ELSEVIER

Journal of Chromatography B, 664 (1995) 393–400

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Stereospecific high-performance liquid chromatographic determination of an S(−)-benzopyran methyl ester derivative (CGP 50 068), its (−)-carboxylic acid metabolite (CGP 55 461) and the related (+)-enantiomer (CGP 54 228) in human and dog plasma

P. Mangoni, A. Sioufi*, J. Godbillon

Bioanalytics and Pharmacokinetics, Ciba-Geigy Laboratories, 2-4 rue Lionel Terray, B.P. 308, 92506 Rueil-Malmaison Cedex, France

First received 1 July 1994; revised manuscript received 7 October 1994

Abstract

The simultaneous determination of CGP 50 068, *S*(−)-enantiomer (I), its (−)-carboxylic acid metabolite CGP 55 461 (II) and the related (+)-enantiomer CGP 54 228 (III) by stereospecific high-performance liquid chromatography, in human plasma, is described. The three compounds and racemic acebutolol, used as internal standard, were isolated from plasma by liquid–solid extraction on disposable C_{18} columns. The resolution and determination of I and the two carboxylic acid enantiomers were achieved by direct chromatography using a Chiral-AGP column refrigerated at 5°C. The mobile phase was tetrabutylammonium iodide in a pH 7 phosphate buffer solution used at a constant flow-rate of 0.5 ml/min. The UV detection wavelength was set at 270 nm. The reproducibility and accuracy of the method were found to be suitable over the concentration range 0.56–28.0 μmol/l for II and III and 2.0–26.7 μmol/l for I.

1. Introduction

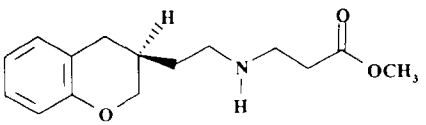
CGP 50 068, *S*(−) - β - alanin - N - [2 - (3,4 - dihydro - 2H - 1 - benzopyran - 3 - yl) - ethyl] methyl ester hydrochloride (I), is a nootropic compound under development. It is biotransformed into a (−)-carboxylic acid metabolite, CGP 55 461 (II) (Fig. 1).

A chiral analytical method was needed for characterization of the pharmacokinetics of these compounds in case of an interconversion between the (−)- and (+)-enantiomers of the carboxylic acid metabolite or in case of racemization.

This paper describes an enantioselective

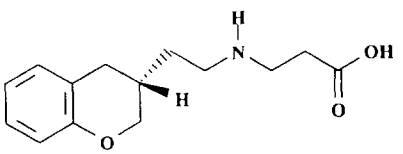
* Corresponding author.

0378-4347/95/$09.50
SSDI 0378-4347(94)00471-4



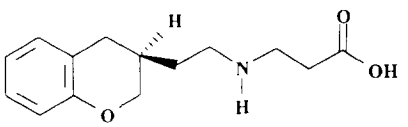
.HCl

CGP 50 068 S(–)



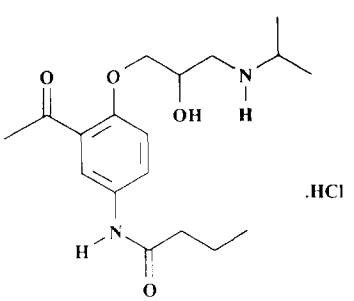
.HCl

CGP 55 461 (–)



.HCl

CGP 54 228 (+)



.HCl

Racemic acebutolol (I.S.)

Fig. 1. Chemical structures of the compounds.

HPLC method for the simultaneous determination of I and the two enantiomers II and III of the carboxylic acid metabolite in human plasma. Direct chromatography on a column containing α_1-acid glycoprotein as the chiral selector was chosen for resolution of the compounds.

2. Experimental

2.1. Chemicals and reagents

S(–)-CGP 50 068 A, ($C_{15}H_{21}NO_3$-HCl, M_r 299.80), (–)-CGP 55 461 A and (+)-CGP 54 228 A, ($C_{14}H_{19}NO_3$-HCl, M_r 285.77) were supplied by Ciba-Geigy (Basle, Switzerland).

