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High-performance liquid chromatographic method for the separation of the optical isomers of γ,γ′-di-*tert.*-butyl-D,L-γ-carboxyglutamic acid and D,L-γ-carboxyglutamic acid

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Abstract

The chemical synthesis of γ,γ′-di-*tert.*-butyl-γ-carboxyglutamic acid is accompanied by extensive racemization, and very careful resolution is needed to obtain D- and L-γ,γ′-di-*tert.*-butyl-γ-carboxyglutamic acids in high chiral purity. A novel method was devised for the separation of enantiomers of γ,γ′-di-*tert.*-butyl-γ-carboxyglutamic acid and γ-carboxyglutamic acid, applying precolumn derivatization with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide and 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate as chiral reagents, with subsequent reversed-phase high-performance liquid chromatographic separation of diastereomeric compounds. The effects of organic modifiers, of the mobile-phase composition and of the pH on the separation of the diastereomers were investigated.

1. Introduction

γ-Carboxyglutamic acid (Gla), an acid-labile derivative of glutamic acid (Glu), exhibits a very strong and specific affinity for calcium ions. A number of Gla residues are present in the calcium-binding region of Gla-containing polypeptides [1–4] and proteins [5], and as a result the interactions between the bivalent ions and the cumulated carboxylic side-chains are extensively enhanced. These proteins and peptides are known to be responsible for many biological functions, and consequently there is a considerable interest in the biological study of Gla-containing peptides. The synthesis of Gla-containing peptides requires protected Gla derivatives, which are not always readily available commercially. In our laboratory γ,γ′-di-*tert.*-butyl-D- and -L-γ-carboxyglutamic acids (DTBGLA) have been synthesized with high efficiency.

According to the literature the chromatographic analysis of Gla can be divided into three main phases: (1) separation on an anion-exchange column, with subsequent postcolumn derivatization with ninhydrin or *o*-phthalaldehyde [5–23] (Low et al. [24] used thin-layer chromatography), (2) RP-HPLC methods, with precolumn derivatization with *o*-phthalaldehyde in the presence of thiols [25–30], phenyl isothiocyanate [31], 1,1-diphenylboronic acid [32] or 4-dimethylamino-4-azobenzenesulfonyl chloride [33] and (3) identification of D- and L-isomers of Gla and Gla derivatives after their conversion to Glu. For the separation of D- and

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L-Glu, Rivier et al. [34] and Nishiuchi et al. [35,36] used 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA) as chiral reagent, while Kurihara et al. [37] used 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC).

The method described in the present paper allows the analysis of the optical isomers of DTBGLA and Gla without conversion to Glu. The amino acids were derivatized directly with FDAA and GITC, and the D- and L-diastereoisomers were separated by an RP-HPLC method.

2. Experimental

2.1. Chemicals and reagents

Chirally pure enantiomers of DTBGLA were synthesized via a seven-step route with preparatively advantageous modification of the general literature procedures [38–41].

D- and L-Gla were synthesized by acidic treatment of DTBGLA with hydrogen fluoride at 0°C for 30 min. The chiral purity of D- and L-DTBGLA and D- and L-Gla was determined by our new method.

GITC was purchased from Aldrich (Steinheim, Germany), FDAA from Pierce Chemical Company (Rockford, IL, USA), potassium dihydrogenphosphate, phosphoric acid of analytical reagent grade, acetonitrile and methanol of HPLC grade from Merck (Darmstadt, Germany). Buffers were prepared by means of dissolving 0.01 mol potassium dihydrogenphosphate in water, adjusting the pH with 5.0 *M* phosphoric acid or 0.5 *M* sodium hydroxide and diluting to the final volume of 1 l.

2.2. Apparatus

The HPLC analyses were performed on an M-600 low-pressure gradient pump fitted with an M-996 photodiode-array detector and a Millennium 2010 Chromatography Manager data system (Waters Chromatography, Division of Millipore, Milford, MA, USA) and on an L-6000 liquid chromatographic pump (Merck Hitachi, Tokyo, Japan) fitted with a UV 308 detector (Labor MIM, Budapest, Hungary) and an HP 3395 integrator (Hewlett-Packard, Waldbronn, Germany).

