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ASSAY OF THE COMBINED FORMULATION OF ERGOMETRINE AND OXYTOCIN BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The present British Pharmacopoeial monograph for the combined formulation of ergometrine and oxytocin requires a spectrophotometric assay for the ergometrine and a biological assay for the oxytocin content. Simultaneous spectrophotometric assay of the two ingredients has not previously been practicable, owing to the widely different amounts of each. Two high-performance liquid-chromatographic separations have been developed by which both ingredients can be assayed at different dilutions in both systems. One separation is on the strong cation-exchange bonded phase Partisil 5 SCX, where reproducibility of injection gave a peak-height coefficient of variation of 0.8% for ergometrine and 2.5% for oxytocin. An alternative system involves use of the ion-pair reagent, sodium tetradecyl sulphate, with a reversed-phase packing, giving a coefficient of variation for repeat injections of 1.0% (peak height) for ergometrine and 2.5% (peak area) for oxytocin.

INTRODUCTION

Combined formulations often require different assay procedures for each active component. However, if each could be quantitated by the same method, assay might be simpler, quicker and more cost-effective. The British Pharmacopoeial monograph for the combined formulation, ergometrine and oxytocin injection, requires a spectrophotometric assay with dimethylaminobenzaldehyde for the alkaloid[1] and a biological assay by milk ejection pressure in the lactating rat for oxytocin[1,2]. Dimethylaminobenzaldehyde is a colorimetric reagent specific for the indole nucleus and so also reacts to give coloured derivatives with breakdown products of ergometrine, which are thus measured by the assay. A high-performance liquid-chromatographic (HPLC) system that resolved ergometrine from these products would provide a more specific and accurate assay of ergometrine activity. The rats needed for the bioassay of oxytocin in the presence of ergometrine must be at the correct stage of lactation (*i.e.*, between 3 and 21 days post partum) and are not always readily available. An HPLC assay for the peptide would be subject neither to such biological constraints, nor to the accompanying ethical considerations. Good correlation has been obtained

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between HPLC assay results and bioassay (rat uterus contraction and cockerel blood pressure) for oxytocin injection[3,4] and may be expected to be applicable to analysis of other preparations. Separate HPLC procedures for both oxytocin and ergometrine have been published[4–6], and in principle the two assays specified in the monograph might be replaced by a single chromatographic analysis. However, two main obstacles to this are, firstly, that ergometrine is present at a 50-fold higher concentration than oxytocin and, secondly, that ergot alkaloids typically form breakdown products on storage and exposure to light[8] in quantities that might interfere with measurement of the oxytocin. This paper reports two HLPC assays that circumvent these problems.

EXPERIMENTAL

Apparatus

An Altex 110 or LDC mini-pump was used with a Cecil 272 or Hewlett-Packard 1030B variable-wavelength UV monitor. Fluorescence was monitored with a Perkin-Elmer 2000 fluorescence monitor. Unless stated otherwise, a Rheodyne 7125 or Waters U6K loop injector was used.

Materials

Partisil 10 ODS2 (Whatman, Maidstone, Great Britain) and μBondapak C_{18} (Waters Assoc., Milford, MA, U.S.A.) were purchased pre-packed. Stainless-steel columns (100 or 250 mm × 4.6 or 5 mm) were slurry-packed at 300 bar with the following reversed-phase packings: LiChrosorb RP-18 (10 μm; Merck, Darmstadt, G.F.R.), Nucleosil C_8 and C_{18} (both 5 μm; Macherey, Nagel & Co., Düren, G.F.R.), Hypersil SAS, Hypersil ODS (Shandon Southern Instruments, Runcorn, Great Britain), Spherisorb S5 ODS (Phase Separations, Queensferry, Great Britain), and HSCP C_{18} (5 μm, not capped; HS Chromatography Packings Ltd., Bourne End, Great Britain) and the following cation-exchange packings: Partisil 5 SCX, Partisil 10 SCX (Whatman), LiChrosorb KAT (10 μm, Merck), Nucleosil SA (5 μm; Macherey, Nagel & Co.) and Vydac cation-exchange (The Separations Group, Hesperia, CA, U.S.A.). A LiChrosorb Si 60 (10 μm, Merck) column was also packed. A pre-column (50 mm × 4.6 mm) containing silica (LiChroprep Si 60, 5–25 μm was used before the injector with all columns.

