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Liquid chromatographic determination of total celiprolol or (*S*)-celiprolol and (*R*)-celiprolol simultaneously in human plasma

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Abstract

A method has been developed for the determination of total celiprolol (sum of enantiomers) or the enantiomers (*R*)-celiprolol and (*S*)-celiprolol in plasma by high-performance liquid chromatography with UV and fluorescence detection. After extraction from alkalinized plasma with methyl-*tert.*-butyl ether and back-extraction into 0.01 *M* HCl (for total celiprolol determination) or after evaporation of the organic phase and derivatisation with *R*(−)-1-(1-naphthyl)ethyl isocyanate (enantiomer determination), total celiprolol or its diastereomeric derivatives were chromatographed on a reversed-phase HPLC column with a mixture of acetonitrile and phosphate buffer pH 3.5 (+0.05% triethylamine). Acebutolol was used as internal standard. Linearity was obtained in the range of 5 to 2000 ng/ml for total and 2.5 to 500 ng/ml for enantiomer determination. Intra-day and inter-day variation was lower than 10%. The method can be applied for analysis of plasma samples obtained from patients treated with oral racemic celiprolol doses.

Keywords: Enantiomer separation; Celiprolol

1. Introduction

(*R,S*)-Celiprolol hydrochloride, 3-[3-acetyl-4-(*tert.*-butylamino - 2 - hydroxypropoxy)phenyl]-1,1-diethylurea hydrochloride, is a β_1-selective adrenergic antagonist with weak vaso- and broncho-dilating effects. Celiprolol was introduced in 1985 and used as racemate. Several pharmacokinetic studies have been performed with the racemic drug [1]; radioligand binding studies however showed that the enantiomers of celiprolol have different pharmacodynamic properties and therefore stereospecific evaluation of the pharmacokinetics is appropriate [2]. The available analytical methods permit quantification of the total celiprolol only [3–5]; only one method describes the stereospecific determination of (*R*)- and (*S*)-celiprolol, using a chiral column without derivatisation of the compounds [6].

The enantiomers of a number of β-adrenoceptor blocking agents were separated using the chiral reagent *R*(−)-1-(1-naphthyl)ethyl isocyanate (NEIC) for derivatisation [7–9]. The naphthyl moiety of NEIC contributes strong UV and fluorescence characteristics to the derivatised molecule [10].

We developed a non-enantiospecific method for total celiprolol and also a stereospecific method for determination of the enantiomers of celiprolol by using a reversed-phase HPLC column after derivatisation of celiprolol to diastereomers with NEIC.

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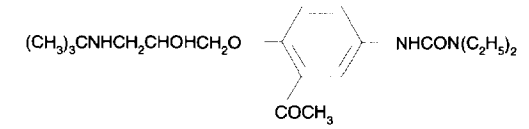
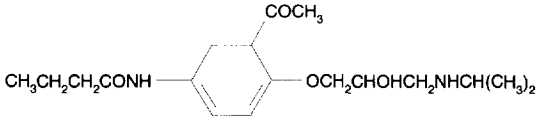


Celiprolol



Acebutolol

Fig. 1. Structures of celiprolol and acebutolol (internal standard).

Structures of celiprolol and the internal standard acebutolol are shown in Fig. 1.

2. Experimental

2.1. Chemicals

Racemic celiprolol hydrochloride, (*R*)-celiprolol hydrochloride and (*S*)-celiprolol hydrochloride were obtained from Hafslund Nicomed Pharma (Linz, Austria). Racemic acebutolol hydrochloride was obtained from Specia (Paris, France). Acetonitrile, methanol, methyl-*tert.*-butyl ether and triethylamine (TEA) were HPLC grade and purchased from Rathburn Chemicals (Walkerburn, UK). *R*(−)-1-(1-Naphthyl)ethyl isocyanate (NEIC) was supplied by Fluka (Buchs, Switzerland) and was claimed to have an optical purity >98%. No further attempts have been undertaken to check this value. All other reagents and solvents were analytical grade and obtained from Merck (Darmstadt, Germany).

2.2. Reference, internal standard and derivatisation reagent solutions

Stock solutions of celiprolol hydrochloride and acebutolol hydrochloride were prepared in methanol at a concentration (as base) of 1 mg/ml and stored at 4°C for one month. Daily dilutions of celiprolol and acebutolol were prepared in a mixture of methanol–water (1:1). Stock solution of NEIC was made in hexane at a concentration of 0.1% (v/v) and stored at −20°C for one month.

Before derivatisation, the stock solution of NEIC was diluted daily to 0.005% in a mixture of hexane (+5% 2-propanol).

