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Resolution of carboxylic acid enantiomers by high-performance liquid chromatography with peroxyoxalate chemiluminescence detection

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ABSTRACT

The peroxyoxalate chemiluminescence (CL) detection of carboxylic acid enantiomers, combined with high-performance liquid chromatography (HPLC), is described. The CL reaction is influenced by various factors (*e.g.*, eluent pH, type of aryl oxalate, relative concentrations of aryl oxalate and hydrogen peroxide and reaction time). Good linearity between CL intensity and injected amounts (5 fmol–5 pmol) of authentic derivatives, DBD-APy-Nap and ABD-APy-Nap, which were synthesized by the reaction with naproxen (Nap) with (+)-4-(N,N-dimethylaminosulphonyl)-7-(3-aminopyrrolidin-1-yl)-2,1,3-benzoxadiazole [(+)-DBD-APy] and (+)-4-(aminosulphonyl)-7-(3-aminopyrrolidin-1-yl)-2,1,3-benzoxadiazole [(+)-ABD-APy], were obtained with the proposed procedures. The reproducibility of the CL intensity during 6 h was also excellent, and no peak decrements were observed. The detection limits (signal-to-noise ratio = 2) of authentic DBD-APy-Nap, ABD-APy-Nap and NBD-APy-Nap {synthesized from naproxen and (+)-4-nitro-7-(3-aminopyrrolidin-1-yl)-2,1,3-benzoxadiazole [(+)-NBD-APyl]} with the bis[4-nitro-2-(3,6,9-trioxadecyloxy)phenyl] oxalate (TDPO)–hydrogen peroxide (H_2O_2) system after separation by HPLC were 0.49, 1.9 and 15 fmol, respectively, whereas those with bis(2,4,6-trichlorophenyl) oxalate (TCPO)–H_2O_2 were 0.74, 2.8 and 29 fmol, respectively. Some carboxylic acid enantiomers were converted on reaction with (+)-DBD-APy into the corresponding fluorescent diastereomers after 2 h at room temperature in the presence of 2,2'-dipyridyl disulphide and triphenylphosphine, activating agents for carboxylic acids. The diastereomers derived from each pair of enantiomers of anti-inflammatory drugs and N-acetylamino acids were efficiently resolved by reversed-phase chromatography with on ODS column and a 0.1 *M* imidazole-NO_3 (pH 7.0)–acetonitrile mixture as the mobile phase. The applicability of the proposed procedure was also evaluated for the detection of racemic ibuprofen (anti-inflammatory drug) added to rat plasma and human urine.

INTRODUCTION

Optical resolution of racemates in high-performance liquid chromatography (HPLC) has been mainly performed by two methods: introduction of an asymmetric environment intramolecularly by conversion into diastereomers and intermolecularly by use of a chiral stationary phase (CSP) [1–4]. Many CSP columns have been successfully applied to the resolution of various kinds of chiral molecules. Compared with the diastereomeric method, however, the CSP method is not advantageous in terms of sensitivity.

In a previous paper [5], we reported the syntheses of the fluorescence chiral reagents [(+)- and (−)-isomers] for the carboxylic acid functional group, 4-(N,N-Dimethylaminosulphonyl)-7-(3-aminopyrrolidin-1-yl)-2,1,3-benzoxadiazole (DBD-APy), 4-(aminosulphonyl)-7-(3-aminopyrrolidin-1-yl)-2,1,3-benzoxadiazole (ABD-APy) and 4-nitro-7-(3-aminopyrrolidin-1-yl)-2,1,3-benzoxadiazole (NBD-APy) (Fig. 1). The reagents were used to resolve carboxylic acid enantiomers by HPLC with fluorescence detection. The diastereomers derived from each pair of enantiomers of drugs and N-acetylami-

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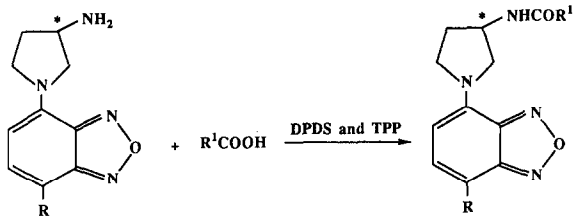

NBD-APy: R=NO_2
ABD-APy: R=SO_2NH_2
DBD-APy: R=$SO_2N(CH_3)_2$

Fig. 1. Labelling reactions of carboxylic acid enantiomers with chiral reagents in the presence of activating agents.

no acids were resolved completely by an ODS column and detected in the 10–30-fmol range (signal-to-noise ratio = 2), The diastereomers produced had the attractive feature of fluorescence at long excitation (460–480 nm) and emission (530–610 nm) wavelengths.

Some fluorescent compounds emit light on chemical reaction without the need for optical excitation with lamps such as the xenon arc. Chemiluminescence (CL) detection is well known to be sensitive and useful for trace analyses for fluorescent compounds [6,7] and hydrogen peroxide (H_2O_2) [8,9]. Among the CL reagents such as luminol and lucigenin, the combination of oxalates and H_2O_2 seems to be suitable as postcolumn CL reagents for the determination of fluorophores, because the reaction conditions at around neutral pH are much milder than those required for other reagents such as luminol.

This paper describes the resolution of carboxylic acid enantiomers (anti-inflammatory drugs and N-acetylamino acids) by HPLC with peroxyoxalate CL detection. Suitable conditions for CL detection were established by investigation of the various factors that affect the CL reaction. Racemic ibuprofen added to rat plasma and human urine was also studied with the recommended procedures.

EXPERIMENTAL

Materials and reagents

The derivatization reagents, (+)-DBD-APy, (+)-ABD-APy and (+)-NBD-APy, were synthesized as described previously [5]. The derivatives of (+)-2-(6-methoxy-2-naphthyl)propionic acid (naproxen) with (+)-DBD-APy, (+)-ABD-APy and (+)-NBD-APy, (designated as DBD-APy-Nap, ABD-APy-Nap and NBD-APy-Nap, respectively), were also synthesized as described [5]. Racemic 2-(6-methoxy-2-naphthyl)propionic acid (*rac*-naproxen), racemic 2-(4-isobutylphenyl)propionic acid (*rac*-ibuprofen) and racemic 2-[*p*-(2-oxocyclopentylmethyl)phenyl]propionic acid (*rac*-loxoprofen) were donated by Tokyo Tanabe Pharmaceutical, Kyowa Hakko Kogyo and Sankyo (all Tokyo, Japan), respectively. N-Acetylamino acid enantiomers were kindly supplied by Ajinomoto (Tokyo, Japan). Bis(2,4,6-trichlorophenyl) oxalate (TCPO) and bis[4-nitro-2-(3,6,9-trioxadecyloxy) phenyl] oxalate (TDPO) (biochemical research grade) were purchased from Wako (Osaka, Japan). Triphenylphosphine (TPP) (Wako), 2,2′-dipyridyl disulphide (DPDS) (Tokyo Kasei, Tokyo, Japan), imidazole (grade for buffer preparation) (Tokyo Kasei) and hydrogen peroxide (H_2O_2) (30% in water) (Wako) were also used as received. Trifluoroacetic acid (TFA), acetonitrile (CH_3CN) and water were of HPLC grade (Wako). All other chemicals were of analytical-reagent grade and were used without further purification.