AnalaR ammonia and orthophosphoric acid were supplied by BDH (Poole, Great Britain). Triethylamine (Et_3N) was obtained from Koch-Light Labs. (Colnbrook, Great Britain) and acetonitrile (far-UV HPLC grade) from Fisons (Loughborough, Great Britain). Methanesulphonic acid was obtained from Aldrich (Milwaukee, WI, U.S.A.), the sodium salts of pentane- and heptane-sulphonic acids and sodium tetradecyl sulphate (STS) from Eastman-Kodak (Rochester, NY, U.S.A.), and sodium dodecyl sulphate (SDS) from BDH. Ephedrine was supplied by Sigma (St. Louis, MO, U.S.A.) and thermolysin by Merck (for use in solution) and by Sigma (for immobilisation).

Oxytocin concentrate, ergometrine and the [D-Tyr^2]-, [D-Gln^4]- and [D-Tyr^2, D-Gln^4]-oxytocin analogues were gifts from Drs. K. Krummen and H. G. Leeman (Sandoz, Basle, Switzerland). Ergometrine and oxytocin injection BP (Sandoz) was purchased locally.

Mobile phases

Reversed-phase system. A calculated volume of ion pairing agent (10%, w/v, in water) was added to the buffer (0.83 m*M* H_3PO_4 adjusted to pH 5.0 with Et_3N) to give a final concentration, after the addition of acetonitrile, of 0.05% (w/v); normally, the acetonitrile concentration was 40% (v/v). The temperature of the mobile-phase reservoir was maintained at 18°C, or above, to keep the ion-pairing agent in solution. Before use, reversed-phase columns were washed with approximately 100 ml of [8.3 m*M* H_3PO_4–Et_3N (pH 5.0)]–acetonitrile (60:40) 0.1% in ion-pair reagent. After use, the reversed-phase columns were washed with 5–10 column volumes of 83 m*M* H_3PO_4–acetonitrile (60:40, v/v) to remove Et_3N.

Cation-exchange system. The mobile phase was 8.3 m*M* H_3PO_4 adjusted to pH 5.0 with NH_3–acetonitrile (88:12).

Dilution of formulations

Reversed-phase system. The formulation was diluted 1:10, and to 1 1-ml portion were added 1.5 ml of water and 2.5 ml of ephedrine solution (0.02 mg ml^{-1}). The injection volume for the quantitation of ergometrine was 80 μl. No internal standard was used for oxytocin, and 50 μl of the undiluted formulation were injected direct.

Cation-exchange system. A 1-ml portion of the formulation was diluted with 2.5 ml of ephedrine solution (0.2 mg ml^{-1}) and 1.5 ml of water, and 50-μl aliquots were injected into the HPLC system to quantitate ergometrine. To another portion of the formulation (950 μl) were added 50 μl of ephedrine solution (0.1 mg ml^{-1}), and 50-μl aliquots were injected to assay oxytocin.

Digestion of oxytocin

Soluble enzyme. A freshly prepared solution (1 mg ml^{-1}) of thermolysin (Merck) in water was added to the formulation, or to a solution of oxytocin and ergometrine in water at the same concentrations, to give a final thermolysin concentration of 125 μg ml^{-1}. Digestion was allowed to proceed at room temperature in the dark for the times noted below.

Immobilised enzyme. Thermolysin (Sigma) was immobilised by linkage to cyanogen bromide activated Sepharose 4B[9,10]. A 0.2-ml volume of a 50% (v/v) suspension of immobilised thermolysin in 0.03 *M* sodium borate–5 m*M* $CaCl_2$ (pH 7.0) was added to an equal volume of formulation (or a control solution of the same concentrations of oxytocin and ergometrine in water), and digestion was allowed to proceed at room temperature in the dark for the stated periods.

RESULTS AND DISCUSSION

Since the concentration of oxytocin in the combined formulation was low (10 μg/ml), it was desirable to monitor the UV absorption at the shortest practicable wavelength. For this reason, the separations developed by Larsen *et al.*[5] and Sondack[6], with acetate in the mobile phase, were not suitable. In addition, to maximise sensitivity for oxytocin, it was preferable for the peptide to be eluted before ergometrine. Investigation of other published systems[4,7] showed that ergometrine emerged close to the solvent front and oxytocin was eluted afterwards, too near the alkaloid for quantitation and sometimes together with an impurity peak.

