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JOURNAL OF CHROMATOGRAPHY A

Journal of Chromatography A, 685 (1994) 349–355

Short communication

Direct enantiomeric separation of the four stereoisomers of nadolol using normal-phase and reversed-phase high-performance liquid chromatography with Chiralpak AD

James P. McCarthy

Analytical Research and Development, Pharmaceutical Research Institute, Bristol-Myers Squibb, Princeton, NJ 08543, USA

First received 28 April 1994; revised manuscript received 19 August 1994

Abstract

Two enantiomeric separations have been developed which resolve the four stereoisomers of nadolol on Chiralpak AD using high-performance liquid chromatography. The normal-phase method uses hexane, ethanol and diethylamine to baseline resolve all four stereoisomers. The reversed-phase method uses ethanol, water and diethylamine. Both methods were optimized by investigating different mobile phase modifiers, flow-rates and column temperatures. Accuracy and reproducibility were determined for quantitating levels of racemate A and racemate B using the normal-phase method.

1. Introduction

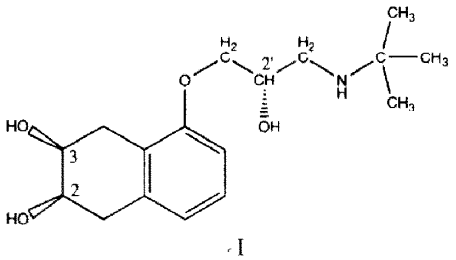
Nadolol, 5 - {3 - [(1,1 - dimethylethyl)amino] - 2 - hydroxypropoxy} - 1,2,3,4 - tetrahydro - *cis* - 2,3 - naphthalenediol is a β-blocker used in the management of hypertension and angina pectoris. Its chemical structure has three chiral centers which allow for eight possible stereoisomers. However, the two hydroxyl substituents on the cyclohexane ring are in the *cis* configuration which precludes four stereoisomers (see Fig. 1). Nadolol is marketed as an equal mixture of the four stereoisomers, designated as the diastereomers "racemate A" and "racemate B". Racemate A is a mixture of stereoisomer I and its enantiomer II, whereas racemate B is a mixture of the most active stereoisomer III and its enantiomer IV.

Many β-blockers have been successfully separated from their enantiomers using Chiralcel OD, a cellulose-based 3,5-dimethylphenyl carbamate HPLC column manufactured by Daicel (Tokyo, Japan) [1–6]. The method developed by Krstulovic et. al. [7] for enantiomeric separation of nadolol used Chiralcel OD to separate nadolol into two peaks. Nadolol however requires an approach different from Chiralcel OD because it contains two diastereomeric pairs of enantiomers, for a total of four compounds to be separated. Most other β-blockers contain only one pair of enantiomers. Daicel's Chiralpak AD HPLC column is amylose based and has a helical structure, whereas Chiralcel OD has a rigid linear structure. Chiralpak AD has the same 3,5-dimethylphenyl carbamate moiety as Chiralcel OD and the same silica support. Therefore, observed differences in separation must be due to the structural differences between cellulose and amylose.

The most recent HPLC methodology for the

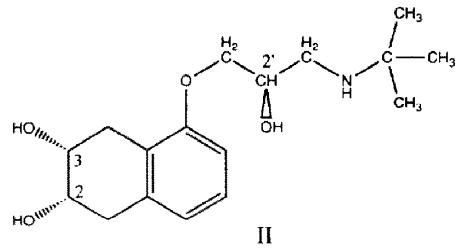
0021-9673/94/$07.00
SSDI 0021-9673(94)00803-5

Racemate A



I

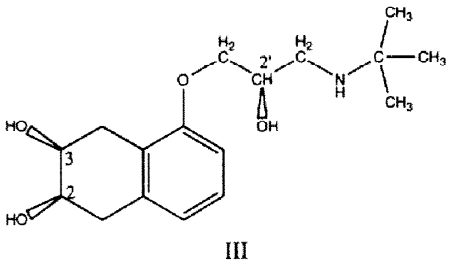
2R,3S,2'R (-) ring, (+) side chain



II

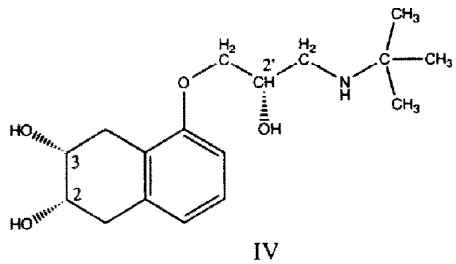
2S,3R,2'S (+) ring, (-) side chain

Racemate B



III

2R,3S,2'S (-) ring, (-) side chain



IV

2S,3R,2'R (+) ring, (+) side chain

Fig. 1. Absolute conformation of the four stereoisomers of nadolol.

