

**Peroxyoxalate chemiluminescence detection for the highly sensitive determination of fluorescence labeled chlorpheniramine with Suzuki coupling reaction**

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## ABSTRACT

A sensitive and selective HPLC-peroxyoxalate chemiluminescence (PO-CL) method has been developed for the simultaneous determination of chlorpheniramine (CPA) and monodesmethyl chlorpheniramine (MDCPA) in human serum. The method combines fluorescent labeling with 4-(4,5-diphenyl-1*H*-imidazole-2-yl)phenyl boronic acid (DPA) using Suzuki coupling reaction with PO-CL detection. CPA and MDCPA were extracted from human serum by liquid-liquid extraction with *n*-hexane. Excess labeling reagent which interfered with trace level determination of analytes was removed by solid phase extraction using a C18 cartridge. Separation of derivatives of both analytes was achieved isocratically on silica column with a mixture of acetonitrile and 60 mM imidazole-HNO<sub>3</sub> buffer (pH 7.2) (=85:15, v/v) containing 0.015% triethylamine. The proposed method exhibited a good linearity with correlation coefficient of 0.999 for CPA and MDCPA within the concentration range of 0.5-100 ng/mL. The limits of detection (S/N=3) were 0.14 and 0.16 ng/mL for CPA and MDCPA, respectively. Using the proposed method, CPA could be selectively determined in human serum after oral administration.

Keywords: Suzuki coupling reaction; Peroxyoxalate chemiluminescence; Chlorpheniramine; Monodesmethyl chlorpheniramine; HPLC

## Abbreviations

CL	Chemiluminescence
CPA	Chlorpheniramine
CPPO	Bis(2,4,5-trichlorophenyl-6-carbopenoxyphenyl)oxalate
DMF	<i>N,N</i> -Dimethylformamide
DPA	4-(4,5-Diphenyl-1 <i>H</i> -imidazole-2-yl)phenyl boronic acid
FL	Fluorescence
MDCPA	Monodesmethyl chlorpheniramine
LOD	Limit of detection
OTC	Over the counter
PO-CL	Peroxyoxalate chemiluminescence
RCI	Relative chemiluminescence intensity
S/N	Signal to noise ratio
TEA	Triethylamine

## 1. Introduction

Suzuki coupling reaction is a palladium-catalyzed cross coupling reaction between aryl boronic acid and aryl halides or aryl triflates [1]. It has been described as the most powerful transformation for the construction of carbon-carbon bonds in modern day organic chemistry [2]. Its advantage over other carbon-carbon bond forming protocols includes its mild reaction conditions [3,4] and tolerance of a wide range of functional groups [4,5]. For analytical application, Suzuki coupling reaction provides a route to selectively label a compound containing aryl halide moiety with a fluorescent boronic acid derivatives to develop selective analytical methods for aryl halide [6]. Its superior selectivity in analysis is derived from minimal interferences because most of biological components are not reacted with phenylboronic acid, in addition to improved sensitivity brought about by the elimination of a halogen atom, which sometimes leads to unexpected quenching of fluorescence (FL) [6]. Lophine derivatives have been identified to possess excellent FL properties [7], so we have designed a boronic acid derivative of lophine, 4-(4,5-diphenyl-1*H*-imidazole-2-yl) phenyl boronic acid (DPA) [8]. In one of our previous reports, we demonstrated the analytical applicability of Suzuki coupling reaction for the labeling of aryl halides [6]. We have since employed Suzuki coupling reaction as a pre-column fluorescent labeling step to develop sensitive and selective methods for drugs analysis using fluorescence detection [9,10].

Compared to FL detection methods, chemiluminescence (CL) affords higher sensitivity brought about by significant reduction in baseline noise due to the lack of external excitation source. Peroxyoxalate CL (PO-CL) as first reported by Chandross in 1963 [11] is an indirect CL reaction. According to the general mechanism, a high

energy intermediate is generated by a reaction between aryloxalate ester and H<sub>2</sub>O<sub>2</sub>, this intermediate transfers its energy to a coexisting fluorophore which becomes excited. The excited fluorophore returns to the ground state with the emission of light [12]. Therefore, fluorescent compounds can be analyzed in an HPLC-PO-CL system by using a mixture of aryloxalate ester and H<sub>2</sub>O<sub>2</sub> as a post column reagent, and measuring the resulting light signal [13]. Sensitive determinations of non-fluorescent compound have been achieved after fluorescent derivatization [14-19]. Therefore, DPA-labeled compounds can be detected sensitively using PO-CL reaction.

