Journal of Chromatography, 545 (1991) 115–122
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 107

Separation and estimation of small amounts of the enantiomers of carbidopa and methyldopa on a chiral stationary phase with L-phenylalanine as selector in ligand-exchange chromatography

B. LAUSECKER*[a]
Central Institute of Organic Chemistry, Department of Central Analytics, Rudower Chaussee 5, O-1199 Berlin (Germany)
and
F.-M. ALBERT
ISIS-Chemie GmbH, Breithauptstrasse 3–5, O-9541 Zwickau (Germany)
(First received November 16th, 1990; revised manuscript received January 4th, 1991)

ABSTRACT

The chiral stationary phase with L-phenylalanine as selector was used for the separation of the enantiomers of carbidopa and methyldopa in ligand-exchange chromatography. The influence of pH, organic modifier and copper(II) salt concentration in the eluent on capacity and separation factors has been shown. The applicability of this method to the detection of small amounts of *S*-methyldopa and *R*-carbidopa in *S*-carbidopa has been demonstrated.

INTRODUCTION

S-Carbidopa [(*S*)-(−)-2-hydrazino-2-(3,4-dihydroxybenzyl)propionic acid] is a drug used in combination with *S*-DOPA [(*S*)-(−)-2-amino-3-(3,4-dihydroxyphenyl) propionic acid] for the therapy of Parkinson disease; the *S*-form of carbidopa is the biologically active one, whereas the *R*-form is toxic. In the manufacturing process *S*-methyldopa [(*S*)-(−)-2-amino-2-(3,4-dihydroxybenzyl)propionic acid] is N-aminated by 3,3-pentamethyleneoxaziridine to give *S*-carbidopa [1]. If *S*-methyldopa contains some *R*-methyldopa or if racemization occurs during the N-amination, *R*-carbidopa may be produced. Gelber and Neumeyer [2] resolved the enantiomeric carbidopa and methyldopa with L-phenylalanine in the eluent on a C_{18} RP column. The disadvantage of this method is the high noise level of the eluent in the detector which is caused by the L-phenylalanine in the eluent. Secondly, impurities of D-phenylalanine in L-phenylalanine may lead to errors in the results of the analysis.

[a] Present address: Fa. Hoffmann–La Roche AG, Department of Clinical Kinetics, Grenzacher Strasse, CH-4002 Basle, Switzerland.

0021-9673/91/$03.50

In this paper we describe the synthesis of a stationary phase with L-phenylalanine as selector using a procedure similar to the procedure employed by Gübitz *et al.* [3]. The data for the phases are given below. The influence of modifier, copper concentration and pH on the capacity and separation factors of the enantiomeric carbidopa and methyldopa are shown. The sensitivity of this method in the detection of 0.03% *S*-methyldopa and 0.03% *R*-carbidopa in 99.97% *S*-carbidopa has been demonstrated with a precision of 25% for *R*-carbidopa and 29% for *S*-methyldopa when 200 μg of *S*-carbidopa were injected.

EXPERIMENTAL

The chiral stationary phase was synthesized in a similar fashion to the procedure of Gübitz *et al.* [3].

L-Phenylalanine was converted into the sodium salt with sodium hydroxide. The sodium phenylalanate was dried over phosphorus pentoxide, and an excess of sodium phenylalanate was placed in a flask with a magnetic stirrer and condenser together with (3-glycidoxypropyl)triethoxysilane in absolute ethanol. The reaction was carried out at 76°C for 2 h. The silica gel was dried in vacuum at 130°C for 12 h. The silica gel and the [3-N-(L)-phenylalanine-2-(*R,S*)-hydroxypropyloxypropyl]triethoxysilane together with dry toluene were then placed in a flask with a stirrer and condenser with water separator and boiled until ethanol was no longer separated. After filtration the silica gel was washed with methanol, acetone, water and again with methanol and then dried in vacuum at 65°C for 5 h. Then the silica gel was again suspended in dry toluene and treated with hexamethyldisilazane for 4 h. The silica gel was filtered and washed as described before.

The elemental analysis of the stationary phase is: C = 11.53%, H = 2.02% and N = 0.50%. The surface coverage is 2.04 μmol/m^2 calculated on the basis of the nitrogen value of the elemental analysis. The stationary phase was filled in a stainless-steel column of 300 × 4 mm I.D. The silica gel was therefore suspended in 2-propanol and packed with ethanol.