2.3. Equipment

An LC3-XP pump (Pye Unicam) was used in combination with an SPD-6A UV detector (Shimadzu) or F-1000 fluorescence detector (Merck–Hitachi) and a 717 plus autosampler (Waters). Data integration was performed by a SP-4290 integrator (Spectra-Physics).

2.4. Sample preparation

2.4.1. Total celiprolol determination

To 1 ml of plasma were added respectively 50 μl of internal standard solution (1.4 μg/ml acebutolol), 1 ml of 0.067 *M* phosphate buffer pH 7.4, 0.2 ml of 1 *M* sodium hydroxide and finally 6 ml of methyl-*tert.*-butyl ether. After shaking for 10 min and centrifuging for 5 min at 1300 *g* at 4°C, the upper organic layer was transferred into a new extraction tube, after freezing the aqueous layer in a cooling mixture of acetone and dry ice. To the organic phase was added 0.2 ml of 0.01 *M* hydrochloric acid; the samples were shaken again for 10 min and centrifuged for 5 min at 1300 *g*.

The organic phase was discarded after freezing the aqueous layer in a mixture of acetone–dry ice.

A cold air stream was blown for 3–4 min over the aqueous layer for elimination of any traces of the organic layer.

Finally, after thawing, 180 μl of the acidic layer were transferred to glass injection vials (300 μl) for automatic injection; the injected volume was 20 μl.

2.4.2. Stereospecific celiprolol determination

To 1 ml of plasma were added 50 μl of internal standard solution (0.4 μg/ml acebutolol), 1 ml of 0.067 *M* phosphate buffer pH 7.4, 0.2 ml of 1 *M* sodium hydroxide and finally 6 ml of methyl-*tert.*-butyl ether. After shaking for 10 min and centrifuging for 5 min at 1300 *g* at 4°C, the upper organic layer was transferred into a conical glass tube (after freezing the aqueous layer in a mixture of acetone–dry ice) and evaporated to dryness with an air stream in a waterbath at 40°C.

To the dry residues was added 100 μl of 0.005% NEIC, vortex-mixed and stoppered. After a reaction time of 30 min the tubes were evaporated again with air at 35°C. The residues were dissolved in 200 μl of the mobile phase for separation of the diastereomers. 180 μl were transferred to glass injection vials (300 μl) for automatic injection; 20 μl were injected onto the column.

2.5. Chromatography

For determination of total celiprolol a reversed-phase HPLC column packed with 5 μm Spherisorb hexyl (150×4.6 mm I.D.) was used (Phase Separations); for separation of the diastereomers of celiprolol a column packed with 5 μm Hypersil BDS C_{18} (250×4.6 mm I.D.) was used (Alltech Associates).

The mobile phase for the separation of total celiprolol and total acebutolol was acetonitrile–15 m*M* potassium dihydrogenphosphate buffer pH 3.5 (+0.05% TEA) (45:55, v/v) and pumped at a flow-rate of 1 ml/min.

For separation of the diastereomers of celiprolol and acebutolol a mixture of acetonitrile and 15 m*M* potassium dihydrogenphosphate buffer pH 3.5 (+0.05% TEA) (50:50, v/v) was prepared and pumped at a flow-rate of 1 ml/min.

Total celiprolol and acebutolol were detected by UV at 238 nm; the diastereomers of celiprolol and acebutolol were detected by fluorescence at 350 nm and 480 nm as excitation and emission wavelength respectively.

2.6. Accuracy and reproducibility

Reproducibility was assessed by spiking plasma samples with 5, 10, 50, 250 and 1000 ng/ml total celiprolol and extracted in six-fold according to the method for total determination or the method for stereospecific determination. Intra-day coefficients of variation were calculated. Inter-day variability was determined at concentrations of 20, 100 and 500 ng/ml of total celiprolol and extracted according to the two methods over a period of six days.

2.7. Recovery

In order to determine the recovery, plasma samples were spiked with 5, 10, 50, 250, and 1000 ng/ml of total celiprolol, extracted in six-fold according to the described methods and the peak heights compared with non-extracted celiprolol and acebutolol dissolved in the respective mobile phases.

2.8. Linearity

Calibration curves were constructed in the range of 5–2000 ng/ml total celiprolol and 2.5–500 ng/ml of each enantiomer of celiprolol. Peak-height ratios of celiprolol (total or each enantiomer) to the internal standard (acebutolol 2) were plotted against concentrations of celiprolol (total or each enantiomer).

