Journal of Chromatography, 350 (1985) 179–185
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 113

APPLICATION OF CHROMATOGRAPHIC CHIRAL STATIONARY PHASES TO PHARMACEUTICAL ANALYSIS

ENANTIOMERIC PURITY OF D-PENICILLAMINE

EBERHARD BUSKER* and KURT GÜNTHER
Chemical Research Department, Degussa AG, Postfach 1345, D-6450 Hanau 1 (F.R.G.)
and
JÜRGEN MARTENS
Pharmaceutical Coordination, Degussa AG, Postfach 110533, D-6000 Frankfurt 11 (F.R.G.)
(First received July 15th, 1985; revised manuscript received August 12th, 1985)

SUMMARY

The enantiomeric purity of the pharmaceutical D-penicillamine (I) has been determined by a novel high-performance liquid chromatographic technique involving the formation of 5,5-dimethylthiazolidine-4-carboxylic acid (III) by reaction of I with formaldehyde and separation of the optical antipodes by means of ligand exchange chromatography using the copper(II) complex of (2*S*,4*R*,2′*RS*)-4-hydroxy-1-(2′-hydroxydodecyl)proline, which is coated on a reversed-phase column. The limit of determination for the L-antipode is *ca.* 0.1%. The validation of the method is accomplished by comparison with an independent gas chromatographic procedure. Thirteen commercially available lots of I are shown to be of equally high enantiomeric purity (*ca.* 99.9%).

INTRODUCTION

The D-isomer of the α-amino acid penicillamine (I) has revealed favourable effects regarding laboratory parameters and clinical aspects of rheumatoid arthritis[1]. D-I is also of interest in the treatment of a wide variety of other illnesses, *e.g.* Wilson's desease[2], cystinuria[3], idiopathic pulmonary fibrosis[4] and scleroderma[5]. On the other hand, L-penicillamine (L-I) has been shown to be toxic[6–8]. Accordingly, the resolution and quantification of penicillamine enantiomers has received a great deal of attention.

An indirect method has been reported for the separation of diastereomeric derivatives of penicillamine by high-performance liquid chromatographic (HPLC)[9]. Lodemann *et al.*[10] desulphurised D-penicillamine (D-I) with Raney nickel to give D-valine (D-II). The L-valine content of the sample was determined by a combination of enzymatic and colorimetric methods. König *et al.*[11] obtained thiazolidin-2-ones by esterification of penicillamine and cyclisation with phosgene. The enantiomers of

0021-9673/85/$03.30

the former were separated by gas chromatography (GC). Usually the optical rotation is used as an indication of the enantiomeric purity of commercial lots of D-I.

The estimation of the enantiomeric excess from its optical purity may give erroneous results. Optical rotations may be very sensitive to experimental conditions and may be affected by unsuspected impurities, *e.g.* the disulphide of penicillamine. Even if the rotation is determined correctly, the optical purity may still be uncertain, because the specific rotation of the pure enantiomer and the chemical purity of the sample may have been determined incorrectly. More accuracy is to be expected from those methods evaluating the discrete content of the optical antipodes.

GC[12–15], liquid chromatography[16–22] and thin-layer chromatography[23] have successfully been used for the resolution of enantiomers and are entering the field of routine analysis. Chiral recognition for the separation of enantiomers is accomplished by optically active stationary or mobile phases.

As part of our laboratory's programme in the development of more sensitive assays for the stereochemical purity of commercial pharmaceuticals, intermediates for asymmetric syntheses[24,25] and amino acids[26,27], we have investigated the applicability of chiral stationary phases to the analytical resolution of penicillamine enantiomers.