Chemicals

L-Phenylalanine was purchased from Reanal (Budapest, Hungary) and hexamethyldisilazane and (3-glycidoxypropyl)triethoxysilane were obtained from Chemiewerk Nünchritz (Nünchritz, Germany); silica gel, obtained from Leuna-Werke (Leuna, Germany), had the following properties: mean particle diameter, 7.6 μm; surface, 250 m^2/g; spheric particles. Methanol, ethanol and toluene were analytical-grade reagents from Laborchemie Apolda (Apolda, Germany) and dried as usual. *R*-Carbidopa and *S*-carbidopa as well as *R*- and *S*-methyldopa were obtained from ISIS-Chemie (Zwickau, Germany) and had the following properties:

Substance	Molecular rotation, $[\alpha]_D 20$	
R-Methyldopa Al 1276	+ 26.35°	*c* = 4.4
R-Carbidopa Al 1278	+ 23.8°	*c* = 1
S-Methyldopa lab. charge	− 26.0°	*c* = 4.4
S-Carbidopa Al 1201	− 24.8°	*c* = 1
S-Carbidopa Al 1279	− 24.86°	*c* = 1
S-Carbidopa 020788	− 24.8°	*c* = 1

All measurements were performed in $AlCl_3$–H_2O (19.65:80.35, w/w) and the pH was adjusted to 1.5 with NaOH (corresponding to United States Pharmacopeia XXI).

Chromatography

The chromatographic system consisted of a Knauer HPLC pump type 64.00, a Knauer injection valve (20 μl), a Knauer variable-wavelength detector set at 280 nm (Dr. H. Knauer, Wiss. Gerätebau, Bad Homburg, Germany) and a CR 6 A integrator from Shimadzu (Duisburg, Germany), or a liquid chromatographic system had been used consisting of two Knauer high-performance liquid chromatography pumps type 64.00, a 20-μl injection valve (Rheodyne type 7215), a Knauer variable-wavelength detector set at 280 nm, an Epson PC AX2e and Knauer software version 2.2. The eluent consisted of methanol–water with 1 m*M* $CuSO_4$. The pH value was adjusted with sulphuric acid and measured with an MV 870 digital pH meter (Präcitronic, Dresden, Germany) with a glass electrode *vs.* Ag/AgCl reference electrode. Solutes were dissolved in 0.1 *M* HCl. The dead time was determined with 0.1 *M* HCl.

Due to the peak asymmetry the discussion is based on the separation factor instead of the resolution.

RESULTS AND DISCUSSION

The chiral stationary phase with L-proline or with L-hydroxyproline as selector is the most common one in ligand-exchange chromatography. All attempts to resolve the enantiomers of carbidopa on this column failed. We varied the pH, the type and amount of organic modifier and the temperature of the chromatographic process. In all cases resolution could not be achieved. Gelber and Neuneyer [2] resolved the enantiomers of carbidopa and methyldopa with L-phenylalanine in the eluent, but not in the same run. While the enantiomers of methyldopa were resolved with methanol–water, those of carbidopa were resolved with 0.1% HCl.

We synthesized a chiral stationary phase with L-phenylalanine as selector similar to the procedure developed by Gübitz *et al.* [3] as described above.

Attempts to resolve the enantiomers of carbidopa with several amounts of acetonitrile as modifier in the eluent did not lead to resolution but with methanol as modifier resolution was achieved. Table I (*R* and *S*-methyldopa) and Table II (*R* and *S*-carbidopa) demonstrate the dependence of the capacity factors k' and the separation factor α on the methanol content in the eluent. Further chromatographic conditions are: eluent containing 1 m*M* $CuSO_4$, pH adjusted to 3.2 with sulphuric acid; temperature, 25°C; flow-rate, 1 ml min^{-1}. It is not possible, however, to take more than 70% methanol in the eluent at pH 3.2 as the copper sulphate is insoluble at this concentration. It must be noted that the k' values and separation factors of enantiomeric carbidopa were more strongly influenced by the increasing amount of methanol than those of enantiomeric methyldopa. In the case of enantiomeric carbidopa good baseline separation was achieved from 30% up to 70% methanol. For enantiomeric methyldopa the separation factor increased up to 1.40 at 65% methanol in the eluent; however, no baseline resolution was achieved. The best compromise between resolution, time consumption and detection limit of small amounts of *R*-carbidopa and *S*-methyldopa seems to be at pH 3.2 with 65% methanol in the eluent. For other chromatographic conditions see above. A typical chromatogram of the

TABLE I

CAPACITY FACTORS (k') AND SEPARATION FACTORS [$\alpha = k'(S)/k'(R)$] OF (R)- AND (S)-METHYLDOPA VERSUS METHANOL CONTENT IN ELUENT

Methanol content (%)	k'		
	R-Methyldopa	S-Methyldopa	$k'(S)/k'(R)$
20	1.23	1.53	1.24
30	1.20	1.50	1.25
40	0.89	1.25	1.40
50	0.90	1.20	1.33
60	0.88	1.19	1.35
65	0.86	1.20	1.40
70	1.80	2.21	1.23

resolution of the enantiomers of carbidopa and methyldopa with 65% methanol in the eluent (other chromatographic conditions as above) is demonstrated in Fig. 1.