The aim of this study was to use Suzuki coupling reaction as a fluorescent labeling step (Fig. 1) in combination with PO-CL reaction to develop a sensitive and selective method for the determination of chlorpheniramine(CPA) and its metabolite, monodesmethyl chlorpheniramine (MDCPA) in human serum. CPA is a potent, highly effective and widely used as an active ingredient in formulations to alleviate symptoms of common cold and allergic conditions [20]. CPA is extensively metabolized via *N*-dealkylation to its monodemethylated metabolite, MDCPA which accounts for about 30% of the oral dose [21].

Various analytical methods have been developed for the determination of CPA, some of these are based on HPLC with FL detection [22,23], HPLC with UV detection [24 - 28], capillary electrophoresis with UV detection [29], sequential injection analysis with electro-chemiluminescence detection [30,31]. However, these methods lack the sensitivity for the determination of CPA in biological samples. Recently, highly sensitive methods using HPLC-mass spectrometry (MS) have been developed for the determination of CPA [32, 33] or the simultaneous determination of both CPA and MDCPA in human plasma [20]. Although these analytical methods employing HPLC-

MS or HPLC-MS/MS afford high sensitivity, the cost of the equipment still limits its application. Therefore, a highly sensitive and cost-effective method is required.

We hereby report the successful combination of fluorescence labeling based on Suzuki coupling reaction with PO-CL detection for the first time. Moreover, we found that the sensitivities for CPA and MDCPA obtained by PO-CL detection were 7 and 18 times higher than those obtained by FL detection. The proposed PO-CL method should become an alternative sensitive and selective method for the simultaneous determination of CPA and MDCPA.

## 2. Experimental

### 2.1. Chemicals

DPA was synthesized as previously reported [6], CPA was purchased from Sigma Chemical (St Louis, USA), MDCPA was kindly donated by Schering-Plough, Japan. *N,N*-Dimethylformamide (DMF), *N,N*-diethylacetamide (DEA), palladium acetate (Pd(OAc)<sub>2</sub>), cesium carbonate (Cs<sub>2</sub>CO<sub>3</sub>), tetrahydrofuran (THF), 10% nitric acid (HNO<sub>3</sub>), acetonitrile and *n*-hexane, were purchased from Nacalai Tesque (Kyoto, Japan). Imidazole, bis(2,4,6-trichlorophenyl)oxalate (TCPO) was bought from Tokyo Chemical Industry (Tokyo, Japan). Tripotassium phosphate (K<sub>3</sub>PO<sub>4</sub>), trisodium phosphate (Na<sub>3</sub>PO<sub>4</sub>), potassium *tert*-butoxide (*t*-BuOK), triethylamine (TEA) and bis[2-(3,6,9-trioxadecanyloxycarbonyl)-4-nitrophenyl]oxalate (TDPO) were obtained from Wako Pure Chemical (Osaka, Japan). Bis(2,4,5-trichlorophenyl-6-carbopenoxyphenyl)oxalate (CPPO) was a kind gift from Lumica (Fukuoka, Japan). All other reagents used were of analytical grade.

### 2.2. Instrument

The HPLC-PO-CL system consisted of two Shimadzu LC-10AT pumps, (Shimadzu, Kyoto, Japan) and a Rheodyne 7125 injector with a 20- $\mu$ L loop (Cotati, CA, USA). Chromatographic separation was achieved isocratically on a Lichrosorb Si100 column (5  $\mu$ m, 250  $\times$  4.0 mm i.d.) (GL Science, Tokyo, Japan) using a mixture of acetonitrile-60 mM imidazole-HNO<sub>3</sub> buffer (pH 7.2) (=85:15, v/v) containing 0.015% TEA as the mobile phase. CL signal was measured with a Shimadzu CLD-10 CL detector using a mixture of 0.8 mM CPPO and 30 mM H<sub>2</sub>O<sub>2</sub> in acetonitrile as the post

column reagent. The mobile phase and the post column reagent were degassed thoroughly before use. For the fluorescence detection of DPA-labeled CPA and MDCPA, Shimadzu RF-550 detector was employed and excitation and emission wavelengths were set at 320 and 410 nm, respectively.

