC system with CL detection. Key: P1, LC pump; SI, sample injection valve; P2, reagent-delivery pump; MC, mixing coil; RC, reaction coil; CLD, CL detector.

HPLC apparatus and its operation

A block diagram of the HPLC system is illustrated in Fig. 1. A Shimadzu LC-6A liquid chromatograph was used, equipped with a Rheodyne 7125 syringe-loading sample injector valve (100-μl loop). A TSK gel ODS-120T column (particle size 5 μm, 250 × 4.6 mm i.d.; Tosoh Co., Japan) was used. The column temperature was ambient (24 ± 4°C). The mobile phase comprised acetonitrile–methanol–imidazole buffer (pH 5.8, 120 mM) (9:2:6, v/v/v) and the flow-rate was 0.8 ml min^{-1}.

In the post-column CL reaction system, 0.3 mM TDPO and 0.3 M hydrogen peroxide were delivered at 0.56 ml min^{-1} each by a SSP PM-2M 1024 pump (Sanuki Kogyo Co., Tokyo, Japan). Both the solutions were combined and mixed by passing through a stainless-steel mixing coil (5 m × 0.5 mm i.d.). The column eluate and the CL reagent stream were mixed through a stainless steel reaction coil (1.6 m × 0.25 mm i.d.). The CL generated in the eluate was monitored with a Lumino-monitor-I MODEL AC-2220 (ATTO Co., Tokyo, Japan) (detection wavelength range: 450 to 700 nm).

Results and Discussion

Screening of diarylethylenediamines

Fourteen DAEs were screened as potential

derivatizing reagents for CAs (Table 1). The reagents screened were selected from the compounds reported [17], on the basis of their having longer fluorescence excitation and emission wavelengths and/or higher fluorescence intensity than that of DPE. Data obtained with the 4 CA derivatives using the screened DAEs which showed higher or comparable CL intensity as that of DPE derivatives are shown in Table 2; the remaining reagents displayed CL intensities less than 50% of that for DPE. Of the DAEs investigated, DCED derivatives of CAs were the most sensitive, followed by m-CED derivatives. Under the stated conditions DAE derivatives of CAs showed longer retention times (t_R) and better separation than for DPE derivatives. As regards solubility, DPE followed by m-CED and p-CED were readily soluble up to 0.1 M in aqueous 60% acetonitrile; however, DCED was almost insoluble at concentrations higher than 10 mM. From the above observations m-CED (pH* 6–7, 50 mM solution in aqueous acetonitrile 60%) was selected as the best reagent and used for further investigations.

Optimization of the derivatization reaction conditions

The optimum apparent pH for the reaction was pH* 6.5 in aqueous acetonitrile as previously investigated [17]. Since the m-CED derivatization reaction is more susceptible to pH of the reaction medium than the DPE reaction. It is necessary to buffer the reaction medium to pH 6.5. The effect of the following buffers (all 0.1 M containing 0.5 M potassium chloride) was studied at pH* values in the range 4–10: Bis–Tris, N,N-bis(2-hydroxyethyl)glycine (Bicine), 2-morpholinoethanesulphonic acid (MES), 2-hydroxy-3-morpholinopropanesulphonic acid (MOPSO), piperazine-1,4-bis-(2-ethanesulphonic acid (PIPES) and 2-[4-hydroxyethyl)-1-piperazinyl]ethanesulphonic acid (HEPES).

The highest CL intensity was observed for Bis–Tris buffer (pH* 6.5). This Bis–Tris buffer did not interfere with the derivatization or the CL reactions.

For potassium hexacyanoferrate(III) concentration (25–200 mM) it was shown that 100–200 mM yielded almost maximum CL intensity; 100 mM was selected as optimum.

The reaction time at 37°C was studied (0–50 min), and the CL response shown to reach maximum after 20 min at 37°C and to remain stable up to 40 min. Thermostatting at 37°C for 25 min was selected as being optimum for reproducible results. At higher temperatures (50–70°C) the reaction proceeds rapidly but the degradation rate of the derivatives also increased.

Thus reaction rate achieved after optimization was approximately 2-fold faster than that of the previously reported reaction [17].