Racemic acebutolol, ($C_{18}H_{28}O_4N_2$-HCl, M_r 372.89) was supplied by Laboratoires Specia (Paris, France).

The solvents and reagents used were all of analytical grade: methanol (414902, Carlo Erba, Italy); acetonitrile for HPLC ultragradient grade (9017, Baker, Deventer, Netherlands); Deionized water filtered and purified on a Milli-Q Reagent Water System; 0.1 *M* sodium acetate solution: 0.82 g sodium acetate anhydrous (6264, Merck, Darmstadt, Germany) are dissolved in 100 ml of water; $2 \cdot 10^{-2}$ *M* dipotassium hydrogen phosphate solution is obtained by dissolving 0.34 g K_2HPO_4 (5101, Merck) in 100 ml of water; pH 7 phosphate solution for the mobile phase: 13.2 g potassium dihydrogen phosphate KH_2PO_4 (471686, Carlo Erba) and 14.9 g disodium hydrogen phosphate anhydrous Na_2HPO_4 (6586, Merck) dissolved in one litre of water; tetrabutylammonium iodide (T-3638, Sigma, St. Louis, MO, USA); Bakerbond SPE-C_{18} columns, 100 mg, 1 ml (Baker 7020-01).

2.2. Internal standard and calibration solutions

The internal standard (I.S.) solution was prepared by dissolving 2 mg of acebutolol hydrochloride in 50 ml of methanol. The stock solution of I (2 mg dissolved in 50 ml of methanol) was ten-fold diluted in methanol. The same procedure was repeated for the solutions of II and III. Stock and diluted solutions were used for the preparation of calibration samples. All solutions were stored refrigerated at 4°C. New solutions were prepared every three weeks.

2.3. Equipment and chromatographic conditions

The chromatographic system consisted of: a pump (Model 590, Waters, Milford, MA, USA),

an automatic variable volume injector (Wisp 712, Waters) equipped with a cooling system set at −5°C to obtain a final temperature of the injector of 0°C, a variable wavelength UV detector (Model SF 783, Kratos, Applied Biosystems, Division of Perkin Elmer, Foster City, CA, USA) used at 270 nm. A workstation NEC supplied with baseline 810 software which performed integration, recording and storage of the data.

A Chiral-AGP column (15 cm × 4 mm I.D.) based on the use of α_1-acid glycoprotein as chiral stationary phase was chosen for the resolution of the enantiomers of the metabolite.

A guard-column (10 × 3 mm I.D.) containing the same phase was placed between the injector and the analytical column. Analytical and guard columns were manufactured by Chromtech AB (Norsborg, Sweden). Both columns were immersed in a water bath containing 20% of methanol and thermostated at 5°C (Model Ministat, Huber, Offenburg Elgersweier, Germany).

The mobile phase, pH 7 phosphate buffer to which were added $2.5 \cdot 10^{-3}$ *M* of tetrabutylammonium iodide as charged modifier, was used at a flow-rate of 0.5 ml/min. The pressure at the top of the precolumn was around 80 bar.

2.4. Sample preparation

The samples were prepared by introducing 50 μl (5.36 nmol) of the internal standard solution in a 10-ml polypropylene tube. For the calibration samples, various aliquots of the stock or diluted solutions of each of the three compounds were also added to the tube. All samples were then treated as described below.

2.5. Extraction procedure

After evaporation of the methanol, 0.5 ml of plasma and 0.5 ml of 0.1 *M* CH_3COONa were added and the tube was shaken on a vortex-mixer for five s and centrifuged at 1600 *g* for 3 min. The diluted plasma was loaded onto a prepacked Bakerbond SPE C_{18} column, set on a liquid–solid extraction device (Extra-Sep), previously conditioned with 2 × 1 ml of methanol and 2 × 1 ml of water. The plasma was aspirated through the column using a high-vacuum pump (Edwards, Model E2M5) the vacuum being applied during 1 min. The column was then washed with 2 × 1 ml of 0.02 *M* K_2HPO_4 and with 0.8 ml of acetonitrile. The vacuum was maintained during 2 min after the second washing step. The four compounds were eluted with 0.5 ml of methanol and the eluate was evaporated to dryness at 45°C under a nitrogen stream. A 0.25-ml volume of mobile phase was added to the dry residue and 50 μl were injected onto the chiral column.

