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Note

High-performance liquid chromatography of some biologically active carbodiimides

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Hydrophobic carbodiimides may be used for the modification and estimation of carboxylic acid groups in proteins[1]. In recent years, the use of dicyclohexylcarbodiimide (DCCD) as an inhibitor of oxidative phosphorylation[2] and for the estimation of membrane proteins involved in proton translocation[3–5] has become widespread. The proteolipid subunit of the proton translocating ATPase responsible for binding DCCD has been isolated from a variety of biomembranes, and has been shown to be covalently labelled at a glutamic or aspartic acid residue[6]. At concentrations of DCCD which cause total inhibition of ATPase activity, only 15–30% of the carbodiimide binding protein is covalently labelled: on this basis it has been suggested that the proteolipid exists as a hexamer, and that modification of only one of the subunits is sufficient for total inhibition. In *Saccharomyces cerevisiae*, binding of DCCD, but not inhibition of mitochondrial ATPase, is dependent on the growth conditions[7].

In view of the known lability and reactivity of carbodiimides[8], and the possibility of biological activity of their hydration products, it is important to know the degree of purity of carbodiimides used for estimation or inhibition of the proteolipid. A number of colorimetric methods for estimation of carbodiimides exist[9,10], but do not provide any information of the nature or quantity of possible contaminants. We present here the development of a simple and rapid method for the resolution of the products formed in solutions of carbodiimides.

MATERIALS AND METHODS

Chemicals

DCCD was purchased from BDH (Poole, Great Britain). N-(2,2,6,6-tetra-

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methylpiperidyl-1-oxyl)-N'-cyclohexylcarbodiimide (NCCD) was synthesised as described by Azzi *et al.*[11]. Hexamethylenebis[N-(N'-cyclohexylcarbodiimide)] (HMCC) was synthesised from hexamethylenediamine via N-hexamethylenebis[N-(N'-cyclohexylurea)] (HMCU). N,N'-Dicyclohexylurea (DCU) and N-(2,2,6,6-tetramethylpiperidyl-1-oxyl(-N'-cyclohexylurea (NCU) were derived from the corresponding carbodiimides by the action of dilute aqueous acid. Stock solutions of these compounds were dissolved in methanol and stored at −20°C.

The carbodiimides and ureas purified by chromatography were identified by IR analysis of the solvents and either by elemental analysis or by mass spectrometry. Additionally the carbodiimides were converted to the corresponding ureas and rechomatographed; the capacity factors of these ureas was compared with those of authentic material.

High-performance liquid chromatography (HPLC)

An Altex Model 110A solvent delivery system was connected to a 25 cm × 5 mm I.D. stainless steel column (HPLC Technology, Wilmslow, Great Britain), packed with various sorbents. Samples were introduced via a 100 μl loop Rheodyne 7125 sample injector. The separation of sample components was monitored at 212 mm with a Cecil Model 212 spectrophotometer fitted with an 8-μl flow cell. Solvents (of A.R. grade) were degassed by passage through a No. 4 glass sinter under vacuum. Columns were either slurry packed with Partisil 10-μm ODS 2, 10-μm ODS 3 or Spherisorb 5-μm ODS, or dry packed with magnesium oxide (BDH), LiChrosorb Alox T 30 μm (Merck), Sephasorb HP (Pharmacia) or LiChrosorb RP-2 (30 μm HPLC Technology). The RP-2, ODS 2 and ODS 3 columns were eluted with methanol at a flow-rate of 2 ml/min. Aluminium oxide and magnesium oxide columns were eluted with hexane—methanol mixtures at 2 ml/min. The Sephasorb HP column was eluted with methanol–water (1:1 v/v) at a flow-rate of 2 ml/min.

Ultraviolet (UV) spectra of carbodiimides were recorded on a Unicam SP1800 spectrophotometer in 1-ml quartz cuvettes with methanol as a reference. Colorimetric assay of carbodiimides was as previously described[10].