### 2.3. Assay procedure for CPA and MDCPA

To a 200- $\mu$ L portion of the serum, 100  $\mu$ L of 0.4 M NaOH was added and vortexed briefly, after which 3 mL of *n*-hexane was added. The mixture was vortexed for 5 min and centrifuged at 3000 $\times$ *g* at 4 °C for 5 min. The organic layer was carefully taken and evaporated to dryness using centrifugal evaporator. The residue was reconstituted in 50  $\mu$ L of DMF and subjected to the fluorescence labeling as follows: 50- $\mu$ L portion each of 10 mM DPA in DMF, 4 mM Pd(OAc)<sub>2</sub> in DMF and 50 mM Cs<sub>2</sub>CO<sub>3</sub> in water were added to the reconstituted residue. The mixture was vortexed for 10 s and deoxygenated by N<sub>2</sub> purge for 20 s. The reaction mixture was heated at 100 °C for 40 min. After the reaction, the reaction mixture was diluted to 1 mL with a mixture of water and methanol (20:80, v/v) containing 0.1 % TEA and applied to a solid-phase cartridge to remove excess DPA. The SPE cartridge (Inertsil Slim C18 B, GL Science) was preliminary washed with 4 mL of methanol and 4 mL of water. The diluted reaction mixture was then applied and washed twice with 1 mL of acetonitrile containing 0.1% TEA, the labeled analytes were eluted with THF containing 0.01% trifluoroacetic acid. The eluate was evaporated to dryness, re-constituted in 100  $\mu$ L DMF and passed through a membrane filter (0.5  $\mu$ m, DISMIC-3, Toyo Roshi, Japan). A 20- $\mu$ L portion of the filtrate was injected into the HPLC-PO-CL system.



#### *2.4. Sample collection*

This section of experiments was approved by the Ethics Committee of the Graduate School of Biomedical Sciences, Nagasaki University (approval number 24) and performed under established guidelines. The healthy volunteer was orally administered over-the-counter (OTC) preparation containing 6 mg of CPA 2 h before sampling. The blood sample was kept on ice and allowed to clot. It was centrifuged at 1000×g at 5 °C for 10 min to obtain serum. The samples were kept at -80 °C in polypropylene tubes.

### 3. Results and discussion

#### 3.1. Optimization of labeling conditions

In this study, the conditions for the labeling reaction were optimized using standard solution of CPA and MDCPA. The yield of the labeling reaction was monitored using the relative FL intensity (RFI). Dioxane, DEA, DMF and isopropanol were investigated as the solvent for the reaction. The highest RFI was observed with DMF which was selected as the solvent for the labeling reaction. The concentration of DPA was optimized over the range of 6-12 mM. The highest RFI was obtained at 10 mM.  $\text{Na}_2\text{CO}_3$ ,  $\text{Cs}_2\text{CO}_3$ ,  $\text{K}_3\text{PO}_4$ ,  $\text{Na}_3\text{PO}_4$  and *t*-BuOK were also investigated as the base for the labeling reaction. The highest RFI for CPA and MDCPA was obtained for  $\text{Cs}_2\text{CO}_3$ . Although we have developed the determination methods for some aryl chloride drugs by Suzuki coupling reaction with DPA [6,9,10],  $\text{Cs}_2\text{CO}_3$  was used for the first time as a base catalyst for Suzuki coupling reaction in this study. The application of  $\text{Cs}_2\text{CO}_3$  to DPA labeling reaction may improve the sensitivities of the method for such aryl chloride drugs because  $\text{Cs}_2\text{CO}_3$  gave higher reactivity. Subsequently, the concentration of  $\text{Cs}_2\text{CO}_3$  was studied over the range of 10-60 mM. The maximum and constant peak heights were obtained with more than 40 mM  $\text{Cs}_2\text{CO}_3$  (Fig. 2); 50 mM was selected as optimum concentration.  $\text{Pd}(\text{OAc})_2$  was decisively selected as the catalyst due to its success for the labeling reaction in our previous reports [6,9,10]. The concentration of  $\text{Pd}(\text{OAc})_2$  was varied from 1 to 5 mM, the optimum RFI was observed over 3 mM; 4 mM was selected. The temperature and the time for the reaction were also investigated. Increase in RFI was observed as temperature increases. At 100 °C, the optimum time for the reaction was 40 min.

After the derivatization step, excess DPA interfered with trace level determination of both analytes. To remove the excess DPA, a sample cleaning step with SPE procedure was incorporated into the experimental procedure as already described in the experimental section. Percentage recoveries (mean $\pm$ SD, n=3) of CPA and MDCPA were 86.0 $\pm$ 3.0% and 87.5 $\pm$ 3.0%, respectively.

