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Short Communication

Resolution of carboxylic acid enantiomers by high-performance liquid chromatography with highly sensitive laser-induced fluorescence detection

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ABSTRACT

Optical resolution of carboxylic acid enantiomers by high-performance liquid chromatography with laser-induced fluorescence detection after chiral derivatization was investigated. Both laser power and time constants influence the detection limit of the fluorophore. The minimum detectable levels of three derivatives of naproxen [4-(aminosulphonyl)-7-(3-aminopyrrolidin-1-yl)-2,1,3-benzodioxazole, 4-(N,N-dimethylaminosulphonyl)-7-(3-aminopyrrolidin-1-yl)-2,1,3-benzoxadiazole and 4-nitro-7-(3-aminopyrrolidin-1-yl)-2,1,3-benzodiazole derivatives] were in the femtomole–attomole ranges, and the lowest detection limit was obtained with the last derivative.

INTRODUCTION

The enantiomeric separation of racemates of various drugs is one of the most important subjects in pharmacokinetic studies. When the pharmacological activity and side-effects are different for each enantiomer, the enantiomers must be determined for the study of the efficiency and safety of drugs. Therefore, a reliable method for the determination of drug enantiomers at trace levels is a prerequisite. There are several methods for the separation of enantiomers, such as gas chromatography (GC) [1], high-performance liquid chromatography (HPLC) [2] and high-performance capillary electrophoresis (HPCE) [3,4]. Of these, the HPLC method is widely employed owing to the development of many efficient chiral stationary phase (CSPs) and to the progress in derivatization techniques. Although a CSP often provides excellent separations of enantiomers, selection of the column is difficult and it may be applicable to only a limited number of racemates. A method based on diastereomer formation, including a derivatization step with a chiral reagent, is favourable for the determination of enantiomers in biological specimens because high sensitivity and selectivity can be achieved.

In a previous paper [5], we described the syntheses of fluorescent chiral tagging reagents for the carboxylic acid functional group [(+)- and (−)-isomers of 4-nitro-7-(3-aminopyrrolidin-1-yl)-2,1,3-benzoxadiazole (NBD-APy), 4-(N,N-dimethylaminosulphonyl)-7-(3-aminopyrrolidin-1-yl)-2,1,3-

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benzoxadiazole (DBD-APy) and 4-(amino sulphonyl)-7-(3-aminopyrrolidin-1-yl)-2,1,3-benzoxadiazole (ABD-APy)] and their application to the resolution of some enantiomeric drugs and N-acetylamino acids by HPLC with conventional fluorescence detection. The reagents react with the carboxylic acid enantiomers at room temperature in the presence of 2,2'-dipyridyl disulphide (DPDS) and triphenylphosphine (TPP) to form fluorescent diastereomers. The diastereomers corresponding to each pair of carboxylic acid enantiomers are separated completely with an ODS column in a single chromatographic run [*e.g.*, R_s = 2.40, *rac*-naproxen derived from (+)-DBD-APy; R_s = 3.45, *rac*-naproxen derived from (+)-NBD-APy]. The resolution by reversed-phase chromatography is advantageous for the determination of carboxylic acid enantiomers in biological samples because complicated pretreatment is not necessary. The detection limits of authentic diastereomers after separation by HPLC is in the 15–45 fmol range (signal-to-noise ratio = 3). Further, all the resulting compounds have relatively long excitation maximum wavelengths (*ca.* 470 nm) and emissions at 570 nm (DBD-APy-naproxen), 580 nm, (ABD-APy-naproxen) and 540 nm (NBD-APy-naproxen) (Fig. 1). As the excitation maximum wavelength is close to the light emission of an argon ion laser (488 nm), the minimum detectable level might be expected to be improved with laser-induced fluorescence (LIF) detection. In this work we evaluated the applicability of the chiral tagging reagents DBD-APy, NBD-APy and ABD-APy to the resolution of carboxylic acid enantiomers when a commercially available LIF detector is used.

