Journal of Chromatography, 297 (1984) 21–29
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 376

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF AMINO ACIDS, PEPTIDES AND PROTEINS

LVIII*. APPLICATION OF REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY TO THE SEPARATION OF TYROSINE-SPECIFIC PHOSPHORYLATED POLYPEPTIDES RELATED TO HUMAN GROWTH HORMONE

B. GREGO
St. Vincent's School of Medical Research, Victoria Parade, Fitzroy, Melbourne, 3065 (Australia)
G. S. BALDWIN and J. A. KNESSEL
Ludwig Institute for Cancer Research, Tumour Biology Unit, Parkville, Melbourne 3050 (Australia)
and
R. J. SIMPSON, F. J. MORGAN and M. T. W. HEARN*
St. Vincent's School of Medical Research, Victoria Parade, Fitzroy, Melbourne, 3065 (Australia)

SUMMARY

Procedures for the purification of native and phosphorylated human growth hormone (hGH), S-carboxymethylated hGH, and hGH tryptic peptides, based exclusively on reversed-phase chromatography have been developed. Combinations of several volatile ion-pairing systems with small- and large-pore *n*-alkylsilicas were exploited in a general strategy, which allowed high recoveries of various hGH-related polypeptides from enzymatic incubations, as well as rapid desalting of samples following chemical modifications of the native protein, such as reductive alkylation in 6 *M* guanidine hydrochloride. The influence of the elution conditions on retention behaviour of phosphorylated hGH and its tryptic peptides in reversed-phase high-performance liquid chromatography is discussed.

INTRODUCTION

The purification of peptides and proteins by high-performance liquid chromatography (HPLC) has been well documented[1–5] in recent years. In particular, rapid, high resolution ultramicro-isolation of polypeptides from biological extracts can now be achieved by reversed-phase HPLC with porous microparticulate *n*-alkylsilica or polystyrene columns usually in conjunction with acidic aqueous–organic eluents. Recently, mobile phases of higher pH and containing various ion-pairing modifiers have gained[2,3,6,7] some popularity owing to the additional options they provide for

* For Part LVII see ref. 30.

0021-9673/84/$03.00

the modulation of polypeptide selectivity. Precise control over selectivity is clearly desirable in the chromatographic analysis of proteins which may undergo, as a prelude to the expression of their biological properties, post-translational enzymatic modifications such as phosphorylation, sulphation, transamidation, etc. Post-translational phosphorylation of proteins, specifically at serine and tyrosine residues, is now recognised to play an important role in the expression of a number of different biological functions in both prokaryotic and eukaryotic cells. For example, the regulation of glutamine synthetase activity in *Escherichia coli* involves[8] a unique phosphotyrosine residue at the adenyl group ligation site, whilst protein phosphorylation by specific tyrosine kinases is now believed[9,10] to be involved in the regulation of normal and malignant growth in mammalian cells. Since tyrosine phosphorylation accounts[9] for less than 0.1% of the phosphoamino acid pool in mammalian cells, sensitive chromatographic methods are required if *in vivo* or *in vitro* phosphorylated proteins, specifically labelled by tyrosine kinases, and their peptide fragments are to be adequately separated and analysed. The present study records our experiences with ultramicropreparative reversed-phase HPLC procedures for the isolation of human growth hormone (somatotropin hGH)-related polypeptides, specifically phosphorylated[10] by the EGF-stimulated kinase of A431 human epidermoid carcinoma cells. In this investigation a variety of *n*-alkylsilica columns have been employed with elution systems that allow high recovery and selectivity for both phosphorylated and non-phosphorylated species. These studies demonstrate that it is possible to recover and purify to homogeneity these growth hormone-related polypeptides solely by reversed-phase HPLC.