analysis of nadolol baseline resolves the four stereoisomers using Ultron ES-OVM with a mobile phase of methanol and phosphate buffer [8]. In order to perform preparative-scale isolations of each stereoisomer of nadolol, Hoshino et al. [8] developed a second separation using Chiralpak AD which did not baseline resolve all four stereoisomers. Prior to the Ultron ES-OVM, a method was not available which baseline resolved all four of the stereoisomers without derivatization.

Daicel's cellulose- and amylose-based carbamate columns have been almost exclusively used under normal-phase conditions. Recently, separations have been developed on Chiralcel OD using reversed-phase conditions ("OD-R") [9]. Ishikawa and Shibata [9] successfully separated the enantiomers of two β-blockers, propranolol and pindolol, using 0.1 *M* aq. $NaPF_6$–CH_3CN. Their article detailed a comprehensive study of the effects different mobile phase additives have on the separation of acidic, neutral and basic compounds from their enantiomers. It was determined that acidic compounds are best resolved at pH < 3, neutral compounds can be separated using water and an organic modifier, and basic compounds require the addition of a chaotropic modifier. The chaotropic modifier has an effect similar to ion pairing. Perchlorate and hexafluorophosphate modifiers were determined to be among the best chaotropes for resolving enantiomers of basic compounds.

Detailed in the following article are the separations of the four stereoisomers of nadolol on Chiralpak AD using normal-phase and reversed-phase conditions. The effects of mobile phase composition, flow-rate and column temperature on resolution are discussed. The research by Ishikawa and Shibata [9] prompted the investigation of Chiralpak AD column under revers-

ed-phase conditions detailed below. The conclusions of Ishikawa and Shibata were applied to nadolol in a similar though less comprehensive study.

2. Experimental

2.1. Reagents

Methanol, 85% *n*-hexane and 2-propanol were all Baker HPLC reagents (Phillipsburg, NJ, USA). Ethanol used was dehydrated 200 proof obtained from Pharmco (Bayonne, NJ, USA). The diethylamine was 98% and obtained from Aldrich (Milwaukee, WI, USA). UV-grade acetonitrile was obtained from Burdick & Jackson (Muskegon, MI, USA). Water used in these experiments was drawn from a Milli-Q water system (Millipore, Milford, MA, USA). The nadolol standards were obtained internally. Nadolol, racemate A, racemate B and standards of each individual stereoisomer were available. Structural conformations of the individual stereoisomer standards were confirmed by X-ray diffraction.

2.2. Apparatus

The HPLC system consisted of a Perkin-Elmer Model Series 4 solvent-delivery system (Norwalk, CT, USA), a Perkin-Elmer ISS-100 autosampler and an Applied Biosystems variable-wavelength UV-absorbance detector Model 785A (Foster City, CA, USA) with the wavelength set at 270 nm. Column temperature was maintained by a Euramark Model EU-255 column thermostat (Mt. Prospect, IL, USA) which utilizes Peltier cooling. The stationary phase used was amylose-based tris-3,5-dimethylphenyl carbamate (Chiralpak AD) coated on 10-μm silica gel, 25.0 × 0.46 cm (Daicel) available from Chiral Technologies (Exton, PA, USA). Data collection and reporting was by a VG Multichrom data acquisition system.

The Chiralpak AD-R (reversed-phase) column was created from a standard Chiralpak AD column received from Chiral Technologies. It was gradually changed over from hexane to 2-propanol, then to methanol, and finally ethanol. A slow gradient and low flow-rate were used to avoid too high pressure.

