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Investigation of the reaction mechanism of the mercurimetric determination of benzylpenicillin[1]

E. Roets*, P. Rappe, M. Heeren, J. Hoebus, A. Verbruggen, J. Hoogmartens

Katholieke Universiteit Leuven, Faculteit Farmaceutische Wetenschappen, Laboratorium voor Farmaceutische Chemie en Analyse van Geneesmiddelen, Van Evenstraat 4, B-3000 Leuven, Belgium

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Abstract

The assay of different penicillins in the European Pharmacopoeia was carried out by mercurimetric titration with potentiometric determination of the end-point. The consecutive formation of reaction products during titration was followed by reversed-phase liquid chromatography (LC) and ultraviolet detection. In one experiment the titration was carried out with ^{14}C-labeled benzylpenicillin and the reaction was followed with LC coupled to radiochemical detection. The identity of the intermediates and final reaction products was deduced from their retention times in comparison with reference products obtained by independent chemical transformation of benzylpenicillin. This allowed one to define for the first time the complete reaction scheme. This involves the isomerization of the natural penicilloic acid followed by decarboxylation, which has not been reported previously. At the end of the titration, only benzylpenilloaldehyde and a 1:1 complex of mercury and penicillamine were present in the solution.

Keywords: Benzylpenicillin; Liquid chromatography; Mercurimetric determination; Reaction products

1. Introduction

Probably no group of drugs has received more attention and been more intensively studied than the penicillins. The differences in the physical, chemical and biological properties of the various penicillins are due to their side chain.

Benzylpenicillin or penicillin G (**1**) (Fig. 1), on which most of the work has been done, was used as a model in this study. Benzylpenicillin is one of at least six naturally occurring penicillins. The other five are pent-2-enylpenicillin or penicillin F ($R = CH_3CH_2CH = CHCH_2-$), *n*-heptylpenicillin or penicillin K ($R = CH_3(CH_2)_6CH_2-$), *n*-amylpenicillin or dihydropenicillin F ($R = CH_3(CH_2)_4CH_2-$), *p*-hydroxybenzylpenicillin or penicillin X ($R = HOC_6H_4CH_2-$) and phenoxymethylpenicillin or penicillin V ($R = C_6H_5OCH_2-$). The formation of benzylpenicillin during fermentation was favored by the use of corn steep liquor in the nutrient medium, rich in phenylacetic acid.

Benzylpenicillin is unstable in acid and contains an aromatic ring, which permits detection under UV radiation. The fused β-lactam–thiazolidine

* Corresponding author.

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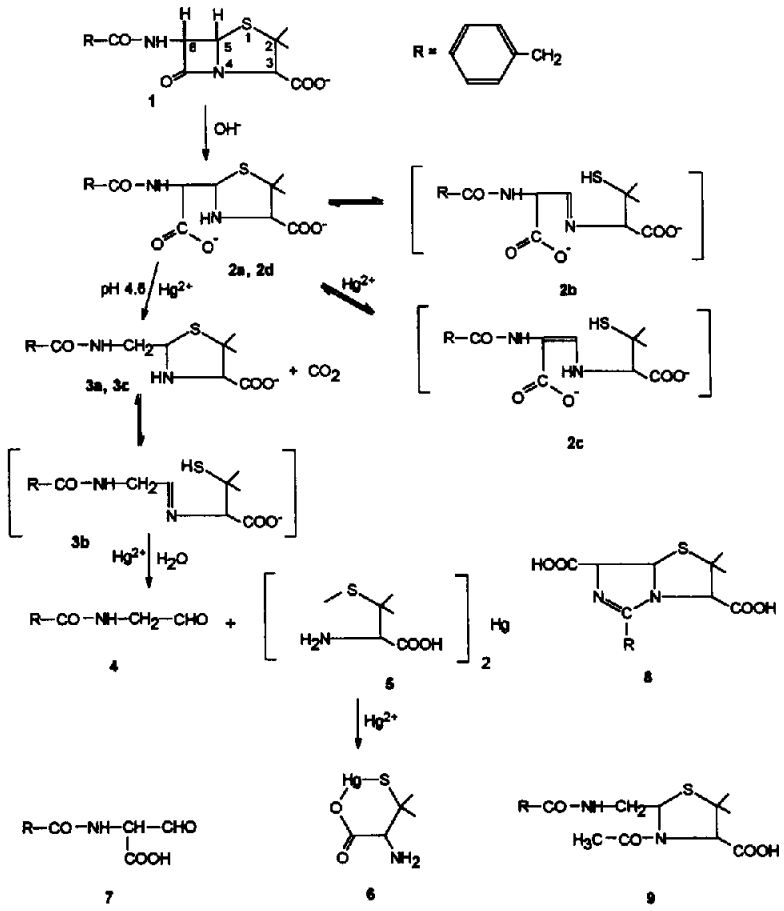