MATERIALS AND METHODS

Chemicals and reagents

Purified human growth hormone (22K hGH) was provided by the Human Pituitary Advisory Committee, Department of Health, Australia. The 20,000 M.W. structural variant of human growth hormone (20K hGH) was a gift from Dr. G. E. Chapman (University of Auckland, New Zealand). Authentic hGH tyrosine-containing peptides used in this study (Table I) were purified and characterised by amino acid composition and N-terminal sequence analysis according to well-established procedures. Epidermal growth factor (EGF-α) was purified as described previously[12]. Trypsin which had been treated with L-(tosylamido-2-phenyl)ethyl chloromethyl ketone was obtained from Worthington (Freehold, NJ), U.S.A.). HPLC grade acetonitrile was from either Burdick & Jackson (Muskegonn, MI, U.S.A.) or Waters Assoc. (Milford, MA, U.S.A.), and HPLC grade 2-propanol was from Ajax Chemicals (Sydney, Australia). Ammonium bicarbonate was also from Ajax Chemicals. Iodoacetic acid was obtained from BDH (Poole, U.K.) and subsequently recrystallised; β-mercaptoethanol was also from BDH.

Equipment

Two gradient liquid chromatographs were used. The Waters system consisted of two Model M6000 A pumps, a Model 660 gradient controller, a Model U6K sample injector and a Model 450 variable-wavelength detector connected to a Model 730 Data Module. The DuPont system consisted of a Model 850 gradient pump

TABLE I

STRUCTURES, RECOVERIES AND CHANGE IN RETENTION FOR PHOSPHORYLATED hGH, REDUCED AND ALKYLATED hGH AND TYROSINE-CONTAINING hGH TRYPTIC PEPTIDES

Polypeptides	*Sequence**	*Percentage recovery*	*Δt_R** (min)*
Native hGH	See refs. 18 and 25	95***,§	1.0
CMC-hGH	See refs. 18 and 25	97***,§	1.0
Tryptic peptide T_4	LHQLAFDTYQEFEEAYIPK§§	65§	15.0
Tryptic peptides T_6	YSFLQNPQTSL(CMC)FSESIPTPSNR§§	85§	6.0
Tryptic peptide T_{10}	SVFANSLVYGASNSDVYDLLK§§	68§	23.0
Tryptic peptide T_{14}	QTYSK§§	79§	4.0
Tryptic peptide T_{16}	NYGLLY(CMC)FR§§	72§	22.0

* The one-letter code for the amino acids is as given by M. O. Dayhoff in *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, Silver Spring, MD, 1972. CMC = carboxymethylcysteine.

** Decrease in retention time of the phosphorylated polypeptide relative to the corresponding non-phosphorylated polypeptide under the same chromatographic conditions.

*** Determined by mass recovery from amino acid composition analysis.

§ Determined as recovered radioactivity.

§§ The sequences of the tryptic peptides are aligned from the N-terminal phenylalanine residue, as determined in refs. 18 and 25.

system with a Rheodyne Model 7120 sample injector and controller, connected to a Waters M450 variable-wavelength detector and a Hewlett-Packard Model 3390A integrator. Chromatographic separations were performed on two reversed-phase columns, a Waters μBondapak C_{18} column (30 × 0.4 cm I.D., d_p 10 μm) and a laboratory-prepared *n*-butylsilica, synthesised by bonding chlorobutyldimethylsilane to Merck LiChrospher Si 500 silica (d_p 10 μm), followed by endcapping with hexamethyldisilazine, and packed under constant pressure into a stainless-steel column (15 × 0.46 cm I.D.). Eluted fractions were collected with either a LKB Multirac 2111 fraction collector or a Pharmacia Frac 100 collector. Amino acid analysis was performed on a Durrum D-502 amino acid analyzer after hydrolysis in 6 *M* hydrochloric acid at 110°C for 22 h. Analysis of phosphoamino acids was performed as described previously[13] by high-voltage paper electrophoresis at pH 1.9. Radioactivity was detected by means of Cerenkov counting of various fractions.