The column used was Lichrospher 100 RP-18 (125 × 4 mm I.D.), 5 μm particle size (Merck, Darmstadt, Germany).

2.3. Derivatization of amino acids for chromatographic analysis

An amount of 2–4 μmol of DTBGLA or Gla was derivatized with FDAA by the Marfey method [42]. The molar ratio of FDAA/amino acid in the mixture was kept at 1.4:1. For derivatization with GITC, 2–4 μmol of DTBGLA or Gla was used, applying the method of Nimura et al. [43]. In both derivatization methods, after completion of the reaction, the reaction mixture was diluted with the eluent 2–10 fold and 10-μl aliquots were used for injection onto the HPLC system.

3. Results and discussion

To keep the ionization of the free carboxylate groups of Gla and DTBGLA at a constant level within the column, and to achieve good run-to-run reproducibility, buffering of the aqueous–organic modifier phase system is required. Protonation of the free carboxylate groups changes the hydrophobicity and hence the retention behaviour. On the other hand, Gla and its derivatives are sensitive to hydrolysis under acidic conditions [17]. Thus, the correct choice of the buffer system and the conditions used can be rather important. The application of potassium dihydrogenphosphate as buffer appeared favourable. Under the conditions used for the analysis of Gla and DTBGLA, any Glu peak could readily be observed in the chromatograms.

3.1. Separation of D,L-DTBGLA–FDAA derivatives

The chromatographic analysis was carried out in different phosphate buffer (pH 2–6)–organic

modifier (acetonitrile or methanol) systems. Data obtained in the phosphate buffer–acetonitrile system indicate that, with a decrease of the acetonitrile content at a given pH, k' increases and the α and R_s values improve. The k' and R_s values increased considerably on decrease of the pH of the aqueous phase from 6 to 2. At a constant phosphate buffer–acetonitrile ratio, e.g. 60:40 (v/v), the k' values of the L- and D-isomers increased from 1.3 to 16.0 and from 2.1 to 24.5, while R_s increased from 1.1 to 4.9 (Fig. 1A). This behaviour is probably correlated with protonation of the α-carboxylate group. The protonation microconstant for the α-carboxylate group of Gla in aqueous solution is $\log k^{\alpha} = 2.66$ [44]. There is no general rule for the conversion of the protonation constants obtained in aqueous solution to those in an aqueous–organic modifier system [45], but the increase in the retention time at low pH is probably connected with the increased hydrophobicity of the adduct. On the other hand, a resolution higher than 2 can also be achieved with a relatively low k' ($k' < 5$) at higher pH, by keeping the acetonitrile content below 40% (v/v).

