Journal of Chromatography, 500 (1990) 629–636
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 668

ENANTIOSELECTIVE DETERMINATION OF METOPROLOL IN PLASMA BY LIQUID CHROMATOGRAPHY ON A SILICA-BONDED α_1-ACID GLYCOPROTEIN COLUMN

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(Received May 26th, 1989)

SUMMARY

The enantiomers of metoprolol were determined in plasma samples after direct resolution on a silica bonded α_1-acid glycoprotein column. Metoprolol was extracted from plasma into a diethyl ether–dichloromethane mixture and after back extraction to dilute phosphoric acid and adjustment of pH the sample was injected on a Chiral-AGP column for separation of *R*- and *S*-metoprolol. It was possible to measure down to 2 nmol per litre plasma with a relative standard deviation of less than 15% by use of gradient elution and fluorescence detection. The analytical method was employed to study the pharmacokinetics of the metoprolol enantiomers after administration of the racemate to humans.

INTRODUCTION

The great difference in pharmacological effect and pharmacokinetics between the two enantiomeric forms of many drugs[1] has in recent years emphasized the need of methods for enantioselective separation and determination in biological samples. For β-adrenoceptor agents such as propranolol the ratio between the concentrations of the *S*- and *R*-forms 2 h after dose may vary from 1 to 2 (ref. 2).

Column liquid chromatography can be employed for the resolution of the enantiomers of metoprolol using different approaches such as derivatization with a chiral reagent to form diastereomers[3], chemically bonded chiral stationary phases[4] and chiral complexing or ion-pairing agents in the mobile phase[5,6]. The methods used for determination in biological samples must beside good resolution also give high sensitivity, which can be achieved by techniques that combine high detectability with a small peak width.

Recently a paper was published on the determination of the metoprolol enantiomers in plasma by derivatization with the chiral reagent 2,3,4,6-tetra-O-acetyl-β-D-glycopyranosyl isothiocyanate and separation of the diastereomers[7]. In a pa-

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per on direct separation of metoprolol enantiomers coupled columns were used with a chiral stationary phase, Chiral-AGP, as the precolumn and two C_{18} columns as analytical columns to enhance both the peak height and detectability[8].

In the present study we used α_1-acid glycoprotein as a chiral stationary phase. A new commercial generation of this material, Chiral-AGP, with a particle size of 5 μm and improved chromatographic performance, was developed by Hermansson *et al.*[9] We applied this phase for the separation of *R*- and *S*-metoprolol in plasma samples after a liquid–liquid extraction procedure. By use of gradient elution and fluorescence detection[10] such a sensitivity was obtained that down to 2 nmol/l of plasma (0.5 ng/ml) of each enantiomer were determined with a relative standard deviation of less than 15%.

EXPERIMENTAL

Chemicals and reagents

Acetonitrile was of HPLC grade S (Rathburn, Walkerburn, U.K.), dichloromethane, 2-propanol and hexane of HPLC grade (Rathburn), diethyl ether of pro analysi quality (May & Baker, Dagenham, U.K.), phosphate buffer substances of p.a. grade (E. Merck, Darmstadt, F.R.G.). *RS*-metoprolol tartrate, the separate *R*- and *S*-enantiomers and the internal standard, 2-dehydroxymetoprolol, were obtained from the Department of Organic Chemistry, Hässle, Mölndal, Sweden.

Chromatographic system

The chromatographic system comprised an autosampler, Waters WISP 710B (Waters Assoc., Milford, MA, U.S.A.), an LC-pump LKB 2150 (Bromma, Sweden), with a gradient unit LKB 2152, a fluorescence detector, Perkin-Elmer LS-4 (Norwalk, CT, U.S.A.) or Jasco 820-FP (Tokyo, Japan), and an integrator Spectra-Physics SP 4270 (San José, CA, U.S.A.). The chiral column Chiral-AGP, 5-μm particles, 100 mm × 4.0 mm, was obtained from ChromTech (Stockholm, Sweden) and the EnantioPac column from LKB. The analytical column was preceded by a Brownlee Aquapore Diol guard column (15 mm × 3.2 mm; Brownlee Labs, CA, U.S.A.). The temperature of the analytical column was controlled by immersing it in a thermostat bath (RMS; Lauda, Königshofen, F.R.G.).

