Journal of Chromatography, 239 (1982) 559–564
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,544

SEPARATION AND DETERMINATION OF NAPROXEN ENANTIOMERS IN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The separation and determination of *d*- and *l*-naproxen in serum by high-performance liquid chromatography (HPLC) is described. The method is based on pre-column derivatization with a newly developed chiral reagent, *l*-1-(4-dimethylamino-1-naphthyl)ethylamine (DANE) to form the diastereoisomeric amides. The diastereoisomers were separated on a normal-phase column by HPLC with fluorescence detection employing *n*-hexane–tetrahydrofuran (80:26) as the mobile phase. The clean-up of naproxen in serum was efficiently attained by the use of a Sep-Pak C_{18} cartridge. The quantitation limit of naproxen by this method was 100 pg. The serum levels of naproxen enantiomers after administration of the racemate or each enantiomer to rabbits were determined by the proposed method.

INTRODUCTION

A reliable method for the simultaneous determination of each enantiomeric drug in biological fluids is a prerequisite for pharmacokinetic studies of the racemate. In previous papers we reported the preparation of chiral derivatization reagents for the resolution of enantiomeric amines[1,2] and their use for the determination of 2,4-dimethoxy-4-methylamphetamine enantiomers in plasma by high-performance liquid chromatography (HPLC)[3]. We also synthesized chiral derivatization reagents, *d*- and *l*-1-(4-dimethylamino-1-naphthyl)ethylamine (DANE), which have a fluorophore that is highly responsive to a fluorescence detector, and demonstrated their applicability to the resolution of carboxylic acid enantiomers by HPLC[4]. This paper deals with the use of these pre-column derivatization reagents for the separation and determination of naproxen enantiomers in serum by HPLC on a normal-phase column.

EXPERIMENTAL

High-performance liquid chromatography

The apparatus was a Waters 6000A solvent delivery system (Waters Assoc., Milford, MA, U.S.A.) equipped with a Hitachi Model 650-10LC fluorescence spectrophotometer (excitation wavelength 320 nm; emission wavelength 410 nm). The test samples were applied to the chromatograph by a Waters U6K sample loop injector

0021-9673/82/0000–0000/$02.75

(Waters Assoc.) with an effective volume of 2 ml. A μPorasil column (1 ft. × 1/4 in. I.D.) was used under ambient conditions. *n*-Hexane–tetrahydrofuran (80:26) was employed as mobile phase at a flow-rate of 0.6 ml/min.

Materials

All of the chemicals employed were of analytical-reagent grade. *dl*-Naproxen was obtained by the known method[5]. The racemate was resolved by fractionally crystallizing the *d*- or *l*-α-methylbenzylamine salt from ethyl acetate. The optical purity of each enantiomer thus obtained was over 99.0% as judged by HPLC. *l*-DANE hydrochloride was synthesized by the method previously reported[4] and its optical purity was determined to be over 99.5%. 3,5-*tert*.-Butyl-4-hydroxybenzaldehyde O-(methoxycarbonylmethyl)oxime, used as the internal standard (IS), was prepared in these laboratories. Solvents were purified by distillation prior to use. A Sep-Pak C_{18} cartridge (Waters Assoc.) was washed thoroughly with ethanol and water before use.