EXPERIMENTAL

Materials and reagents

(+)-4-(Aminosulphonyl)-7-(3-aminopyrrolidin-1-yl)-2,1,3-benzoxadiazole [(+)-ABD-APy], (+)-4-nitro-7-(3-aminopyrrolidin-1-yl)-2,1,3-benzoxadiazole [(+)-NBD-APy] and (+)-4-(N,N-dimethylaminosulphonyl)-7-(3-aminopyrrolidin-1-yl)-2,1,3-benzoxadiazole [(+)-DBD-APy] were synthesized as described previously [5]. Racemic 2-(6-methoxy-2-naphthyl)propionic acid (*rac*-naproxen) was donated by Tokyo Tanabe Pharmaceutical (Tokyo, Japan). The derivatives of (+)-naproxen with (+)-ABD-APy, (+)-NBD-APy and (+)-DBD-APy were also synthesized according to the previous paper [5]. TPP (Wako, Osaka, Japan) and DPDS (Tokyo Kasei, Tokyo, Japan) were used as received. Acetonitrile and water were of HPLC grade (Wako). All other chemicals were of analytical-reagent grade and were used as received.

HPLC–LIF detection

The high-performance liquid chromatograph consisted of two LC-9A pumps and an SCL-6B system controller (Shimadzu, Kyoto, Japan). Sample solutions were injected by a SIL-6B autoinjector (Shimadzu). The analytical columns were an Inertsil ODS-2 (150 × 4.6 mm I.D., 5 μm) (GL Sciences, Tokyo, Japan), a TSK-gel ODS-80TM (150 × 4.6 mm I.D., 5 μm) (Tosoh, Tokyo, Japan) and a TSK-gel PTH Pak (250 × 2.0 mm I.D. 5 μm) (Tosoh). The column was maintained at 40°C with a 655A-52 column oven (Hitachi, Tokyo, Japan). A Tosoh LF-8010 monitor equipped with a 5-μl flow cell was employed for detection. The peak areas obtained

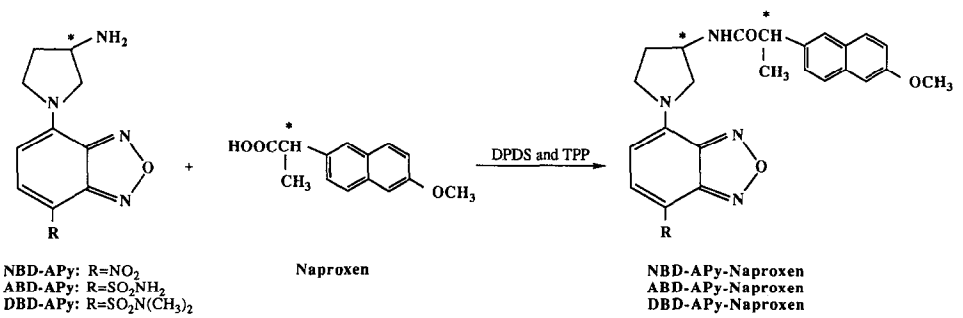

Fig. 1. Reaction of naproxen with chiral tagging reagents in the presence of activation agents.

from the LIF monitor were calculated with a C-R4A Chromatopac (Shimadzu). All mobile phases were degassed with a DGU-3A on-line degasser (Shimadzu).

RESULTS AND DISCUSSION

Table I lists the detection limits (signal-to-noise ratio = 3) of the three authentic derivatives obtained from (+)-naproxen and the (+)-isomers of the chiral tagging reagents under various conditions. Although the peak heights of the derivatives were slightly reduced with increasing time constants, the background noise level was also decreased. As a result, lower detection limits were obtained from longer time constants. In addition, the laser power has the greatest influence on detection. Although the baseline noise levels increased with increasing laser power, the peak heights of the fluorescent derivatives increased considerably. Hence lower detection limits were obtained with higher laser power (Table I). However, the sensitivity of NBD-APy-naproxen was essentially the same at 200 and 500 mW. The ratio of peak heights at 500 and 200 mW was *ca.* 2.4, while that of the background noise was 2.2. Therefore, the difference in detection limits between 200 and 500 mW was not great (0.69 *versus* 0.62 fmol). Greater variation of the baseline noise, based on emission of impurities etc., in the eluent, may be the main reason for the low sensitivity in spite of the use of a high power laser, compared with the use of a low-power laser at 5–15 mW. However, the results in Table I indicate that both a high-power laser and long time constants provide low detection limits.