### *3.2. Optimization of PO-CL conditions*

The CL conditions for the determination of CPA and MDCPA were optimized using the HPLC-PO-CL system described in the experimental section. Optimum conditions were selected by monitoring the relative CL intensity (RCI) and signal-to-noise ratio (S/N) for the peaks of CPA and MDCPA. Imidazole has been identified as the best catalyst for the PO-CL reaction; it has been established that imidazole catalyses the slow 1, 2-dioxethanedione generation reaction between the aryloxalate and H<sub>2</sub>O<sub>2</sub>. Imidazole acts not only through basic catalysis but also nucleophilic catalysis pathway [34, 35]. Imidazole solution was used as the aqueous portion of the mobile phase. The concentration of imidazole was optimized over the range of 0-100 mM. The highest RCI and S/N was observed at 40 and 60 mM; the optimum concentration was 60 mM. The acid used in combination with imidazole has also been found to significantly influence the CL signal. Several acids were tried including HClO<sub>4</sub>, CF<sub>3</sub>COOH, HNO<sub>3</sub> and CH<sub>3</sub>COOH. Maximum RCI and S/N ratio were observed with the use of imidazole-HNO<sub>3</sub> combination (Fig. 3). This result is consistent with previously reports [17,36,37]. The pH of the mobile phase was also optimized over the range of 6.8-7.6. This range was selected because lower pH values lead to loss of retention of the analytes. There is also risk of dissolution of the silica gel with the use of aqueous

mobile phase with pH above 8, and the consequent distortion of column efficiency [38]. The addition of TEA in the mobile phase is very necessary since it acts as masking agent on the silica gel to minimize interaction between basic analytes and the silica gel, which generally causes tailing of peaks and results in longer retention times [39]. For these reasons, 0.015% was added to the mobile phase prior to the adjustment of the pH with HNO<sub>3</sub>. The optimum pH of the mobile phase was 7.2. The type of aryloxalate was also optimized. Aryloxalates with electron withdrawing groups are known to provide highest quantum yield [40]. The effects of CPPO, TDPO and TCPO on RCI and S/N were investigated. CPPO gave the best RCI and S/N ratio for both CPA and MDCPA. The concentration of CPPO was varied from 0.1 to 1.0 mM. The optimum concentration of CPPO was 0.8 mM (Fig. 4). The concentration of H<sub>2</sub>O<sub>2</sub> was also optimized from 0.1 to 40 mM. The best RCI and S/N were at 30 mM. Finally, the flow rate of the CL reagent was varied from 0.2 to 1.4 mL min<sup>-1</sup>, increase in signal intensity commensurate flow rate, however, flow rate above 1.3 mL min<sup>-1</sup> led to an increase in background noise. The flow rate of the CL reagent was set at 1.2 mL min<sup>-1</sup>, since the best RCI and S/N ratio were best at this point. A chromatogram of standard solution of CPA and MDCPA after optimization of FL labeling conditions and PO-CL conditions is shown in Fig. 5.

### *3.3. Method validation*

Under the optimized conditions, linear relationship was observed by plotting a concentration of analytes (ng/mL) against CL intensity using spiked human serum samples. The calibration range, correlation coefficient and limits of detection (LOD) for the calibration curve (n=3) are shown in Table 1.

A good linearity was observed with a correlation coefficient of 0.999 for both analytes in the concentration range of 0.5-100 ng/mL with 8 calibration points. The LODs (S/N=3) for CPA and MDCPA were 0.14 and 0.16 ng/mL, respectively. On the other hand, the LODs (S/N=3) for CPA and MDCPA obtained by FL detection were 2.5 and 1.1 ng/mL, respectively. This results indicated that the application of PO-CL for the detection of DPA labeled compound could improve the sensitivity. Fig. 6 (A) and (B) shows representative chromatograms of blank human serum and human serum spiked with a standard solution of CPA and MDCPA. Since DPA could react specifically with aryl halides and did not react with most of serum components, both analytes can be detected clearly without interference on the chromatogram.

Accuracy and precision of the proposed method were accessed at three different concentration levels: low (2 ng/mL), intermediate (20 ng/mL) and high (80 ng/mL) concentration on three different days for both analytes. The results of these experiments are illustrated in Table 2. The intra- and inter-day accuracy of CPA ranged from 89.3% to 103.5% with precision values less than 10.8%. MDCPA had intra- and inter-day accuracy ranging between 92.2% and 103.5% with precision values less than 10.2%. These results indicate that the proposed method showed good accuracy and precision. The proposed method has sensitivity >300 fold that of HPLC-UV [25,28,27] and >15 fold that of other CL methods [30,31] for the determination of CPA. Also, the detection limit of the proposed method was almost same as that of HPLC-MS methods [20,32,33]. However, the sample amount subjected to analysis of the proposed method (200  $\mu$ L) was smaller than that of the HPLC-MS methods (0.5 or 1.0 mL). In addition, the proposed HPLC system can be constructed inexpensively compared with those of

HPLC-MS methods. Therefore, proposed HPLC-CL method should become a cost-effective alternative for the analysis of CPA and MDCPA.