HPLC separation

The derivatives of NE, E, DA and IP in the reaction mixture were separated on a reversed-phase HPLC column (TSK gel ODS-120T) with isocratic elution. The column eluate was successfully mixed post-column with TDPO and with hydrogen peroxide solution for CL detection (Fig. 1). Using the same mobile phase as that reported for the separation of DPE derivatives of CAs [16] resulted in long separation times, so it was necessary to modify

Table 2
Retention times (t_R, min)*, relative peak heights (RPH) and detection limits (DL, fmol on-column)† of 1,2-diarylethylenediamine derivatives of catecholamines and isoproterenol (IP)

1,2-Diarylethylenediamine	NE			E			DA			IP		
	t_R	RPH	DL	t_R	RPH	DL	t_R	RPH	DL	t_R	RPH	DL
m-CED	8.4	50	0.15	14.4	100	0.05	21.2	60	0.10	24.4	80	0.08
p-CED	8.6	30	0.20	14.5	73	0.07	20.7	36	0.15	24.4	56	0.10
DCED	12.2	75	0.10	22.1	112	0.03	35.1	65	0.10	38.7	83	0.08
NED	14.5	31	0.30	17.0	69	0.10	24.9	53	0.15	29.5	57	0.10
FED	6.8	12	0.80	10.2	38	0.20	13.6	40	0.40	16.0	33	0.30
BED	12.6	28	0.40	23.5	53	0.10	35.0	25	0.40	42.0	27	0.30
MOED	9.0	36	0.60	14.0	35	0.20	24.0	21	0.30	33.0	33	0.30
HED	8.6	23	0.70	14.0	43	0.30	—‡	—	—	24.3	35	0.30
DPE	4.8	20	0.50	9.2	52	0.20	12.2	25	0.40	14.4	21	0.40

*The peak height of m-CED derivative of epinephrine was taken as 100.
†$S/N = 3$.
‡Undetectable.