Sample preparation

A serum sample (20–100 μl) was diluted with 0.1 *M* phosphate buffer (pH 2.0, 2 ml) and passed through a Sep-Pak C_{18} cartridge. After successive washing with water (10 ml) and 70% ethanol (0.5 ml), the desired fraction was obtained by elution with 70% ethanol (2 ml). After evaporation of the solvent, the residue was dissolved in 0.05 *M* sodium carbonate buffer (pH 10, 1 ml) and washed with *n*-hexane (three 2-ml volumes). The aqueous layer was acidified with 1 *N* hydrochloric acid (200 μl) and then extracted with ethyl acetate (three 2-ml volumes). After addition of 1-hydroxybenzotriazole (HOBT) (5 μg) and IS (300 ng), the solution was evaporated under a stream of nitrogen. To the residue were added *l*-DANE hydrochloride (30 μg) in pyridine (20 μl) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (WSC) (100 μg) in dichloromethane (200 μl), and the solution was allowed to stand at 4°C for 45 min. After removal of the solvent under a stream of nitrogen, the residue was dissolved in *n*-hexane–ethyl acetate (3:1) (500 μl). The solution was washed with 5% sodium hydrogen carbonate solution and then applied to a silica gel column (3 cm × 4 mm I.D.). After washing with *n*-hexane–ethyl acetate (3:1) (700 μl), the desired fraction was obtained by elution with ethyl acetate (2 ml). The eluate was dried and dissolved in the mobile phase (400 μl), and a 5–10-μl aliquot of the solution was injected into the HPLC system.

Recovery test for naproxen added to human serum

The test samples were prepared by dissolving 25, 50, 100 or 250 ng of *d*- and *l*-naproxen in human serum (0.1 ml). Clean-up by the use of a Sep-Pak C_{18} cartridge and derivatization with *l*-DANE followed by determination of each enantiomer by HPLC were carried out according to the procedure described above.

Administration of naproxen to rabbits

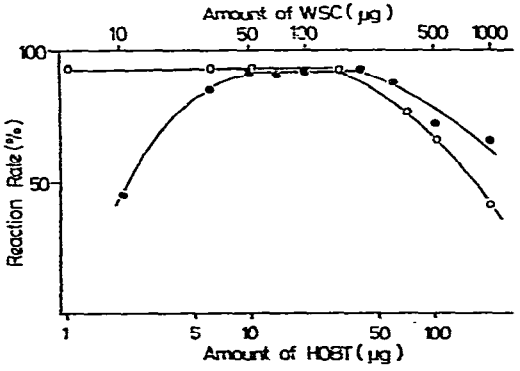
d- or *l*-naproxen (5 mg/kg each) or *dl*-naproxen (10 mg/kg) was dissolved in 0.05 *M* sodium carbonate buffer (pH 10, 1.3 ml) and saline (0.7 ml), and the solution was injected intravenously into a male albino rabbit weighing 3–4 kg. The blood was withdrawn at 5, 10, 20 and 40 min and 1, 2, 3, 4, 5, 6 and 8 h after administration and centrifuged for 20 min at 1500 *g* to separate serum.

RESULTS AND DISCUSSION

Derivatization of naproxen to diastereoisomers with l-DANE

Resolution of the enantiomers by liquid chromatography has been developed in two ways: introduction of an asymmetric environment intramolecularly by conversion to diastereoisomers[6,7] and intermolecularly by the use of a chiral stationary or mobile phase[8–11]. The former method is more favourable for pharmacokinetic studies of a racemic drug with respect to resolution and sensitivity. In a previous paper, we reported the preparation of *d*- and *l*-DANE as derivatization reagents and their utility for the resolution of carboxylic acid enantiomers by HPLC[4].

In this study, suitable conditions were first investigated for the coupling of *l*-DANE with naproxen through its carboxylic acid function. There are several methods available for the formation of a peptide bond, but most are not necessarily suitable for the quantitative derivatization of carboxylic acid groups owing to the lack of reproducibility. The combined use of WSC and HOBT was chosen for condensation of naproxen with *l*-DANE. Initially, the effects of the concentrations of these two reagents on the formation of diastereoisomeric amides were examined. *dl*-Naproxen, *l*-DANE and various amounts of HOBT and WSC were dissolved in dichloromethane–pyridine (200:20), and the reaction mixture was allowed to stand at 4°C for 45 min. A portion of the resulting solution was then used for HPLC. As shown in Fig. 1, the concentrations of HOBT and WSC exerted a significant influence on the reaction rate. When naproxen was treated with these reagents at 4 or 50°C, the yield of the diastereoisomeric amide increased with increasing reaction time up to 30 min and reached a plateau as illustrated in Fig. 2. The quantitative formation of a peptide bond was attained at 4°C, but a lower reaction rate was observed at 50°C. In addition, without HOBT the reaction rate was depressed to 60% and hence HOBT was concluded to be a useful catalyst for amide formation. No racemization of naproxen or *l*-DANE took place during the condensation reaction.



