

DETERMINATION OF THE L-ISOMER IN D-PENICILLAMINE BY DERIVATIZATION LIQUID CHROMATOGRAPHY

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SUMMARY

A method for a fast quantitative determination of the L-isomer in D-penicillamine is described. L-Leucine is coupled to D,L-penicillamine and the resulting diastereomers separated by reversed phase liquid chromatography. The mild conditions for the derivatization cause no racemization. For detection the UV-absorption of the derivatives at a wavelength of 215 nm is used. A linear signal was gained in a concentration range of 0.05-10% L-isomer in D-penicillamine. One complete determination can be carried out in 1.5-2 h. A comparison of products from different manufacturers resulted in the detection of L-penicillamine in the material of only one producer.

INTRODUCTION

D-Penicillamine (D-PA) is used in relatively high dosage forms as a long-term therapeutic. The purity of the substance, especially regarding by-products that can cause secondary effects, is of great importance. High secondary effects are due to the presence of L-penicillamine (L-PA). Studies on microorganisms showed that L-PA competes with L-valine and L-isoleucine in protein synthesis (Wacker et al., 1971). This is the basis for the high toxicity of L-PA (Kienel, 1972).

The most frequently used method for determining the L-isomer is NMR spectroscopy after derivatization (Cockerill et al., 1974). The disadvantage of this technique is its great demand on time and the detection limit of 0.5% L-PA which is not satisfactory.

Lately there have been different trends towards a chromatographic separation of amino acid enantiomers. One possibility is the gas chromatographic separation of volatile derivatives on chiral stationary phases (Howard and Parr, 1974; Charles et al., 1975; Frank et al., 1978; Nicholson et al., 1979). Two of the described chiral materials are commercially available (Gas-chrom. Newsletter (1978); Bulletin, Supelco, 1976). For penicillamine the derivatization procedure is complicated due to the free thiol-function

of the molecule (Frank et al., 1978). An alternative is the use of conventional phases after synthesis of diastereomeric compounds which is described for many amino acids and amines (Whelan, 1975; Hasegawa and Matsubara, 1975; Nambara et al., 1974; Kruse et al., 1979).

The same two possibilities are open in liquid chromatography. Most chiral stationary phases described until now are polymers with proline or hydroxyproline as the active centres. After complexation with Cu-(II)-ions a high stereoselectivity is gained (Lefebvre et al., 1976/77; Jozefonvicz et al., 1978; Davankov et al., 1978a and 1978b). High speed separations have not previously been possible with this technique. Furthermore this method is not applicable for penicillamine, because this substance forms stable complexes with Cu-(II). Recently the synthesis of a similar chiral stationary phase based on silica was described (Gübitz et al., 1979). As the complexation with Cu-(II) is also necessary for a separation of DL-amino acids, this method cannot be applied for D,L-penicillamine. For other types of chiral phases the separation of enantiomeric amino acids has been demonstrated only after derivatization (Hara and Dobashi, 1979; Pirkle and House, 1979). The second possibility is the separation of diastereomeric derivatives. For non-polar amino acid esters effective separations have been performed with silica gel columns (Furukawa et al., 1975; Furukawa et al., 1977; Goto et al., 1977). On the other hand, it was shown that diastereomeric dipeptides and tripeptides can also be separated by reversed phase liquid chromatography (Kroeff and Pietrzyk, 1978). As a reagent for preparing diastereomeric peptides, *t*-butyloxy-L-leucine-N-hydroxysuccinimidester (BOC-L-Leu-SU) proved to be optimal (Mitchell et al., 1979). The racemization under the mild conditions of this reaction is below 0.1% (Mitchell et al., 1978; Goodman et al., 1977).

In this paper a simple derivatization technique for D,L-penicillamine, using BOC-L-Leu-SU, combined with the determination of the resulting diastereomers after separation by reversed phase liquid chromatography is described.