2.3. Chromatographic conditions

All sample solutions were approximately 2 mg/ml dissolved in ethanol. Sample solutions were stable for at least 24 h. A 25μ-l aliquot was injected. The Chiralpak AD column was investigated using both normal-phase (hexane, ethanol, 2-propanol and diethylamine) and reversed-phase (water, ethanol, methanol, 2-propanol, acetonitrile and diethylamine) at various flow-rates and column temperatures. Final normal-phase conditions used a mobile phase of 800 ml hexane, 200 ml ethanol and 3 ml diethylamine. The flow-rate was 1.2 ml/min at 23°C. The reversed-phase conditions used the same column and sample solutions. The mobile phase consisted of 750 ml water, 250 ml ethanol and 5 ml diethylamine. A flow-rate of 0.2 ml/min was used and the column temperature was maintained at 0°C.

2.4. Determination of elution order

All four stereoisomers were analyzed by HPLC individually and compared to analyses of nadolol and racemates A and B. The elution orders are shown in Fig. 2 and Fig. 3. The elution order of the four stereoisomers is the same in both normal- and reversed-phase systems.

3. Results and discussion

Both the normal- and the reversed-phase systems resolve the four stereoisomers of nadolol. Fig. 2 shows the normal-phase separation. All four compounds are baseline resolved. The reversed-phase separation is shown in Fig. 3. Two of the four compounds are baseline resolved. Since the normal-phase separation was superior, it was evaluated for accuracy and reproducibility.

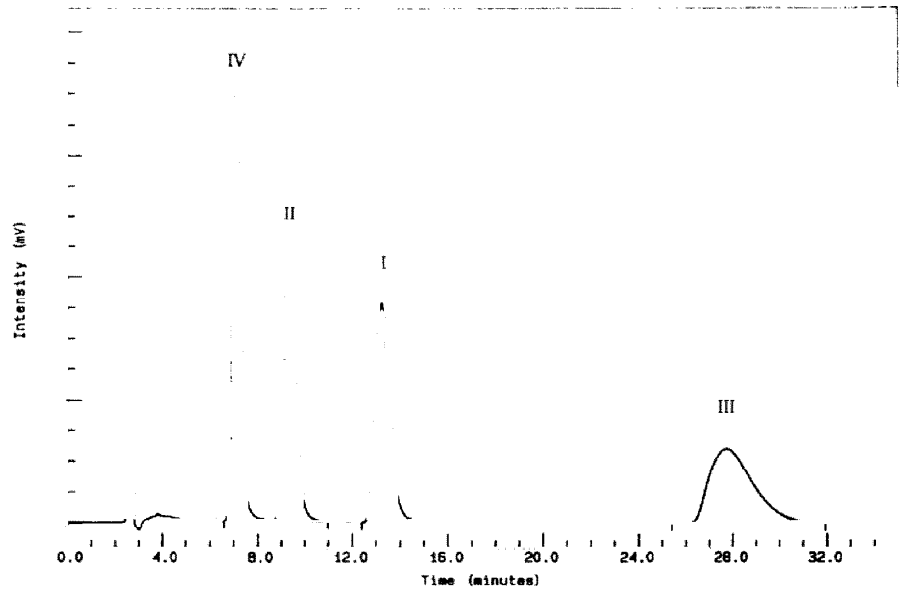


Fig. 2. Resolution of the four stereoisomers of nadolol in normal-phase mode using hexane–ethanol–diethylamine (80:20:0.3).

3.1. Normal-phase separation

During development of the normal-phase system, alcohol type, alcohol concentration, diethylamine concentration, flow-rate and column temperature were varied to determine their effect on resolution. Many combinations baseline resolved all four stereoisomers.

Alcohol type and concentrations were varied in mobile phases containing hexane and diethylamine. Ethanol, 2-propanol and combinations of the two were investigated. A systematic ap-