Another approach to sensitive detection is the use of a microbore column. The chromatogram in Fig. 2 illustrates the separation and detection of a mix-

TABLE I

DETECTION LIMITS OF DIASTEREOMERS BY HPLC WITH LIF DETECTION

Diastereomer	Conditions[a]	Retention time (min)	Detection limit (fmol)[b]	Laser power (mW)	Time constant (s)
ABD-APy-naproxen	A	27.0	265	5	0.5
	D	5.27	42	10	3
	D	5.27	29	15	3
	E	6.64	10	10	3
	E	6.64	6.6	15	3
DBD-APy-naproxen	B	20.8	33	5	0.5
	D	11.4	18	10	3
	D	11.4	11	15	3
	E	11.4	2.7	10	3
	E	11.4	1.7	15	3
NBD-APy-naproxen	C	24.2	14	5	0.5
	C	24.2	7.2	10	0.8
	C	24.2	5.1	10	1.5
	D	7.72	4.4	10	3
	D	7.72	2.9	15	3
	E	9.05	1.0	10	3
	E	9.05	0.66	15	3
	C	24.2	0.69	200	4
	C	24.2	0.62	500	4

[a] Eluent: A, water–acetonirile (65:35); B, 0.1 *M* phosphate buffer (pH 6.8)–acetonitrile (55:45); C, water–acetonitrile (60:40); D, water–acetonitrile (50:50); E, water–acetonitrile (40:60). Column for A, B and C, TSK-gel ODS-80TM (150 × 4.6 mm I.D., 5 μm); column for D, Inertsil ODS-2 (150 × 4.6 mm I.D., 5 μm); column for E, TSK-gel PTH Pak (250 × 2.0 mm I.D., 5 μm). Column temperature, 40°C. Flow-rate for A, B, C and D, 1.0 ml/min; flow-rate for E, 0.2 ml/min. Amount injected, 10 μl. Interference filter for A, B and C, 540 ± 10 nm; Interference filter for D and E, 540 ± 20 nm.

[b] Signal-to-noise ratio = 3.

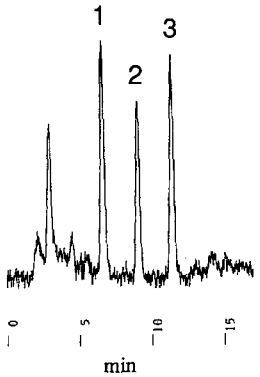

Fig. 2. Chromatogram of diastereomers separated using a microbore column. HPLC conditions: column, TSK-gel PTH Pak (250 × 2.0 mm I.D., 5 μm) at 40°C; eluent, water–acetonitrile (40:60); flow-rate, 0.2 ml/min; detection, argon ion laser at 15mW; interference filter, 540 ± 20 nm. Peaks: 1 = ABD-APy-naproxen (40 fmol); 2 = NBD-APy-naproxen (3 fmol); 3 = DBD-APy-naproxen (10 fmol).