#### *3.4. Determination of CPA and MDCPA in human serum*

The proposed method was applied to the determination of CPA and MDCPA in human serum samples after oral administration of CPA. The blood sample was collected from a subject who used OTC preparations that contain CPA. Fig. 7 shows the chromatogram of serum sample of the subjects after a single oral administration of 6 mg of CPA. The concentration of CPA in this chromatogram corresponds to 3.5 ng/mL, and the value was similar to previous studies [20, 32-33]. On the other hand, MDCPA was not detected on the chromatogram in the present study. This might be due to the presence of concentrations that are lower than the LOD. To determine MDCPA, increasing of sample amount subjected to analysis will be required.

#### **4. Conclusion**

Suzuki coupling reaction as a fluorescent labeling step has been successfully applied to PO-CL detection to develop a sensitive and selective method for the simultaneous determination CPA and MDCPA in human serum. The derivatization principle employed reduces interference since naturally occurring aryl halides in biological samples are limited. The sensitivity of the proposed method is many folds better than HPLC-UV and CL methods, and is comparable to HPLC-MS methods with smaller sample amounts. This method has been shown to be able to determine CPA in human serum after oral administration.

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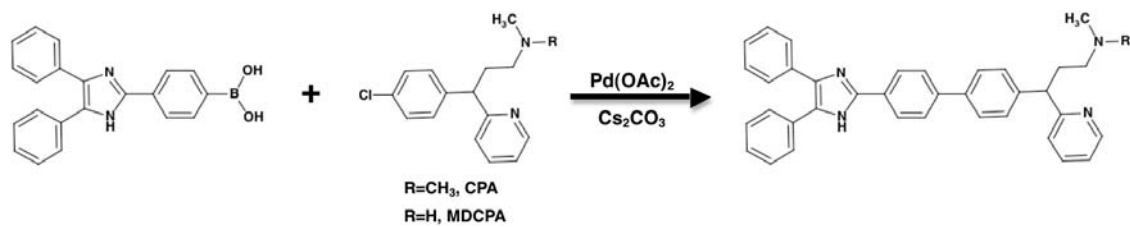
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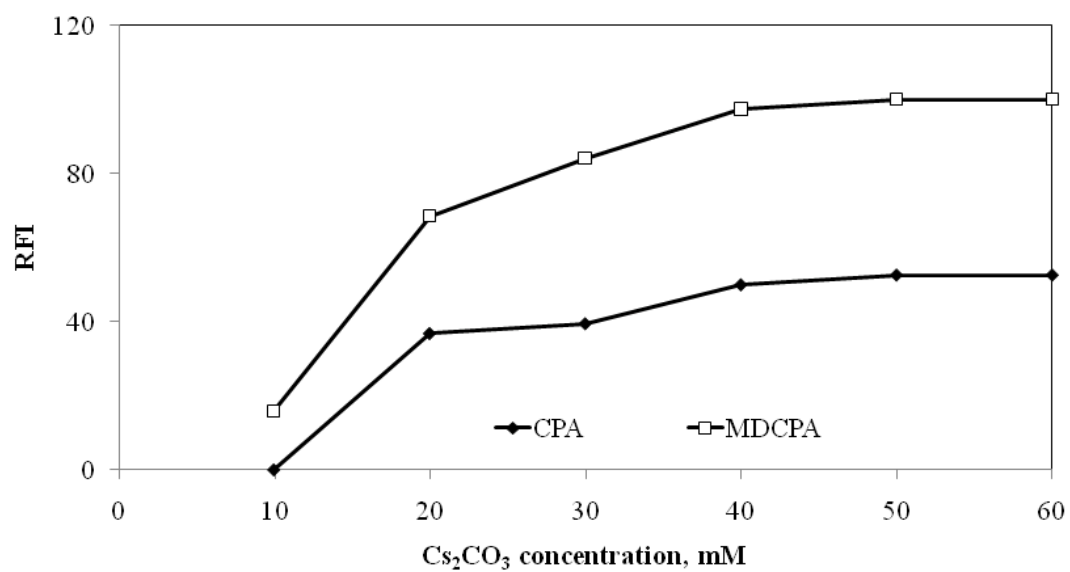
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## Figure captions