3. Results and discussion

For separation of total celiprolol and acebutolol and of the formed diastereomers of celiprolol and acebutolol a reversed-phase column was used. Back-extraction into acid resulted in clean and interference free chromatograms for total celiprolol and acebutolol determination. Compared to the direct determination of the enantiomers on a chiral column, our method shows good resolution between the formed diastereomers of celiprolol and acebutolol within 20 min; our internal standard acebutolol is structurally similar to celiprolol with the same extraction and fluorescence properties as celiprolol; the diastereomers after derivatisation are stable for at least 48 h and no interferences were seen coming from the derivatisation reagent NEIC. Purity of NEIC was checked by derivatising blank plasma or water extracts followed by injection onto the column. No interfering or late eluting peaks due to NEIC derivatisation were seen, as was the problem in

another published method [9] where a back-extraction step was needed. The reason for these interference-free chromatograms after NEIC derivatisation is the use of a very specific wavelength setting for the fluorescence detector.

Chromatograms of the separation of total celiprolol and acebutolol and of the separation of the enantiomers of celiprolol and acebutolol are shown in Fig. 2 and Fig. 3. The two diastereomeric derivatives of acebutolol were well separated, but we did not have reference substances of (*S*)-acebutolol and (*R*)-acebutolol; so we could not identify both enantiomers. Consequently we called the two compounds acebutolol 1 and acebutolol 2 in order of increasing retention time. Acebutolol 2 peak was used as an internal standard in all calculations, because a small interfering peak was seen with the same retention time as acebutolol 1.

Retention times of (*R*)-celiprolol and (*S*)-celiprolol were determined by injection of the diastereomeric derivatives of racemic celiprolol and the diastereomeric derivative of (*S*)-celiprolol separately. The (*R*)-celiprolol elutes before (*S*)-celiprolol.

A series of currently used β-adrenergic antagonists and anti-arrhythmic drugs like propranolol, oxprenolol, atenolol, alprenolol, metoprolol, propafenone and flecainide were subjected to the extraction and derivatisation procedures. None of these drugs showed any interference with the formed diastereomers of celiprolol and acebutolol because of the lack of fluorescence at the wavelength settings for celiprolol and acebutolol. None of the above mentioned drugs showed interference in the determi-

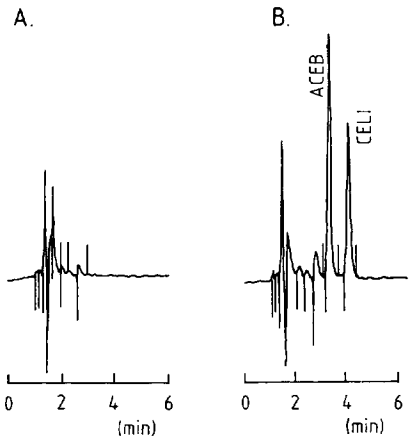


Fig. 2. Chromatograms of stereospecific determination of celiprolol: (A) blank plasma; (B) blank plasma spiked with 250 ng/ml total celiprolol (=125 ng/ml of each enantiomer) and 20 ng/ml total acebutolol (=10 ng/ml of each enantiomer).

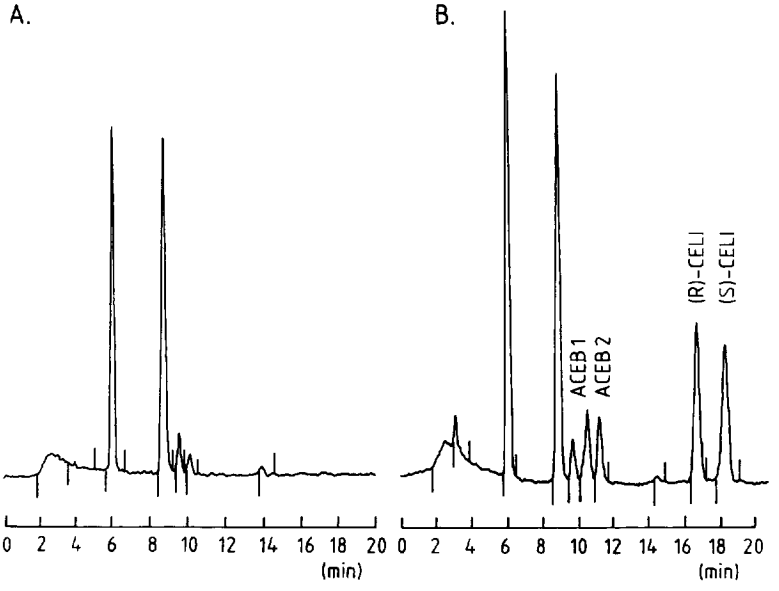


Fig. 3. Chromatograms of total celiprolol determination: (A) blank plasma; (B) blank plasma spiked with 50 ng/ml total celiprolol and 70 ng/ml total acebutolol.

nation of total celiprolol where UV-detection was used.

The analytical recovery of total celiprolol averaged 75.8±4.7% (mean±S.D.) over the whole concentration range (5–2000 ng/ml) of the calibration curve and for total acebutolol 83.3±4.3%.