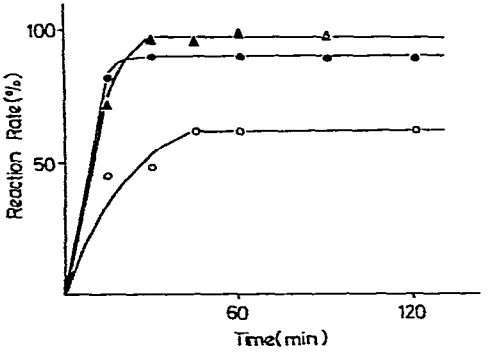


Fig. 1. Effects of concentrations of HOBT and WSC on the formation of diastereomeric amides. ○, HOBT; ●, WSC.

Fig. 2. Time course for derivatization of naproxen with *l*-DANE. ▲, At 4°C with HOBT; ●, at 50°C with HOBT; ○, at 50°C without HOBT.

Clean-up of naproxen in serum

The recovery of a drug in blood is significantly influenced by the clean-up procedure employed. For this purpose, deproteinization with an alcohol or acid and extraction with the organic solvent are widely used prior to HPLC. These procedures, however, proved not to be applicable to serum naproxen owing to the insufficient recovery rate and the occurrence of interfering peaks on the chromatogram. In previous papers, we demonstrated the utility of a Sep-Pak C_{18} cartridge for the clean-up of 2,5-dimethoxy-4-methylamphetamine[3], bile acids[12] and their sulphates[13] in biological fluids. Therefore, the use of this cartridge was attempted for the purification of naproxen in serum. Naproxen was readily adsorbed on Sep-Pak C_{18} and quantitatively recovered by elution with 70% ethanol. This procedure was found to be effective for the removal of co-existing polar substances in serum. Elimination of the neutral materials in biological fluids was then carried out. The eluate was dissolved in alkali and the resulting solution was washed with *n*-hexane. The aqueous layer was then acidified and extracted with ethyl acetate, providing the desired naproxen quantitatively. The efficient clean-up procedure for HPLC of naproxen enantiomers in serum gave an excellent chromatogram without any interfering peaks (Fig. 3). No racemization of naproxen occurred during this clean-up.

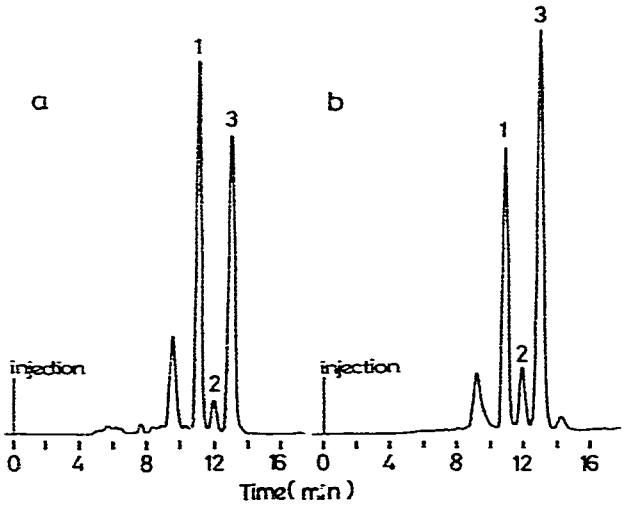


Fig. 3. Chromatogram of *d*- and *l*-naproxen. (a) Standard sample; (b) serum sample obtained 2 h after administration of *dl*-naproxen. 1, *l*-Enantiomer; 2, IS; 3, *d*-enantiomer.

