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Porous graphitic carbon for the chromatographic separation of O-tetraacetyl-β-D-glucopyranosyl isothiocyanate-derivatised amino acid enantiomers

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Abstract

Amino acid enantiomers, following facile N-derivatisation with O-tetraacetyl-β-D-glucopyranosyl isothiocyanate, have been separated by high-performance liquid chromatography on a porous graphitic carbon column (Hypercarb S) using a binary 0.1% (v/v) aqueous trifluoroacetic acid–acetonitrile gradient elution profile. Resolution of fourteen pairs of derivatised D,L-amino acid diastereoisomers (apparent $\alpha = 1.02$–1.63) was achieved, including the simultaneous analysis of common protein-derived amino acid enantiomers.

1. Introduction

High-performance liquid chromatographic (HPLC) separation of common amino acid enantiomers has been achieved previously by the use of chiral derivatisation reagents or chiral ligands [1–4] on silica-based stationary phases. To date, one of the most facile chiral derivatisation reagent is O-tetraacetyl-β-D-glucopyranosyl isothiocyanate (GITC), which gives relatively stable diastereomeric glucopyranosyl thiourea derivatives [2,5]. The condensation reaction, outlined in Fig. 1, is most rapid under basic conditions. Efficient separation of these thiourea derivatives (GITC-amino acids) have been demonstrated on reversed-phase silica using a complex ternary solvent system [5]. In addition to the complexity of this elution profile, alkyl-bonded silica stationary phases suffer from a number of drawbacks, including poor stability at extremes of pH and a variety of unwanted interactions due to surface heterogeneity [6].

Porous graphitic carbon (PGC, Hypercarb) was recently introduced as an alternative for HPLC [7,8]. This material, consisting of pure carbon micro-particles, has several advantages, most notably its physical and chemical stability as well as superior selectivity towards diastereoisomers and geometric isomers [9–13]. We now report, for the first time, efficient separation of GITC-D,L-amino acid diastereoisomers on graphitic carbon-HPLC using the commercially available Hypercarb S column.

2. Experimental

2.1. Chemicals

Amino acids, GITC and other reagents were purchased from Sigma (Poole, UK) and Aldrich (Gillingham, UK). HPLC-grade acetonitrile,

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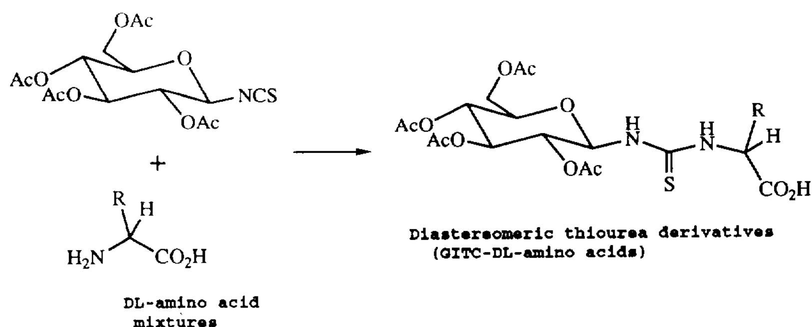

Fig. 1. Derivatisation of amino acids with the isothiocyanate GITC.

purchased from Fisons (Loughborough, UK) and double-distilled water (using Elgastat Spectrum) were used for all sample preparations and chromatographic separations. The pre-packed graphitic carbon column (Hypercarb S, 200 × 4.6 mm, particle diameter 7 μm) was supplied by Shandon HPLC (Runcorn, UK). All experiments were performed at ambient temperature.

2.2. Chromatographic analysis

The HPLC apparatus (Pharmacia LKB, Milton Keynes, UK) was equipped with a binary solvent-delivery system comprised of two 2150 HPLC pumps, a 2152 controller and 2151 variable-wavelength monitor. The eluents used were: (A) 0.1% (v/v) aqueous trifluoroacetic acid (TFA) and (B) 0.1% (v/v) TFA in 90% (v/v) aqueous acetonitrile. The elution step program is as follows: isocratic 30% B for 15 min, followed by linear gradient from 30 to 36% B in 10 min, then 36% B for 3 min, then 36 to 39% B in 5 min, then 39% B for 2 min, then 39 to 45% B in 10 min, then 45% B for 5 min, then 45 to 100% B in 33 min, then 100% B for 10 min, and 100 to 30% B in 3 min (total run time 96 min). The Hypercarb S column was eluted at a flow-rate of 1.10 ml min^{-1} and the eluate was monitored at 250 nm. Using acetone, the t_0 for the column was found to be 2.0 min.