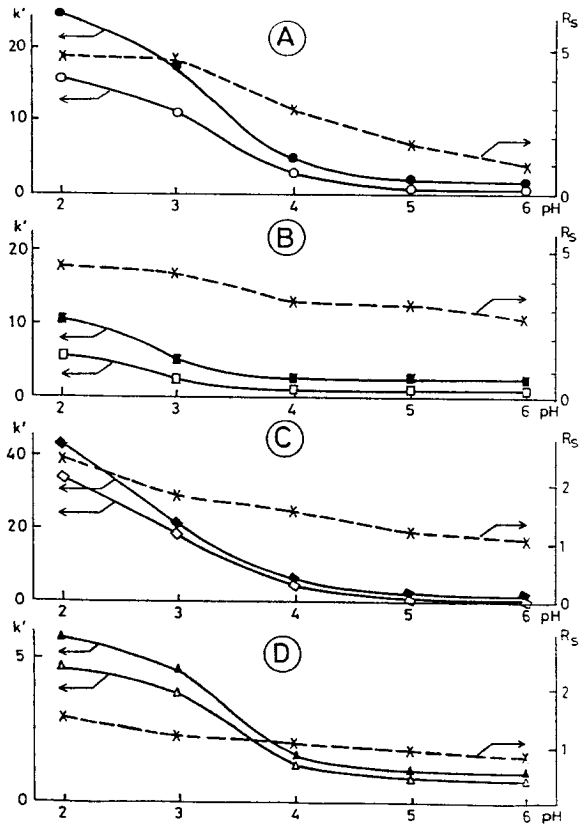

Fig. 1. Dependence of retention factor (k') and resolution (R_s) of DTBGLA–FDAA and DTBGLA–GITC derivatives on pH at a given eluent composition. Column, Lichrospher 100 RP-18; flow-rate, 0.8 ml/min. (A,B) DTBGLA–FDAA derivatives; (C,D) DTBGLA–GITC derivatives. Mobile phase, 0.01 *M* potassium dihydrogenphosphate (pH 2–6)–organic modifier; (A,C) buffer–acetonitrile 60:40 (v/v), (B,D) buffer–methanol 35:65 (v/v); (○, □, ◇, △) retention factor (k') of L-isomer, (●, ■, ◆, ▲) retention factor (k') of D-isomer; (×) R_s value of L/D isomers.

The general observations for the phosphate buffer–methanol system are similar to those obtained with acetonitrile as organic modifier. With respect to the separation capabilities of the two systems, the phosphate buffer–methanol system seems more efficient than the acetonitrile-containing one. To compare the two systems (see Fig. 1A and B), in the methanol-containing system a resolution of higher than 2.5 can be achieved at every pH with low values of the corresponding retention factor. Figs. 2A and 2B show characteristic chromatograms of DTBGLA–FDAA derivatives obtained in the acetonitrile- or methanol-containing eluent systems. The elution sequence on the chromatograms is L-isomer before D-isomer. The described method is suitable for the determination of a content of the minor enantiomer of less than 0.05% of that of the major enantiomer.

3.2. Separation of D,L-DTBGLA–GITC derivatives

The results of the separation in the phosphate buffer–acetonitrile or methanol systems of the GITC derivatives show that with a decrease in the organic modifier concentration, k', α and R_s increase at every pH. The effect of pH at a given phosphate buffer–organic modifier ratio is similar to that observed for the FDAA derivatives (Fig. 1C,D). With regard to separations carried out with the two derivatizing reagents: FDAA and GITC at the same eluent composition, phosphate buffer–acetonitrile 60:40 (v/v), and at the same pH, the GITC derivatives generally