Stock solutions

A 0.2 *M* buffer solution was prepared by dissolving 13.616 g of imidazole in 950 ml of water, adjusted to pH 6.0–8.0 with 61% HNO_3 and then diluted to 1 l with water. The solution was further diluted with water to prepare buffer solutions of 5 m*M*–0.1 *M*. The reagent solution [10 m*M* (+)-DBD-APy], the authentic derivatives (1 m*M* DBD-APy-Nap, ABD-APy-Nap or NBD-APy-Nap), anti-inflammatory drugs (1 m*M* naproxen, ibuprofen and loxoprofen) and N-acetylamino acids (1 m*M* N-acetylphenylalanine, -tryptophan, -tyrosine, -valine, -methionine, etc.) were also prepared in acetonitrile. The reagent solutions were diluted to appropriate concentrations with acetonitrile. The activation agents (DPDS and TPP) for carboxylic acids were prepared with acetonitrile just prior to use. Fresh solutions of CL reagents (aryl oxalate and H_2O_2) were also prepared every day. All stock solutions except imidazole buffer were stored in a refrigerator at −20°C.

HPLC–CL detection

The high-performance liquid chromatograph consisted of two LC-9A pumps (Shimadzu, Kyoto, Japan) and an SCL-6B system controller (Shimadzu). Sample solutions were injected with a SIL-6B autoinjector (Shimadzu). The analytical column was a 5-μm Inertsil ODS-2 (150 × 4.6 mm I.D.) (GL Sciences, Tokyo, Japan). The column and rotating mixing device (KZS-1) (Kyowa Seimitsu, Tokyo, Japan) were maintained at 30°C with a Model 655A-52 column oven (Hitachi, Tokyo, Japan). A stainless-steel tube (40 cm × 0.1 mm I.D.) was used as a reaction delay coil. A Shodex CL-2 chemiluminescence monitor (single photon counting type) (Showa Denko, Tokyo, Japan) equipped with a 120-μl spiral flow cell was employed for the detection of emitted light. The peak areas corresponding to CL intensity were calculated with a C-R4A Chromatopac (Shimadzu). All mobile phases and chemilumigenic reagents were degassed with an online degasser (DGU-3A; Shimadzu). The flow-rate of the eluent was 0.5 ml/min. The instrumental setup for HPLC–CL analysis is shown in Fig. 2.

The CL intensities of authentic DBD-APy-Nap and ABD-APy-Nap were determined after separation by HPLC under various CL reaction conditions. The signal-to-noise ratios (S/N) were calculated from the difference between the peak height of each diastereomer and the variation of the baseline noise.

The recommended HPLC–CL conditions were as follows: eluent, 0.1 *M* imidazole-NO_3 buffer (pH 6.5)–acetonitrile (2:3) for TDPO–H_2O_2 CL detection system and 0.1 *M* imidazole-NO_3 buffer (pH 7.0)–acetonitrile (2:3) for TCPO–H_2O_2 CL detection system; concentrations of CL reagents, 0.5 m*M* oxalate (TDPO or TCPO) and 15 m*M* H_2O_2 in acetonitrile; and flow-rate of CL reagents, 1.5 ml/min.

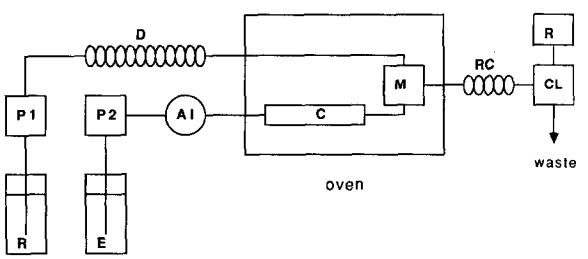

Fig. 2. Schematic flow diagram of the HPLC–CL detection system. P = Pump; D = damper coil; AI = autoinjector; C = column; E = eluent reservoir; R = reagents (oxalate and H_2O_2) reservoir; M = rotating mixing device; RC = reaction delay coil; CL = chemiluminescence monitor; R = recorder

Linearity of the response under the recommended CL detection conditions

Volumes of 5 μl of the mixed solution of DBD-APy-Nap and ABD-APy-Nap (1 n*M*–1 μ*M* concentration) were injected on to the column and detected with the CL monitor under the recommended procedures. The CL intensities of individual peaks were plotted against the injected amount of the diastereomers.

Optical resolution of carboxylic acid enantiomers labelled with (+)-DBD-APy

(+)-DBD-APy (0.25 m*M*) in 0.5 ml of CH_3CN reacts with anti-inflammatory drugs or N-acetyl-amino acids (*ca.* 1 mg each) at room temperature in the presence of the activation agent consisting of DPDS (4 m*M*) and TPP (4 m*M*). After a 2-h reaction period, an aliquot (5 μl) of the solution was injected into the column, separated with 0.1 *M* imidazole-NO_3 (pH7.0)–CH_3CN (2:3) and detected chemilumigenically with TCPO–H_2O_2. The capacity factor (k'), separation factor (α) and the resolution (R_s) were caluculated from the following equations:

$$k' = (t_R - t_0)/t_0$$

$$\alpha = k'_2/k'_1$$

$$R_s = 2(t_{R_2} - t_{R_1})/(w_1 + w_2)$$

where t_R, t_{R_1} and t_{R_2} are the retention times of the peaks, t_0 is the void volume of the column (2.7 min) and w_1 and w_2 are the bases of triangles derived from the peak.

Determination of carboxylic acid enantiomers in plasma and urine

In general, profens such as naproxen and ibuprofen in biological specimens are separated and extracted well using a solid-phase column such as Sep-Pak C_{18} [10]. Therefore, Bond Elut Certify II columns (developed for acidic pharmaceuticals) were employed for the extraction of *rac*-ibuprofen in rat plasma and human urine.

A 1.0-ml sample of human urine spiked with 8 μl (4 nmol of each enantiomer) of 1 m*M* *rac*-ibuprofen

was adjusted to pH 1–2 with 36% HCl (*ca.* 10 μl), then 3 ml of 10 m*M* sodium acetate buffer (pH 2.0) were added. The acidified urine was applied to a Bond Elut Certify II LRC extraction column (Varian, Harbor City, CA, USA), which was washed with 2 ml of methanol and 2 ml of 10 m*M* sodium acetate buffer (pH 2.0). The column was washed sequentially with 2 ml of 10 m*M* sodium acetate buffer (pH 2.0) and 2 ml of 10% acetic acid. The column was dried under reduced pressure with an aspitator for 5 min. All washings were discarded. *rac*-Ibuprofen was eluted with 2 ml of 0.1% trifluoroacetic acid (TFA)–CH_3CN (1:1). The eluate was evaporated to dryness and the residue was dissolved in 1.0 ml of CH_3CN (urine extraction sample).

A 0.5-ml sample of rat plasma spiked with 2 μl (1 nmol of each enantiomer) of 1 m*M* *rac*-ibuprofen was also adjusted to pH 1–2 with 36% HCl (*ca.* 5 μl), then 3.5 ml of 10 m*M* sodium acetate buffer (pH 2.0) were added. The acidified plasma was applied to the solid-phase column and treated in the same manner as described for the urine sample. The dried eluate containing *rac*-ibuprofen was dissolved in 0.5 ml of CH_3CN (plasma extraction sample).