Fig. 1. Reaction scheme of the mercurimetric titration of benzylpenicillin (1). Compounds: Benzylpenicilloic acid, **2a**, **2d**; benzylpenamaldic acid imine tautomer, **2b**; benzylpenamaldic acid enamine tautomer, **2c**; benzylpenilloic acid, **3a**; benzylpenilloic acid open imine tautomer, **3b**; benzylpenilloaldehyde, **4**; penicillamine–mercury (2:1) sulfide, **5**; penicillamine–mercury (1:1) chelate, **6**; benzylpenaldic acid, **7**; benzylpenillic acid, **8**; *N*-acetylbenzylpenilloic acid, **9**. The pathway goes through **2**, **3** to **4**, **5** and **6**. Benzylpenaldic acid (**7**) is not an intermediate. Benzylpenillic acid (**8**) and *N*-acetylbenzylpenilloic acid (**9**) are only used as model compounds to study the titration.

two-ring system contains three chiral centers with the absolute configuration 3*S*, 5*R* and 6*R*. A surprisingly large number of different rearrangement, reaction and fission products were found to be produced under various conditions [1]. Because of the known lability of the β-lactam ring, many assay methods have been described. The first developed method was iodimetric titration, which is based on the observation that penicillin does not react with iodine, whereas penicilloic acid (**2a**), formed after hydrolysis with alkali or treatment with penicillinase, consumes iodine [2]. The disadvantage of the method is the lack of a constant stoichiometry, due to variation of the iodine consumption depending on parameters such as pH, temperature and reaction time. For this reason, simultaneous analysis of a reference compound is necessary. The reaction mechanism of iodine with degraded penicillins is exceedingly complex and a large number of reaction products have been identified [3,4].

The European Pharmacopoeia (Ph. Eur.) now describes a titrimetric assay for the determination of penicillins, which is based on the reaction of mercury(II) nitrate with penicilloic acid, produced from the penicillin by the action of alkali. A direct

titration without hydrolysis measures the amount of degradation products present in the sample. The end-point is detected potentiometrically with a mercury or platinum working electrode and a silver chloride or mercury(I) sulfate reference electrode. In contrast with the iodimetric assay, the stoichiometry of the mercury(II) titration is well defined [5]. This titration has been optimized thoroughly [6]. The second inflection of the potentiometric curve is equivalent to equimolar amounts of the penicillin and mercury(II) ion present. The method thus has the advantage of being an absolute method requiring no standard. The complete reaction mechanism has not been unambiguously elucidated. Levine [7] proposed that when the β-lactam ring is opened, a sulfhydryl group may be formed from the free thiazolidine group. It was postulated that the mercury(II) ion reacts with the penicilloate to form penamaldate complexes. On this basis, the titrimetric method was developed.

A later study suggested that fission of the penicilloic acid occurs, with formation of benzylpenilloaldehyde (**4**), carbon dioxide and the mercaptide of penicillamine (2-amino-β-mercaptobutanoic acid) (**5**) [8]. A complete reaction scheme was never proposed. In the intact penicillin, the fused β-lactam-thiazolidine ring, an acylated thiazolidine derivative, is stable and no fission of the aminothioacetal function can occur to form a thiol group. In order to identify the intermediates and the final reaction products formed during the mercurimetric titration of benzylpenicillin, two different LC systems were used. Reference products which are stable enough to be isolated were prepared by independent chemical transformation starting from benzylpenicillin. The formation of unstable intermediates was investigated by changes in the UV spectra of the titrated solutions. This allowed one to define for the first time the complete reaction pathway from **1** to **6**, as shown in Fig. 1.

2. Experimental

The titrations were performed according to the Ph. Eur. [9]; for the radioactive compound, all amounts used were 50 times smaller.

2.1. Chemicals and reagents

All reagents were of Ph. Eur. quality. Acetate buffer (pH 4.6) was prepared by dissolving 5.4 g of sodium acetate in water, adding 2.4 g of glacial acetic acid and diluting to 100.0 ml with water. Mercury(II) nitrate solution (0.02 M) was prepared by dissolving 6.85 g of mercury(II) nitrate in 20 ml of 1 M nitric acid and diluting to 1000.0 ml with water. Benzylpenicillin potassium and penicillamine were commercial products of the highest purity available. [^{14}C]Benzylpenicillin, labeled on the carbonyl group of the phenacetyl side chain, was a product from the Radiochemical Centre (Amersham, UK).