We have previously reported the enantiomeric separation of D,L-cysteine[22], and here we describe the development of a direct HPLC method for the determination of the optical purity of D-penicillamine. Our approach involves condensation of penicillamine with formaldehyde to form the enantiomeric dimethylthiazolidinecarboxylic acids D-III and L-III (Fig. 1), followed by resolution of the isomers by HPLC on a chiral stationary phase developed in our laboratory[22]. The chiral selector (Fig. 2) is (2*S*,4*R*,2′*RS*)-4-hydroxy-1-(2′-hydroxydodecyl)proline (IV). The selector IV is used as a mixture of two diastereomers and was synthesised according to the literature[28], starting from optically pure (2*S*,4*R*)-4-hydroxyproline and the corresponding racemic C_{12} terminal epoxide.

In order to check our new method we desulphurised[10] penicillamine (Fig. 1) to give valine (II), and separated the enantiomers of valine by GC after derivatization following a similar procedure reported by Frank *et al.*[12].

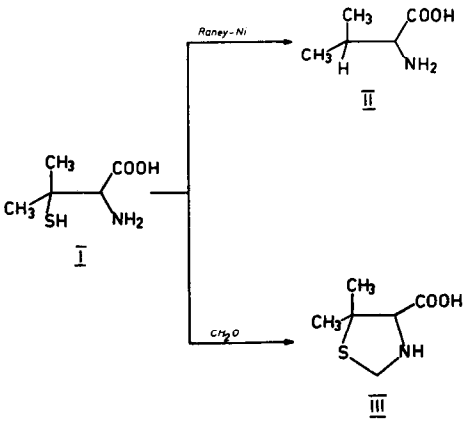
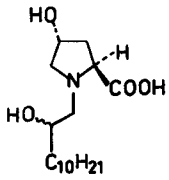


Fig. 1. Derivatisation of penicillamine for determination of the optical purity by means of chromatography.



IV

Fig. 2. Formula of the chiral selector.

EXPERIMENTAL

Materials and methods

The D-penicillamine samples were either of semisynthetic origin (prepared from sterically homogeneous penicillin) or products of an optical resolution of a totally synthetic process starting from isobutyraldehyde, ammonia and sulphur[29]. L-Penicillamine was obtained from the pharma division of our company.

High-performance liquid chromatography. Thiazolidinecarboxylic acids (III) were prepared by the following procedure. Penicillamine (150 mg) was treated with 100 μl of 37% (w/w) aqueous formaldehyde solution and 4 ml of water. After stirring for 2 h at 50°C the reaction mixture is diluted with water to give a total volume of 25 ml. Then 10 μl of the resulting solution is injected for chromatographic analysis. The separation of the enantiomers of dimethylthiazolidinecarboxylic acid (III) was accomplished on a 125 × 4 mm I.D. LiChrosorb RP-8 column (E. Merck, Darmstadt, F.R.G.) coated with the copper(II) complex of (2*S*,4*R*,2′*RS*)-4-hydroxy-1-(2′-hydroxydodecyl)proline (IV). The coating procedure was described in ref. 22. By means of a water-bath the temperature of the columns was held at 50°C.

The mobile phase was composed of 12% methanol and 88% water containing $1 \cdot 10^{-4}$ mol/l copper sulphate; the pH was adjusted to 4.5 by the addition of small amounts of orthophosphoric acid. The instrumentation consisted of a Gynkotek M600 pump (flow-rate, 3 ml/min), a Rheodyne 7105 injection valve, a UV detector LC-85 (235 nm) from Perkin-Elmer and an integrator SP 4100 from Spectra-Physics.

Gas chromatography. Penicillamine (I) was desulphurised with Raney nickel[30] to give valine (II). The solution containing 10–12 mg of II is evaporated to dryness and treated with 2 ml of 3 *N* hydrochloric acid in anhydrous 1-propanol. A 0.5-ml volume of this solution is heated to 110°C for 45 min. The excess of reagent is blown off with a stream of helium at 50°C. The resulting residue is treated with 0.2 ml of methylene chloride and 0.2 ml of trifluoroacetic anhydride and kept at 110°C for 10 min. After careful removal of excess reagent under a gentle stream of helium, the sample is dissolved in 50 μl of methylene chloride. A 1-μl volume of this solution is injected into the gas chromatograph at a split ratio of 100:1.