RESULTS AND DISCUSSION

Hitherto, we have used thin-layer chromatography on alumina with either benzene–ethyl acetate or hexane–ethyl acetate as eluent to monitor the purity of carbodiimide solutions[12]. The method is relatively insensitive however. Using the UV absorbance of carbodiimides and ureas (Fig. 1) as the method of detection, we examined a variety of supports for HPLC of hydrophobic carbodiimides.

Carbodiimides are acid labile and the attempted separation of DCCD from DCU on silica supports resulted in conversion of the carbodiimide to the urea due to the acidic nature of the silanol groups. Chromatography on adsorbants with a basic nature such as alumina or magnesium oxide was successfully employed in separating DCU from DCCD using hexane methanol mixtures as eluents. Conversion of carbodiimide to urea did not occur at significant rates using these systems, but retention times for the ureas were very long. Separation of carbodiimides and ureas could also be achieved by chromatography on Sephasorb, a cross-linked hydroxypropyldextran, using methanol–water (1:1 v/v) as eluent (Table I). Although useful

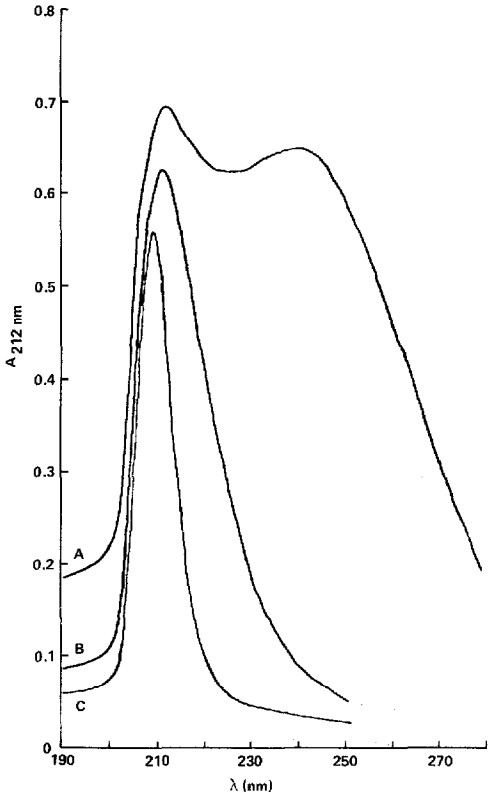

Fig. 1. Ultraviolet absorbance of carbodiimides and ureas in methanol. Curves: A, NCCD (0.63 μM); B, DCCD (1.03 μM); C, DCU (0.43 μM).

TABLE I

CAPACITY FACTORS OF CARBODIIMIDES AND UREAS

HPLC of carbodiimides and ureas by adsorption (Sephasorb) or partition (ODS 2 and ODS 3) was performed as outlined in Materials and Methods. Capacity factors, k', were calculated according to Snyder and Kirkland[13].

Compound	k'		
	ODS 2	*ODS 3*	*Sephasorb*
DCCD	1.60	1.39	5.77
DCU	0.68	0.78	1.31
NCCD	1.00	1.06	0.54
NCU	0.52	0.43	0.20
HMCC	1.56	1.39	—
HMCU	0.48	0.78	1.31