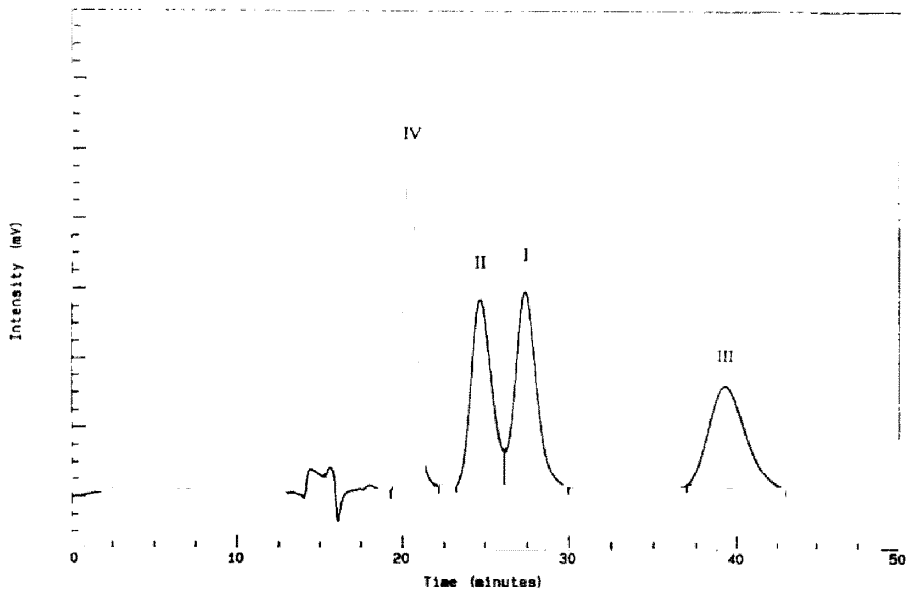


Fig. 3. Resolution of the four stereoisomers of nadolol in reversed-phase mode using ethanol–water–diethylamine (25:75:0.5).

proach was taken varying the alcohol concentrations in 2% increments from hexane–ethanol–2-propanol (75:25:0) to (75:0:25). As the concentration of ethanol decreased and 2-propanol increased, compounds IV and II eluted closer together. Hexane–ethanol (80:20) and hexane–ethanol–2-propanol (77:15:8) baseline resolved all four stereoisomers. When hexane–2-propanol was used without ethanol, compounds IV and II merged and compounds I and III were only partially resolved.

Diethylamine was necessary to separate the four stereoisomers. At concentrations less than 0.3%, the peaks tailed into one another and could not be quantitated. At levels higher than 0.3%, up to 1.0%, neither peak shape nor resolution improved.

Various flow-rates and column temperatures were also investigated. Several combinations of mobile phases at different flow-rates and temperatures baseline resolved all four stereoisomers. Table 1 shows examples of mobile phase/flow-rate/column temperature combinations that baseline resolved all four stereoisomers. As expected, lower column temperatures and less alcohol increased retention. However these changes did not always improve resolution (R_s) or separation (α) of the critical pair (the closest eluting pair of stereoisomers). By increasing the ethanol concentration to 25% and cooling the column to 10°C, resolution and separation of the critical pair compounds IV and II were increased. Since column coolers are sometimes inconvenient and not universally used, the normal-phase conditions at room temperature were studied further.

Accuracy and reproducibility of this method to quantitate levels of the four stereoisomers in synthetic mixtures were determined. Mixtures at ratios of approximately 80:20, 60:40, 40:60 and 20:80 of racemates A:B were weighed and the experimental results were compared to the theoretical levels. Detector response was measured for the individual stereoisomers and corrections were made for contamination of the individual stereoisomers with the other stereoisomers. The averages of four replicate analyses of each mixture are summarized in Table 2. All experimental results were within 99.1–101.5% of theoretical racemate A levels. The mixture of A–B (80:20) was injected five times and statistically analyzed for reproducibility. The relative standard deviations (R.S.D.s) ranged from 0.18 to 1.36%.

In several batches of the individual stereoisomers which were synthesized, trace levels of the other three stereoisomers have been quantitated. Levels as low as 0.10%, measured by area counts, have been quantitated. Linearity, however, was not determined.

3.2. Reversed-phase separation

Nadolol is classified as a basic drug, similar to propranolol and pindolol. Therefore, mobile phases containing chaotropic modifiers, as suggested by Ishikawa and Shibata [9], were investigated. In addition, diethylamine was investigated because it reduces tailing in other normal-phase and reversed-phase chromatographic applications.

Various organic modifiers were evaluated in combination with water. Ethanol was the only modifier which resolved the stereoisomers satisfactorily. Methanol, 2-propanol and acetonitrile were substituted for ethanol and did not resolve nadolol into four peaks. Diethylamine was necessary at a level of 0.5% to reduce tailing and resolve the four peaks. Lowering the column temperature and flow-rate retained nadolol longer and improved the separation. Lowering the concentration of ethanol was investigated as a means of increasing retention but did not improve resolution. The combination of a high concentration of ethanol, low column temperature and low flow-rate were necessary to resolve nadolol. The four stereoisomers were best resolved with ethanol–water–diethylamine (25:75:0.5) at a flow-rate of 0.2 ml/min and column temperature maintained at 0°C. However, compounds II and I were not baseline resolved. The R_s was calculated to be 1.0.