EXPERIMENTAL

Reagents and materials

D-Penicillamine reference substance was produced by Biochemie GmbH, Kundl, Austria. L-Penicillamine was provided by Serva, Heidelberg, G.F.R. and *t*-butyloxy-L-leucine-N-hydroxysuccinimidester (BOC-L-Leu-SU) puriss CHR by Fluka, Buchs, Switzerland. The reagent solution (90 mg BOC-Leu-SU/10 ml methanol) must be prepared freshly every day. LiChrosorb RP 8, particle sizes 5 μ m and 10 μ m, were used as stationary phases in HPLC. All solvents except acetonitrile were of analytical grade quality (Merck); acetonitrile was used in LiChrosolv quality (Merck).

Instruments

For the HPLC determinations a high pressure pump, Altex 110, in combination with a Uvikon LCD 725 spectrophotometric detector was used. The injection system was a Rheodyne 7120 loop injector. Peak integrations and calculations of the data were carried out with a laboratory data system 3353 (Hewlett Packard).

Derivatization

As a result of the optimization studies the following procedure can be given. All

solvents that were needed for the derivatization reaction were treated with a stream of nitrogen before use to remove absorbed oxygen. Then 0.5 ml of the test or reference solution in water containing 9 mg penicillamine was mixed with 0.5 ml of NaHCO_3 solution (56 g NaHCO_3 /1 H_2O) and 1.0 ml reagent solution was added. The reaction was quantitative after 20 min at room temperature. The mixture was rinsed into a 50 ml round-bottomed flask by means of 25 ml of absolute ethanol. The solvent was evaporated with a rotavapor (50°C) and the residue was dissolved in 2.0 ml of an ice-cooled mixture of trifluoroacetic acid/dichloromethane (50/50, v/v). After 20 min reaction in an ice-bath the solution was concentrated on a rotavapor (50°C), the oily residue was diluted with 2.0 ml sodium acetate buffer (140 g $\text{CH}_3\text{COONa} \cdot 3 \text{H}_2\text{O}$ /1 H_2O) and injected immediately into the chromatograph.

RESULTS AND DISCUSSION

A presupposition for the derivatization of amino acids with free mercapto groups in most cases is the protection of this function, for example, by benzylation. The necessary reaction conditions could cause a racemization, however. Therefore it was tried to use penicillamine in its free, unprotected form for all investigations.

Synthesis of the dipeptides

The reaction of penicillamine with BOC-L-Leu-SU is shown in Fig. 1. If the sterical conditions are taken into consideration the enantiomers of D,L-penicillamine result in diastereomeric dipeptides.

Mitchell et al. (1978) describe this reaction in a mixture of NaHCO_3 -solution/THF. The reaction time is 60 min. THF seemed to not be optimal in the case of penicillamine because of the possibility of forming peroxides and the following oxidation of the

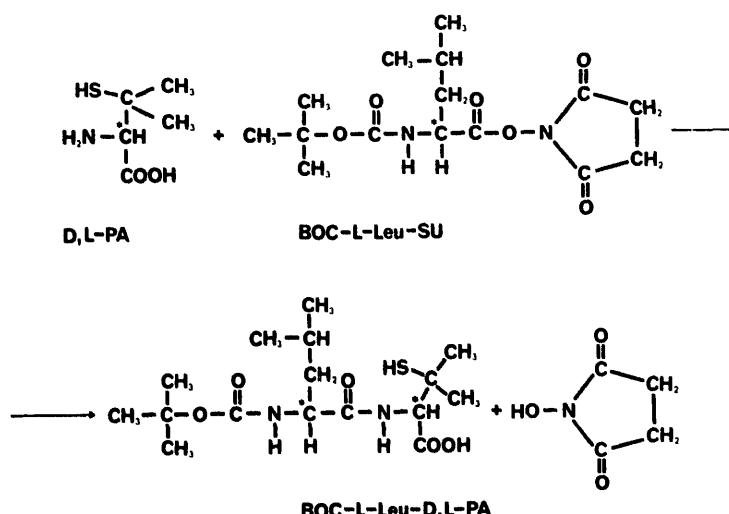


Fig. 1. Reaction of D,L-PA with BOC-L-Leu-SU.