The volumes injected were between 5 and 200 μl. The flow-rate of the pump was 0.5 and 0.3 ml/min for Chiral-AGP and EnantioPac respectively. The excitation and emission wavelengths for the fluorescence detector were 228 or 272 and 306 nm respectively. The mobile phases for the gradient system contained phosphate buffer pH 7.0 (ionic strength, $I = 0.02$) with 0 (phase A) and 10% (phase B) of acetonitrile. The linear gradient profile was: 1–51% of B in 0–12.5 min, then 51% of B for another 12.5 min and then back to 1% of B and finally equilibration for 10 min before the next injection. The dead volume of the gradient system was 2.0 ml, which was taken into account by making the sample injection coincide with the gradient reaching the column inlet. Isocratic elution used a mobile phase containing 2.2% of acetonitrile in phosphate buffer pH 7.0 ($I = 0.02$) (22% of B).

Standard solutions and plasma standards

A standard solution of *RS*-metoprolol was prepared in 0.01 *M* hydrochloric

acid at a concentration corresponding to 4 μmol/l of each enantiomer. The solution can be stored for up to 6 months in a refrigerator. Plasma standards were prepared daily by adding 100 μl of the standard solution to 1 ml of drug-free human plasma, the concentration of each enantiomer being 400 nmol/l of plasma. Quality control samples containing 200 nmol/l of each enantiomer in drug-free pooled human plasma were prepared. The quality controls can be stored for up to 1 year at −18°C without any degradation. The internal standard solution contained 1.5 μmol/l of 2-dehydroxymetoprolol in 0.01 *M* HCl.

Analytical procedure

A 1-ml volume of plasma was mixed with 100 μl of internal standard solution and 100 μl of 1 mol/l NaOH. The compounds were extracted into 5 ml of diethyl ether–dichloromethane (4:1) with a reciprocating shaker for 15 min at 250 rpm. After centrifugation at 2000 *g*, the aqueous phase was frozen in a dry ice–ethanol. The organic extract was poured into a new tube containing 200 μl of 0.01 *M* phosphoric acid. After agitation for 5 min and centrifugation, the aqueous phase was frozen and the organic phase discarded. The aqueous phase was washed by shaking with 1 ml of hexane for 1 min, followed by centrifugation, freezing and discarding of the organic phase. The pH was adjusted to about 7.0–7.5 by adding 30 μl of 0.5 mol/l disodium hydrogenphosphate. Volumes of 50–100 μl were injected onto the LC column.

Quantification and quality control

The ratio of the peak height of each compound to that of the internal standard in the plasma standards was measured and the median value used for daily calibration. A full standard plot, covering the range 2–800 nmol/l, for examination of the linearity was made intermittently. The limit of determination was defined as the concentration giving a signal-to-noise ratio of 5 or more and a relative standard deviation of 10–15%.

The intra-day variability was determined by performing replicate analysis (n = 10) of spiked plasma samples containing 2, 10, 50 and 250 nmol/l. The inter-day variability was determined by assaying two quality control samples each day of analysis, and calculating the relative standard deviation for the whole batch of control samples (n = 24).

Determination of recovery

The absolute recovery of the analytical procedure was determined by comparison with aqueous solutions injected directly into the chromatograph.

RESULTS AND DISCUSSION

A comparison between the two α_1-acid glycoprotein phases EnantioPac and Chiral-AGP is shown in Table I, where data for the separation of *R*- and *S*-metoprolol using isocratic elution are presented. The separation factor, α, was lower with the new packing material Chiral-AGP than with EnantioPac, but the much higher theoretical plate number for the new material gave improved resolution, R_s, despite the higher flow-rate.