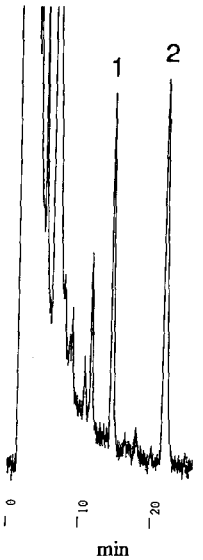

Fig. 3. HPLC separation of *rac*-naproxen labelled with (+)-NBD-APy. Racemic naproxen (1 μ*M* each) and (+)-NBD-APy (1 m*M*) in acetonitrile reacted at room temperature in the presence of DPDS (1 m*M*) and TPP (1 m*M*). After 2 h of reaction, the sample solution was diluted with acetonitrile and injected on to the column. HPLC conditions: column, Inertsil ODS-2 (150 × 4.6 mm I.D., 5 μm) at 40°C; eluent, water–acetonitrile (57:43); flow-rate, 1.0 ml/min; detection, argon ion laser at 15mW; interference filter, 540 ± 20 nm. Peaks: 1 = (+)-naproxen; 2 = (−)-naproxen. Each peak coresponds to 50 fmol.

ture of three derivatives, ABD-APy-naproxen (40 fmol), NDB-APy-naproxen (3 fmol) and DBD-APy-naproxen (10 fmol), using a microbore ODS column with an LIF monitor at 15 mW. Compared with a conventional column, the detection limits were decreased by a factor of 4–7 (Table I). Those of DBD-APy-naproxen (11 fmol) and ABD-APy-naproxen (29 fmol), separated by the conventional column, were in same range as those obtained with a conventional fluorescence detector (DBD-APy-naproxen, 15 fmol; ABD-APy-naproxen, 45 fmol) [5]. Among the fluorophores, NBD-APy-naproxen showed the highest sensitivity with LIF detection. It is well known that the fluorescence intensity depends on the light intensity (I_0), concentration of fluorophore (c), cell length (l), molar absorptivity (ε) and fluorescence quantum yield (ϕ). When I_0, c and l remain the same, the intensity changes according to the ε and ϕ values of the compound. The values of ε at the absorption maximum wavelengths of NBD-APy-naproxen, DBD-APy-naproxen and ABD-APy-naproxen were 22 800 (493 nm), 12 400 (450 nm) and 10 400 (450 nm), respectively, in acetonitrile–water (1:1), whereas the values at 488 nm (light emission of the argon ion laser) were *ca.* 22 400, 5400 and 5000, respectively. As the ε value of NBD-APy-naproxen is about four times larger than those of the other two derivatives, the higher sensitivity of NBD-APy-naproxen may be due to the larger ε value at 488 nm. Further, the interference filter (540 nm) used in the LIF detector fits the emission maximum of NBD-APy-naproxen (543 nm) [5]. The large difference in sensitivity between DBD-APy-naproxen and ABD-APy-naproxen cannot be explained solely by ε, as the values of ε are almost equal and the absorption spectra at 300–700 nm are almost superimposable (data not shown). As described previously [5], the fluorescence intensity of DBD-APy-naproxen was approximately three times stronger than that of ABD-APy-naproxen. The results suggest that the ϕ value of DBD-APy-naproxen is larger than that of ABD-APy-naproxen. Hence the lower detection limit of DBD-APy-naproxen with LIF detection might be mainly caused by ϕ. On the other hand, the interference filter of 540 nm employed in the LIF detector was not suitable for the detection of ABD-APy-naproxen and DBD-APy-naproxen because the derivatives

have emission maxima at *ca.* 585 and 580 nm, respectively [5].

Fig. 3 shows the resolution of *rac*-naproxen using the conventional ODS column after derivatization with (+)-NBD-APy. Excellent separation and high sensitivity were obtained with LIF detection. The detectability is better than that of conventional fluorescence detection using a xenon arc lamp. NBD-APy is a useful reagent for the resolution of carboxylic acid enantiomers with sensitive LIF detection. Although the sensitivity of the DBD-APy derivative is lower than that of the NBD-APy derivative, it might be improved by the use of a high-power laser source and a suitable interference filter at *ca.* 580 nm. Hence the proposed technique should provide an ultratrace analytical method for carboxylic acid enantiomers. Further studies of optical resolution using DBD-APy and NBD-APy with real samples are in progress.

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