Phosphorylation conditions

Phosphorylation of peptides and proteins was carried out with [γ-^{32}P]ATP as described[10,13] with the EGF-stimulated tyrosine kinase obtained from membrane preparations[14,15] of the human epidermoid carcinoma cell line A431[16]. Phosphorylated products were purified from the reaction mixture by batch fractionation[2,3,13,17] on octadecylsilica, followed by reversed-phase HPLC.

Fragmentation conditions

The hGH, phospho-hGH and related proteins were S-carboxymethylated in 6 *M* guanidine hydrochloride, as described elsewhere[17]. After desalting of the reaction mixture on *n*-butyl LiChrospher Si 500, with a 100 m*M* ammonium

bicarbonate–propanol–acetonitrile–water (35:35:30) gradient, the S-carboxymethyl proteins including the [^{32}P]phospho-hGH were digested with trypsin by established methods[18].

Chromatographic conditions

All chromatograms were developed at ambient temperature. Mobile phases were degassed and filtered through 0.22-μm filters from Millipore (Bedford, MA, U.S.A.). Chromatographic conditions for the reversed-phase HPLC analysis of tryptic peptides of hGH were based on previously reported procedures[11,18].

RESULTS AND DISCUSSION

Although selectivity for polypeptides and proteins in reversed-phase HPLC is primarily dependent on the relative hydrophobicity of the solutes, a variety of other factors associated with secondary solution equilibria can nevertheless affect the separation and recovery. Often, advantage can be taken of the pronounced influence which the organic solvent modifier has on the retention behaviour of globular proteins, as reflected by the relationship between the isocratic desorption parameters (S-value)[19,20], the gradient steepness parameters (b-value)[19,20], peak capacity, and solute recovery. Under appropriate gradient conditions, similar reversed-phase HPLC procedures can often be employed with the same column for the recovery of proteins directly from biological extracts as well as for the desalting of samples following chemical modification experiments over a very wide range of sample loadings.

As part of our current investigations on hGH-related polypeptides, we required methods which would exploit in a systematic manner the subtle changes in protein or peptide polarity that arise on phosphorylation or other post-translational events. As a consequence, a sequential isolation protocol was developed, which allows the analysis of intact phosphorylated hGH as well as related polypeptides derived from chemical modification and tryptic digestion. Because only trace amounts of hGH related polypeptides were available from cell incubation experiments, it was essential that high recoveries be maintained throughout the extraction and purification steps. For example, significant loss of polypeptide and protein material can occur at these low levels of abundance, owing to adsorption on plastic or glass surfaces of flasks, tubes, etc., during handling, concentration, or other manual manipulations involving chemical or enzymatic modifications. The general approach we have used to circumvent some of these difficulties involved micropreparative reversed-phase HPLC purification of the protein, carboxymethylation, reversed-phase HPLC desalting, concentration, tryptic digestion and reversed-phase HPLC purification of the tryptic peptides. Small- and large-pore n-alkylsilicas were employed at different reversed-phase HPLC stages with combinations of gradient elution conditions containing volatile eluents. Further, crude reaction mixtures were loaded directly onto the reversed-phase columns, allowing the same protein sample to be sequentially analysed at each stage of the fractionation scheme and thus keeping handling and concentration steps to a minimum. From preliminary experiments it was evident that phosphorylated 22K hGH, the 20K hGH variant, and their corresponding S-carboxymethylated derivatives could be recovered in excellent yield from the A431 membrane incubation medium directly by chromatography on a n-butyl LiChrospher Si-500 stationary

phase by using a 100 m*M* ammonium bicarbonate–water–acetonitrile gradient elution system. The stationary phase (mean effective pore diameter *ca.* 35 nm, *n*-butyl ligand coverage *ca.* 2.8 μmole/m^2) used for these investigations was selected on the basis of criteria established[21,22] in our previous studies on the relationship between ligand density, surface area and retention for polypeptides of molecular weight up to *ca.* 30,000. The present data with hGH-related polypeptides were consistent with earlier experiences with other polypeptides, confirming the favourable chromatographic features (high recoveries, column stability and efficiency) of this *n*-alkylsilica support.