Metoprolol was eluted with acetonitrile as a modifier. 2-Propanol may also be

TABLE I

COMPARISON OF THE ENANTIOPAC AND CHIRAL-AGP COLUMNS

Aqueous solutions of *R,S*-metoprolol (10 μl, containing 3000 and 1000 nmol/respectively) were injected. The theoretical plate number, N, α and R_s are given. Temperature: 13°C. The mobile phase content of acetonitrile was 1.25 (I) and 1.65% (II). pH 7.0. Flow-rate 0.3 (EnantioPac) and 0.5 ml/min (Chiral-AGP).

	N	α	R_s
(I) EnantioPac	400	1.7	1.7
(II) Chiral-AGP	2000	1.4	3.3

used, however, the concentration expressed as volume percent may then be halved to give the same retention. No significant difference in separation factor, column efficiency or resolution was noticed between the two organic modifiers.

On the Chiral-AGP column metoprolol is eluted with baseline separation at a low content of organic modifier in the mobile phase. In a solvent gradient elution the separation factor of the enantiomers is decreased. Studies have shown that the decrease in the difference between the retention times is balanced by the peak compression effect, when the gradient is moderately steep[10], but the R_s value is still the same as in an isocratic elution at comparable retention times. In a properly designed gradient elution system a better sensitivity is obtained compared with isocratic elution at the same resolution.

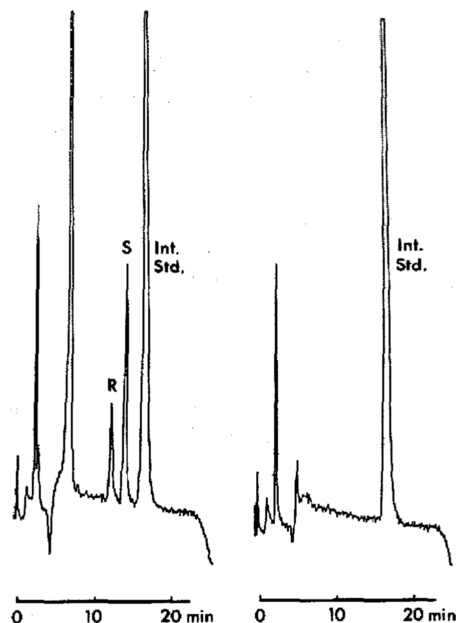

Fig. 1. Gradient elution on a Chiral-AGP column of *R*- and *S*-metoprolol and the internal standard (Int. Std.) extracted from an authentic plasma sample and a plasma blank according to the analytical procedure. The concentration of the *R*- and *S*-enantiomers in the sample are 9 and 22 nmol/l of plasma respectively. A 50-μl volume of the sample extract was injected. Temperature: 20°C. Detector: Perkin-Elmer LS-4, 228/306 nm.

With a linear solvent strength gradient it was found that the sensitivity was the same for all the solutes eluted during gradient conditions, quite in accordance with the relationships between sensitivity, steepness of the gradient and k' of solute bands leaving the column given by Snyder[11]. This implies that the enantiomers in a racemate give peaks of equal height. The gradient technique can be applied to extracts from plasma to increase the sensitivity for both the enantiomers but it is of vital importance that factors like temperature, pH and purity of the sample are well controlled. A chromatogram obtained with gradient elution of an extract from a plasma sample taken from a patient is given in Fig. 1. An isocratic elution of the same sample is seen in Fig. 2.

The separation on the α_1-acid glycoprotein phases Enantiopac and Chiral-AGP is strongly dependent on temperature. Results obtained on Chiral-AGP are shown in Table II. Capacity factors, k', α and R_s increase at lower temperature. However, if the resolution is sufficient, higher sensitivity is obtained at higher temperature. It is obviously of great importance to thermostat the Chiral-AGP column in order to get results with good precision, if the temperature in the laboratory fluctuates.