2.6. Calibration curves

Plasma calibration samples (six to eight different concentrations) were processed according to the sample preparation procedure described above. The calibration curves for I, II and III were obtained by plotting the peak-height ratio (I, II or III/I.S.) versus the concentration of the corresponding compound in the sample.

The equation was calculated by the least squares method using weighted linear regression with a weighting factor of $1/conc^2$. The range of concentrations in the calibration samples was 0.56–28.0 μmol/l (0.16–8.0 μg/ml) for II and III, and 2.0–26.7 μmol/l (0.60–8.0 μg/ml) for I.

3. Results and discussion

3.1. Chromatography

An example of a chromatogram showing the resolution of the metabolite enantiomers after extraction from plasma is given in Fig. 2. The two enantiomers of the metabolite eluted first at ca. 9.5 min for II and 11.2 min for III. The internal standard, racemic acebutolol, gave only one peak at ca. 17 min and the methyl ester, I, eluted at 28 min as the (+)-enantiomer and their racemate. The chromatographic run time was 35 min. The chromatogram of an extract of drug-free human plasma exhibited no interference for the four components.

Several attempts were made to resolve I, II

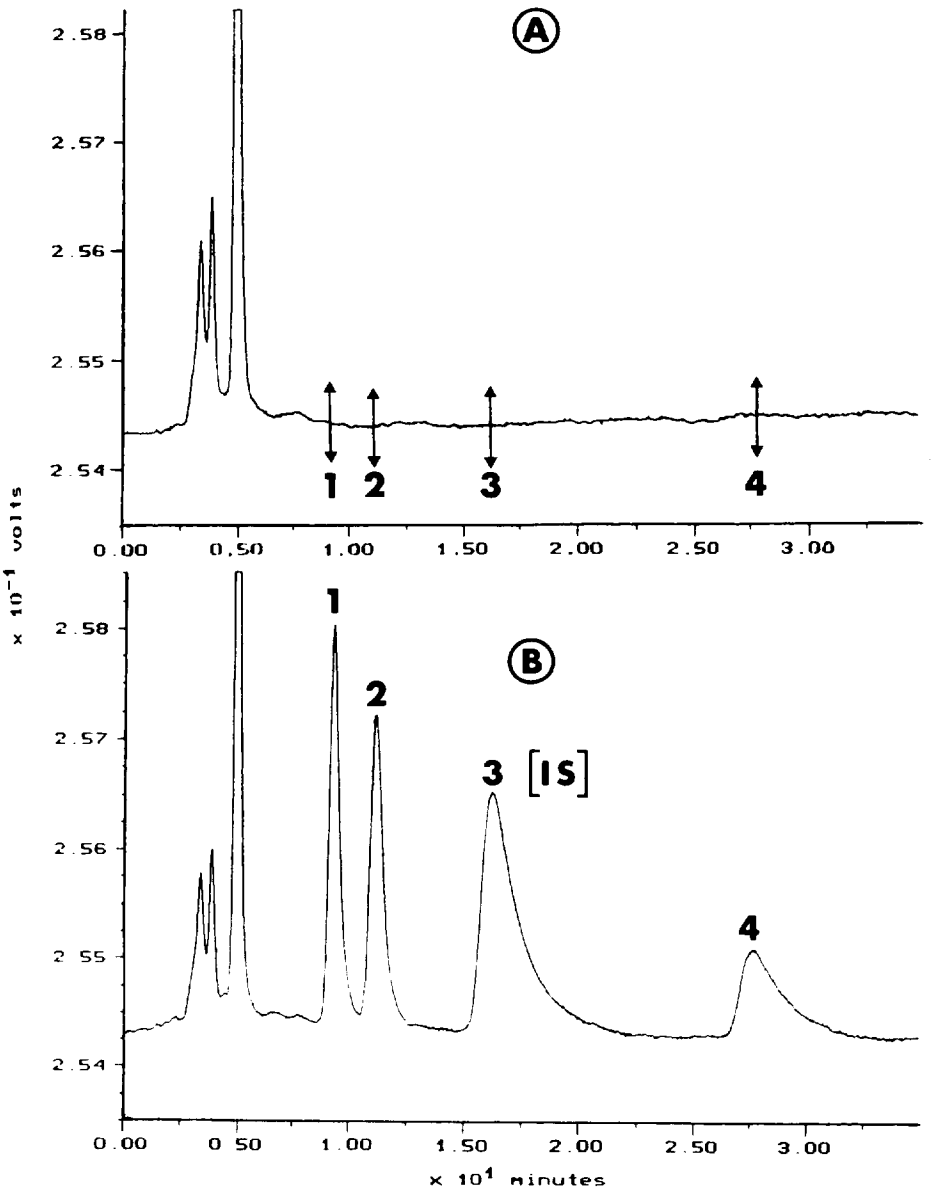


Fig. 2. Example of chromatograms obtained from an extract of 0.5 ml of plasma. (A) 0.5 ml drug-free human or dog plasma. (B) spiked human plasma sample containing 11.2 μmol/l of II and III, 10.7 μmol/l of I and 10.7 μmol/l of acebutolol. Peaks: 1 = II acid; 2 = III acid; 3 = racemic acebutolol (I.S.); 4 = I methyl ester.

and III on BSA, Chiralcel OD-R and Pirkle-type columns. The interactions of the two enantiomers, II and III, of the metabolite with the stationary phases of these columns did not differ sufficiently to enable satisfactory resolution.

Complete separation of the enantiomers was achieved on a α_1-AGP column. This type of column has already been used for the resolution and the determination in plasma of various drugs [1–8]. The enantioselectivity and retention can