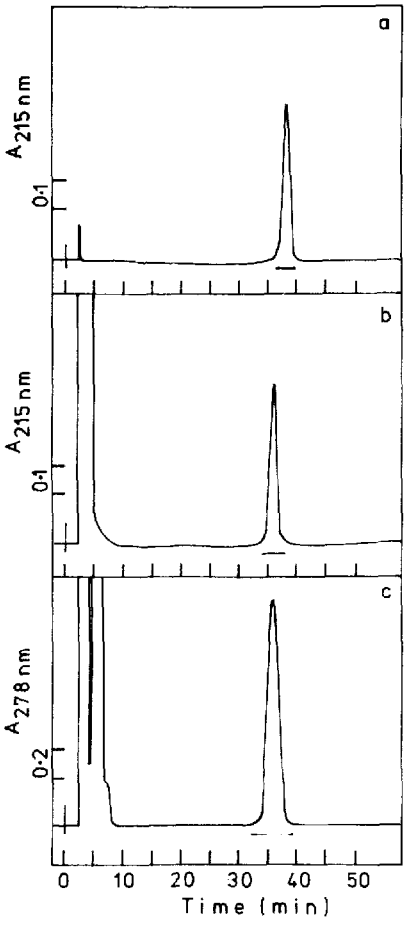

Fig. 1. Reversed-phase HPLC profile of native and S-carboxymethylated human growth hormone (hGH). Chromatographic conditions were: *n*-butyl LiChrospher Si-500 column (15 cm × 0.4 cm I.D., d_p 10 μm, 35 nm effective pore diameter); flow-rate, 1.0 ml/min; linear 30.-min gradient from 0 to 100% B where solvent A was aqueous 100 m*M* ammonium bicarbonate, pH 7.8, and solvent B was acetonitrile–water (60:40)–100 m*M* ammonium bicarbonate. Samples chromatographed were (a) 50 μg of native hGH in 10-μl injection volume, (b) 50 μg of crude S-carboxymethylated hGH in 25-μl injection volume, (c) 3 mg of S-carboxymethylated hGH from the reductive alkylation mixture in 1500-μl injection volume. The bars under the profiles indicate regions of the chromatograms collected for amino acid analysis and assay. Recoveries for native hGH and S-carboxymethylated hGH were 95% and 97% respectively.

Figs. 1a and 1b show the chromatographic profiles of 22K hGH, and its S-carboxymethylated derivatives (50 μg each). The elution profiles for the corresponding tyrosine phosphorylated derivatives eluted under gradient conditions are shown in Fig. 2. Based on amino acid analysis and recovered radioactivity, the protein recoveries for native 22K hGH and the S-carboxymethylated 22K hGH, located in the fractions which was eluted around 35 min, were 95% and 97%, respectively. In view of the high affinity of the hGH-related peptides for this *n*-butylsilica support, the same chromatographic system was also used in circumstances where maximal peak capacity was not essential, such as in the semi-preparative fractionation of the crude reaction product in 6 *M* guanidine hydrochloride from the reductive alkylation of 22K hGH. Fig. 1c shows a typical example with sample loads more than 100 times greater than those used in Fig. 1b. In common with several other small, globular