The dependence of the capacity and separation factors of enantiomeric methyldopa and enantiomeric carbidopa on the pH value is demonstrated in Tables III and IV. For enantiomeric carbidopa at pH 2.7, baseline separation is achieved and the capacity factors for R- and S-carbidopa increased up to 6.14 and 13.71, respectively, at pH 3.5, whereas for enantiomeric methyldopa at pH values from 2.7 to 2.9 no separation has been achieved. From pH 3.0 to 3.5 the capacity and the separation factors slightly increased, leading to k' values of 1.35 and 1.78 for R- and S-methyldopa, respectively, and to a separation factor of 1.32. The chromatographic conditions for these experiments were 65% methanol and 1 mM $CuSO_4$. Using an eluent consisting of 50% methanol with a pH of 4.5 (for other conditions see above) the k' of the methyldopa enantiomers increased to 3.36 and 5.1 for R and S, respectively, but the R- and S-carbidopa became unstable in this pH range and eluted as three identical peaks at k' ranging from 3.0 to 6.2. Therefore, it is necessary to use an acidic eluent within the pH range 3.0–3.3 to separate both the enantiomers of carbidopa and the enantiomers of methyldopa.

TABLE II

CAPACITY FACTORS (k') AND SEPARATION FACTORS [$\alpha = k'(S)/k'(R)$] OF (R)- AND (S)-CARBIDOPA VERSUS METHANOL CONTENT IN ELUENT

Methanol content (%)	k'		
	R-Carbidopa	S-Carbidopa	$k'(S)/k'(R)$
20	1.60	2.20	1.38
30	1.76	2.80	1.59
40	2.44	4.26	1.75
50	2.43	4.73	1.95
60	2.42	5.83	2.41
65	2.70	6.61	2.45
70	6.33	14.12	2.23

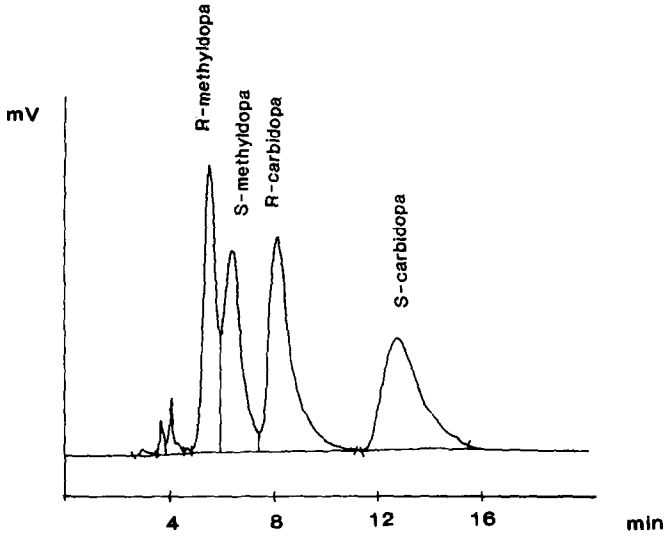

Fig. 1. Separation of the enantiomers of carbidopa and methyldopa. Amounts found: 2.5 μg of *R*-methyldopa, 2.5 μg of *S*-methyldopa, 3 μg of *R*-carbidopa, 3 μg of *S*-carbidopa.