Detection of diastereoisomers formed from naproxen with l-DANE

Elution of the diastereoisomeric amides in HPLC was monitored by fluorescence detection at 320 nm, the limit of quantitation being 100 pg. A calibration graph was constructed by plotting the ratio of the peak area of *d*- or *l*-naproxen to that of 3,5-*tert*.-butyl-4-hydroxybenzaldehyde O-(methoxycarbonylmethyl)oxime against the amount of naproxen enantiomers, a linear response to each enantiomer being observed in the range 5–2000 ng.

Determination of d- and l-naproxen in serum

The applicability of the method to the simultaneous determination of *d*- and *l*-

TABLE I

RECOVERY OF *d*- AND *l*-NAPROXEN ADDED TO HUMAN SERUM

Enantiomer	*Naproxen (ng)*		*Recovery ± S.D. (%)**
	Added	*Found*	
d	25	24.5	97.8 ± 3.4
	50	49.0	98.0 ± 3.8
	100	95.7	95.7 ± 3.2
	250	249.5	99.8 ± 2.4
l	25	23.9	95.7 ± 4.2
	50	47.7	95.3 ± 4.3
	100	95.3	95.3 ± 2.5
	250	242.5	97.0 ± 3.4

* $n = 8$.

naproxen in serum was examined. Known amounts (25–250 ng) of *d*- and *l*-naproxen was added to human serum and the recoveries were determined. As shown in Table I, the recoveries of *d*- and *l*-naproxen were 95–100% with a standard deviation of 2.4–4.3%. It is evident that the proposed method is applicable to the quantitation of naproxen enantiomers in serum with satisfactory accuracy and precision.

The simultaneous determination of serum levels of the enantiomers was carried out according to the procedure thus established. Blood specimens were collected at 5, 10, 20 and 40 min and 1, 2, 3, 4, 5, 6 and 8 h following intravenous injection of *d*-, *l*- or *dl*-naproxen to rabbits. A typical chromatogram of naproxen enantiomers in serum obtained at 2 h is shown in Fig. 3. The half-life of *d*-naproxen was found to be longer than that of *l*-enantiomer when *dl*-naproxen was injected intravenously (Fig. 4). Moreover, the formation of a significant amount of the *d*-enantiomer was recognized after administration of *l*-naproxen. On the other hand, the *l*-enantiomer was not detected when *d*-naproxen was administered in a similar manner (Fig. 5). The stereo-

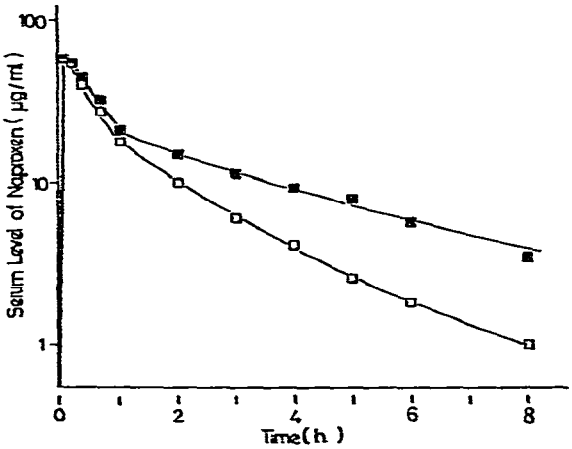


Fig. 4. Serum levels of *d*- and *l*-naproxen after administration of the racemate. ■, *d*-Naproxen; □, *l*-naproxen.