2.2. Reference products

Benzylpenillic acid, (5*R*,6*R*)-benzylpenicilloic acid and (5*R*)- and (5*S*)-benzylpenilloic acids were prepared according to the literature [10]. (5*S*,6*R*)-Benzylpenicilloic acid was prepared by epimerization of the natural 5*R*,6*R* epimer [11]. N^4-Acetylbenzylpenilloic acid was prepared as described for N^4-acetylphenylpenilloic acid in the literature [12]. The structure of the reference products was confirmed by IR and ^{1}H and ^{13}C NMR spectra.

2.3. LC apparatus and chromatographic conditions

The liquid chromatograph consisted of a Milton Roy Minipump (Laboratory Data Control, Riviera Beach, FL), a Valco injector, Model CV-6-UHPa-N60, equipped with a 20 μl loop (Houston, TX), a Waters Model 440 UV detector (Milford, MA) set at 254 nm and a sensitivity of 0.08 a.u.f.s. and a Kipp Model BD 40 recorder (Kipp en Zonen, Delft, Netherlands) with a chart speed of 5 mm min^{-1}. For the detection of the radioactive peaks a β-scintillation detector was used (Berthold, Wildbad, Germany). The flow rate was 1 ml min^{-1}.

A Hypersil C_{18} column (250 × 4.6 mm i.d., particle size 5 μm) (Shandon, Runcorn, UK) was used under ambient conditions with methanol–0.2 M phosphate buffer (pH 4.6)–sodium

methanesulfonate–water (20:5:0.2:75, v/v/w/v) as the mobile phase (LC system I). A Zorbax C_8 column (250 × 4.6 mm i.d., particle size 8 μm) (Du Pont, Wilmington, DE) was used under ambient conditions with methanol–0.2 M phosphate buffer (pH 7.0)–water (30:5:65, v/v/v) (LC system II) as the mobile phase. Samples from the titration vessel were injected directly.

2.4. Spectrophotometry

A PU 8700 UV/VIS scanning spectrophotometer (Philips, Cambridge, UK) was used with 1 cm cells.

2.5. Nuclear magnetic resonance spectrometry

All ^1H and ^{13}C NMR spectra were recorded on a Jeol FX909 spectrometer. The samples were run in DMSO-d_6 or D_2O. ^1H and ^{13}C chemical shifts were referenced to the solvent peaks.

3. Results and discussion

The mercurimetric titration of benzylpenicillin according to the Ph. Eur. was followed by direct analysis of the reaction mixture by LC. It was possible to identify the following reaction steps.

3.1. Hydrolysis of benzylpenicillin

It was observed with LC system I that a reaction time of 15 min at room temperature in 0.5 M sodium hydroxide as prescribed by the Ph. Eur. titration insures the quantitative conversion of 1 into benzylpenicilloic acid (**2a**). It has been reported that in alkaline medium benzylpenicillin is hydrolyzed at the β-lactam ring to benzylpenicilloic acid [13]. This hydrolytic reaction is described as proceeding without the formation of substances other than penicilloic acid [14]. The reaction at constant pH and temperature is first order with respect to penicillin and is directly proportional to the hydroxyl concentration. Benzylpenicillin is extremely susceptible to nucleophilic attack owing to the non-planarity of the penicillin nucleus and is thus much more reactive than simple β-lactams [12,15].

3.2. Epimerization of the natural epimer

During the hydrolysis, it was observed that the initially formed natural (5*R*,6*R*)-benzylpenicilloic acid (**2a**) was partially transformed into the corresponding (5*S*,6*R*)-benzylpenicilloic acid (**2d**). The newly formed epimer was eluted before the natural product in LC system I (Fig. 2c). A third, small peak was eluted between the two epimers. This is probably the (5*R*,6*S*)-benzylpenicilloate, called the 6-epimer. It has been reported that the natural (5*R*,6*R*)-benzylpenicilloic acid (**2a**) is susceptible to slow epimerization in the pH range