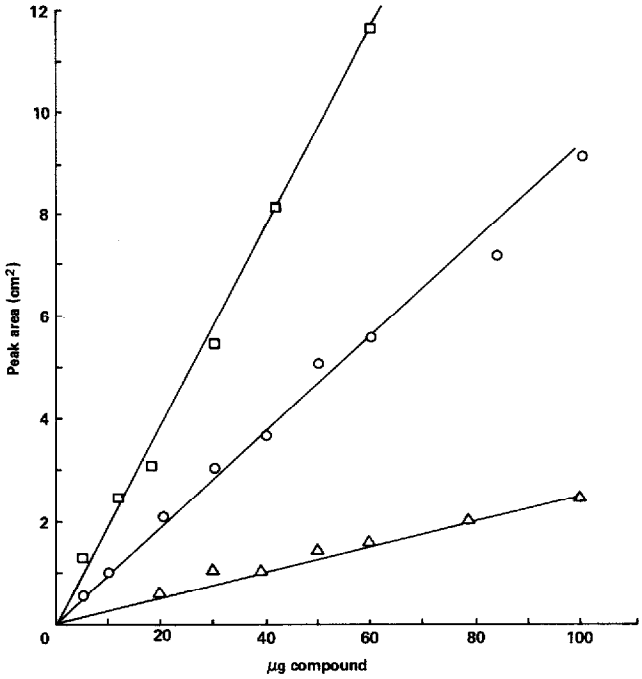

Fig. 2. Linearity of detection of carbodiimides and ureas by HPLC. Aliquots of stock solutions of NCCD (□), DCCD (○) and DCU (△) were injected into a Partisil ODS 3 column and eluted at a methanol flow-rate of 2 ml/min as described in Materials and Methods. Peak area (detected at 212 nm) was estimated by weighing.

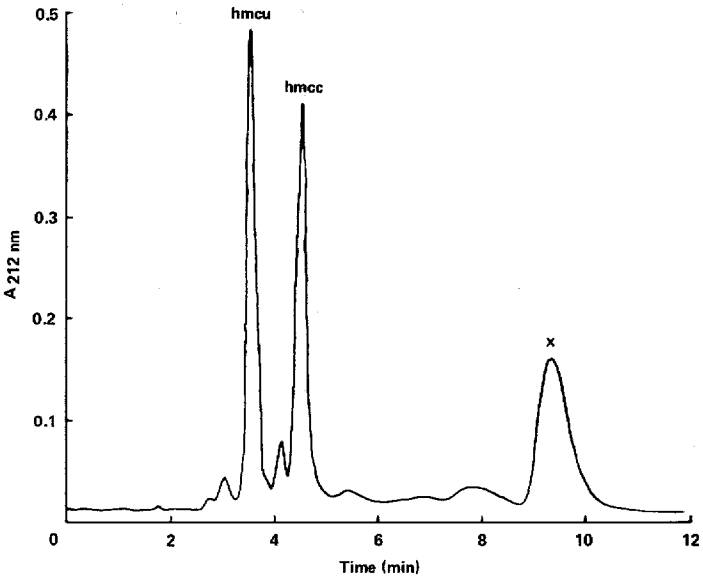

Fig. 3. Analytical HPLC of HMCC and hydration products on Partisil ODS 3. Methanol flow-rate was 2 ml/min. Sample was 100 μg of a 2-month-old stock solution of HMCC in methanol. X = Unknown material which appears on storage of stock solutions of HMCC.

for preparative column chromatography, separation of the basis of adsorption was not pursued further as an analytical technique due to (a) the long retention times of the ureas, (b) the asymmetry of the eluted peaks and (c) the long equilibration times required on changing eluent.

Resolution of the hydration and polymerisation products of carbodiimides was much improved when separation was based on reversed-phase partition (Table I). Solutions of hydrophobic carbodiimides which were separated into only two peaks by adsorption chromatography were resolved into as many as six components by elution from a Partisil ODS 2 column with methanol. Rechromatography of the individual components gave single peaks, indicating that conversion of carbodiimide to other products was not a problem in this separation. Similar results were obtained