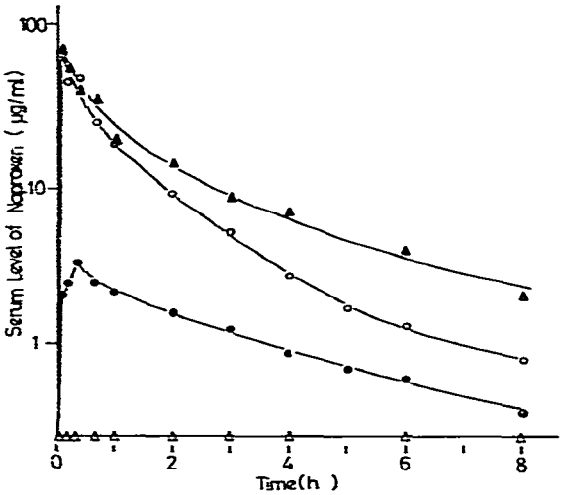


Fig. 5. Serum levels of *d*- and *l*-naproxen after administration of each enantiomer. ▲, *d*-Naproxen after administration of *d*-enantiomer; △, *l*-naproxen after administration of *d*-enantiomer; ●, *d*-naproxen after administration of *l*-enantiomer; ○, *l*-naproxen after administration of *l*-enantiomer.

specific inversion of *l*-naproxen to the *d*-enantiomer in living animals has been unequivocally demonstrated. These results are in good accord with the findings reported in previous papers[14–16].

It is hoped that the availability of a new method for the simultaneous determination of enantiomeric drugs in biological fluids with satisfactory reliability and sensitivity may provide more precise information on the pharmacokinetics of α-arylpropionic acid anti-inflammatory drugs.

ACKNOWLEDGEMENTS

This work was supported in part by a grant from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1 J. Goto, M. Hasegawa, S. Nakamura, K. Shimada and T. Nambara, *J. Chromatogr.*, 152 (1978) 413.
2 T. Nambara, S. Ikegawa, M. Hasegawa and J. Goto, *Anal. Chim. Acta*, 101 (1978) 111.
3 J. Goto, N. Goto, A. Hikichi and T. Nambara, *J. Liquid Chromatogr.*, 2 (1979) 1179.
4 J. Goto, N. Goto, A. Hikichi, T. Nishimaki and T. Nambara, *Anal. Chim. Acta*, 120 (1980) 187.
5 I. T. Harrison, B. Lewis, P. Nelson, W. Rooks, A. Roszkowski, A. Tomolonis and J. H. Fried, *J. Med. Chem.*, 13 (1970) 203.
6 T. Tamegai, M. Ohmae, K. Kawabe and M. Tomoeda, *J. Liquid Chromatogr.*, 2 (1979) 1229.
7 T. Kinoshita, Y. Kasahara and N. Nimura, *J. Chromatogr.*, 210 (1981) 77.
8 P. E. Hare and E. Gil-Av, *Science*, 204 (1979) 1226.
9 B. Lefebvre, R. Audebert and C. Quivoron, *J. Liquid Chromatogr.*, 1 (1978) 761.
10 S. Hara and A. Dobashi, *J. Chromatogr.*, 186 (1979) 543.
11 C. Gilon, R. Leshem and E. Grushka, *Anal. Chem.*, 52 (1980) 1206.
12 J. Goto, H. Kato, Y. Saruta and T. Nambara, *J. Liquid Chromatogr.*, 3 (1980) 991.
13 J. Goto, H. Kato, Y. Saruta and T. Nambara, *J. Chromatogr.*, 226 (1981) 13.
14 D. G. Kaiser, G. J. Vangiessen, R. J. Reischer and W. J. Wechter, *J. Pharm. Sci.*, 65 (1976) 269.
15 T. Tamegai, T. Tanaka, T. Kaneko, S. Ozaki, M. Ohmae and K. Kawabe, *J. Liquid Chromatogr.*, 2 (1979) 551.
16 J. M. Kemmerer, F. A. Rubio, R. M. McClain and B. A. Koechlin, *J. Pharm. Sci.*, 68 (1979) 1274.