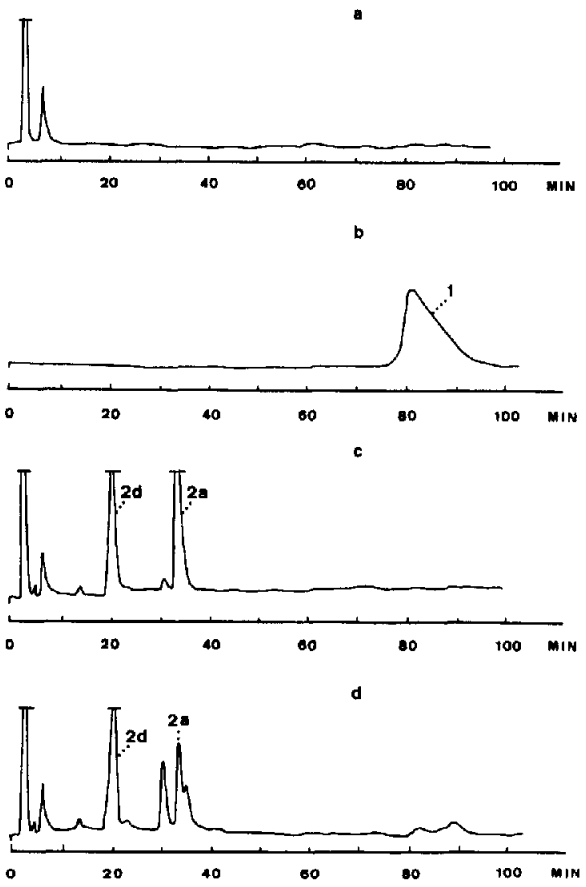

Fig. 2. Chromatograms of the mercurimetric titration mixture (LC system I). (a) Blank titration without penicillin added; (b) benzylpenicillin solution before titration; (c) reaction mixture after alkaline hydrolysis for 15 min at room temperature; (d) reaction mixture after neutralization and addition of a small amount of mercury(II) nitrate solution.

4–12. It is transformed partly into the corresponding (5*S*,6*R*)-benzylpenicilloic acid (**2d**). The alkaline epimerization process involves the benzylpenamaldic acid imine tautomer **2b** as an intermediate. This intermediate has an open thiol form [13]. It is also known that penicillins with a tertiary amide side chain or acylalkylamino substituents in the side chains epimerize under the influence of strong base to their 6-epimers [16,17]. Benzylpenicilloic acid does not decarboxylate in mildly alkaline solution [11].

At the end of the hydrolysis period, the Ph. Eur. titration prescribes neutralization of the alkaline reaction mixture with nitric acid and addition of excess acetate buffer (pH 4.6).

3.3. Changes due to the presence of mercury(II) ions

The addition of a small amount of mercury(II) nitrate solution influences strongly the pathway of the reaction. It was observed that the (5*S*,6*R*)-benzylpenicilloate (**2d**) becomes the predominant species by epimerization (Fig. 2d). Besides the natural epimer **2a**, two small peaks are visible; they are probably the two 6-epimers. After addition of mercury(II) ions the UV spectrum of the solution shows a transient absorption band at 280 nm which is an indication for 6-epimerization (Fig. 3). It has been reported that the mercury(II) ion promotes the opening of the thiazolidine ring of benzylpenicilloic acid. This gives the appearance of the enamine structure of benzylpenamaldate **2c**, which is a tautomer of the imine structure of opened benzylpenicilloic acid **2b** [12]. The enamine structure is responsible for the absorbance at 280 nm. Deuterium experiments in an NMR study corroborated that this epimerization in the presence of mercury(II) ions proceeds via the enamine tautomer of benzylpenamaldate [18].

3.4. Decarboxylation to benzylpenilloic acid

During the titration, further addition of mercury(II) nitrate solution promotes the decarboxylation of the benzylpenicilloic acid mixture (Fig. 4a). The fact that the four peaks disappear with the formation of penilloic acid is a supplementary

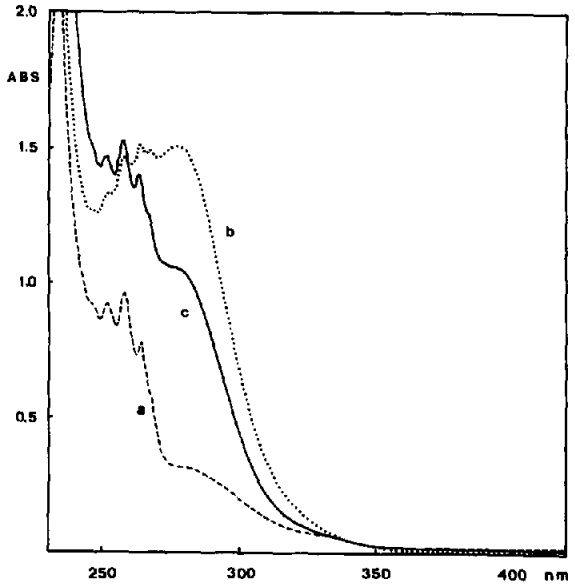

Fig. 3. UV spectrum showing the intermediate formation of benzylpenamaldate after addition of mercury(II) nitrate solution to benzylpenicilloic acid at pH 4.6. (a) Before addition; (b) immediately after addition; (c) after a reaction time of 15 min.