mercapto group. Therefore the derivatization was studied in a mixture NaHCO_3 -solution/methanol (50/50, v/v). The solutions were analyzed by direct injection into the chromatograph. A typical chromatogram is shown in Fig. 2. The conditions were optimized for a selective determination of BOC-L-Leu-D-PA. The excess reagent and the impurities are separated from the interesting derivative. The result of the optimization study can be seen in Fig. 3. The reaction was measured by calculating the peak area of BOC-L-Leu-D-PA after chromatographic determination according to Fig. 2. The derivatization became quantitative within a short time. In the case of a great reagent excess the derivatization is finished within 10 min. Four mg of D-PA reacts quantitatively

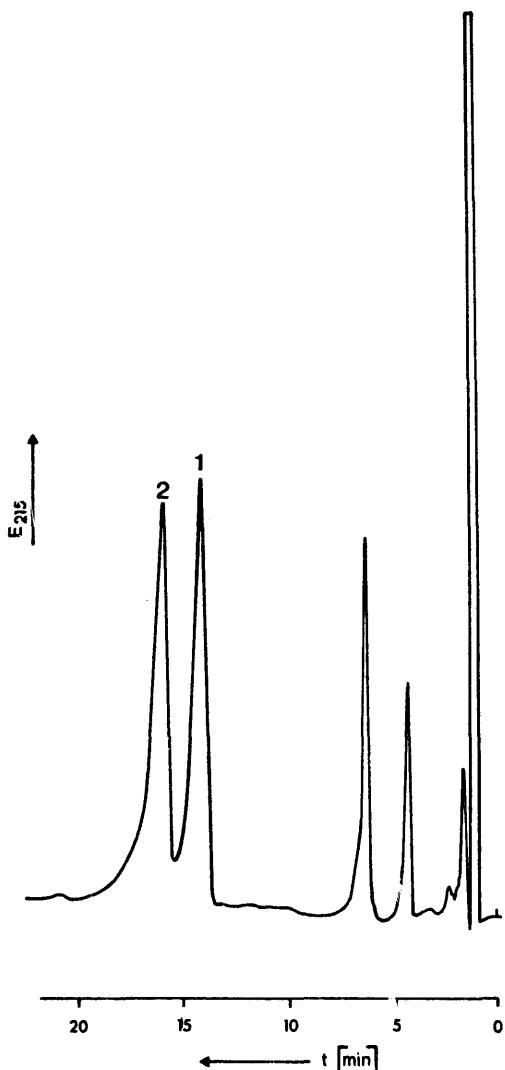


Fig. 2. Determination of BOC-L-Leu-D-PA in the reaction mixture. 1: BOC-L-Leu-D-PA. 2: BOC-L-Leu-SU (excess reagent). Conditions used: column — LiChrosorb RP 8, 10 μm , 25 cm \times 3.2 mm i.d.; solvent system — phosphate buffer (0.015 M)/ CH_3CN (7/3); flow rate — 1.5 ml/min at 140 bar; temperature — 22°C.