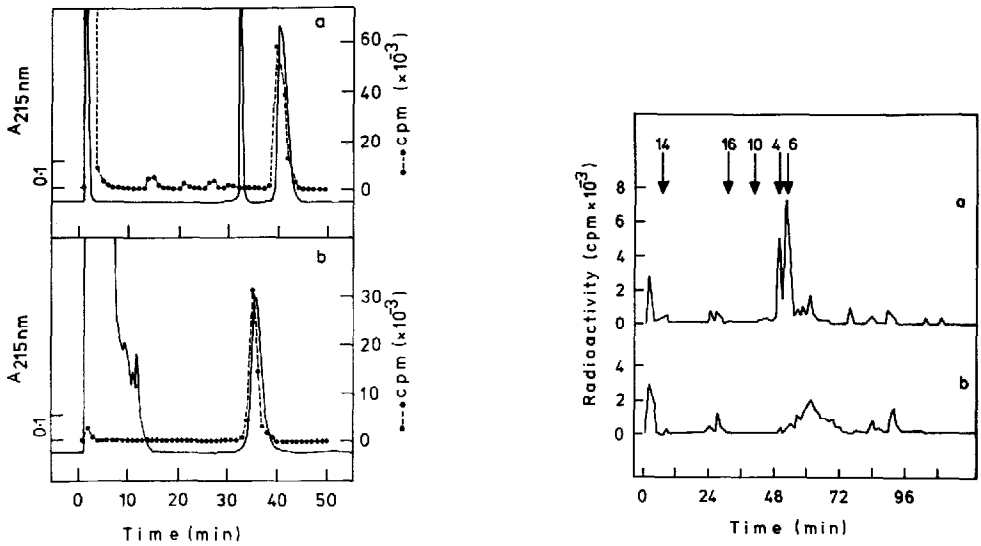

Fig. 2. Reversed-phase HPLC profiles of the crude reaction product from (a) native hGH or (b) S-carboxymethylated hGH, specifically phosphorylated in the presence of EGF-α with the EGF-stimulated tyrosine kinase obtained from human epidermoid carcinoma A431 cell membranes. Chromatographic conditions: column, *n*-butyl LiChrospher Si-500; flow-rate, 1 ml/min; mobile phase, a 30-min linear gradient from aqueous 100 m*M* ammonium bicarbonate to 2-propanol–acetonitrile–water (35:35:30)–100 m*M* ammonium bicarbonate. The chromatographic profiles of the ^{32}P-labelled proteins are shown as the dashed lines. The ^{32}P-labelled phospho-hGH was recovered from the chromatogram shown in (a) by lyophilisation and, following reduction and carboxymethylation in 6 *M* guanidine hydrochloride, the crude reaction mixture containing ^{32}P-labelled S-carboxymethylated phospho-hGH was purified under the same reversed-phase conditions. A similar sequential procedure was used to purify the unlabelled S-carboxymethylated hGH. See text for additional details.

Fig. 3. Separation by reversed-phase HPLC of the ^{32}P-labelled tryptic peptides of (a) S-carboxymethylated hGH and (b) S-carboxymethylated 20K hGH variant. Chromatographic conditions: column, μBondapak C_{18}; flow-rate, 1.0 ml/min; mobile phase, a 120-min linear gradient from 100 m*M* ammonium bicarbonate to acetonitrile–water(50:50)–100 m*M* ammonium bicarbonate. The elution positions of authentic ^{32}P-labelled tryptic peptides T_{14}, T_{16}, T_{10}, T_4, and T_6 are indicated by arrows. See Table I for amino acid sequences of these tyrosine-containing peptides.