TABLE III

CAPACITY FACTORS (k') AND SEPARATION FACTORS [$\alpha = k'(S)/k'(R)$] OF (*R*)- AND (*S*)-METHYLDOPA VERSUS pH

pH	k'		
	R-Methyldopa	*S*-Methyldopa	$k'(S)/k'(R)$
2.7	0.06	0.06	1.00
2.9	0.21	0.21	1.00
3.1	0.68	0.93	1.37
3.2	0.86	1.20	1.40
3.3	1.16	1.50	1.29
3.5	1.35	1.78	1.32

TABLE IV

CAPACITY FACTORS (k') AND SEPARATION FACTORS [$\alpha = k'(S)/k'(R)$] OF (*R*)- AND (*S*)-CARBIDOPA VERSUS pH

pH	k'		
	R-Carbidopa	*S*-Carbidopa	$k'(S)/k'(R)$
2.7	0.31	0.91	2.94
2.9	0.85	2.00	2.35
3.1	2.86	7.00	2.45
3.2	3.46	7.93	2.29
3.3	4.70	10.65	2.27
3.5	6.14	13.71	2.23

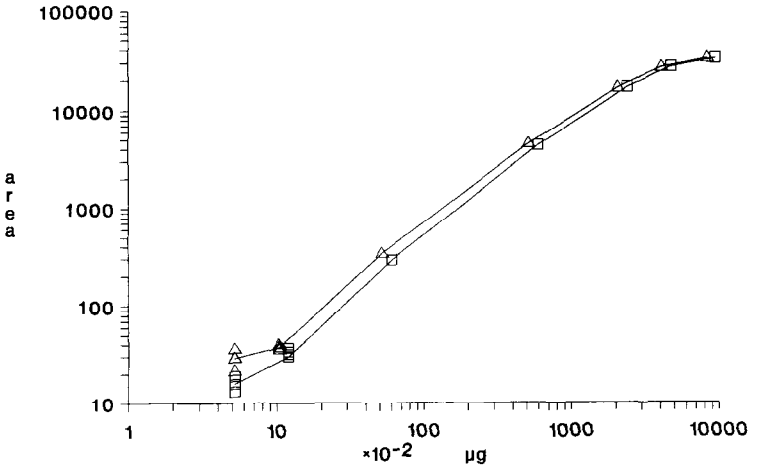

Fig. 2. Calibration curve for the estimation of (△) *S*-methyldopa and (□) *R*-carbidopa.

We also investigated the influence of the concentration of copper(II) salt in the eluent on the separation factors. We varied the concentration from 0.01 to 1 m*M* copper sulphate and found that with 0.01–0.1 m*M* copper(II) in the eluent (other conditions: methanol–water, 65:35, v/v, pH 3.2, adjusted with sulphuric acid) no resolution of enantiomeric methyldopa was achieved. Enantiomeric carbidopa was slightly resolved with 0.1 m*M* copper(II). With 0.25 m*M* copper in the eluent both enantiomers of carbidopa and methyldopa were resolved. We increased the concentration of copper(II) up to 1 m*M* but the transparency of the eluent decreased drastically so that 0.25 m*M* seems to be the optimal concentration for the estimation of small amounts.

In Fig. 2 the calibration curve for *S*-methyldopa and *R*-carbidopa in the range 0.05–100 µg is shown. At 50–100 µg the signal is not proportional to the concentra-

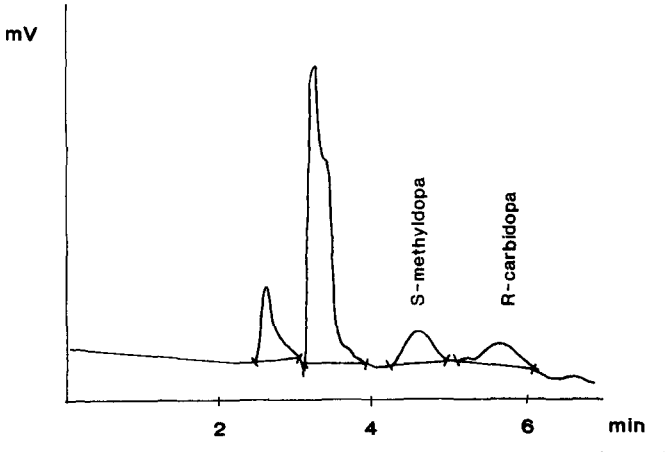

Fig. 3. Separation of 0.103 µg of *S*-methyldopa and 0.1035 µg of *R*-carbidopa.