The GC analyses were carried out on a Dani Model 3900 gas chromatograph. The separation of the enantiomers was achieved on a 25 m × 0.3 mm I.D. glass capillary coated with Chirasil-val (Applied Science), with helium as carrier gas at 0.7 bar. The temperature programme was 90°C isothermal for 1 min, rising by 5 K/min to 200°C. Peak integration was effected with the laboratory automation system 3357 from Hewlett-Packard.

RESULTS

The applicability of the analytical system is demonstrated in Fig. 3, which shows the separation of racemic 5,5-dimethylthiazolidinecarboxylic acid. The excellent selectivity (separation factor $\alpha = k'_{(D)}/k'_{(L)} = 3$) and the fact that the L-antipode is eluted first, permits the exact determination of L-penicillamine in D-penicillamine far below a relative content of 1%.

The chromatographic system described here had to be optimised for speed of analysis and long-term stability. One important parameter controlling the capacity factors and the peak shape is the amount of chiral selector coated on the reversed-phase column. By varying the concentration of the selector in the methanolic coating solution, and by testing different types of silanised silica, we were able to establish a reliable and reproducible column-preparation technique. An elemental analysis of a coated silica prepared according to ref. 22 did not show a significant increase of the carbon content. So we conclude that only microgram amounts of the selector are adsorbed on the stationary phase.

The dimethylthiazolidinecarboxylic acid enantiomers show similar high capacity factors during ligand exchange chromatography on reversed-phase columns, *e.g.* the lipophilic amino acid phenylalanine[19], when using eluents without an organic modifier. Addition of methanol to the mobile phase led to a reduction in the retention of L- and D-III. In order to avoid column bleeding, which becomes a problem if the percentage of methanol in the mobile phase exceeds 15%, we restricted the methanol content to 12%. For the same reason the upper limit for the column temperature was 50°C.

Another striking quantity effecting retention is the pH value[19], since the amino acids have to release one proton when forming the chelate complex with the copper(II) ion bound to the chiral selector. In a slightly acidic medium (pH 4.5), the complexation and therefore the retention were diminished. The observed loss in selectivity of enantiomer separation was compensated by a narrower peak shape.

To achieve a short analysis time we used a column only 125 mm long, and

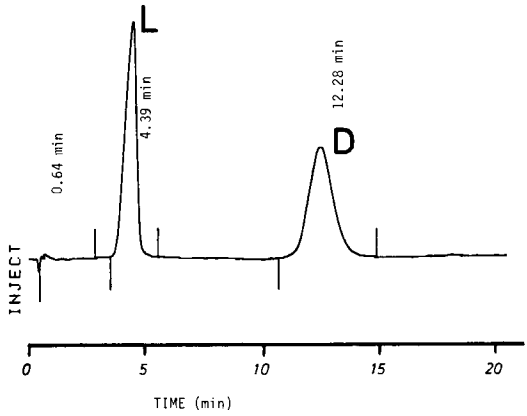


Fig. 3. HPLC separation of D,L-5,5-dimethylthiazolidinecarboxylic acid derived from D,L-penicillamine. For chromatographic conditions see Experimental.