A 0.25-ml volume of the prepared urine or plasma extract, 0.1 ml of (+)-DBD-APy (2 m*M*) in CH_3CN and 0.15 ml of a mixed solution of DPDS (2 m*M*) and TPP (2 m*M*) in CH_3CN were thoroughly mixed in a 1.5-ml glass vial. The vials were capped and allowed to stand for 2 h at room temperature (20–30°C). An aliquot (5 μl) of the reaction solution, diluted appropriately 100–200-fold with CH_3CN, was injected into the column for HPLC–CL analysis. A blank urine or plasma sample without (+)-DBD-APy was treated in the same manner.

RESULTS AND DISCUSSION

Peroxyoxalate CL detection

Peroxyoxalate chemiluminescence (CL) is based on the reaction of an oxalate and H_2O_2, which was reported by Rauhut *et al.* in 1967 [11]. The CL reaction has been used with HPLC for the determination of fluorophores such as DNS-amines [12,13], OPA-amines [14] and polycyclic aromatic hydrocarbons [15,16]. Detection limits for these compounds in the picomole and sub-femtomole range have been achieved with CL detection. Aryl oxalates and H_2O_2 react with each other to produce unstable and energy-rich intermediates, which cause fluorescent compounds to reach an excited state. The excited fluorescent compounds return to the ground state with the emission of light which has same maximum wavelength as when photochemical excitation is used. Therefore, the compounds excited easily at low-energy show stronger CL intensity. In other words, compounds with long excitation wavelengths provide the most satisfactory results; however, some exceptions have been noted [6]. The CL intensity is also dependent on the fluorescence quantum yield (ϕ_f), the concentration of the fluorophore and the concentration of the energy-rich intermediates produced by CL reaction. When the same fluorescent chromophore is bonded to a variety of compounds, the final detection limits in CL detection depend on the concentration of the excited intermediates that are generated by the CL reaction conditions.

Various aryl oxalates have been reported; TCPO and TDPO were selected for this work. Bis(2,6-dinitrophenyl) oxalate (DNPO) is commonly used, but it and its hydrolysate exhibit strong quenching effects and a high level of background emission in comparison with other oxalates [17,18]. TDPO is more soluble oxalate than TCPO [19] in water-miscible solvents such as CH_3CN. Although ethyl acetate has been employed as a solvent for oxalates, its use should be avoided owing to the biohazard. The highest CL intensity and persistence are obtained with CH_3CN relative to other solvents such as ethyl acetate and acetone [12]. Therefore, the oxalates and H_2O_2 were dissolved in CH_3CN. Imidazole buffer was selected for the separation of the diastereomers, as the catalytic effect of imidazole in the CL reaction is stronger than that of phosphate buffer, which is commonly used as an eluent [20,21]. The purity of imidazole has a great influence on both the level of background emission and the variation of baseline noise. Therefore, high-quality imidazole and water must be used for the preparation of the buffers. The buffer pH was adjusted to 6.0–8.0 with HNO_3 instead of HCl and HBr, as NO_3^- does not quench the CL intensity, where Cl^- and Br^- do [17]. As thorough mixing of the effluent from the column and CL reagents is an important factor in obtaining a stable baseline and reproducible peaks, a rotating mixing device was adopted instead of usual T-type mixer [22]. Stainless-steel

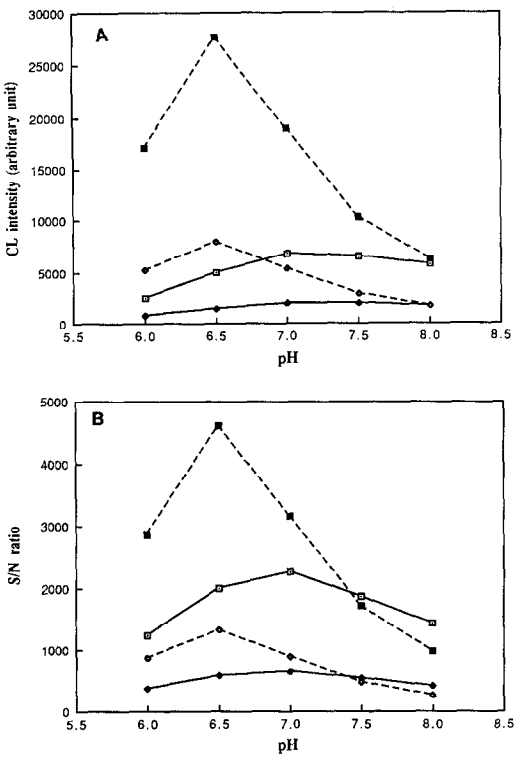

Fig. 3. Effect of eluent pH on CL reaction. (A) Effect on CL intensity; (B): effect on S/N. □ = DBD-APy-Nap with TCPO–H_2O_2; ◆ = ABD-APy-Nap with TCPO–H_2O_2; ■ = DBD-APy-Nap with TDPO–H_2O_2; ◇ = ABD-APy-Nap with TDPO–H_2O_2. Conditions: eluent, 0.1 M imidazole-NO_3 (pH 6–8)–CH_3CN (2:3); CL reagent flow-rate, 2.0 ml/min; For other conditions, see Experimental.

tubes of 40 cm × 0.1 mm I.D. and 100 cm × 0.1 mm I.D. used as a reaction delay coil and a damper coil for the CL reagent solution, respectively. The CL reaction increases with increasing temperature, but the signal-to-noise ratio was optimum at around room temperature. Therefore, the mixing device was maintained at 30°C in an air oven together with the separation column. The inlet and outlet connections of the detection cell in the CL monitor were made with stainless-steel connectors and tubing to prevent light from entering through the connectors and tubing. Utilizing these conditions, the various factors that influence the CL reaction were investigated with authentic DBD-APy-Nap and ABD-APy-Nap.

Initially, the effect of pH on the CL reaction was examined with TCPO–H_2O_2 and TDPO–H_2O_2 detection systems. As shown in Fig. 3, the optimum pH was different with each oxalate. The CL intensity of the TDPO–H_2O_2 reaction was strongest at pH 6.5, whereas the highest intensity with the TCPO–H_2O_2 reaction was achieved at pH 7.0–7.5 (Fig. 3A). The optimum pH for the TCPO–H_2O_2 reaction was 7.0 as determined by the S/N (Fig. 3B). The CL intensities of both diastereomers at the optimum pH with the TDPO–H_2O_2 reaction were approximately three times those of the TCPO–H_2O_2 reaction (Fig. 3A). As both the level of background emission and the variation of the baseline noise were higher in the reaction with TDPO–H_2O_2, the differences in S/N obtained from both oxalates were less than threefold (Fig. 3B). The results suggest that the reaction pH is one of the most important factors influencing the CL reaction using oxalate and H_2O_2.