proteins, such as lyzozyme, the retention of the native 22K hGH on the *n*-butylsilica column decreased only slightly following reductive alkylation (Δt_R = 1 min) with this ammonium bicarbonate-based gradient system. The close similarity in retention on the *n*-butylsilica as well as on other *n*-alkylsilicas, shown by the native globular 22K hGH and the fully dissociated S-carboxymethylated derivative, suggests that the hydrophobic contact domains for both solutes are also similar under these reversed phase HPLC conditions. Circular dichroism spectral analysis[23,24] of native and fully reduced and carboxymethylated hGH indicates that a significant and essentially equivalent percentage of the amino acids residues of these two polypeptides is in an α-helical conformation. This analysis further predicts 57 ± 5% α-helix for hGH with the polypeptide chain, when folded into an α-helix, forming a cylindrical column, one side of which is essentially hydrophobic and the other predominantly hydrophilic. The observed retention behaviour could thus be accounted for if the interaction between stationary phase and protein were to involve predominately region(s) of 22 K hGH within the large disulphide loop from cysteine 53 to cysteine 165 and in particular region(s) between residues 73 to 93, 103 to 107 and 156 to 163, which are known from amino acid sequence analysis to be rich in hydrophobic amino acids. The involvement of these segment of the hGH structure in interactions with the non-polar stationary phase would also account for the observation[22] that the 20K hGH variant, which differs[17,25] from the usual 22K hGH form by delections of amino acid residues 32 to 45 inclusive, *i.e.* between phenylalanine 31 and asparagine 46 in the 22K hGH sequence, cannot be readily separated from the 22K hGH protein under a variety of reversed-phase HPLC conditions, despite the significant change in acidic amino acid content. With the 100 m*M* ammonium bicarbonate primary eluent, essentially complete ionisation of the S-carboxymethyl groups of fully reduced and alkylated 22K hGH is anticipated. However, the small decrease in retention evident after reductive alkylation of 22K hGH (and attenuated further by phosphorylation of this polypeptide) is in accord with only slight changes in molecular polarity. This suggests that the value of the group retention coefficient (χ_i)[26] for the carboxymethylcysteinyl moiety under these chromatographic conditions is small and negative. In contrast, with low-pH eluents the χ_i value for the carboxymethylcysteinyl group is positive, large, and of a magnitude similar to that of phenylalanine or tryptophan[27]. In fact, S-carboxymethylated proteins often show enhanced retention with low-pH eluents, compared with the corresponding native protein, a situation which may be reversed, as in the case of S-carboxymethylated 22K hGH, with appropriate choice of mobile phases of higher pH.

The specific sites of tyrosine phosphorylation on hGH were located by tryptic mapping by means of similar reversed-phase HPLC procedures. In Fig. 3 are shown the elution radioactivity profiles for the tryptic peptides of the S-carboxymethylated phosphorylated 22K hGH and 20K hGH. In each case, carboxymethylation and tryptic digestion was carried out after phosphorylation of the protein with the EGF-dependent tyrosine kinase from the human epidermoid carcinoma cell line A 431. As reported elsewhere[10], comparison of the tryptic peptides, derived from phosphorylated hGH, with the products from the phosphorylation of authentic, tyrosine-containing, hGH-related peptides, confirmed that the major sites of phosphorylation in native 22K hGH were either tyrosine 28 or tyrosine 35 and tyrosine 42. These residues are known[28] from other studies on the chemical modification of 22K

hGH to be accessible at the surface of the protein. A more complex elution profile of radioactivity was observed with the product from the phosphorylation of the tryptic peptide T_4 (see Table I for amino acid sequence). This difference is believed to arise as a consequence of preferential phosphorylation of both tyrosine residues of the peptide T4 (corresponding to tyrosine-28 and tyrosine-35 in native 22K hGH). Again, it is noteworthy that the retention of each phosphorylated peptide was less than that of the corresponding unphosphorylated peptide counterpart (see Table I).

In summary, this paper illustrates the use of reversed-phase HPLC in the isolation and analysis of phospho-polypeptides, related to 22K hGH. Although the use throughout of volatile mobile phases considerably facilitated the recovery and subsequent analysis of the various phosphorylated and non-phosphorylated peptides, other ionic modifiers can prove equally effective. For example, similar combinations of volatile and non-volatile ion pairing systems, such as the alkylsulphonates and the tetra-alkylammonium salts can be employed[2,3,7,29] with small- and large-pore *n*-alkylsilicas in general strategies that allow the rapid micropreparative purification, including multi-step desalting, of the intact, the S-carboxymethylated forms, and the enzymatically derived fragments of other pituitary proteins, including the glycoprotein hormones.

ACKNOWLEDGEMENTS

These investigations were supported by the National Health and Medical Research Council of Australia and the Human Pituitary Advisory Committee.