TABLE I

DETERMINATION OF L-PENICILLAMINE IN COMMERCIAL D-PENICILLAMINE SAMPLES

Sample (Producer)	*HPLC*		*GC*	
	L-Enantiomer (%)	*Standard deviation (n = 12)*	*L-Enantiomer (%)*	*Standard deviation (n = 12)*
1 (A)	<0.1	0.05	0.08	0.01
2 (B)	<0.1	0.05	0.08	0.01
3 (C)	<0.1	0.05	0.07	0.01
4 (C)	<0.1	0.05	0.07	0.01
5 (C)	<0.1	0.05	0.07	0.01
6 (C)	<0.1	0.05	0.07	0.01
7 (C)	<0.1	0.05	0.07	0.01
8 (C)	<0.1	0.05	0.07	0.01
9 (C)	<0.1	0.05	0.08	0.01
10 (C)	<0.1	0.05	0.09	0.01
11 (C)	<0.1	0.05	0.08	0.01
12 (C)	<0.1	0.05	0.08	0.01
13 (C)	<0.1	0.05	0.08	0.01

selected an unusually high flow-rate of 3 ml/min. The column performance could be maintained for at least 50 replicate injections. In our experience a loss in selectivity is less a question of column bleeding than a problem of the irreversible adsorption of impurities blocking the active sites in the stationary phase. Such a contaminated column can be washed with an acidic methanolic solution and regenerated several times.

The results of the evaluation of the optical purity from thirteen different commercially available samples are shown in Table I. The materials tested, from three different suppliers, were found to be of equally high enantiomeric purity, at least

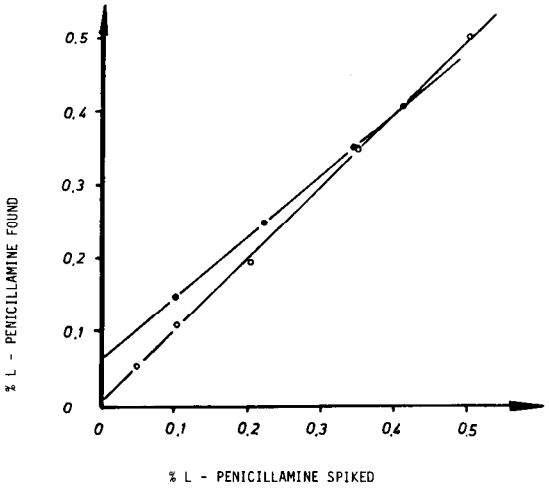


Fig. 4. Quantitative determination of L-penicillamine impurities in D-penicillamine spiked with different amounts of L-penicillamine by HPLC (○) and by GC (●).

99.9%. Our values indicate an even better purity than reported previously for five other batches[9], and they agree well with the results published by Lodemann *et al.*[10] who determined the relative content of L-penicillamine in commercially available samples *ca.* 0.1%. Since the evaluation of enantiomeric purity by means of chromatography may be falsified by unknown coeluting compounds, the control with a chromatographic system of a different separation mechanism is often recommended. Therefore we referred to the above described GC procedure and we found that the results accorded well with the values from the HPLC determinations.

In order to test the linearity and accuracy of the HPLC and GC methods, samples of D-penicillamine were spiked with 0–0.5% of L-penicillamine. The resulting calibrants were treated as described above. Fig. 4 shows the percentage of L-penicillamine found plotted against the expected values. Each value represents the mean of at least three injections. Thus, in both methods the observed values follow a linear correlation with a regression coefficient better than 0.999. However, a small intercept of 0.07% for the GC procedure was observed. This cannot be explained with certainty, since two opposing factors have to be taken into account. The GC procedure using the flame ionization detector shows a detection limit of 0.01%, compared with 0.05% for the HPLC method which is based on the UV detection of the eluting amino acid copper(II) complexes. Since the limit of determinations is higher by at least a factor of 2.5, the range below 0.1% cannot be validated by means of HPLC with the necessary precision. Otherwise it is reported that a partial racemisation of optically pure amino acids has been observed during the GC derivatisation reactions[31], which demands temperatures above 100°C. The formation of the HPLC derivative thiazolidine carboxylic acid, however, is less likely to induce racemisation, since it works at a neutral pH and a relative low temperature. In the range from 0.2 to 0.5%, the values from the two methods coincide very well.