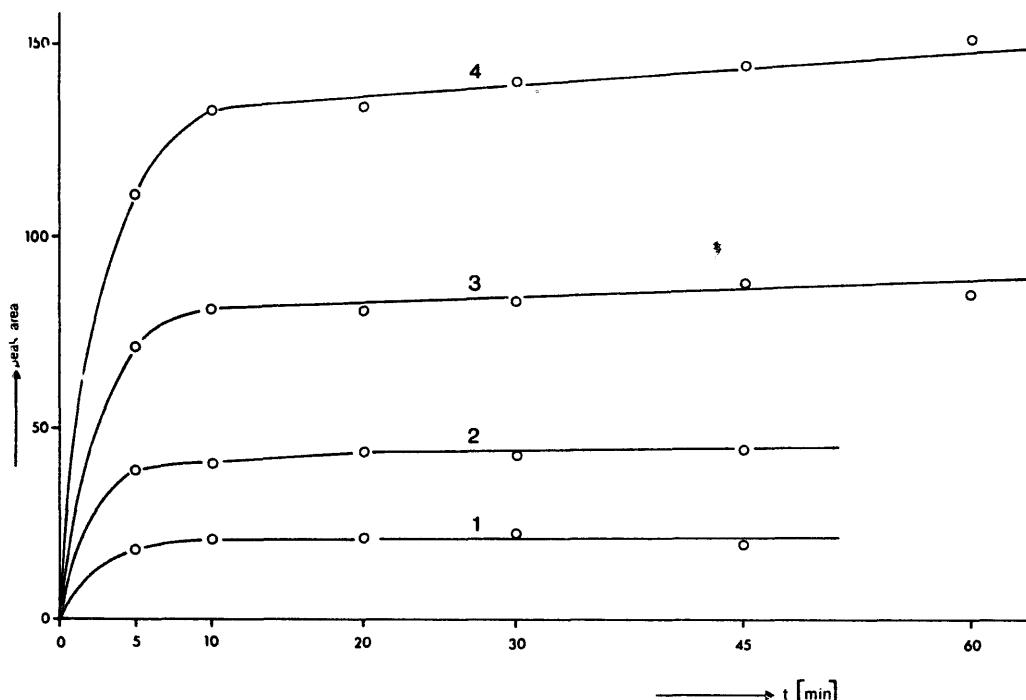


Fig. 3. Reaction of D-PA with BOC-L-Leu-SU. Conditions used: Reaction volume – 2 ml; Amount of reagent – 20 mg; Amount of D-PA – 1 mg (1), 2 mg (2), 4 mg (3), 8 mg (4).

with 20 mg of reagent within 20 min, which means a 2–3-fold molar excess of reagent. Eight mg of D-PA cannot be quantitatively derivatized. Analogous investigations for L-PA showed no difference in the reactivity of the two enantiomers.

A sufficient separation of D- and L-PA in the form of their BOC-L-Leu-derivatives was not possible in spite of systematic investigations. The best result is shown in Fig. 4. A quantitative determination of small amounts of the L-isomer in D-PA is impossible under these conditions. We therefore attempted to increase the separation efficiency by splitting off the BOC-group of the protected dipeptides. In all reagent mixtures of the optimization study (Fig. 3), by-products were formed during the deprotection step. The reason for the formation of these by-products was found to be the excess reagent in the derivatization mixture. This problem can be overcome by exchanging the molar amount of reagent and substrate. If penicillamine is present in a 2.2–2.5-fold molar excess as described under 'Experimental' no by-products are formed. The kinetics of the reaction are not changed by this variation.

Optimization of the chromatographic conditions

Due to the optical characteristics of the derivatives – the optimal detection wavelength used was 215 nm – only solvents, that are highly transparent in this wavelength region, can be used for the mobile phase. For a separation of the various peptides, mixtures of phosphate buffer containing acetonitrile proved to be well suited (Krummen