easily be regulated by pH and buffer concentration and charged or uncharged modifiers [9–12] due to the different binding characteristics of the chiral centers. Temperature can also affect retention and resolution but very few results were reported [13].

Factors which specially influenced the resolution of the enantiomers II and III were the following:

- concentration and pH of the buffer: the resolution increased with the concentration of the buffer. pH lower than 7 resulted in incomplete resolution or no resolution at all;
- use of charged modifiers to perform ion-pair chromatography: tetraethyl and tetrapropylammonium bromide gave partial resolution, whereas tetrabutylammonium iodide led to total resolution of II and III.
- temperature of the analytical column: the resolution increased when the temperature of the column decreased from ambient to 5°C.

3.2. Extraction yield

The extraction yield was determined by comparison of the peak height of II, III and I after extraction from a plasma sample spiked with the three compounds and after direct injection of a calibration solution. The yield ranged from 80 to 90% for the three compounds.

3.3. Within-day reproducibility

Various concentrations were measured on the same day with three plasma samples per concentration. The mean recovery [(found/given)·100] ranged from 92 to 112% for II, 89 to 112% for III and 96 to 110% for I. The results of the within-day reproducibility are shown in Table 1.

3.4. Day-to-day reproducibility

The day-to-day reproducibility was assessed from the concentration values measured on different days (day 1, 2, 4, 7 and 9) in plasma samples spiked with different amounts of the three compounds using the calibration curves established on day 0. Individual recoveries ranged from 80.9 to 134% for II, 78.2 to 137% for III and 66.5 to 136% for I. Mean recoveries ($n = 3$) are presented in Table 2.