REFERENCES

1 S. Stein, in E. Gross and J. Meienhofer (Editors), *The Peptides*, Academic Press, New York, 1981, pp. 73–94.
2 M. T. W. Hearn, *Advan. Chromatogr.*, 20 (1982) 1.
3 M. T. W. Hearn, in Cs. Horváth (Editor), *HPLC: Advances and Perspectives*, Academic Press, New York, 1983, Vol. 3, pp. 87–155.
4 M. T. W. Hearn, *Methods Enzymol.*, 104 (1984) 190.
5 M. T. W. Hearn, F. E. Regnier and C. T. Wehr (Editors), *HPLC of Proteins and Peptides*, Academic Press, New York, 1983, pp. 1–267.
6 H. J. P. Bennett, *J. Chromatogr.*, 266 (1983) 501.
7 M. T. W. Hearn, S. J. Su and B. Grego, *J. Liquid Chromatogr.*, 4 (1981) 1547.
8 T. M. Martensen and E. R. Stadman, *Proc. Nat. Acad. Sci. U.S.*, 79 (1982) 6458.
9 T. Hunter and B. M. Sefton, *Proc. Nat. Acad. Sci. U.S.*, 77 (1980) 1311.
10 G. S. Baldwin, B. Grego, M. T. W. Hearn, J. A. Knesel, F. J. Morgan and R. J. Simpson, *Proc. Nat. Acad. Sci. U.S.*, 80 (1983) 5276.
11 B. Grego, F. Lambrou and M. T. W. Hearn, *J. Chromatogr.*, 266 (1983) 89.
12 A. W. Burgess, J. A. Knesel, L. G. Sparrow, N. A. Nicola and E. C. Nice, *Proc. Nat. Acad. Sci. U.S.*, 79 (1982) 5753.
13 G. S. Baldwin, J. A. Knesel and J. M. Monckton, *Nature (London)*, 301 (1983) 435.
14 D. Cassel and L. Glaser, *J. Biol. Chem.*, 257 (1982) 9845.
15 D. L. Brantigan, P. Bornstein and B. Gallis, *J. Biol. Chem.*, 256 (1981) 6519.
16 G. Carpenter, L. King and S. Cohen, *J. Biol. Chem.*, 254 (1979) 4884.
17 M. T. W. Hearn and W. S. Hancock, in J. Hawk (Editor), *Biomedical/Biological Applications of Liquid Chromatography*, Marcel Dekker, New York, 1979, pp. 243–265.
18 M. T. W. Hearn, B. Grego and G. E. Chapman, *J. Liquid Chromatogr.*, 6 (1982) 215.
19 M. T. W. Hearn and B. Grego, *J. Chromatogr.*, 255 (1983) 125.

20 L. Snyder, in Cs. Horváth (Editor), *HPLC: Advances and Perspectives*, Academic Press, New York, 1980, Vol. 1, pp. 207–316.
21 M. T. W. Hearn and B. Grego, *J. Chromatogr.*, 282 (1983) 541.
22 M. T. W. Hearn and B. Grego, *J. Chromatogr.*, 296 (1984) 61.
23 T. A. Bewley and C. H. Li, *Arch. Biochem. Biophys.*, 138 (1970) 338.
24 T. A. Bewley and C. H. Li, *Biochemistry*, 11 (1972) 884.
25 G. E. Chapman, K. M. Rogers, T. Brittain, R. A. Bradshaw, O. J. Bates, C. Turner, P. D. Cary and C. Crane-Robinson, *J. Biol. Chem.*, 256 (1981) 2395.
26 S. J. Su, B. Grego, B. Niven and M. T. W. Hearn, *J. Liquid Chromatogr.*, 4 (1981) 1745.
27 T. Sasagawa, T. Okuyama and D. C. Teller, *J. Chromatogr.*, 240 (1982) 329.
28 L. Ma, J. Brovetto-Cruz and C. H. Li, *Biochim. Biophys. Acta*, 165 (1974) 255.
29 C. Turner, P. D. Cary, B. Grego, M. T. W. Hearn and G. E. Chapman, *Biochem. J.*, 213 (1983) 107.
30 P. G. Stanton, B. Grego and M. T. W. Hearn, *J. Chromatogr.*, 296 (1984) 189.