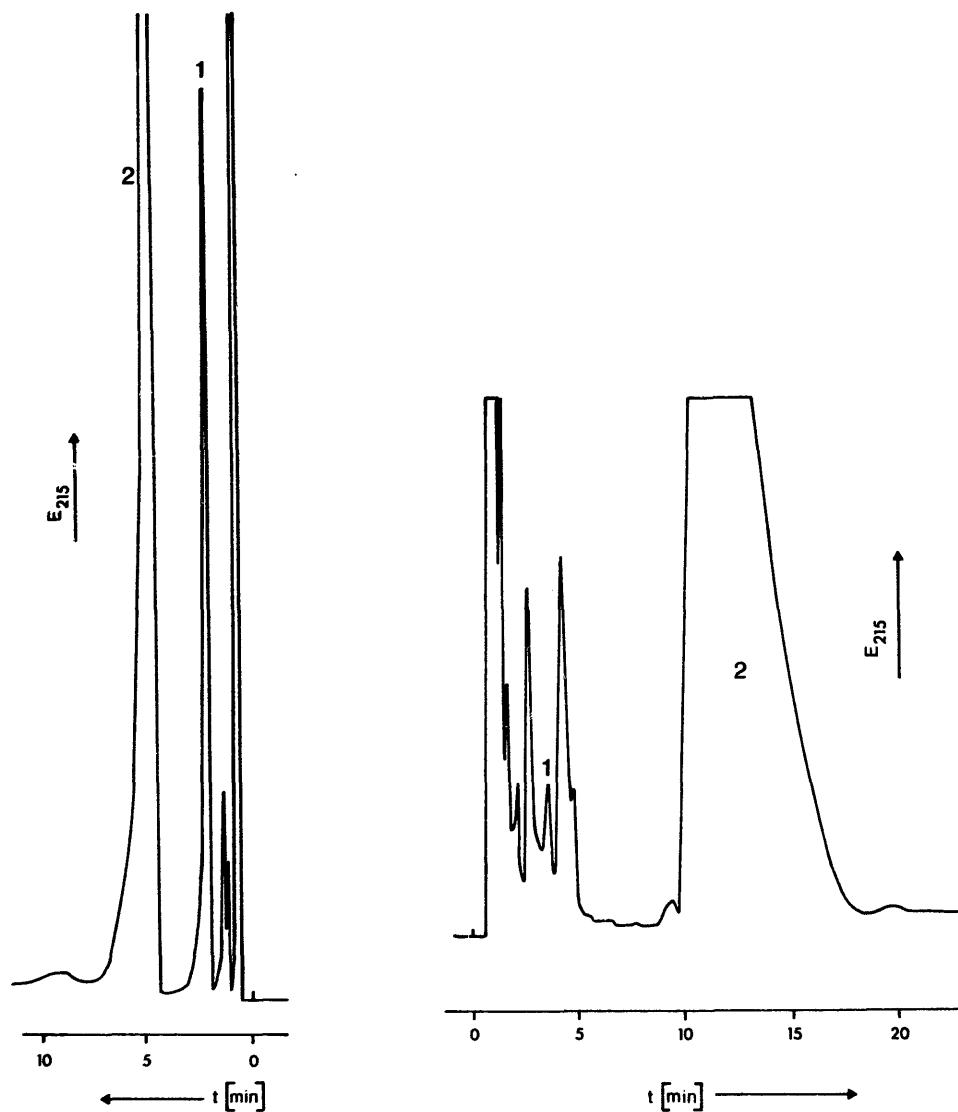


Fig. 4. Separation of BOC-L-Leu-L-PA (1) and BOC-L-Leu-D-PA (2). Conditions used: column – LiChrosorb RP 8, 5 μ m, 15 cm \times 3.2 mm i.d.; solvent system – phosphate buffer (0.015 M) pH 7/CH₃CN (8/2); flow rate – 1.0 ml/min at 180 bar; temperature – 40°C.

Fig. 5. Determination of 0.5% L-PA as L-Leu-derivatives. 1: L-Leu-L-PA. 2: L-Leu-D-PA. Conditions used: column – LiChrosorb RP 8, 5 μ m, 15 cm \times 3.2 mm i.d.; solvent system – methanesulfonic acid 0.1%/CH₃CN (9/1) + 0.01% Na-EDTA; flow rate – 1.2 ml/min at 200 bar; temperature – 50°C.

and Frei, 1977a and 1977b; Krummen et al., 1979; Nachmann, 1979). Similar mixtures were tried for this separation problem. The pH value of the mobile phase greatly influenced the retention of the derivatives (Table 1). Decreasing the pH caused an increase in retention time, if the anion of the buffer solution was kept constant.