The separation of metoprolol on Chiral-AGP was performed at pH 7.0. Even a small change of pH will influence the retention on the protein phase[10] and a lower pH in the injected sample may cause peak distortion. The sample preparation includes back extraction into an acidic aqueous phase which has to be adjusted to pH 7.0–7.5 before injection in order to match the mobile phase. Under these conditions sample volumes of up to 150 μl may be injected onto the column without significant decrease (10%) in column efficiency compared with a 10-μl injection. If the back-extraction step is replaced by evaporation of the organic phase and redissolution in the mobile phase, peaks from plasma components may disturb the metoprolol enantiomers.

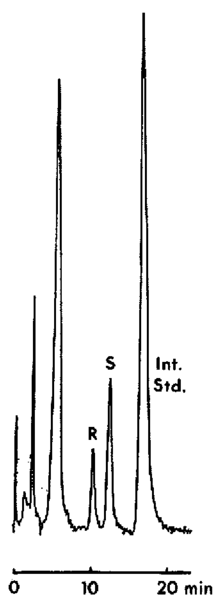

Fig. 2. Isocratic elution on a Chiral-AGP column of *R*- and *S*-metoprolol extracted from a plasma sample taken from a patient according to the analytical procedure. Other details as in Fig. 1.

TABLE II

INFLUENCE OF TEMPERATURE ON RETENTION TIMES, t_R, N, α AND R_s DETERMINED ON CHIRAL-AGP

Experimental conditions as in Table I.

Temperature (°C)	*R-Metoprolol*		*S-Metoprolol*		α	R_s
	t_R	N	t_R	N		
7	14.2	1620	19.6	1830	1.44	3.3
10	13.6	1770	18.6	2080	1.43	3.4
13	13.1	1780	17.7	2120	1.42	3.3
16	12.8	1910	16.8	2260	1.38	3.1
19	12.5	1700	16.4	2330	1.37	3.1
22	12.1	2160	15.6	2390	1.34	3.0
25	11.8	2100	14.9	2580	1.32	2.8
28	11.5	2260	14.2	2650	1.30	2.6

The metoprolol enantiomers are detected by their fluorescence at 306 nm (ref. 10). The Perkin-Elmer fluorescence detector (illuminated cell volume 3 μl) gave by excitation at 228 nm a signal-to-noise ratio which was four times higher than by excitation at 272 nm. The Jasco detector equipped with a microcell (6.5 μl) has about the same signal-to-noise ratio at 228 and 272 nm as the Perkin-Elmer detector at 228 nm. The background disturbance from the gradient may be troublesome in the low wavelength region, if the solvents are not pure enough. The column efficiency is decreased by the Jasco detector in particular with the normal cell (16 μl) and the gradient programme has to be slightly modified by a less steep gradient to counteract this. With the larger cell the Jasco detector gives 3–4 times higher sensitivity at 272 nm. Morover it has, irrespective of cell volume, a much larger linearity range compared with the Perkin-Elmer detector.

Quantitative evaluation

The method for quantitation of *R*- and *S*-metoprolol in plasma samples includes liquid–liquid extraction at pH 12 with a mixture of diethyl ether and dichloromethane (4:1, v/v), back extraction into 0.01 *M* phosphoric acid, washing with hexane

TABLE III

RELATIVE STANDARD DEVIATION (R.S.D., n = 10) AND ABSOLUTE RECOVERY OF *R*- AND *S*-METOPROLOL IN PLASMA SAMPLES

Column: Chiral-AGP. Gradient elution was used. Temperature: 22°C.