indication for their benzylpenicilloic acid structure. When about 25% of the theoretical amount of mercury(II) nitrate solution is added, the penicilloic acids are nearly completely transformed into the penilloic acids **3a** and **3c**. A mixture of the two 5*R* and 5*S* isomers is formed in nearly equal proportions. By comparison with the reference products, it can be concluded that the 5*S* epimer is eluted before the natural 5*R* epimer. Decarboxylation occurs at room temperature and at pH 4.6; this has not been described previously. Normally the transformation of benzylpenicilloic acid into benzylpenilloic acid is carried out by heating at about 70–80°C and at a pH lower than 4 [10]. It has been reported that benzylpenilloic acid does not form an enamine thiol tautomer because no stabilization of the enamine by conjugation with a carbonyl group is possible. The epimerization of benzylpenilloic acid takes place through the imine tautomer **3b** [19]. Indeed, after addition of mercury(II) ions to benzylpenilloic acid in sodium acetate buffer (pH 4.6), no UV absorption band at about 280 nm is formed.

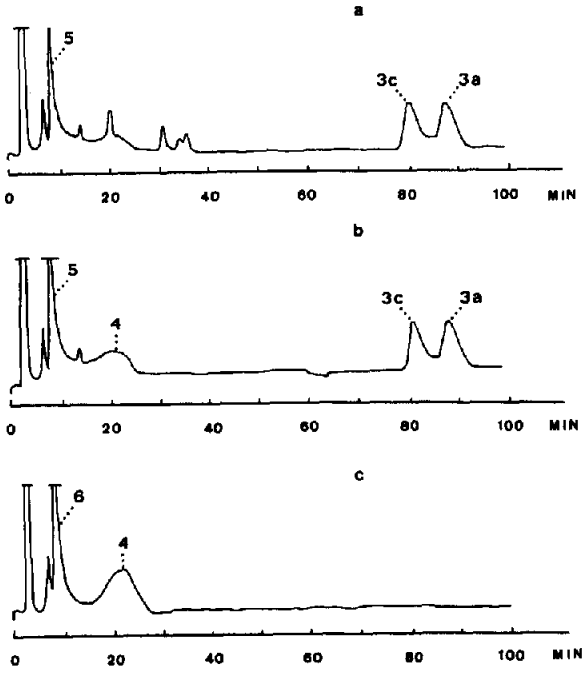

Fig. 4. Chromatograms of the mercurimetric titration mixture (LC system I). (a) After addition of 25% of the theoretical amount of mercury(II) nitrate solution; (b) after addition of 50% of the theoretical amount of mercury(II) nitrate solution; (c) at the end of the titration.

3.5. Fission of benzylpenilloic acid

On further addition of titrant, the surface of the peaks of benzylpenilloic acids descreases while two new peaks appear (Fig. 4b). At about 6 min an asymmetric peak appears and at about 22 min a large broad peak is eluted. These peaks correspond to products **5** and **4** respectively, as shown below. It has not been reported before that the transformation of **3a** or **3c** into **4** and **5** proceeds through **3b**.

3.6. Formation of benzylpenilloaldehyde

It has been reported that benzylpenilloaldehyde (**4**) is formed from benzylpenicilloic acid by reaction with mercury(II) ions in yields as high as 74% [20]. Benzylpenilloaldehyde is unstable in aqueous solutions, even at room temperature [21]. The presence of **4** as a final product of the titration was indicated as follows. At the end of the mercurimetric titration (Fig. 4c), the unstable penilloaldehyde **4** was extracted with ethyl acetate. Its presence was proved by TLC on silica gel with dichloromethane–methanol (96:4, v/v) as eluent. The extracted compound had an R_f value of 0.20, was visible by UV detection and gave a yellow spot after spraying with 2,4-dinitrophenylhydrazine–sulfuric acid solution. This proves that the product has an aromatic side chain and an aldehyde frunction. The addition of the same 2,4-dinitrophenylhydrazine solution directly to the titrated solution yielded a precipitate of benzylpenilloaldehyde 2,4-dinitrophenylhydrazone. TLC with the same mobile phase showed a yellow spot with R_f 0.46. After purification by column chromatography on silica gel with the same solvent mixture and recrystallization from alcohol, the product had an m.p. of 193–194°C and showed an absorption maximum at 355 nm and a shoulder at 254 nm in methanol. The structure was confirmed by ^1H and ^{13}C NMR spectra and the

Table 1
Assignments of resonances in the ^1H and ^{13}C NMR spectra of benzylpenilloaldehyde 2,4-dinitrophenylhydrazone