The results show that the calibration curves obtained for each of the three compounds could be used around one week. However, as the chemical properties of the chiral column slightly changed from day to day, probably due to

Table 1
Within-day reproducibility of II acid, III acid and I methyl ester

Added (μmol/l)	Mean recovery (%)		Added (μmol/l)	Mean recovery (%)
	II acid	III acid		I methyl ester
0.560	97.3 (5.1)[a]	97.1 (8.5)	2.00	110 (19)
0.700	92.1 (6.6)	89.4 (12.5)	5.34	96.8 (11.3)
2.10	106 (7)	106 (6)	12.0	95.9 (8.3)
2.24	109 (1)	112 (3)	21.3	110 (2)
5.60	112 (3)	108 (6)	24.0	101 (4)
12.6	103 (5)	100 (6)	26.7	97.8 (5.7)
22.4	112 (2)	107 (2)		
25.2	107 (5)	101 (4)		
28.0	106 (2)	101 (2)		

[a] Values in parentheses are coefficients of variation (%).

Table 2
Day-to-day reproducibility of II acid, III acid and I methyl ester

Added (μmol/l)	Recovery (%)				
	Day 1	Day 2	Day 4	Day 7	Day 9
Compound II					
0.56	118 (4)[a]	113 (4)	105 (4)	121 (10)	93.5 (7.8)
0.84	107 (6)	107 (8)	97.0 (6.3)	127 (4)	97.3 (10.5)
5.60	110 (1)	102 (9)	98.9 (3.8)	120 (3)	84.3 (3.7)
11.2	110 (1)	109 (5)	101 (2)	122 (2)	93.8 (3.8)
21.0	107 (3)	109 (1)	113[b] (3)	107 (2)	97.1 (2.6)
28.0	103 (3)	105 (2)	109 (5)	107 (1)	102 (1)
Compound III					
0.56	125 (6)	120 (8)	114 (7)	124[b] (4)	92.1 (15.1)
0.84	115 (4)	117 (4)	109 (3)	126 (8)	98.5 (7.1)
5.60	108 (2)	104 (7)	98.6 (3.8)	117 (3)	84.3 (5.4)
11.2	108 (1)	105 (6)	95.8 (1.1)	118 (2)	91.6 (5.6)
21.0	104 (3)	103 (2)	107 (1)	101 (5)	91.9 (3.1)
28.0	97.5 (2.6)	97.5 (1.8)	102 (4)	100 (3)	93.9 (1.4)
Compound I					
2.00	91.0 (1.9)	89.0 (17.9)	110 (8)	99.0 (34.7)	88.7 (10.6)
2.67	93.6 (18.3)	104 (11)	100 (1)	93.5 (27.1)	94.4 (2.6)
5.34	79.6 (15.9)	95.1 (12.2)	91.8 (10.9)	86.6 (7.3)	73.5 (13.1)
10.7	95.3 (4.0)	96.3 (2.6)	101 (4)	102 (5)	84.2 (14.3)
20.0	83.3 (5.2)	99.8 (5.6)	102 (5)	88.5 (2.9)	83.3 (3.9)
26.7	83.8 (4.4)	96.9 (1.9)	93.3 (13.5)	95.0 (5.0)	90.5 (4.5)

[a] Values in parentheses are coefficients of variation (%).
[b] Mean of 2 values.

interactions between the chiral phase and the charged modifier added to the mobile phase, it is recommended to prepare calibration curves twice a week.

3.5. Chiral column life-time

The resolution between the peaks of the enantiomers remained quite good for 2 months of daily use, corresponding to ca. 500 injections. The chiral column can, if necessary, be successfully regenerated by reversing the flow direction of the mobile phase.

3.6. Limits of quantitation

The limits of quantitation defined as the lowest concentration measured with a mean recovery between 80 and 120% and a coefficient of variation lower than 20% were around 0.56 μmol/l (0.16 μg/ml) for II and III and 2.0 μmol/l (0.60 μg/ml) for I.

3.7. Stability

Methanolic solutions of II, III and I were stable for at least 3 weeks at 4°C. This was determined by comparison of freshly prepared solutions and solutions used daily for three weeks.

The stability of the three compounds in plasma extracts kept on the injector refrigerated at 0°C was demonstrated (Table 3). Under such conditions, the compounds were stable for at least 10 h before injection of the extracts.