The analytical recovery of the enantiomers of celiprolol and acebutolol was determined by comparing peak heights after extraction and derivatisation with the peak heights obtained after adding celiprolol and acebutolol to the final organic extract and derivatisation. The mean recovery for (*R*)-celiprolol and (*S*)-celiprolol was 73.6% and 73.2% and for acebutolol 1 and acebutolol 2, 73.3% and 75.2%. respectively.

The percentage of underivatised celiprolol and acebutolol could be measured directly in the same chromatograms of the diastereomers by comparing peak heights with a solution of racemic standards in the mobile phase. For celiprolol 4.8% was underivatised and for acebutolol 7.6%.

Linearity for total celiprolol was found in the range of 5–2000 ng/ml ($y=0.006+0.0124x$, $r=0.9993$) and for the enantiomers (*R*)-celiprolol and (*S*)-celiprolol in the range of 2.5–500 ng/ml ($y=0.005+0.017x$, $r=0.9977$ and $y=0.033+0.019x$, $r=0.9980$ respectively).

The lowest limit of quantitation was 5 ng/ml for the total determination and 2.5 ng/ml for each enantiomer of celiprolol; these limits were determined as being the lowest concentrations which could be determined with a coefficient of variation lower than 10%.

Table 1
Intra-day and inter-day accuracy and precision for determination of total celiprolol in plasma

Concentration added (ng/ml)	Intra-assay ($n=6$)		Inter-assay ($n=6$)	
	Concentration found (ng/ml)	C.V. (%)	Concentration found (ng/ml)	C.V. (%)
5	5.0	3.8		
20			20.3	4.6
50	49.8	1.2		
100			100.7	2.2
250	254.3	3.2		
500			494.8	3.0
1000	983.4	1.5		

Table 2
Intra-day and inter-day accuracy and precision for determination of (*R*)-celiprolol and (*S*)-celiprolol in plasma

Concentration added (ng/ml)	Intra-assay ($n=6$)		Inter-assay ($n=6$)	
	Concentration found (ng/ml)	C.V. (%)	Concentration found (ng/ml)	C.V. (%)
(R)-Celiprolol				
2.5	2.5	7.4		
5	4.9	5.8		
10			10.4	5.2
25	24.5	3.5		
50			50.9	7.8
125	126.1	2.7		
250			250.2	7.0
500	506.9	3.1		
(S)-Celiprolol				
2.5	2.6	2.9		
5	4.6	6.5		
10			10.3	7.7
25	25.0	1.6		
50			51.5	7.8
125	126.2	3.2		
250			249.4	6.3
500	506.9	4.1		

Intra-day and inter-day accuracy and precision for the total celiprolol and for respectively (*R*)-celiprolol and (*S*)-celiprolol extraction from plasma are given in Table 1 and Table 2.

4. Conclusion

Comparing our method to the methods published in the literature, we can say that our method is very reproducible and has good linearity and recovery. The diastereomers formed after derivatisation with NEIC are very stable and can be chromatographed within 20 min of run time. No interfering peaks from endogenous plasma constituents or from the chiral derivatisation reagent are seen.

The use of a reversed-phase HPLC column is less expensive than the chiral columns used in other methods; other advantages are the shorter retention times and the use of an internal standard with analogous chemical structure and physical properties.

In conclusion we can say that we developed a robust HPLC method for determination of total celiprolol or the enantiomers (*R*)-celiprolol and (*S*)-

celiprolol, which is fast, accurate and sensitive; it has applications for the analysis of celiprolol plasma samples in pharmacokinetic studies.

References

[1] J.G. Riddel, R.G. Shanks and R.N. Brogden, Drugs, 34 (1987) 438.
[2] P.S. Wolf, R.D. Smith, A. Khandwala, R.G. van Inwegen, R.J. Gordon, W.S. Mann, D.V. Romano and T.P. Pruss, Br. J. Clin. Pract., 39 (1985) 5.
[3] J.N. Buskin, R.A. Upton, F. Sörgel and R.I. Williams, J. Chromatogr., 230 (1982) 454.
[4] D.R. Rutledge, A.H. Abadi and L.M. Lopez, J. Pharmac. Biomed. Anal., 12 (1994) 134.
[5] D.R. Rutledge, A.H. Abadi and L.M. Lopez, J. Chromatogr. Sci., 32 (1994) 153.
[6] C. Hartmann, D. Krauss, H. Spahn and E. Mutschler, J. Chromatogr., 496 (1989) 387.
[7] G. Gubitz and S. Mihellyes, J. Chromatogr., 314 (1984) 462.
[8] A. Darmon and J.P. Thenot, J. Chromatogr., 374 (1986) 321.
[9] R. Mehvar, J. Chromatogr., 527 (1990) 79.
[10] I.S. Krull, Adv. Chromatogr., 16 (1978) 175.