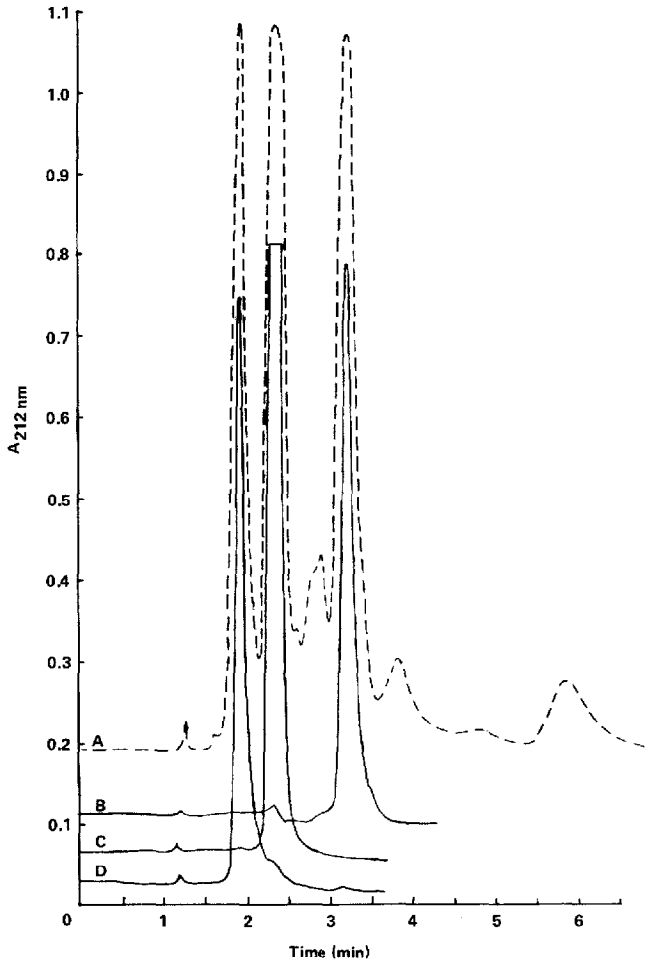

Fig. 4. Analysis and purification of a stock solution of HMCC on a semi-preparative column of Partisil ODS 2. 100 μl of a methanolic solution containing 5 mg of crude HMCC were applied to a 8 mm I.D. × 25 cm column of Partisil ODS 2 eluted with methanol (4 ml/min). The eluent corresponding to the mean peaks were collected and the component identified in the solution rechromatographed on this column. Trace: A, analysis of crude solution; B, HMCC; C, HCU; D, HMCU.

with Spherisorb ODS, but chromatography on LiChrosorb RP-2 failed to resolve DCU from DCCD.

Although hydration of carbodiimides to ureas occurred on silica columns, the presence of silanol groups on the ODS 2 support did not cause a similar degradation. Interaction of the carbodiimide with underivatised silanol groups undoubtedly occurs, as shown by comparison of the capacity factor, k'[13], obtained using ODS 2 (underivatised) or ODS 3 (in which the silanol groups have been capped with trimethylsilane).

In all cases the capacity factor is reduced when separations were performed on ODS 3 columns, indicating that adsorption contributed to the separation process on ODS 2 columns. The peaks obtained by reversed-phase partition chromatography did not show the "tailing" effect found in adsorption chromatography and thus resulted in improved resolution. Since the peak shape is optimal on ODS 3 columns we have chosen this support for analytical studies.

The relationship between peak area and amount of compound is linear over a range of 0–100 μg (Fig. 2). The chromatographic assay method is as sensitive as the colorimetric hydroxamate assay but an order of magnitude less sensitive than the aniline method[10]. The capacity of the assay may be extended up to 1 mg of carbodiimide by increasing the wavelength of detection to 220 nm, at the expense of sensitivity. HPLC also provides the advantage of assessment of contaminants, especially in the case of the bis(carbodiimides) (Fig. 3) where detection of the partial hydration product N-cyclohexyl-N^2-[hexamethylene-6-(carbodiimide-N^4-cyclohexyl)] urea (HCU) is possible.

We have routinely used this method for the small scale preparation of pure carbodiimides prior to their use as inhibitors of biological function. In Fig. 4, trace A shows the components to be found in a stored stock solution of HMCC. It can be seen that there are considerable amounts of HMCU and HCU together with other unknown degradation products. The major peaks were collected, rechromatographed to give traces B, C and D and were subsequently shown to be HMCC, HCU and HMCU respectively. This demonstrates the ability of this method to purify carbodiimides and related compounds.

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