NMR	δ (ppm)[a]
^1H (DMSO-d_6–$CDCl_3$)	3.51 (s, CH_2), 4.02 (t, $J = 4.9$ Hz, CH_2–CH), 7.26 (s, C_6H_5), 7.76 (d, $J = 9$ Hz, 6′-H), 7.92 (t, $J = 4.5$ Hz, CH =), 8.25 (dd, $J = 3.5$ and 9 Hz, 5′-H), 8.3–8.4 (br, CO–NH), 8.84 (d, $J = 3.5$ Hz, 3′-H), 11.40 (s, NH–N). After addition of D_2O to the solution the signals at 8.3–8.4 and 11.4 ppm disappear.
^{13}C	40.5 (CH_2–N), 42.3 (CH_2–C_6H_5), 116.3 (6′-C), 122.7 (3′-C), 126.2 (4-C), 128.0 (3 and 5-C), 128.8 (2′-C), 128.9 (2- and 6-C), 129.3 (5′-C), 135.9 (1-C), 136.9 (4′-C), 144.7 (1′-C), 150.1 (CH =), 170.3 (C = O).

[a] Abbreviations: s, singlet, d, doublet; t, triplet; br, broad signal.

data are presented in Table 1. The benzylpenilloaldehyde was also isolated from the broad peak in the chromatogram (Fig. 4c). The peak was collected several times after injecting 100 μl amounts in LC system I. The volume was reduced in vacuo below 30°C and 2,4-dinitrophenylhydrazine solution was added. The precipitated product was isolated and identified by TLC and UV spectrophotometry.

The fact that benzylpenilloaldehyde is eluted as a broad peak suggests that the product is in equilibrium with its enol form. The same enolization was observed for a substituted acetaminoaldehyde formed during degradation of the cephalosporin cefadroxil [22].

3.7. Formation of penicillamine

The asymmetric peak, corresponding to **5**, that appeared at the beginning of the chromatogram (Fig. 4c) must be assigned to the reaction product between penicillamine, the other half of the split benzylpenicilloic acid, and mercury(II) ions.

During our experiments, the titration curves for hydrolyzed benzylpenicillin and penicillamine were very similar and showed a first inflection at about 50%. Titration with mercury(II) solutions is well established for sulfur-containing compounds such as thiols. Only one inflection is obtained for the titration of simple thiols. The curves obtained now had two inflections. At the first inflection the molar ratio of hydrolyzed penicillin to the mercury(II) ion is 2:1; at the second inflection, equimolar amounts of the species are present in the solution. Similar results were obtained by Billabert et al. [23] in the titration of penicillamine. The first inflection was reported to correspond to the reaction of two molecules of penicillamine with one mercury(II) ion to form a sulfide (**5**). The stability constant was very high. The second inflection corresponded to the reaction of one molecule of penicillamine with one mercury(II) ion to form a chelate (**6**) [23]. The carboxyl group formed the chelate, as was proved by titration of *N*-acetylcysteine and thioglycolic acid, which also gave titration curves with two inflections [24].

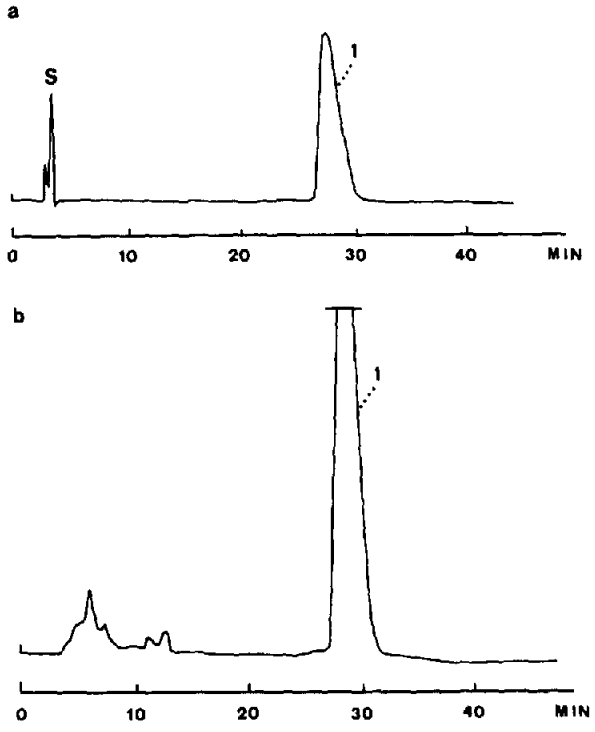

Fig. 5. Chromatograms of radioactive benzylpenicillin (LC system II). (a) Starting material with UV detection; (b) starting material with radioactivity detection. S = solvent; see Fig. 1 for key.

The two penicillamine–mercury derivatives **5** and **6** are eluted at the beginning of the chromatograms obtained with the two chromatographic systems and correspond to the peaks obtained by LC of the titration mixture (Fig. 4b). The sulfide **5** is eluted closely before the chelate derivative **6**.

