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Note

Preparative separation of the enantiomers of the cholecystokinin antagonist (3*S*)-(±)-N-(2,3-dihydro-1-([$^3H_3$]methyl)-2-oxo-5-phenyl-1H-1,4-benzodiazepine-3-yl)-1H-indole-2-carboxamide by high-performance liquid chromatography

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(3*S*)-(−)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepine-3-yl)-1H-indole-2-carboxamide (L-364,718; **I**) (Fig. 1) is a potent, non-peptidal antagonist of the peptide hormone and proposed neurotransmitter cholecystokinin[1] possessing high peripheral cholecystokinin receptor selectivity.[2] Tissue binding studies have shown the (−)-3*S* enantiomer of compound **I** to be approximately 100 times more biologically active than the (+)-3*R* enantiomer[3] and optically pure (−)-**I** has been prepared on a multigram scale by a resolution-racemization sequence involving compound **I** precursor[4,5]. In order to obtain (+) and (−) enantiomers of the drug in high optical purity for use in radioligand receptor binding assays, a procedure that would resolve commercially available (±)-[N-methyl-$^3H_3$]-**I** was desired. An high-performance liquid chromatographic (HPLC) system employing a chiral stationary phase was developed to separate microgram amounts of the enantiomers of [$^3H_3$]-**I** without the need for chemical derivatization.

EXPERIMENTAL

Chemicals

n-Hexane (Fisher Scientific), chloroform (EM Science), and absolute ethanol (Midwest Solvents of Illinois) were filtered through a Millipore Durapore 0.45 μm membrane filter and degassed before use. (±)-[N-methyl-$^3H_3$]-**I** (87 Ci/mmol) was purchased from New England Nuclear Research Products in ethanol solution (0.0047

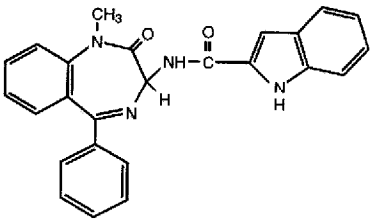

Fig. 1. Structure of (±)-**I**.

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mg/ml). A reference sample of (−)-**I** was obtained from Merck Sharp & Dohme Research Laboratories (West Point, PA, U.S.A.).

High-performance liquid chromatography

Separations were carried out using a Waters 6000 A solvent delivery system, a Rheodyne 7125 injector, and a Kratos Spectroflow 757 variable-wavelength absorbance detector operated at 290 nm. The chiral HPLC column used was a YMC (Mt. Freedom, NJ, U.S.A.) A-K03 250 × 4.6 mm I.D. column packed with a (+)-naphthylethylamine polymer bonded to spherical silica (particle size 5 μm). A mobile phase consisting of *n*-hexane–chloroform–ethanol (75:22:3) at a flow-rate of 1 ml/min was used.

Injection volumes of the drug in ethanol ranged from 10–30μl corresponding to sample amounts of 0.5–1 μg. Enantiomeric purity estimates based on chromatography peak integrations are ±3%.

RESULTS AND DISCUSSION

The enantiomers of [N-methyl-$^3H_3$]-**I** were resolved on a YMC A-K03 chiral column as shown in the chromatogram in Fig. 2a. The (+) enantiomer was eluted first at 8.9 min and was followed by the (−) enantiomer at 9.5 min, a retention time identical to that of authentic (−) enantiomer standard. A number of different solvent system combinations of *n*-hexane–chloroform, ethyl acetate, ethylene dichloride, methylene chloride, or *tert.*-butyl methyl ether–ethanol or isopropanol were examined; however, none surpassed the partial separation (α = 1.08; R_s = 0.8) achieved with *n*-hexane–chloroform–ethanol (75:22:3). It should be noted that a Resolvosil (Macherey-Nagel) bovine serum albumin chiral column was also used to partially resolve the [$^3H_3$]-**I** enantiomers [α = 1.38, (−) enantiomer eluted first]. However, the high organic content of the methanol–0.1 *M* monobasic potassium phosphate (15:85)