Concentration (nmol/l)	*Absolute recovery (%)*		*R.S.D. (%)*	
	R-	*S-*	*R-*	*S-*
2	102	89	15.1	11.8
11	91	93	5.8	8.0
47	91	91	4.0	2.4
235	95	95	1.7	1.5

to remove dissolved extraction solvent and finally adjustment to pH 7.0–7.5 and injection onto the Chiral-AGP column (*cf.* ref. 12). The absolute recovery, ranging from 90 to 95% for each enantiomer and the intra-day variability, determined at four different concentration levels, are given in Table III. With a good column and using gradient elution, about 2 nmol/l (0.5 ng/ml) of each enantiomer can be determined with a relative standard deviation of less than 15%.

The inter-day variability was calculated after analysis of plasma quality control samples, two samples a day, during 2 months. The found concentration of each enantiomer was 99–100% of the nominal concentration at a level of 200 nmol/l and the variability was 4.7 and 4.2% for *R*- and *S*-metoprolol respectively. The method has been used for the analysis of 500 samples in a pharmacokinetic study where the concentration range was 0–1000 nmol/l. An example of a plasma curve is given in Fig. 3.

Column maintenance

The efficiency of the Chiral-AGP column expressed as the theoretical plate number decreased after some use but the separation factor, α, remained constant. If the injected solutions were freed from plasma residue particles and kept at an appropriate pH, as many as 150–200 injections were made before a decrease in efficiency was noticed. Reactivating the column by a low flow of 10% 2-propanol in 0.01 *M* phosphoric acid was often successful[13]. This was regularly performed when analysing a large number of plasma extracts, in order to keep the column in good condition. Injections of metoprolol dissolved in mobile phase did not cause any change in efficiency.

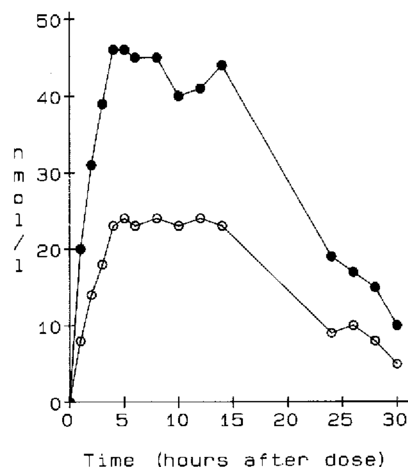

Fig. 3. Plasma concentrations of *R*- (○) and *S*-metoprolol (●) after administration of a controlled release tablet containing 200 mg of the racemate.

REFERENCES

1 E. J. Ariens, *Eur. J. Clin. Pharmacol.*, 26 (1984) 663.
2 C. von Bahr, J. Hermansson and K. Tawara, *Br. J. Clin. Pharmacol.*, 14 (1982) 79.
3 A. J. Sedman and J. Gal, *J. Chromatogr.*, 278 (1983) 199.
4 J. Hermansson and G. Schill, in M. Zief and L. J. Crane (Editors), *Chromatographic Chiral Separation*, Marcel Dekker, New York, 1987, p. 245.
5 C. Pettersson and M. Josefsson, *Chromatographia*, 21 (1986) 321.
6 C. Pettersson and G. Schill, *J. Liq. Chromatogr.*, 9 (1986) 269.
7 D. Schuster, M. Woodruff Modi, D. Lalka and F. M. Gengo, *J. Chromatogr.*, 433 (1988) 318.
8 A. Walhagen and L.-E. Edholm, *J. Chromatogr.*, 473 (1989) 371.
9 J. Hermansson, K. Ström and R. Sandberg, *Chromatographia*, 24 (1987) 520.
10 K. Balmér, B. A. Persson and G. Schill, *J. Chromatogr.*, 477 (1989) 107.
11 L. R. Snyder, in Cs Horváth (editor), *High Performance Liquid Chromatography — Advances and Perspectives*, Vol. 1, Academic Press, New York, 1980, p. 207.
12 K. Balmér, Y. Zhang, P.-O. Lagerström and B.-A. Persson, *J. Chromatogr.*, 417 (1987) 357.
13 I. Hermansson, personal communication.