Benzylpenaldic acid (**7**) is not formed as an intermediate in the mercurimetric titration.

3.8. Results obtained during titration of labeled benzylpenicillin

The titration of radioactive benzylpenicillin gave results identical with those for the non-labeled product. The sensitivity of radioactivity detection is much higher than that of UV detection. Figs. 5–8 show the chromatograms obtained in the different steps of the reaction sequence. The radioactivity is present, as expected, in the ben-

zylpenicilloic acids, eluted as one peak in this LC system, the two benzylpenilloic acid epimers and finally in the benzylpenilloaldehyde. No formation of other substances was observed.

3.9. Results obtained during titration of model compounds

Titrations were carried out on several sulfur-containing model compounds. The results obtained for intermediates such as natural benzylpenicilloic acid, (5*R*)- and (5*S*)-benzylpenilloic acid and penicillamine were identical with or without prior treatment with alkali. The titration of benzylpenillic acid (**8**), a potential acid degradation product of benzylpenicillin, gave two inflections with identical results with or without alkaline hydrolysis. All these substances will therefore be titrated during the mercurimetric titration of the decomposition products according to the Ph. Eur. In this titration no alkali is added so that intact penicillin does not react. N^4-Acetylbenzylpenilloic acid (**9**) did not react with mercury(II) nitrate solutions with or without prior treatment with alkali, as described in the titration method, which is not sufficient to hydrolyze the amide. Only reactive *N*-acylated thiazolidine derivatives, such as penicillins, liberating completely the amino group of the thiazolidine ring by reacting with alkali at room temperaure, will be titrated with mercury(II) nitrate solution after alkaline hydrolysis. This proves the selectivity of the mercurimetric titration.

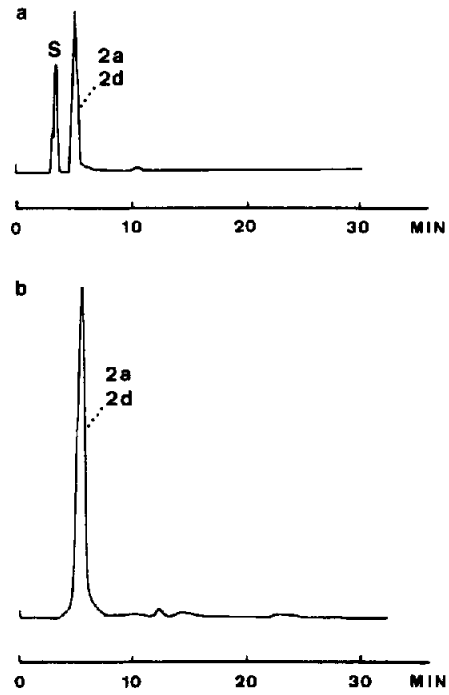

Fig. 6. Chromatograms of the mercurimetric titration mixture of radioactive benzylpenicillin after alkaline hydrolysis for 15 min at room temperature (LC system II). (a) UV detection; (b) radioactivity detection. S = solvent; see Fig. 1 for key.

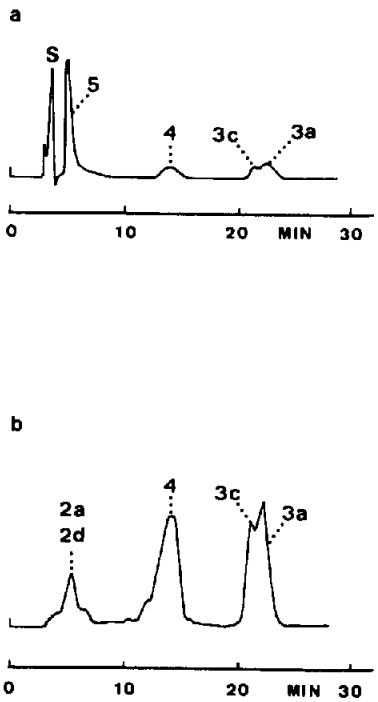

Fig. 7. Chromatograms of the mercurimetric titration mixture of radio-active benzylpenicillin after addition of 25% of the theoretical amount of mercury(II) nitrate solution (LC system II). (a) UV detection; (b) radioactivity detection. S = solvent; see Fig. 1 for key.