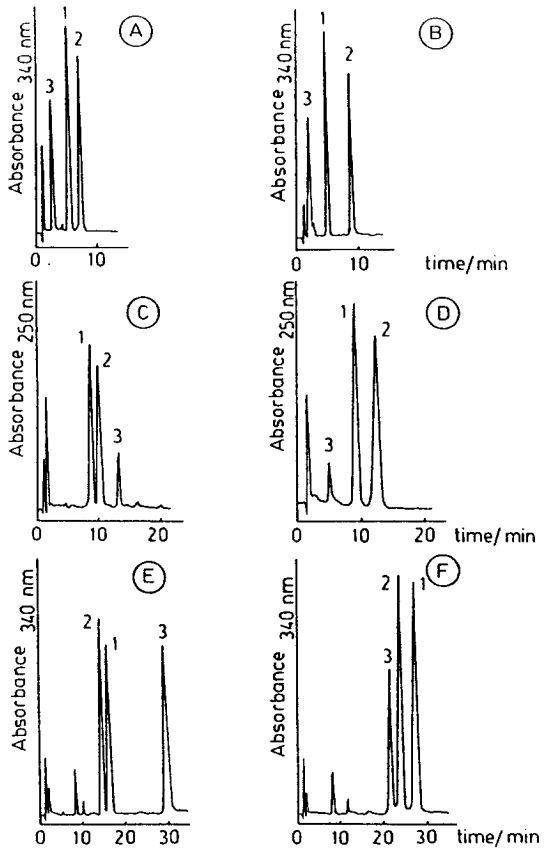

Fig. 2. Chromatograms of D,L-DTBGLA and D,L-Gla derivatives. Column, Lichrospher 100 RP-18; flow-rate, 0.8 ml/min; detection, 340 nm (FDAA derivatives), 250 nm (GITC derivatives). (A,B) D,L-DTBGLA–FDAA derivatives; (C,D) D,L-DTBGLA–GITC derivatives; (E,F) D,L-Gla–FDAA derivatives. Mobile phase, A, phosphate buffer (pH 3)–acetonitrile 50:50 (v/v); B, phosphate buffer (pH 3)–methanol 35:65 (v/v); C, phosphate buffer (pH 5)–acetonitrile 65:35 (v/v); D, phosphate buffer (pH 6)–methanol 45:55 (v/v); E, phosphate buffer (pH 2)–acetonitrile 80:20 (v/v); F, phosphate buffer (pH 2)–methanol 70:30 (v/v). Peaks: 1 = L-isomer, 2 = D-isomer, 3 = unreacted reagent. Molar ratio of D/L isomers is 1:1.

have higher k' and lower R_s values than the FDAA derivatives (Fig. 1A,C). In the phosphate buffer–methanol system at a given eluent composition of 35:65 (v/v) and at the same pH, the GITC derivatives have lower k' and R_s than the FDAA derivatives (Fig. 1B,D). On the basis of these results, a baseline separation ($R_s > 1.5$) of DTBGLA–GITC derivatives can be obtained with relatively low k' values ($k' < 5$). For this purpose the pH of the phosphate buffer should be kept at a relatively high value (pH 5–6) and the organic-modifier content of the eluent at a relatively low concentration (Fig. 2C,D). The elution sequence of the isomers on the chromatograms is L-isomer before D-isomer. The chiral purity of DTBGLA enantiomers can also be determined by application of GITC as derivatizing reagent. The detection limit is lower than 0.05% of the minor isomer in the presence of the major enantiomer.

3.3. Separation of D,L-Gla–FDAA and D,L-Gla–GITC derivatives

The separations were carried out in phosphate buffer–acetonitrile or methanol systems (Fig. 2E,F). The separation of D- and L-diastereoisomers could be achieved only when the pH of the phosphate buffer was near 2. The distribution curves of the Gla species [44] indicate that the formation of more hydrophobic (protonated) species is favourable at low pH ($pH \ll 2$) in aqueous solution.

In the aqueous–organic modifier system, the pK values of the carboxylate groups of Gla are higher, but the increase at 20–30% organic-modifier content is probably not considerable [45]. Thus the observed chromatographic behaviour of Gla derivatives is in agreement with the expectations.

Of the two organic modifier systems, acetonitrile and methanol, the acetonitrile-containing system seems more efficient. A similar resolution in acetonitrile can be achieved at lower k', and the main peak of the unreacted reagent elutes very close to the D-diastereoisomer in methanol. The elution sequence is the reverse of that observed for the DTBGLA–FDAA derivatives: the D-isomer elutes before the L-isomer. The application of this separation method allows the determination of a minor enantiomer content less than 0.05% of that of the major isomer.

The separation experiments for D,L-Gla–GITC derivatives were carried out in different phosphate buffer–acetonitrile or methanol systems.

Both systems were unsuccessful with respect to the separation of GITC derivatives.

4. Conclusion

The described procedure can be applied for the separation of optical isomers and for the determination of the enantiomeric content of DTBGLA and Gla without conversion to Glu. In the case of DTBGLA, FDAA seemed more suitable than GITC as derivatizing reagent, and a better resolution could be achieved within a shorter time. Concerning the two organic modifiers, the methanol-containing systems seemed more favourable than the acetonitrile-containing ones. In the separation of Gla enantiomers, FDAA was much more favourable as derivatizing reagent than GITC, while of the two organic modifiers, acetonitrile was more advantageous than methanol.

The pH of the phosphate buffer was a very important factor in the separation. The elution sequence for the L- and D-diastereomers of DTBGLA was the opposite of that for Gla.

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