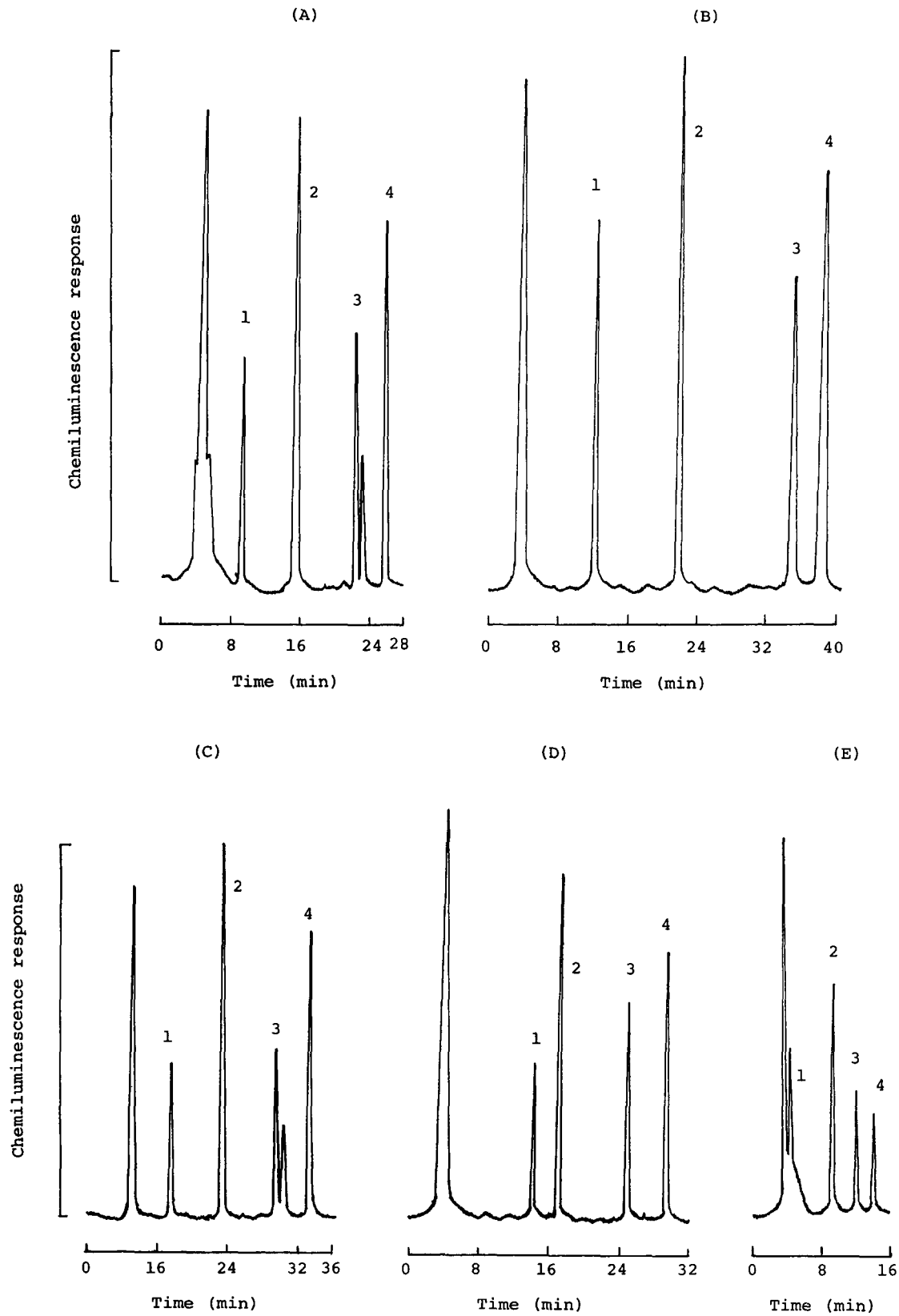

Figure 2
Chromatograms obtained with: (A) m-CED, (B) DCED, (C) p-CED, (D) NED and (E) DPE for derivatives of CAs and IP (4.45 fmol per 100 μl injection). Key: 1 = NE; 2 = E; 3 = DA; 4 = IP; other peaks are from the reagent blank.

the mobile phase to obtain the best separation in a short time. A mixture of imidazole buffer (pH 5.8, 120 mM)–methanol–acetonitrile (6:2:9, v/v/v) gave the desired separation. Methanol was necessary for an efficient separation; without methanol, the peaks of DA (peak 3) and IP (peak 4) in Fig. 2(a) overlapped. The chromatograms obtained with the CL derivatives (Fig. 2a) showed that the chemiluminescent peaks were completely separated within 26 min. There was a delay in the retention time (t_R) of the derivatives of diarylethylenediamine, compared with DPE, which enabled good separation of NE from the early eluting peaks (system peaks) and the reagent blank (cf. Fig. 2).

CL reaction in HPLC

Imidazole was necessary for development of the peroxyoxalate CL reaction with the diarylethylenediamine derivatives of CAs. In the HPLC system, the optimum pH of the imidazole buffer was pH 5.8 (Fig. 3), which was slightly higher than that for the DPE derivatives in the previous work [16]. Studies on the effect of imidazole concentration on the development of CL showed that increasing the concentration of imidazole buffer (pH 5.8) resulted in an increase in CL intensity for the CA derivatives with m-CED (Fig. 4); 120–160 mM yielded almost maximum CL intensity, so 120 mM was selected because this gave the highest signal-to-noise ratios for the CA peaks.

The optimum concentrations of TDPO and hydrogen peroxide

The optimum concentrations of TDPO and hydrogen peroxide were found to be 0.3 mM and 0.3 M, respectively; this was almost the same as for the DPE derivatives (Fig. 5a and b).

HPLC determination

The calibration graphs plotted with the CL

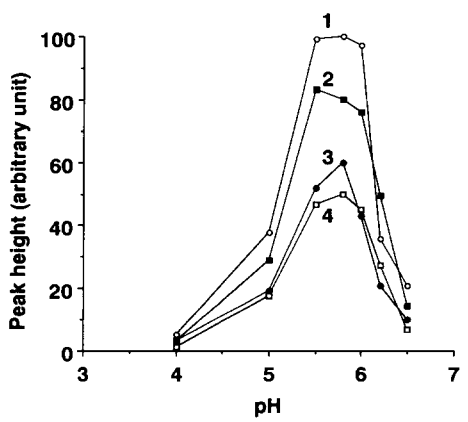

Figure 3
Effect of imidazole buffer pH on CL peak height for m-CED derivatives of CAs and IP. Key to curves (mean of duplicate experiments): 1, E; 2, IP; 3, DA; and 4, NE.