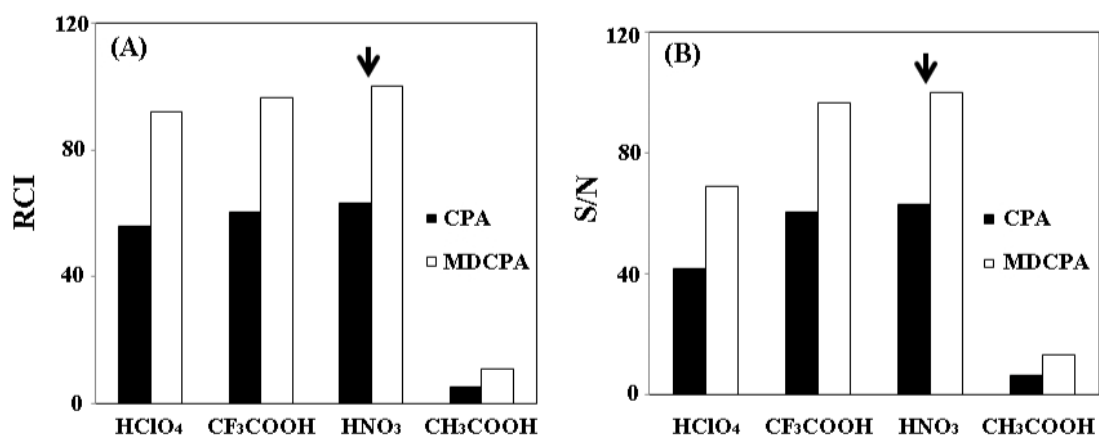


**Fig. 1** Fluorescence labeling reaction of CPA and MDCPA with DPA based on Suzuki coupling reaction



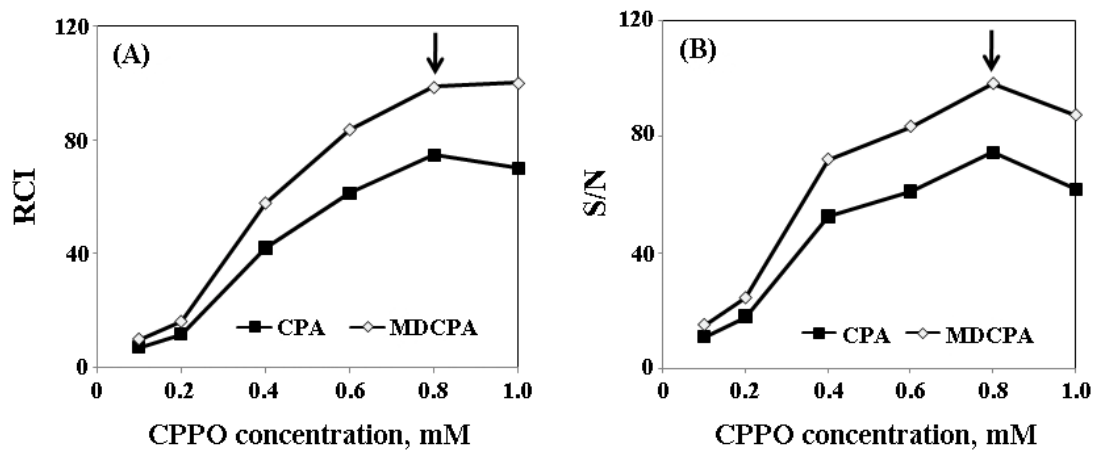
**Fig. 2** Effect of Cs<sub>2</sub>CO<sub>3</sub> concentration on RFI of CPA and MDCPA.

Labeling conditions: concentrations of analyte, DPA and Pd(OAc)<sub>2</sub> were 5 μg/mL, 10 mM and 3 mM, respectively. Reaction temperature and time were 100 °C for 30 min.