The separation of nadolol on the AD-R column did not behave similarly to propranolol and pindolol on the OD-R column. The chaotropic modifiers $NaPF_6$ and $NaClO_4$ had little or no

Table 1
Resolution and capacity factors under normal-phase conditions

Mobile phase (%)			Flow-rate (ml·min)	Column temperature (°C)	Capacity factors				Critical pair	R_s critical pair	Separation of critical pair
Hexane	Ethanol	2-Propanol			Peak IV	Peak II	Peak I	Peak III			
84	16	0	1.1	23	2.84	3.91	7.06	13.61	IV, II	2.78	1.38
80	20	0	1.2	23	1.71	2.55	4.01	9.46	IV, II	2.94	1.49
80	20	0	1.2	10	2.35	3.41	6.58	13.03	IV, II	2.84	1.45
75	25	0	1.2	0	1.63	2.67	4.74	10.65	IV, II	2.23	1.64
75	25	0	1	10	1.25	2.02	3.17	6.85	IV, II	3.22	1.61
75	25	0	1	19	1.13	1.91	2.49	6.58	II, I	2.5	1.31
75	25	0	1	23	1.13	1.86	2.41	6.4	II, I	2.1	1.29
84	6	10	1.1	23	2.31	2.31	3.99	4.91	IV, II		–
78	11	11	1.1	23	1.53	2.02	3.75	6.14	IV, II	1.58	1.33
77	15	8	1.1	23	1.57	2.31	4.24	8.6	IV, II	2.53	1.47
73	18	9	1	23	1.15	1.77	2.98	6.98	IV, II	1.86	1.54
77	20	3	1.1	23	1.57	2.17	4.29	8.06	IV, II	2.74	1.39

Table 2
Accuracy of normal-phase method to quantitate levels of racemates A and B

Racemate A			Racemate B	
Theoretical	Experimental	Accuracy (%A)	Theoretical	Experimental
77.66	78.8	101.47	22.34	21.2
58.86	59.44	100.99	41.14	40.56
40.06	39.71	99.13	59.94	60.29
21.26	21.09	99.20	78.74	78.91

effect resolving nadolol. A complete study of AD-R similar to Ishikawa and Shibata's has not yet been conducted to compare it to OD-R and determine the full versatility of AD-R. Nevertheless, AD-R has been used successfully in this laboratory for applications which were unsuccessful using OD-R. Typically, acetonitrile or methanol and water yield the best separations.

4. Conclusions

Nadolol was best resolved into its four stereoisomers on Chiralpak AD under normal-phase conditions. All four compounds were baseline resolved using hexane–ethanol–diethylamine (80:20:0.3) at room temperature. The final conditions resulted in an R_s value of 2.94 with an α value of 1.49 for the critical pair, compounds IV and II. Cooling the column and increasing the ethanol concentration improved resolution of the critical pair. Resolution of the four stereoisomers was dependent on alcohol type and the presence of diethylamine. The accuracy and reproducibility of this method were determined to be acceptable. This method can be used for determining levels of all four stereoisomers in current nadolol drug substance and can be used to determine trace levels of other stereoisomers in the individual stereoisomers synthesized.

Using the reversed-phase conditions of ethanol–water–diethylamine (25:75:0.5), nadolol could be resolved into four distinct peaks. Compounds IV and III were baseline resolved and compounds II and I had an R_s value of 1.0. Using Chiralpak AD in reversed-phase mode has advantages over normal-phase even though nadolol was baseline resolved for only two of the four stereoisomers. The reversed-phase separation replaces highly flammable hexane with water for utilization in laboratories that prefer to avoid using hexane. This reversed-phase separation may also be adaptable to LC–MS. Utilizing Chiralpak AD in the reversed-phase mode is novel and still in the investigational stage. However, it has been successful in this laboratory for several other applications in which OD-R was unsuccessful. Work is continuing with AD-R.

Acknowledgements

I wish to thank Ms. Rita Fox for synthesizing each of the four stereoisomers of nadolol. Additionally, I wish to thank Dr. Jack Gougoutas for determining the absolute conformations of the stereoisomers by X-ray diffraction.

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