Initially, all the derivatised amino acid enantiomers were analysed separately (in duplicate) to determine the elution order, and subsequently the enantiomer mixtures were analysed. In the latter analysis, including that obtained for the HPLC trace shown in Fig. 2, the mixtures were acquired by mixing freshly prepared respective GITC-amino acids of similar but not identical concentrations. The mean apparent capacity factor (k') and separation factor (α) in gradient elution chromatography were calculated from multiple injections of the enantiomer mixtures. The degree of correlation between the resulting retention data and with previous data on the separation of GITC-derivatised amino acids on an ODS silica column [5] was determined using linear least squares analysis.

2.3. GITC derivatisation of amino acids

To a solution of each amino acid (2.0 μmol; except for Lys whereby 1.0 μmol was used) in 50% (v/v) aqueous acetonitrile (100 μl) was added triethylamine (2 μl) followed by GITC in acetonitrile (20 μl, 40 mg ml^{-1}). The mixture was allowed to stand for about 30 min at room temperature, and was then quenched with 1.0 *M* aqueous hydrochloric acid (50 μl) and 25% (v/v) aqueous acetonitrile (830 μl). An aliquot of 5–20 μl of the resultant mixture was analysed.

Preliminary quantitative analysis using proline confirmed that the GITC-derivatisation reaction is complete within 20 min at room temperature.

3. Results and discussion

GITC has previously been used for derivatisation of both D,L-amino acids and N^{α}-methyl-D,L-

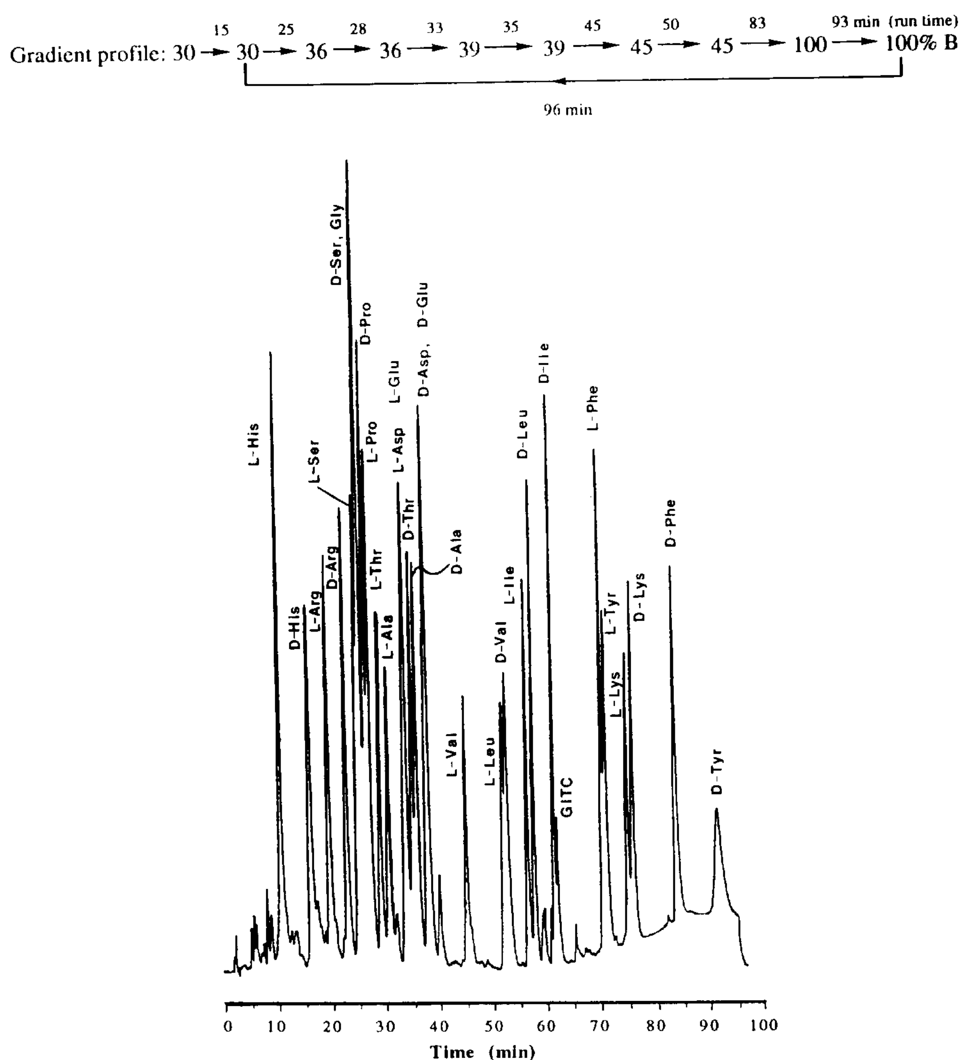

Fig. 2. Elution profile of GITC-D,L-amino acid diastereoisomers. See Experimental section for conditions.