The effect of the concentration of imidazole, which functions as a buffer component for the HPLC eluent and as a base catalyst for the CL reaction, is shown in Fig. 4. When TCPO was used as the oxalate in the CL reaction, the intensity increased with increasing buffer concentration (Fig. 4A). However, 0.1 M imidazole was optimum, as determined by studying the S/N (Fig. 4B). In the reaction with TDPO and H_2O_2, the highest values of both the intensity and S/N were obtained with 0.1 M imidazole. Consequently, 0.1 M imidazole buffers (pH 6.5 for the TDPO–H_2O_2 system and pH 7.0 for the TCPO–H_2O_2 system) were selected for the CL reactions.

The concentrations of oxalate and H_2O_2 also affect the CL reaction. As depicted in Fig. 5, the CL intensity is greatly dependent on the oxalate concentration in the range tested (2.5 μM–1.0 mM) (Fig. 5A), but the variation of the baseline noise also increases. As a result, the optimum TDPO concentration is 0.5 mM (Fig. 5B). In contrast, a high TCPO concentration is the predominant factor for the CL reaction judging from the S/N data (Fig. 5B). As the solubility in CH_3CN is not great, 0.5 mM TCPO was also adopted here. With respect to H_2O_2 concentration, 15 mM gave the strongest intensity with TCPO (Fig. 6A). On the other hand, relatively high CL intensities with TDPO were observed at H_2O_2 concentrations between 5 and 15 mM. Based on these studies of CL intensity, 0.5

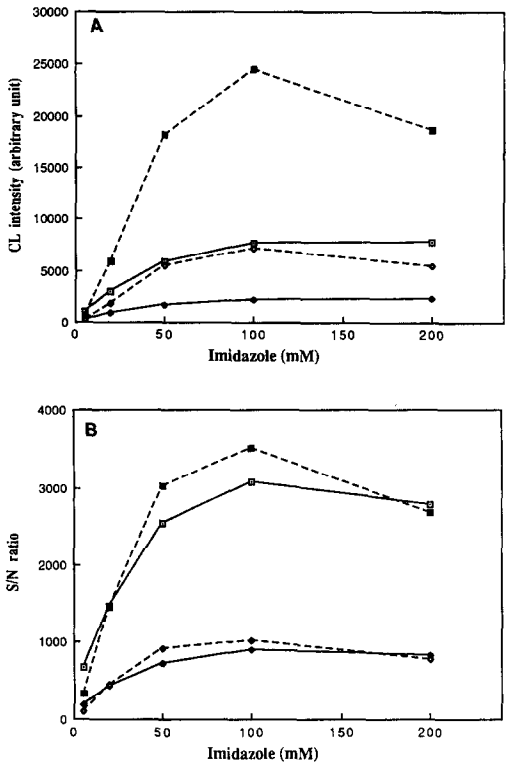

Fig. 4. Effect of imidazole concentration on CL reaction. Symbols as in Fig. 3. Conditions: eluent, 5–200 m*M* imidazole (pH 7.0 for TCPO–H_2O_2, pH 6.5 for TDPO–H_2O_2)–CH_3CN (2:3). For other conditions, see Experimental.

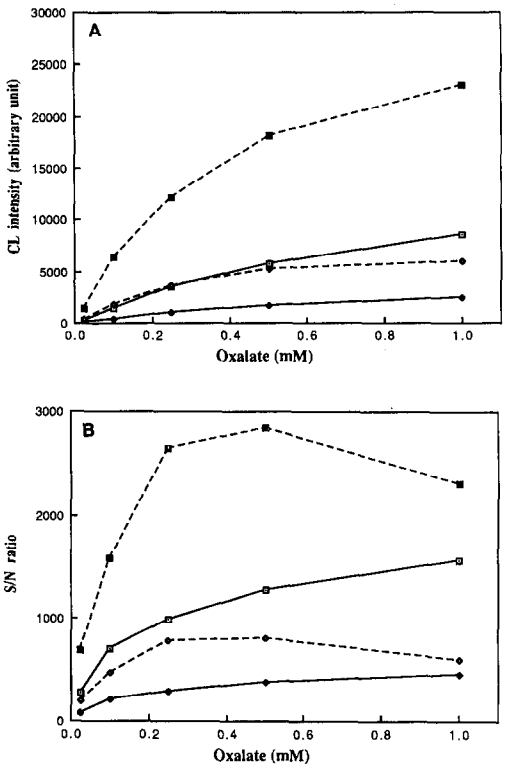

Fig. 5. Effect of aryl oxalate concentration on CL reaction. Symbols as in Fig. 3. Conditions: concentration of TCPO or TDPO, 2.5 μM–1.0 m*M*; eluent, 0.1 *M* imidazole (pH 7.0)–CH_3CN (2:3). For other conditions, see Experimental.

m*M* oxalate and 15 m*M* H_2O_2 in CH_3CN were used as the CL reagent solution.

Finally, the effect of flow-rate of the CL reagent on the CL reaction was tested under the optimum conditions described above. The flow-rates of the eluent and reagent solution affect the CL intensity. When the flow-rate of the eluent was fixed at 0.5 ml/min, the intensity is greatest with a TDPO–

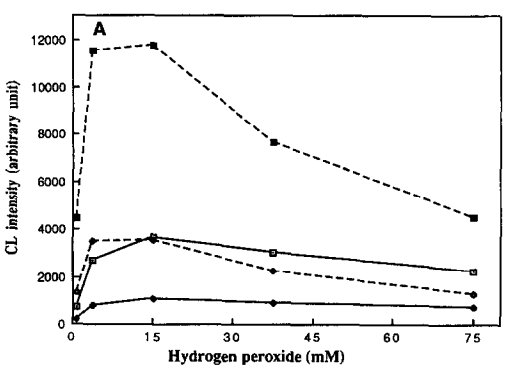

Fig. 6. Effect of H_2O_2 concentration on CL reaction. Symbols as in Fig. 3. Conditions: concentration of H_2O_2, 0.75–75 m*M*; concentration of TCPO or TDPO, 0.25 m*M*. For other conditions, see Experimental.

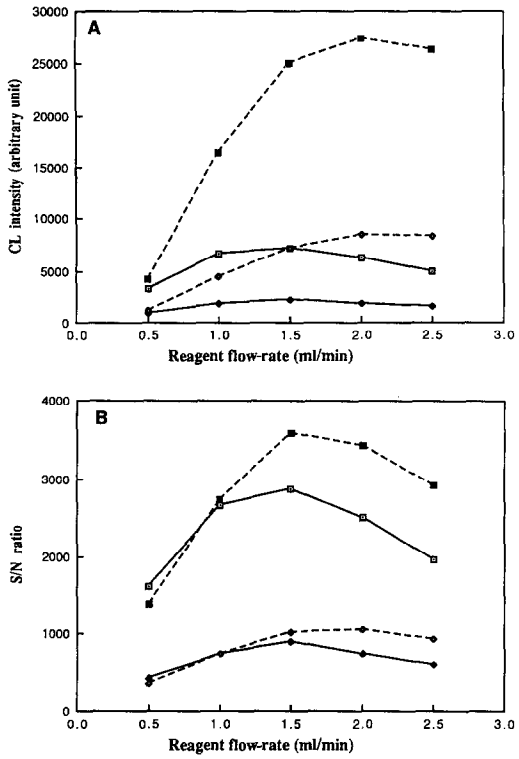

Fig. 7. Effect of flow-rate of CL reagents on CL reaction. Symbols as in Fig. 3. Conditions: flow-rate of CL reagent, 0.5–2.5 ml/min. For other conditions, see Experimental.