TABLE V

S-METHYLDOPA CONTENT IN COMMERCIAL *S*-CARBIDOPA

Brand of *S*-carbidopa	Amount injected (μg)	*S*-Methyldopa found				
		Area			μg	%
		Mean ($n=4$)	S.D.	R.S.D. (%)		
Al 1279	203	444.4	26.2	5.9	0.58	0.3
Al 1201	197	31.3	7.6	24.3	0.06	0.03
020788	201	753.1	38.4	5.1	1.0	0.5

tion because of the limited linearity of the detector, and at about 0.05 μg the precision decreased. The standard deviation increased to 25% for *R*-carbidopa and 29% for *S*-methyldopa at a concentration of 0.05 μg. At 0.1 μg the standard deviation decreased to 5.9% for *R*-carbidopa and to 5.1% for *S*-methyldopa. Fig. 3 shows a chromatogram of the mixture of 0.103 μg of *S*-methyldopa and 0.1035 μg of *R*-carbidopa used for calibration. Chromatographic conditions for these experiments were: eluent, methanol–water (65:35, v/v) containing 0.25 m*M* copper(II), pH 3.13, adjusted with sulphuric acid; temperature, 35°C, flow-rate, 1 ml/min.

Finally, we investigated some samples of *S*-carbidopa from ISIS-Chemie Zwickau produced by N-amination of *S*-methyldopa as described above. The results are shown in Table V. In these investigations we could not find any *R*-carbidopa at a concentration of more than 0.03% in the *S*-carbidopa. In Figs. 4 and 5 two chromatograms of commercial products are shown designated as Al 1279 and Al 1201 with 0.3 and 0.03% *S*-methyldopa in *S*-carbidopa, respectively. As can be seen, no adsorption was detected for *R*-carbidopa at a retention time of 5 min 35 s.

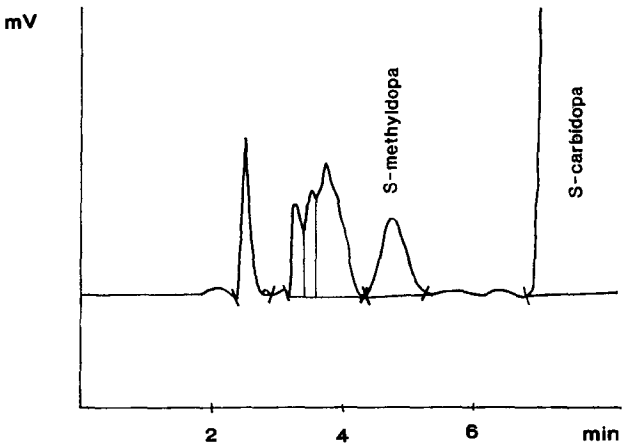

Fig. 4. Chromatogram of the commercial product Al 1279, containing 0.58 μg of *S*-methyldopa (see text for chromatographic conditions).

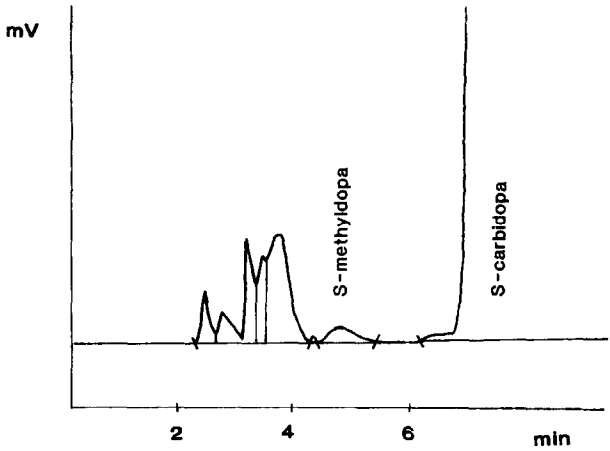

Fig. 5. Chromatogram of the commercial product Al 1201, containing 0.06 μg of *S*-methyldopa (see text for chromatographic conditions).

CONCLUSIONS

Ligand-exchange chromatography with L-phenylalanine as selector on the stationary phase is a sensitive method for the determination of small amounts of *S*-methyldopa and *R*-carbidopa in *S*-carbidopa. With this method it is possible to estimate 0.05 μg of *R*-carbidopa and 0.05 μg of *S*-methyldopa in 200 μg of *S*-carbidopa injected. It has been shown that in the products investigated no *R*-carbidopa and only very small amounts of *S*-methyldopa could be found. This means that the *S*-methyldopa used in the synthesis did not contain *R*-methyldopa above the detection limit of 0.03%. Moreover, no racemization was observed in the course of synthesis.

REFERENCES

1 S. Andreae, E. Schmitz, S. Schramm, F.-M. Albert and D. Lohman, *G.D.R. Pat.*, 230 865 (1986); *C.A.*, 105 (1986) 227 301j.
2 L. R. Gelber and J. N. Neumeyer, *J. Chromatogr.*, 257 (1983) 317–326.
3 G. Gübitz, W. Jellenz and W. Santi, *J. Liq. Chromatogr.*, 4 (1981) 701–712.