amino acids to give lipophilic thiourea diastereoisomers which are separated by reversed-phase HPLC [5,14]. We have now accomplished similar separations (Fig. 2) of GITC-D,L-amino acids on a recently introduced graphitic carbon HPLC column. In addition, our chromatographic conditions allow simultaneous resolution of thiourea derivatives of all common proteinaceous amino acid enantiomers, except tryptophan (not eluted), obtainable by acid hydrolysis (Fig. 2, Table 1). With the exception of three pairs, excellent baseline resolutions are obtained for all thiourea D,L-amino acid diastereoisomers with apparent α values >1.1. Furthermore, the resolutions for the aromatic amino acid derivatives GITC-D,L-Phe and GITC-D,L-Tyr (apparent α, 1.2 and 1.3 respectively) are notably good. The strong retention of aromatic amino acids by the PGC column was confirmed by the failure to elute GITC-derivatised tryptophan enantiomers, which had an apparent k' value of >50 under the analytical conditions used. This behaviour of the aromatic amino acids is not unexpected since hydrophobic compounds are known to interact

Table 1
Separation of GITC-D,L-amino acids on Hypercarb S column

Amino acid	Apparent capacity factor, k'		
	D-isomer	L-isomer	α
His	7.15	4.38	1.63
Arg	10.54	8.92	1.18
Ser	11.89	11.60	1.03
Pro	12.43	12.78	1.03
Thr	16.67	13.71	1.22
Ala	17.07	14.49	1.18
Asp	17.97	16.03	1.12
Glu	17.97	16.03	1.12
Val	25.52	21.69	1.18
Leu	28.02	25.17	1.11
Ile	29.9	27.41	1.09
Phe	41.0	34.27	1.20
Lys	37.17	36.65	1.02
Tyr	45.04	34.67	1.30

more strongly with the PGC compared to ODS silica materials [9]. The order of elution of the GITC-derivatised enantiomers is consistently L-isomer before D-isomer, with the sole exception of GITC-proline, for which the D-isomer elutes before the L-isomer. The reason for this change of elution order is not known.

A detailed comparison of our data with those obtained using ODS-bonded silica [5], with respect to apparent k' values, revealed a linear correlation ($R^2 = 0.8$; Fig. 3). This suggests that the lipophilic tetraacetylglucopyranosyl moiety of these GITC-D,L-amino acids interacts with the graphitic carbon surface in a manner similar or comparable to the reversed-phase silica. However, for GITC-aromatic amino acids such as tyrosine, this is further enhanced by the stronger hydrophobic interactions with the graphitic carbon, giving comparatively longer retention times.

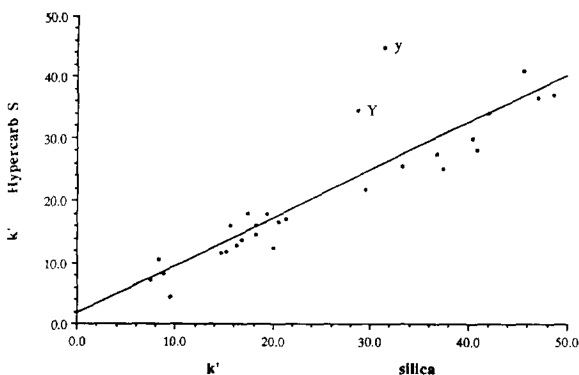

Fig. 3. Correlation between GITC-D,L-amino acids apparent k' obtained on Hypercarb S and on reversed-phase (C_{18}) silica. Reversed-phase silica data re-plotted from Ref. [5]. Y = GITC-L-Tyr; y = GITC-D-Tyr.

In conclusion, we have demonstrated a rapid and simple procedure for analysis of amino acid enantiomers in peptides and proteins by graphitic carbon-HPLC. This methodology clearly has broader applications, e.g. for the quality control of starting materials and analysis for racemisation during chemical synthesis of peptides. Since PGC was observed to have improved diastereo-selectivity for aromatic-containing compounds, we are currently exploring the separation of amino acid enantiomers by derivatisation with N^{α}-(2,4-dinitro-5-fluorophenyl)-L-alaninamide [15].

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