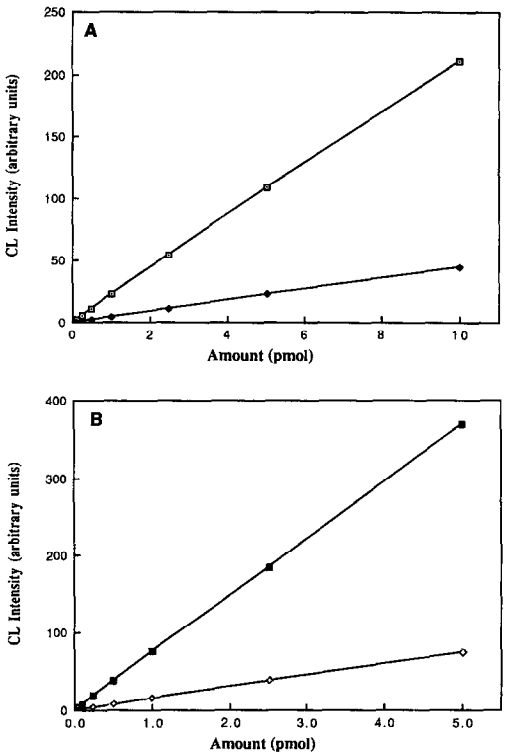

Fig. 8. Linearity of CL intensity under the recommended procedures. Symbols as in Fig. 3. (A) With TCPO–H_2O_2; (B) with TDPO–H_2O_2. For conditions of HPLC separation and CL detection, see Experimental.

H_2O_2 flow-rate of 2.0 ml/min, whereas with TCPO–H_2O_2 it is greatest at 1.5 ml/min (Fig. 7A). However, the optimum *S/N* is realized when the flow-rate of the CL reagent solution is 1.5 ml/min, as shown in Fig. 7B.

Based on the results described above, the CL intensities of the diastereomers, corresponding to the carboxylic acid enantiomers, were measured after separation with an Inertsil ODS-2 column under the following conditions: eluent buffer, 0.1 *M* imidazole-NO_3–CH_3CN (2:3); eluent pH, 6.5 for the TDPO–H_2O_2 and 7.0 for the TCPO–H_2O_2 system; CL reagent concentration, 0.5 m*M* oxalate and 15 m*M* H_2O_2 in CH_3CN; flow-rate, 0.5 ml/min for the eluent and 1.5 ml/min for the CL reagents; mixing temperature, 30°C; and CL reaction time, *ca.* 1 s.

Under the recommended conditions, the relationship between CL intensity and concentration (5 fmol–5 pmol) in the injected solution was linear with a correlation coeffcient (γ) >0.999 (Fig. 8). The slope and the intercept of the linear equations for DBD-APy-Nap were 73.59 and 0.61, respectively, with the TDPO–H_2O_2 system and 21.16 and 0.37, respectively, with the TCPO–H_2O_2 system, whereas those for ABD-APy-Nap were 14.99 and 0.10, respectively, with the TDPO–H_2O_2 system and 4.49 and 0.03, respectively, with the TCPO–H_2O_2 system. With regard to the sensitivity of detection for the diastereomers, DBD-APy-Nap can be measured at roughly one-fifth of the level of ABD-APy-Nap. The proposed postcolumn CL detection is carried out on a one-pump system by using an acetonitrile mixture of oxalate and H_2O_2. Therefore, the peak (corresponding to the CL intensity) may decrease steadily throughout the day owing to the decomposition of oxalates. Imaizumi *et al.* [20] reported that the stability of TCPO depended on the solvent, the temperature of the CL

reagent reservoir and the concentration of H_2O_2. Among the solvents tested, acetonitrile provided the most stable mixture of TCPO and H_2O_2. A lower temperature of the CL reagent mixture and a lower concentration of H_2O_2 also stabilized TCPO in the reservoir. Judging from the data [20], it seems that the decomposition of the oxalates under the proposed conditions is negligible. To confirm this, the variation of the CL peak (1 pmol of DBD-APy-Nap or ABD-APy-Nap) was determined with TCPO and TDPO in the presence of H_2O_2. Although the amount of oxalate decomposition is unknown, the relative CL intensity was independent of the age of the solution for at least 6 h after preparation (less than 3%). Consequently, both oxalates are usable in acetonitrile solution with H_2O_2.

The detection limits (S/N = 2) of DBD-APy-Nap, ABD-APy-Nap and NBD-APy-Nap were determined under the proposed HPLC–CL detection conditions. Fig. 9A and B shows the typical chromatograms of the three mixed diastereomers (corresponding to 10 fmol of DBD-APy-Nap, 30 fmol of ABD-APy-Nap and 200 fmol of NBD-APy-Nap).

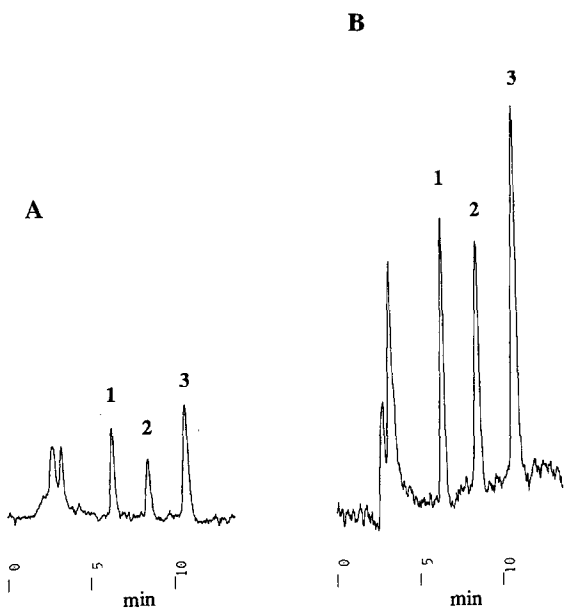

Fig. 9. Chromatograms of authentic diastereomers with CL detection. (A) with TCPO–H_2O_2; (B) with TDPO–H_2O_2. Peaks: 1 = ABD-APy-Nap (30 fmol); 2 = NBD-APy-Nap (200 fmol); 3 = DBD-APy-Nap (10 fmol). Eluent, 0.1 M imidazole-NO_3 (pH 7.0)–CH_3CN (2:3). For other conditions of HPLC separation and CL detection, see Experimental.

The peak heights obtained with the TCPO–H_2O_2 system were 3–4 times lower than those for the TDPO–H_2O_2 system. The corresponding detection limit for the TCPO–H_2O_2 calculated from the S/N was approximately half of that for TDPO–H_2O_2 system, because the variation of the baseline noise also decreased. The detection limits of DBD-APy-Nap, ABD-APy-Nap and NBD-APy-Nap on the chromatograms were 0.49, 1.9 and 15 fmol, respectively, with the TDPO–H_2O_2 system and 0.74, 2.8 and 29 fmol, respectively, with the TCPO–H_2O_2 system. The minimum detectable levels of DBD-APy and ABD-APy-Nap were roughly one order of magnitude lower than those previously reported with fluorescence detection [5]; however, the detection limits for NBD-APy-Nap are in the same range for both detection systems. The results indicate that the DBD-APy moiety is preferable for the highly sensitive detection with peroxyoxalate CL.