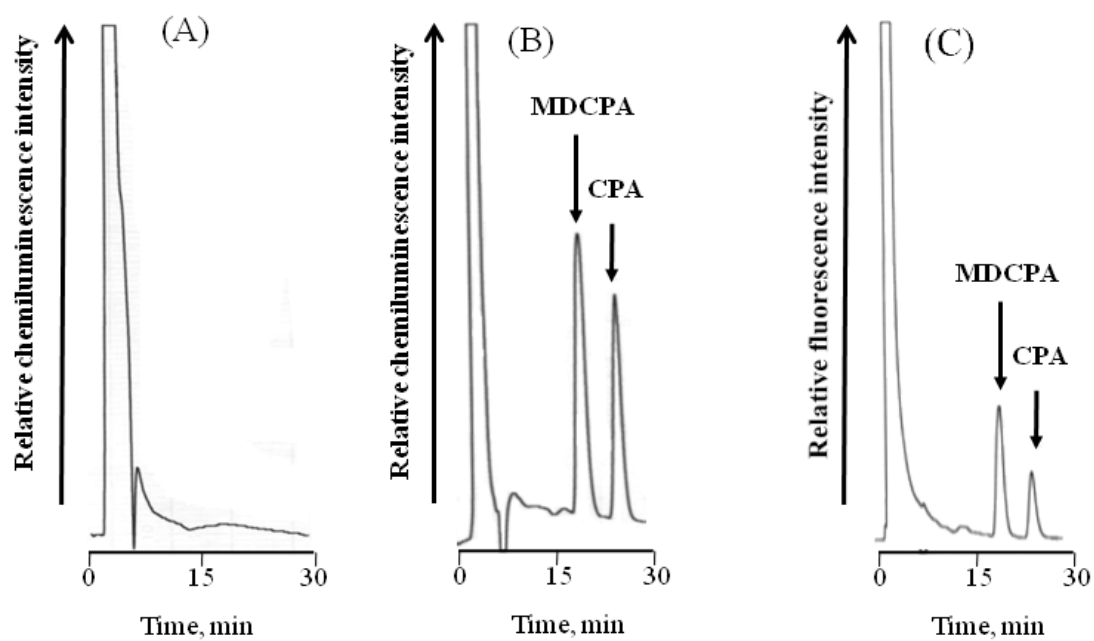
**Fig. 3** Effect of acid modifier on (A) RCI and (B) S/N of CPA and MDCPA.

Labeling conditions: same as Fig. 2. Eluent (flow rate, 1.0 mL/min): acetonitrile-60 mM imidazole solution (85:15, v/v) containing 0.015% TEA, (pH 7.4). CL reagent (flow rate, 1.0 mL/min): 0.5 mM TCPO and 30 mM H<sub>2</sub>O<sub>2</sub> in acetonitrile.



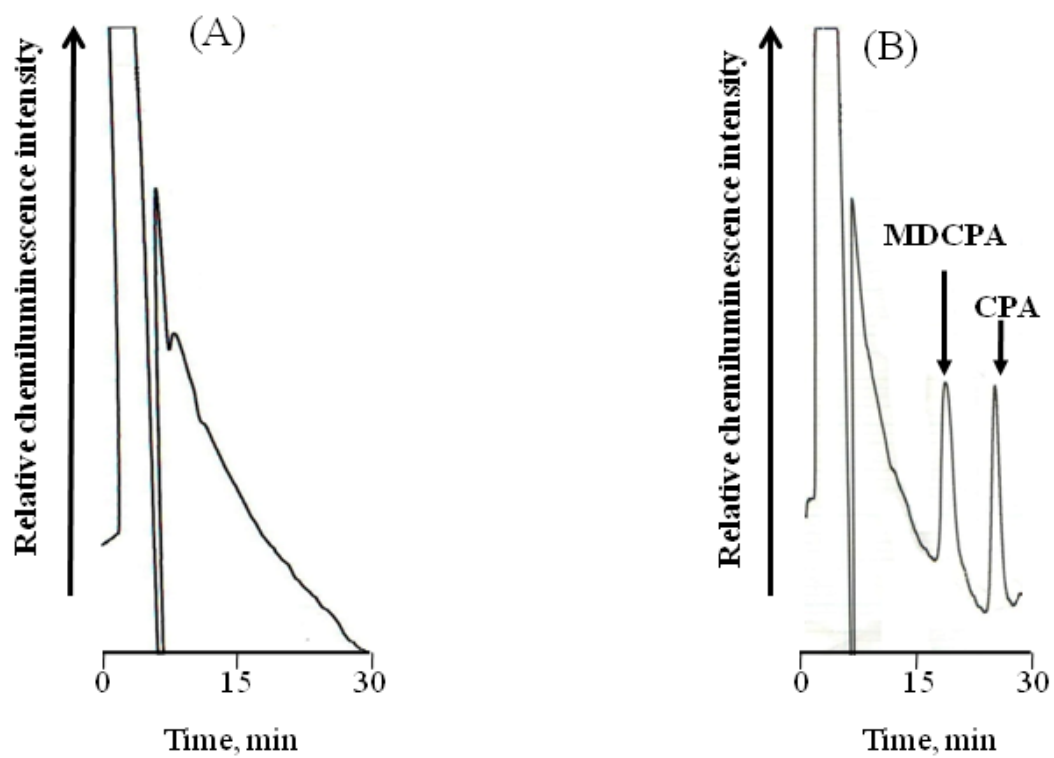
**Fig. 4** Effect of CPPO concentration on (A) RCI and (B) S/N of CPA and MDCPA.