4. Conclusions

These experiments showed that the reaction of hydrolyzed benzylpenicillin with mercury(II) nitrate solution induces extensive epimerization of the natural benzylpenicilloic acid. At the same

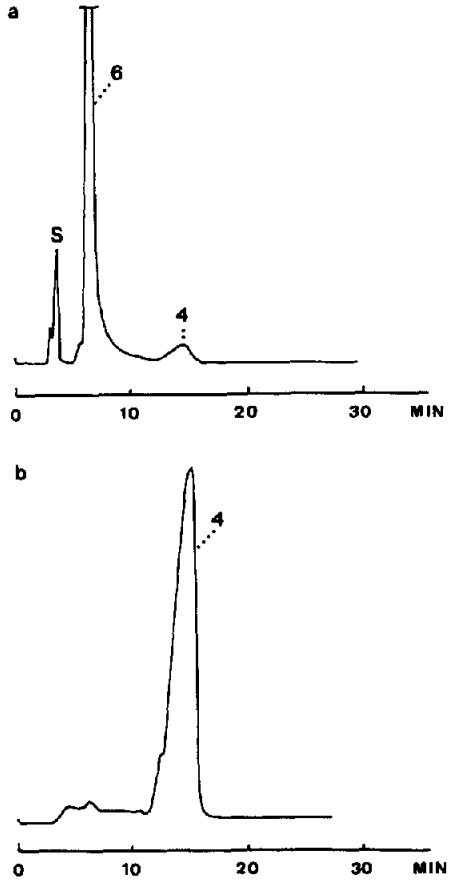

Fig. 8. Chromatograms of the mercurimetric titration mixture of radioactive benzylpenicillin at the end of the titration (LC system II). (a) UV detection; (b) radioactivity detection. S = solvent; see Fig. 1 for key.

time, complete decarboxylation of these benzylpenicilloic acid epimers leads to the two benzylpenilloic acids. They are then cleaved into benzylpenilloaldehyde and penicillamine. The latter is responsible for the titration curve with two inflections; the first corresponds to the formation of a sulfide and the second to the formation of a cyclic chelate.

References

[1] H.T. Clarke, J.R. Johnson and R. Robinson (Eds.), The Chemistry of Penicillin, Princeton University Press, Princeton, NJ, 1949.
[2] J.F. Alicino, Ind. Eng. Chem., Anal. Ed., 18 (1946) 619–620.
[3] K.W. Glombitza an D. Pallenbach, Arch. Pharm. (Weinheim), 302 (1969) 695–700..
[4] D. Pallenbach and K.W. Glombitza, Arch. Pharm. (Weinheim), 302 (1969). 863–868.
[5] B. Karlsberg and U. Forsman, Anal. Chim. Acta, 83 (1976) 309–316.
[6] A.E. Bird and C.E. Redrup, Proc. Anal. Div. Chem. Soc., 14 (1977) 285–288.
[7] B.B. Levine, Nature (London) 186 (1960) 939–940.
[8] T. Paal and M. Molnar, Gyogyszereszet, 20 (1976) 8–13.
[9] European Pharmacopoeia, Maisonneuve, Sainte Ruffine, 2nd edn., 1989, Monograph 113.
[10] A.C. Munro, M.G. Chainey and S. Woroniecki, J. Pharm. Sci., 67 (1978) 1197–1204.
[11] I. Ghebre-Sellassie, S.L. Hem and A.M. Knevel, J. Pharm. Sci., 73 (1984) 125–128.
[12] R. Mozingo and K. Folkers, in The Chemistry of Penicillin, 1949, pp. 535–656.
[13] J.R. Johnson, R.B. Woodward and R. Robinson, in The Chemistry of Penicillin, 1949, pp. 440–454.
[14] K.W. Mooz, H. Knieps and H. Lehmann, Pharmazie, 20 (1965) 764–770.
[15] M.I. Page, Acc. Chem. Res., 17 (1984) 144–151.
[16] D.A. Johnson, D. Mania, C.A. Panetta and H.H. Silvestri, Tetrahedron Lett., 126 (1968) 1903–1905.
[17] A. Vlietinck, E. Roets, P. Claes, G. Janssen and H. Vanderhaeghe, J. Chem. Soc., Perkin Trans. 1, (1973) 937–942.
[18] J. Haginaka and J. Wakai, Chem. Pharm. Bull., 33 (1985) 2605–2608.
[19] H. Rolli and C.H. Schneider, Arzneim.-Forsch., 34 (1984) 1247–1249.
[20] E.V. Brown, in The Chemistry of Penicillin, 1949, pp. 473–534.
[21] R.L. Peck and K. Folkers, in The Chemistry of Penicillin, 1949, pp. 52–75.
[22] C. Hendrix, E. Roets, S. Roesems, R. Busson, J. Rozenski, G. Janssen and J. Hoogmartens, Arch. Pharm. (Weinheim), 327 (1994) 805–807.
[23] A. Billabert, M. Callanquin and M. Hamon, Analusis, 3 (1975) 258–262.
[24] A. Billabert and M. Hamon, Analusis, 5 (1977) 29–33.