HPLC separation and CL detection of diastereomers derived from (+)-DBD-APy

The enantiomeric separation of the racemates of some carboxylic acids, after derivatization with the chiral reagents in the presence of DPDS and TPP, was successfully accomplished with a reversed-phase ODS column, as described previously [5]. Femtomole detection (10–30 fmol) was achieved by HPLC with fluorimetric detection. Among the fluorophores, the DBD-APy structure was chemilumigenically suitable for ultra-trace analysis judging from the chromatograms in Fig. 9. The separations of optical isomers of anti-inflammatory drugs and N-acetylamino acids labelled with (+)-DBD-APy were studied by HPLC under the recommended CL reaction conditions with TCPO–H_2O_2. Fig. 10 shows the chromatograms of the diastereomers derived from carboxylic acid enantiomers and (+)-DBD-APy. The resulting diastereomers of the drugs and the N-acetylamino acids were separated efficiently on the ODS column with 0.1 M imidazole-NO_3 (pH 7.0)–CH_3CN mixture. Differences in peak heights were shown for each pair of some carboxylic acid enantiomers (N-acetyltryptophan and N-acetylvaline), the peaks derived from the (+)-enantiomers being larger than those from the (−)-enantiomers. When (−)-DBD-APy was used instead of (+)-DBD-APy as the tagging reagent for N-acetyltryptophan, the peak of the (−)-

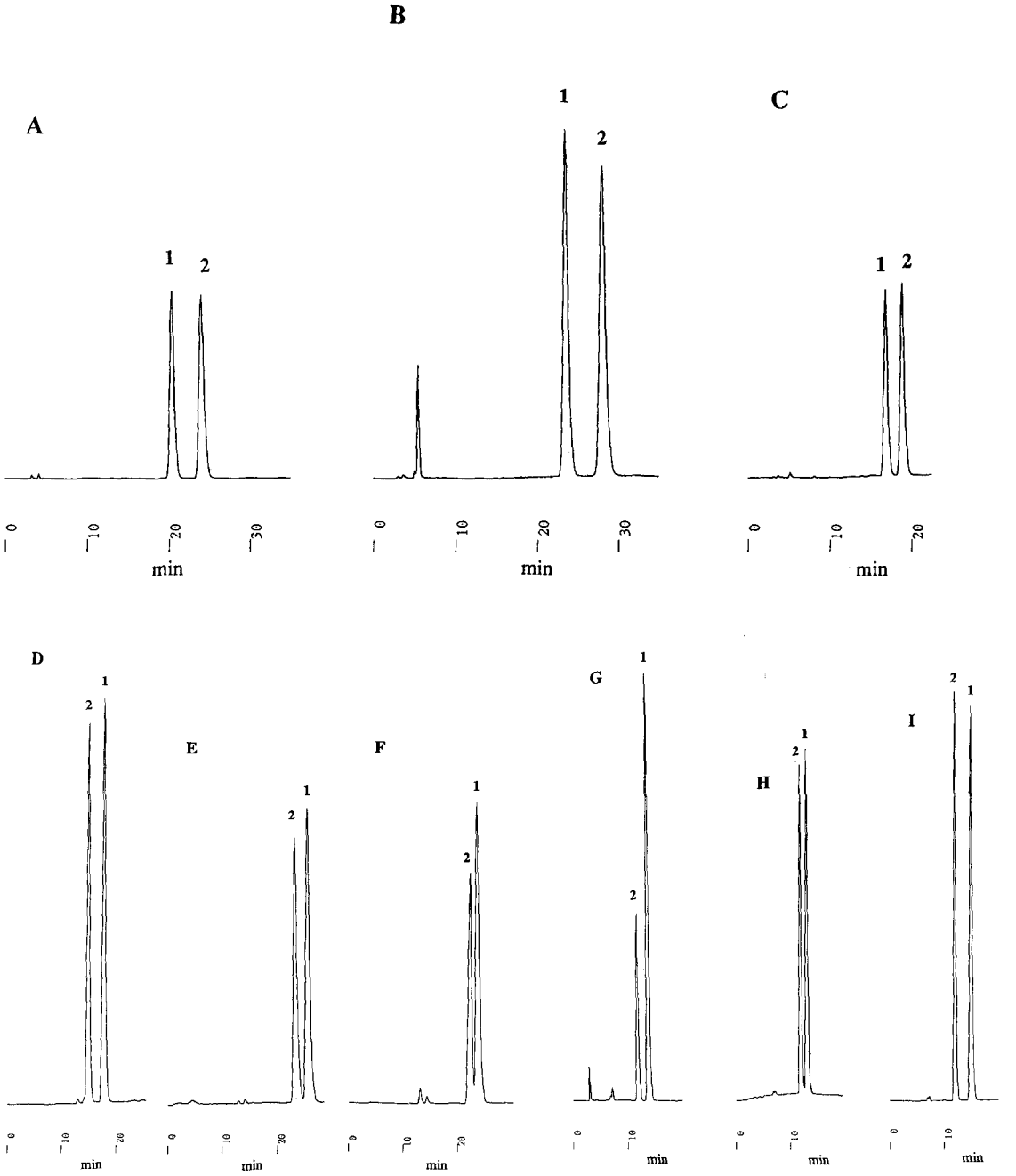

Fig. 10. HPLC separation of carboxylic acid enantiomers labelled with (+)-DBD-APy. (A) ibuprofen; (B) naproxen; (C) loxoprofen; (D) N-acetyltyrosine; (E) N-acetylmethione; (F) N-acetylvaline; (G) N-acetyltryptophan; (H) N-acetylleucine; (I) N-acetylphenylalanine. Peaks: 1 = (+)-enantiomer; 2 = (−)-enantiomer. Eluent: (A) 0.1 *M* imidazole-NO_3 (pH 7.0)–CH_3CN (2:3); (B and C) 0.1 *M* imidazole-NO_3 (pH 7.0)–CH_3CN (1:1); (D, E and F) 0.1 *M* imidazole-NO_3 (pH 7.0)–CH_3CN (7:3); (G, H and I) 0.1 *M* imidazole-NO_3 (pH 7.0)–CH_3CN (3:2). For other conditions of HPLC separation and CL detection, see Experimental.

TABLE I

RESOLUTION OF PAIRS OF CARBOXYLIC ACID ENANTIOMERS WITH CL DETECTION

CL reagents, 0.5 m*M* TCPO and 15 m*M* H_2O_2 in CH_3CN; flow-rate, 1.5 ml/min. For other HPLC–CL detection conditions, see Experimental.