After two freeze–thaw cycles, no degradation of II, III or I was observed (Table 4).

Table 3
Stability of II, III and I in plasma extracts kept at 0°C on the injector

Compound	Time before injection (h)	Added (μmol/l)	Recovery (%)
II	0 [a]	2.80	84.6
	3.60	14.0	95.0
	7.13	2.80	87.9
	10.7	14.0	86.4
III	1.25	2.80	93.9
	4.77	14.0	104
	8.32	2.80	102
	11.9	14.0	103
I	2.42	2.67	94.4
	5.95	13.3	111
	9.50	2.67	84.6
	13.1	13.3	109

[a] First injection.

3.8. Application

The method was applied to the determination of plasma concentrations of I and its carboxylic acid metabolite, II, in dogs after i.v. (10 mg/kg) and p.o. (10 and 300 mg/kg) administrations of ^{14}C-I. Compound I was not detected but the metabolite II appeared rapidly in plasma. No inversion between the (−)- and (+)-enantiomers of the metabolite or racemization occurred.

The drug, I, is a pure enantiomer. According to previous data with its racemate, the drug acts as a prodrug, the demethylation occurring as soon as absorption starts. Thus no circulating I and (+)-enantiomer could be detected in plasma.

Moreover, at the position of I in the chromatogram, no peak appeared after ^{14}C-I administration to dogs. This peak could be I or its (+)-enantiomer or the racemate of these two enantiomers, because the α_1-AGP chiral column used does not allow separation of the prodrug and its two enantiomers despite the fact that the chiral center is not affected by the biotransformation.

The absence of any peak at the location of I could result form the fact that there is no interconversion or racemization for the drug/prodrug as demonstrated for metabolite II.

4. Conclusions

The present method enables the simultaneous determination in human plasma, of I, its carboxylic acid metabolite II, and the related (+)-enantiomer III, by stereospecific high-perform-

Table 4
Stability of II, III and I in spiked human plasma samples after two freeze–thaw cycles

Compound	Added (μmol/l)	Recovery (%)	
		First thawing	Second thawing
II	3.50	86.9	97.4
		84.0	93.4
	17.5	92.6	93.7
		90.9	92.6
III	3.50	92.6	108
		92.0	93.7
	17.5	97.1	97.7
		102	101
I	6.67	93.7	101
		86.2	96.0
	17.5	100	95.1
		103	95.1

ance liquid chromatography, with suitable reproducibility and accuracy.

References

[1] J. Hermansson and M. Eriksson, *J. Liq. Chromatogr.*, 9 (1986) 621.
[2] S. Menzel-Soglowek, G. Geisslinger and K. Brune, *J. Chromatogr.*, 532 (1990) 295.
[3] K.J. Pettersson and A. Olsson, *J. Chromatogr.*, 563 (1991) 414.
[4] A.M. Vermeulen, M.T. Rosseel and F.M. Belpaire, *J. Chromatogr.*, 567 (1991) 472.
[5] O. Beck, L.O. Boreus, P. Lafolie and G. Jacobsson, *J. Chromatogr.*, 570 (1991) 198.
[6] G. Geisslinger, S. Menzel-Soglowek, O. Schuster and K. Brune, *J. Chromatogr.*, 573 (1992) 163.
[7] J.V. Andersen and S.H. Hansen, *J. Chromatrogr.*, 577 (1992) 362.
[8] N. Mork and H. Bundgaard, *Pharm. Res.*, 9 (1992) 492.
[9] J.E. Drouin and M. Broquaire, *J. Chromatogr.*, 605 (1992) 19.
[10] Z. Feng, R. Gollamudi and G. Han, *J. Chromatogr.*, 609 (1992) 187.
[11] J. Hermansson and I. Hermansson, *J. Chromatogr. A*, 666 (1994) 181.
[12] E. Arvidsson and S.O. Jansson, *J. Chromatogr.*, 591 (1992) 55.
[13] R. Gollamudi and Z. Feng, *Chirality*, 3 (1991) 480.