The great increase in separation efficiency that was gained by splitting off the protect-

TABLE 1

RETENTION OF THE L-DERIVATIVES OF D,L-PENICILLAMINE IN DIFFERENT SOLVENT SYSTEMS

Conditions: column – LiChrosorb RP 8, 5 μ m, 15 cm \times 3.2 mm i.d.; flow rate – 0.6 ml/min

Solvent system ^a	Retention (min)	
	L-Leu-L-PA	L-Leu-D-PA
Phosphate buffer pH 5/CH ₃ CN (9/1)	1.8	8.7
Phosphate buffer pH 4/CH ₃ CN (9/1)	2.3	11.0
Phosphate buffer pH 3/CH ₃ CN (9/1)	4.5	22.0
Methanesulfonic acid 0.1%/CH ₃ CN (9/1)	3.7	15.0

^a To each solvent system 0.01% Na-EDTA was added.

ing group was remarkable (see Fig. 4). Na-EDTA (0.01%) was added to each solvent mixture in order to avoid interferences that could be caused by the reaction of traces of heavy metals that might have been present with the free thiol-function of the derivatives. If L-PA was present in the per cent-range, any of the mobile phases of Table 1 could be used. The limiting factor for a determination of quantities of L-PA in a concentration range below 1% was interferences with by-products originating from reagent impurities. In this respect the best separation was achieved with a mobile phase consisting of methanesulfonic acid 0.1%/acetonitrile (9/1) and an addition of 0.01% Na-EDTA at a temperature of 50°C. A typical chromatogram, that was gained for D-PA with an addition of 0.5% of the L-isomer is shown in Fig. 5.

Quantitative determinations

Applying the reaction conditions described under 'Experimental' a quantitative reaction of D,L-penicillamine is not possible. Accurate quantitative results can only be expected, if the reactivity of the enantiomers is identical and no racemization occurs. Therefore recovery studies were performed. To a constant amount of reference substance D-PA 0.5–10% L-PA was added before derivatization. After HPLC-separation the percentage of the peak area of L-Leu-L-PA was calculated relative to the peak area of L-Leu-D-PA. The results are shown in Table 2. The reference substance, D-penicillamine, gives no signal with a retention time identical with the L-isomer, which means that the presence of the L-form and a racemization is not detectable (<0.05%). The recovery for the added amounts of the L-isomer is 90–120%. The reproducibility of the method was tested for 0.5 and 5.0% L-PA using the described peak area calculation. For 8 determinations a relative standard deviation of 2.9% and 2.0% was gained. In a concentration range below 0.5% L-PA, a standard addition method combined with the evaluation of peak heights gives better results than the peak area calculation. With this technique quantitative determinations of as little as 0.05% L-PA in D-PA are possible.

TABLE 2
RECOVERY OF L-PENICILLAMINE IN D-PENICILLAMINE

L-PA added (%)	L-PA found (%)	% recovery
0.0	<0.05	—
0.5	0.6	120
1.0	0.9	90
2.0	1.7	85
10.0	9.9	99

Samples originating from different manufacturers were analyzed according the described method. Using D-penicillamine of 5 producers, the content of the L-isomer was below the detection limit of 0.05%, and only in 2 tested batches of one manufacturer were 0.18% and 0.40% of L-penicillamine found.

CONCLUSIONS

The synthesis of diastereomeric dipeptides from D,L-PA with BOC-L-Leu-SU following a determination of the dipeptides by reversed phase liquid chromatography proved to be a sensitive and simple technique for the analysis of small quantities of the L-isomer in D-PA. The method could also be used for finished dosage forms. The described technique seems to be applicable to other interesting enantiomeric peptides such as the iodinated thyronines. Studies concerning this are in progress.

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