Carboxylic acid	(+)-Enantiomer		(−)-Enantiomer		α	R_s	Eluent[a]
	t_R (min)	k'	t_R (min)	k'			
Ibuprofen	20.41	6.56	24.11	7.93	1.21	3.16	A
Naproxen	10.80	3.00	12.02	3.45	1.15	1.73	A
Naproxen	23.62	7.75	28.19	9.44	1.22	3.32	B
Loxoprofen	8.50	2.15	9.08	2.36	1.10	1.00	A
Loxoprofen	16.74	5.20	18.82	5.97	1.15	2.08	B
N-Acetyl-Tyr	17.46	5.47	14.70	4.44	1.23	2.70	C
N-Acetyl-Met	23.22	7.60	25.46	8.43	1.11	1.63	C
N-Acetyl-Val	23.28	7.62	22.10	7.18	1.06	0.94	C
N-Acetyl-Trp	13.76	4.10	11.86	3.39	1.21	2.38	D
N-Acetyl-Leu	13.01	3.82	11.83	3.38	1.13	1.47	D
N-Acetyl-Phe	14.80	4.48	11.85	3.39	1.32	3.47	D

[a] Eluent: (A) 0.1 *M* Imidazole-NO_3 (pH 7.0)–CH_3CN (2:3); (B) 0.1 *M* Imidazole-NO_3 (pH 7.0)–CH_3CN (1:1); (C) 0.1 *M* Imidazole-NO_3 (pH 7.0)–CH_3CN (7:3); (D) 0.1 *M* Imidazole-NO_3 (pH 7.0)–CH_3CN (3:2). Flow-rate, 0.5 ml/min.

enantiomer was larger than that of the (+)-enantiomer. The results with N-acetyltryptophan suggest that the peaks of the (+)-enantiomer with (+)-DBD-APy and the (−)-enantiomer with (−)-DBD-APy are larger than those of the (−)-enantiomer with (+)-DBD-APy and (+)-enantiomer with (−)-DBD-APy. Therefore, the peak difference might be due to the difference in the CL intensity based on the fluorescence quantum yield (ϕ_f) and/or the difference in the derivatization yield. Table I lists the capacity factors (k'), separation factors (α) and resolutions (R_s) for each pair of diastereomers. Complete resolution was achieved with all the carboxylic acids tested. The (+)-enantiomers of the drugs and the (−)-enantiomers of the N-acetylamino acids were eluted faster than the corresponding opposite enantiomers. Their elution orders were the same as those with the 0.1% TFA–CH_3CN eluent described previously [5]. The carboxylic acid enantiomers, having a chiral centre at the α-position to a COOH functional group, were well separated and detected with the proposed HPLC–CL system, as shown in Fig. 10 and Table I. Judging from the results, the separation of carboxylic acid enantiomers when the COOH group is adjacent to a chiral centre may be possible using the proposed system. However, the resolution of racemates containing a COOH group at a β- or γ-position seems to be difficult, because the distances between the chiral centres in the diastereomer, derived from the carboxylic acid and the reagent, increase. The effect of the distance between an asymmetric carbon and a COOH group on separation should be investigated.

Determination of ibuprofen enantiomers after addition of rac-ibuprofen to rat plasma and human urine

The usefulness of the proposed HPLC–CL detection method for the resolution of *rac*-ibuprofen in rat plasma and human urine was examined as a preliminary step in a pharmacokinetic study. Fig. 11A and B shows the chromatograms obtained from rat plasma with the TCPO–H_2O_2 and TDPO–H_2O_2 systems, and Fig. 12A and B those from human urine. Highly sensitive detection of each pair of ibuprofen enantiomers is possible in both biological samples. Further, no interference of endogenous compounds in the samples was observed (data not shown). The proposed HPLC–CL method provides ultra-sensitive detection of carboxylic acid enantiomers.

The proposed methods are the first to be reported

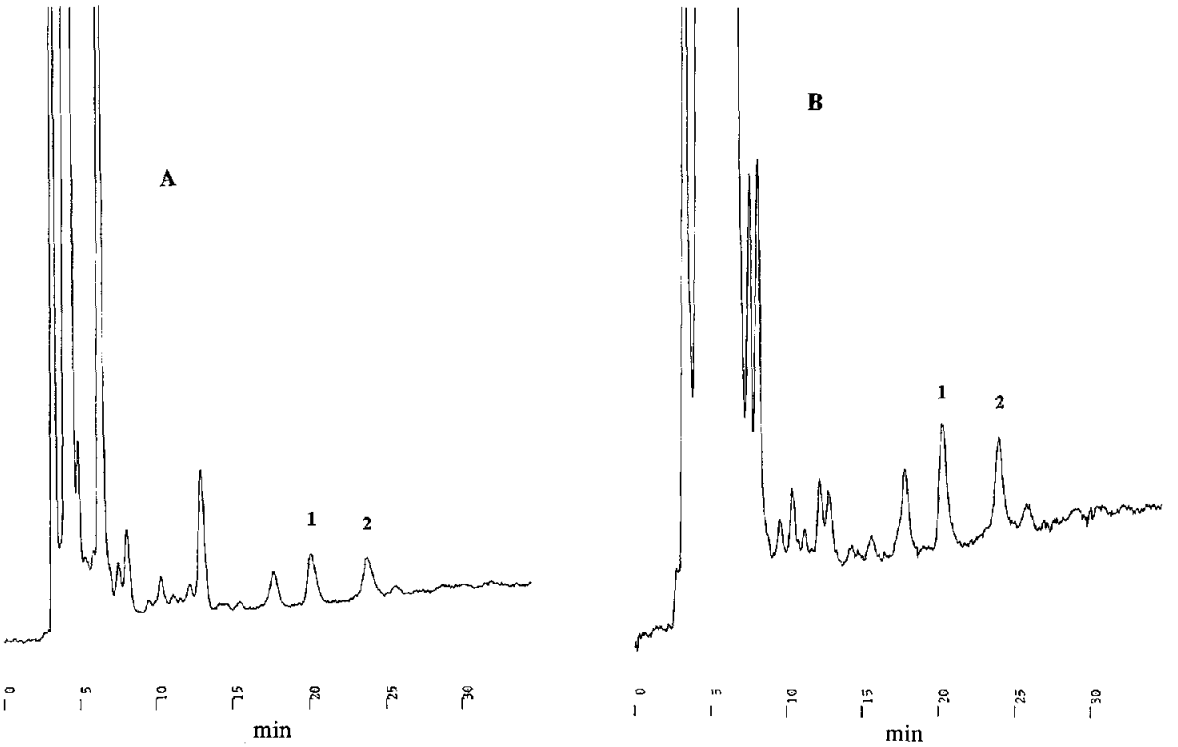

Fig. 11. Chromatograms obtained from rat plasma with CL detection. (A) with TCPO–H_2O_2 (50 fmol each); (B) with TDPO–H_2O_2 (25 fmol each). Peaks: 1 = (+)-ibuprofen; 2 = (−)-ibuprofen. For conditions of HPLC separation and CL detection, see Experimental.