REFERENCES

1 J. A. Jaffe, *Arthritis Rheum.*, 13 (1970) 436.
2 I. Sternlieb and I. H. Scheinberg, *J. Am. Med. Assoc.*, 189 (1964) 748.
3 J. C. Crawhall, E. F. Scowen and R. W. E. Watts, *Br. Med. J.*, 1 (1963) 588.
4 R. I. Henkin, H. R. Keiser, J. A. Jaffe, I. Sternlieb and I. H. Scheinberg, *Lancet*, (1967) 1268.
5 E. D. Harris Jr. and A. Sjoerdsma, *Lancet*, (1966) 996.
6 J. E. Wilson and V. du Vigneaud, *J. Biol. Chem.*, 184 (1950) 63.
7 R. M. Blair and H. V. Aposhian, *Biochim. Biophys. Acta*, 30 (1958) 214.
8 A. Wacker, E. Heyl and P. Chandra, *Arzneim. Forsch.*, 7 (1971) 971.
9 F. Nachtmann, *Int. J. Pharm.*, 4 (1980) 337.
10 E. Lodemann, Z. H. M. El-Kirdassy and A. Wacker, *Arzneim. Forsch.*, 30 (1980) 395.
11 W. A. König, E. Steinbach and K. Ernst, *J. Chromatogr.*, 301 (1984) 129.
12 H. Frank, G. Nicholson and E. Bayer, *J. Chromatogr.*, 146 (1978) 197.
13 W. A. König, I. Benecke and H. Brettig, *Angew. Chem.*, 93 (1981) 688; *Angew. Chem., Int. Ed. Engl.*, 20 (1981) 693.
14 P. E. Hare and E. Gil-Av, *Science*, 204 (1979) 1226.
15 K. Günther, J. Martens and M. Messerschmidt, *J. Chromatogr.*, 288 (1984) 203.
16 V. A. Davankov, *Adv. Chromatogr.*, 18 (1983) 139.
17 W. Lindner, *Chimia*, 35 (1981) 294.
18 G. Blaschke, *Angew. Chem.*, 92 (1980) 14; *Angew. Chem., Int. Ed. Engl.*, 19 (1980) 13.
19 V. A. Davankov, A. S. Bochkov, A. A. Kurganov, P. Roumeliotis and K. K. Unger, *Chromatographia*, 13 (1980) 677.
20 H. Kitahara, *Bunseki Kagaku*, 33 (1984) 386.

21 W. H. Pirkle, C. J. Welch, *J. Org. Chem.*, 49 (1984) 138.
22 E. Busker and J. Martens, *Fresenius' Z. Anal. Chem.*, (1984) 319, 907.
23 K. Günther, J. Martens and M. Schickedanz, *Angew. Chem.*, 96 (1984) 514; *Angew. Chem., Int.·Ed. Engl.*, 23 (1984) 506.
24 K. Drauz, A. Kleemann and J. Martens, *Angew. Chem.*, 94 (1982) 590; *Angew. Chem., Int. Ed. Engl.*, 21 (1982) 584.
25 J. Martens, *Topics Cur. Chem.*, 125 (1984) 165.
26 B. Hoppe and J. Martens, *Chem. unserer Zeit.*, 17 (1983) 41.
27 B. Hoppe and J. Martens, *Chem. unserer Zeit.*, 18 (1984) 73.
28 J. Martens, H. Weigel, E. Busker and R. Steigerwald, *Ger. Pat., Appl.*, 31 43 726 (1982) Degussa AG.
29 W. M. Weigert, H. Offermanns and P. Scherberich, *Angew. Chem.*, 87 (1975) 372; *Angew. Chem., Int. Ed. Engl.*, 14 (1975) 330.
30 E. Lodemann, *Naturwissenschaften*, 66 (1979) 462; and references cited therein.
31 H. Frank, W. Woiwode, G. Nicholson and E. Bayer, *Liebigs Ann. Chem.*, (1981) 354.