Labeling conditions and eluent: same as Fig. 3 CL reagent: CPPO and 30 mM H<sub>2</sub>O<sub>2</sub> in acetonitrile. Flow rate 1.0 mL/min.



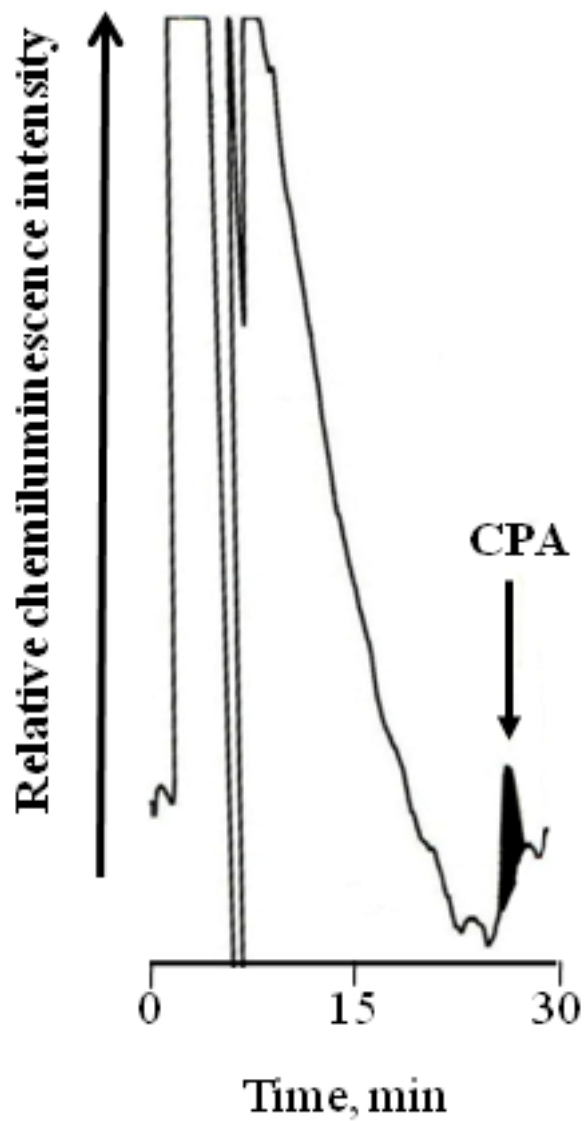
**Fig. 5** Chromatograms of (A) reagent blank, (B) standard solution of CPA and MDCPA ( $0.5 \mu\text{g/mL}$ ) with PO-CL detection, and (C) standard solution of CPA and MDCPA ( $0.5 \mu\text{g/mL}$ ) with FL detection.

Conditions: as mentioned in experimental section.



**Fig. 6** Chromatograms of (A) blank human serum and (B) human serum spiked with CPA and MDCPA (20.0 ng/mL). Conditions: as mentioned in experimental section.





**Fig. 7** Chromatogram of extract from human serum 2 h after oral administration of 6 mg CPA. (Detector response is 4 times higher than that of Fig. 6.

Conditions: as mentioned in experimental section.

**Table 1** Calibration curves and detection limits of CPA and MDCPA in human serum

Sample	Range, ng mL <sup>-1</sup>	Equation (n=3)*	Linearity	LOD** ng mL <sup>-1</sup> , fmol inj <sup>-1</sup>
CPA	0.5-100	$y = 1.52 \pm 0.02 x - 0.87 \pm 1.05$	0.999	0.14, 5.2
MDCPA	0.5-100	$y = 1.42 \pm 0.03 x - 0.72 \pm 1.97$	0.999	0.16, 6.1

\*y = peak height (cm); x = sample concentration (ng mL<sup>-1</sup>); mean ± SD

\*\*S/N=3

**Table 2** Intra- and inter-day accuracy and precision of the proposed method for determination of CPA and MDCPA in human serum

Sample, ng mL <sup>-1</sup>	Intra-day (n=3)		Inter-day (n=3)	
	Accuracy (%) <sup>a</sup>	Precision (RSD)	Accuracy (%) <sup>a</sup>	Precision (RSD)
CPA				
2	100.9 ± 5.1	6.1	89.3 ± 7.6	10.8
20	100.4 ± 5.0	5.1	94.5 ± 2.6	7.9
80	98.9 ± 3.3	3.4	103.5 ± 2.2	2.1
MDCPA				
2	96.7 ± 8.1	10.2	93.6 ± 7.2	9.4
20	100.0 ± 2.0	2.1	103.5 ± 5.4	5.3
80	98.2 ± 4.3	4.3	92.2 ± 6.1	6.7

<sup>a</sup>Mean ± SE