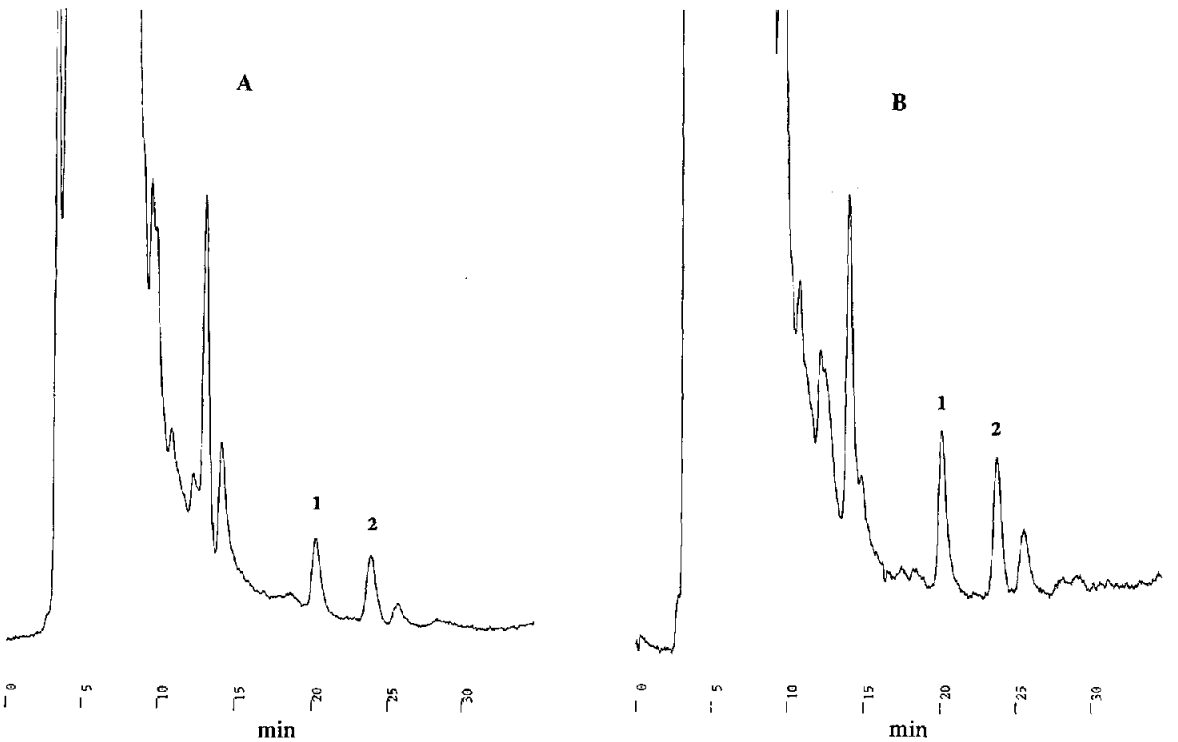

Fig. 12. Chromatograms obtained from human urine with CL detection. (A) with TCPO–H_2O_2 (100 fmol each); (B) with TDPO–H_2O_2 (50 fmol each). Peaks: 1 = (+)-ibuprofen; 2 = (−)-ibuprofen. For conditions of HPLC separation and CL detection, see Experimental.

for the optical resolution of racemates with CL detection. The detection limits of the carboxylic acids after derivatization with (+)-DBD-APy are in the attomole range. The minimum detectable range is lower than that previously reported for the optical resolution of carboxylic acid enantiomers [23-25]. Further, the use of reversed-phase chromatography using an ODS column instead of a normal-phase system is an advantage. Among the fluorophores, the DBD-APy moiety is recommended for the CL detection of carboxylic acid enantiomers because of its high sensitivity. On the other hand, both TDPO and TCPO in the presence of H_2O_2 are applicable in peroxyoxalate CL as described here. The proposed method for the ultra-sensitive and simultaneous determination of enantiomeric drugs in biological fluids may provide more precise information on the pharmacokinetics of drugs such as anti-inflammatories (*e.g.*, ketoprofen and loxoprofen). Further studies are in progress.

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REFERENCES

1 W. H. Pirkle and T. C. Pochopsky, *Chem. Rev.*, 89 (1989) 347.
2 M. Zief and L. J. Crane (Editors), *Chromatographic Chiral Separation*, Marcel Dekker, New York, 1988.
3 A. M. Krstulovic (Editor), *Chiral Separation by HPLC*, Ellis Horwood, Chichester, 1989.
4 P. Camilleri, *Biomed. Chromatogr.*, 5 (1991) 128.
5 T. Toyo'oka, M. Ishibashi and T. Terao, *Analyst (London)*, 117 (1992) 727.
6 K. Imai, K. Miyaguchi and K. Honda, in K. van Dyke (Editor), *Bioluminescence and Chemiluminescence: Instruments and Application*, Vol. II, CRC Press, New York, 1985, p. 65.
7 K. Imai, *Methods Enzymol.*, 133B (1986) 435.
8 P. V. Zoonen, D. A. Kamminga, C. Gooijer, N. H. Velthorst and R. W. Frei, *Anal. Chim. Acta*, 167 (1985) 249.
9 M. S. Abdel-Latif and G. G. Guilbault, *Anal. Chem.*, 60 (1988) 2671.
10 T. Toyo'oka, M. Ishibashi and T. Terao, *Biomed. Chromatogr.*, 6 (1992) 143.
11 M. M. Rauhut, L. J. Bollyky, B. G. Roberts, M. Loy, R. H. Whitman, A. V. Iannotta, A. M. Semsel and R. A. Clarke, *J. Am. Chem. Soc.*, 89 (1967) 6515.
12 S. Kobayashi and K. Imai, *Anal. Chem.*, 52 (1980) 424.
13 K. Miyaguchi, K. Honda, T. Toyo'oka and K. Imai, *J. Chromatogr.*, 352 (1986) 255.
14 G. Mellbin and B. E. F. Smith, *J. Chromatogr.*, 312 (1984) 203.
15 K. W. Sigvardson and J. W. Birks, *Anal. Chem.*, 55 (1983) 432.
16 M. L. Grayeski and A. J. Weber, *Anal. Lett.*, 17 (1984) 1539.
17 K. Honda, J. Sekino and K. Imai, *Anal. Chem.*, 55 (1983) 940.
18 R. S. Givens, R. L. Schowen, J. Stobaugh, T. Kuwana, F. Alvarez, N. Parekh, B. Matuszewski, T. Kawasaki, O. S. Wong, M. Orlovic, H. P. Chokshi and K. Nakashima, in M. C. Goldberg (Editor), *Peroxyoxalate Chemiluminescence Reaction: Luminescence Application in Biological, Chemical, Environmental and Hydrological Sciences (ACS Symposium Series, Vol. 383)*, American Chemical Society, Washington DC, 1989, p. 127.
19 K. Imai, H. Nawa, M. Tanaka and H. Ogata, *Analyst (London)*, 111 (1989) 209.
20 N. Imaizumi, K. Hayakawa, M. Miyazaki and K. Imai, *Analyst (London)*, 114 (1989) 161.
21 N. Hanaoka, R. S. Givens, R. L. Schowen and T. Kuwana, *Anal. Chem.*, 60 (1988) 2193.
22 S. Kobayashi and K. Imai, *Anal. Chem.*, 52 (1980) 1548.
23 J. Goto, M. Ito, S. Katsuki, N. Saito and T. Nambara, *J. Liq. Chromatogr.*, 9 (1986) 683.
24 J. Goto, N. Goto, A. Hikichi, T. Nishimaki and T. Nambara, *Anal. Chim. Acta*, 120 (1980) 187.
25 J. Goto, N. Goto and T. Nambara, *J. Chromatogr.*, 239 (1982) 559.