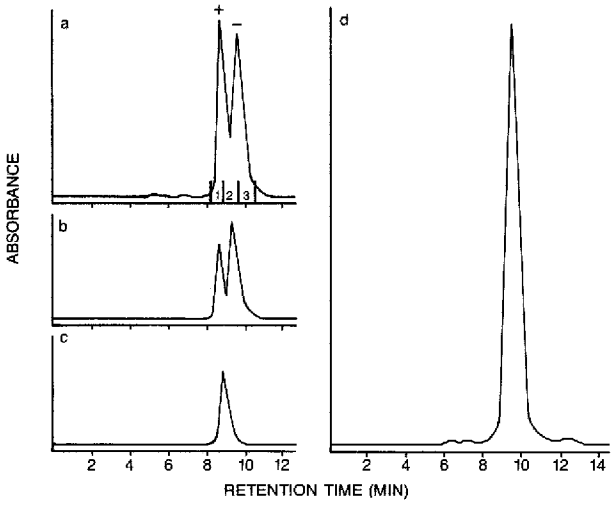

Fig. 2. Preparative HPLC of [N-methyl-$^3H_3$]-**I**. (a) Racemic mixture; (b) 65:35 (+)/(−) mixture; (c) (+) enantiomer; (d) (−) enantiomer.

mobile phase required for resolution caused significant column stationary phase denaturation after only two preparative sequences.

In order to obtain research quantities of the [$^3H_3$]-**I** enantiomers, twelve *ca.* 20-μl injections (1.829 mCi total) of radiochemically pure racemic drug were performed and three fractions were collected for each injection. As indicated in Fig. 2a, fraction 1 was collected from the beginning of the elution of the first peak, the (+) enantiomer, to the peak apex while fraction 2 was then collected to the apex of the second peak. Fraction 3, consisting primarily of the pharmacologically active (−) enantiomer was collected from the apex of the second peak until drug elution ceased. The enantiomeric purity, as determined by rechromatography, and total radioactivity for each of these fractions are listed in row A of Table I. Mixed fraction 2A, consisting of 65% of the (−) enantiomer as shown by the chromatogram in Fig. 2b, was further purified by a second round of chromatography (five *ca.* 20-μl injections). Once again, three fractions were collected with fraction 2 consisting of a mixed fraction collected between the peak apices. As detailed in row B of Table I, fraction 1B was essentially pure (+) enantiomer as shown in the chromatogram in Fig. 2c. Fraction 3B contained 95% of the (−) enantiomer. Fraction 3B and fraction 3A from the first injection series, containing 90% of the (−) enantiomer, were obtained and further purified by rechromatography (six *ca.* 20-μl injections). Three fractions were collected as usual with mixed fraction 2C between peak apices. Fraction 3C (row C, Table I) consisted of essentially 100% of the (−) enantiomer as shown by the chromatogram in Fig. 2d. A final total of 542 μCi of [^3H]$_3$-(−)-**I** was obtained, corresponding to an overall recovery of 60% from the amount of (−) enantiomer present in the racemic mixture.

In conclusion, the simple preparative procedure described above allows isolation of microgram quantities of (−)- and (+)-[N-methyl-$^3H_3$]-**I** in high enantiomeric purity and good yield for cholecystokinin antagonist studies. Although attention was directed at maximization of the yield of the pharmacologically active (−) enantiomer, the yield of the inactive (+) enantiomer could easily be increased by rechromatography of mixed fractions 1A, 2A and 2B. The number of replicate injections required could be reduced by the use of a larger, semi-preparative chiral column. Additionally,

TABLE I

CHROMATOGRAPHY DATA FROM PREPARATIVE RESOLUTION OF (+)-[N-METHYL-$^3H_3$]-**I**

		Fraction 1	*Fraction 2*	*Fraction 3*	*Recovery (%)*
A	Chromatography of racemic [$^3H_3$]-**I**	704 μCi 90% (+) 10% (−)	661 μCi 35% (+) 65% (−)	454 μCi 10% (+) 90% (−)	1.829 μCi injected 1.819 μCi recovered 99%
B	Rechromatography of mixed fraction 2A	116 μCi 100% (+)	230 μCi 40% (+) 60% (−)	279 μCi 5% (+) 95% (−)	661 μCi injected 625 μCi recovered 95%
C	Rechromatography of fractions 3A + 3B	39 μCi 62% (+) 38% (−)	72 μCi 35% (+) 65% (−)	542 μCi 100% (+)	733 μCi injected 653 μCi recovered 89%

a chiral column consisting of the analogous (−)-naphthylethylamine polymer stationary phase (currently not commercially available) would be expected to elute the desired (−) enantiomer first, potentially increasing the yield of the active enantiomer and reducing the number of replicate injections.

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