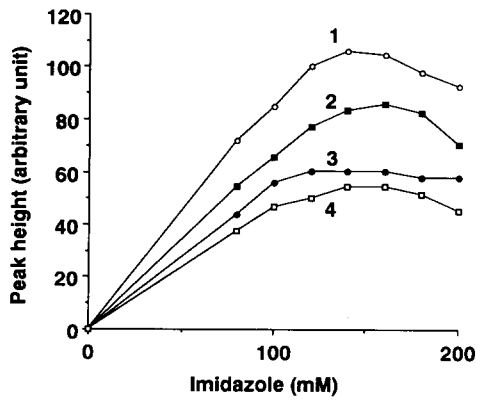

Figure 4
Effect of imidazole concentration on CL peak heights for m-CED derivatives of CAs and IP. Key to curves (mean of duplicate experiments): 1, E; 2, IP; 3, DA; and 4, NE.

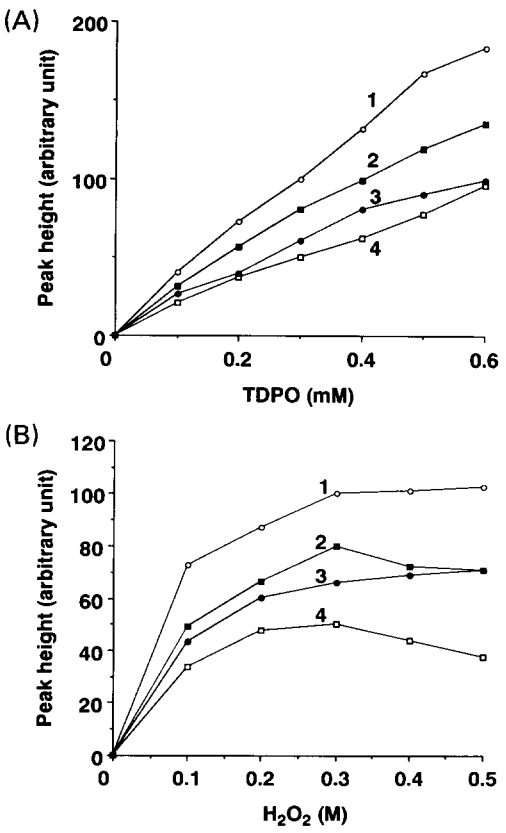

Figure 5
Effects of the concentration of (A) TDPO and (B) hydrogen peroxide on the CL peak heights of the m-CED derivatives of CAS and IP. Curves (mean of duplicate experiments): 1, E; 2, IP; 3, DA; and 4, NE.

intensities for NE, E, DA and IP were all linear (0.5–100 fmol each for a 100-μl injection volume; each point was the mean value of duplicate determinations). The correlation coefficient for each regression was in the range 0.980–0.995.

The detection limits for the CAs were approximately 40–120 amol (attomole) for a signal-to-noise ratio of 3. The within-day (n = 10) and between-day (5 different days) reproducibilities of CL detection were evaluated from the results of repeated measurements of a 2.0 fmol quantity in an injection volume of 100 μl. The relative standard deviations at 2.0 fmol (n = 5) were 2.4, 2.1, 4.3 and 4.8 (within-day) and 3.4, 2.8, 5.4 and 5.2 (between-day) for NE, E, DA and IP, respectively.

Conclusions

The sensitivity of the proposed CL detection system is approximately 4 times higher than that of the earlier method [16]. The latter had the highest sensitivity and lowest detection limits based on peroxyoxalate CL of the DPE derivatives of CAs; detection sensitivity was 96 times higher than that by fluorometric detection using the DPE reaction [12], and higher than that for CL detection using dansyl or fluorescamine derivatives [13, 14] by 190–300 times, and 12 times higher than for proposed ethylenediamine derivatives [15].

This proposed method using the new fluorogenic reagent, m-CED, permits the ultrasensitive determination of CAs at the amol level and should prove useful for biological and medical investigations of CAs.

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