Ion-pairing on reversed-phase

Knox and Jurand[11] and Sood *et al.*[12] obtained good separations of ionizable compounds by incorporating hydrophobic pairing ions containing alkyl groups into the mobile phase, and this approach has since found many applications[13,14]. The addition of a counter-anion containing such a group to the mobile phase reversed the order of elution of ergometrine and oxytocin from reversed-phase packings[15]. The retentions of the nonapeptide and ergometrine increased with the length of the alkyl group attached to the ion-pair (Fig. 1) as has been observed by other investigators[13,14,16,17]. The deviation in retention of oxytocin in the presence of STS may have been due to impurities present in the surfactant[18,19]. Good resolution of ergometrine and oxytocin in the formulation was obtained with a mobile phase containing 0.01% (w/v) SDS and 0.25 m*M* phosphate–Et_3N (pH 5). However, with repeated injections, the retention of the oxytocin peak drifted towards that of trace amounts of chlorbutol (a preservative in the oxytocin concentrate used to prepare the formulation), which progressively interfered with quantitation. This drift was not eliminated by thermostatic control of the system. A similar separation could be obtained on HSCP C_{18} and Partisil ODS at a higher ionic strength (0.1% SDS, 8.3 m*M* phosphate–Et_3N at pH 5) without such drifting, but with other packings the separation was inadequate. Substitution of STS (0.05%, w/v) for SDS in the mobile phase gave good separation of the two drugs at higher ionic strength (0.83 m*M* phosphate–Et_3N). However extra peaks, attributed to breakdown products of ergometrine and to chlorbutol were eluted close to oxytocin.

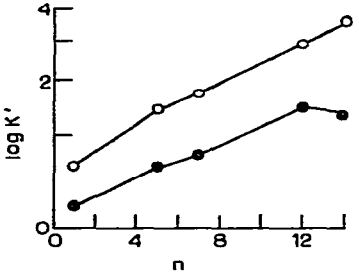

Fig. 1. Relationship between the chain length of counter-ion and capacity ratio (k'). C_1, C_5 and C_7 alkyl chain lengths as sulphonates, the remainder as sulphates. Chromatographic conditions: support ODS Hypersil (50 × 4.6 mm; a freshly packed column for each homologue); mobile phase: 0.05% (w/v) STS, 0.83 m*M* H_3PO_4–Et_3N pH 5.0, 40% (w/v) acetonitrile. Flow-rate: 0.5 ml min^{-1}. ○ = Ergometrine; ● = oxytocin.

When the relative retentions of the two drugs were compared on different commercial packings (Fig. 2), the variations between packings were less pronounced for STS than for SDS at the lower ionic strength[15]. Such differences in behaviour between reversed-phase packings from different manufacture have been widely observed and commented upon[20–22]. Simultaneous monitoring by UV absorption and fluorescence of the separation on Nucleosil C_{18} using STS demonstrated that no fluorescent peak typical of ergometrine breakdown products was co-eluted with oxytocin. A formulation was digested with a preparation of immobilised thermolysin in order to check that oxytocin was not co-eluted with other peaks on three different commercial packings. No peak of significant height was co-eluted with the peptide

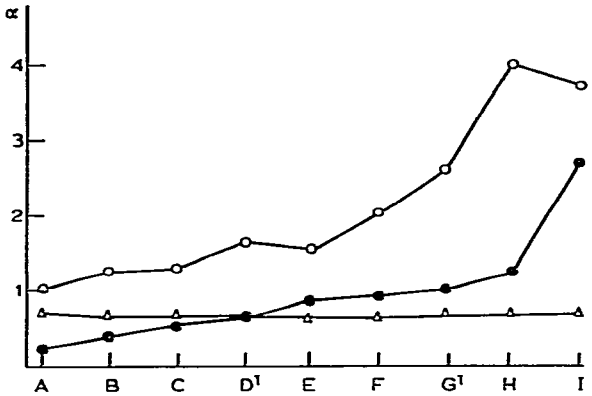

Fig. 2. Relative capacity ratios (α) with respect to chlorocresol of different commercial packings. A, Partisil 10 ODS; B, LiChrosorb 10 RP-18; C, μBondapak 10, C_{18}; D, Nucleosil 5 C_8; E, Nucleosil 5 C_{18}; F, ODS Hypersil; G, SAS Hypersil; H, Spherisorb S5 ODS; I, HSCP C_{18}. Mobile phase as in Fig. 1; flow-rate 1 ml min^{-1}. ○ and ● as in Fig. 1; △ = chlorbutol. Superscript 1, 25% (v/v) of acetonitrile.

from μBondapak C_{18}; however, with Nucleosil C_{18} and Spherisorb ODS, it was necessary to reduce the acetonitrile concentration (to 37% and 35%, respectively) to prevent significant interference.

Three diastereoisomers, [D-Tyr²]-, [D-Gln⁴]- and [D-Tyr²,D-Gln⁴]-oxytocin, were either partially or wholly resolved from oxytocin on Nucleosil C_{18} (Table I). The order of elution was the reverse of that obtained on LiChrosorb RP-8 and μBondapak C_{18} with no hydrophobic ion-pair reagent[3,23]. Resolution of oxytocin from these diastereoisomers was not affected by inclusion in a formulation.

TABLE I

SEPARATION OF OXYTOCIN DIASTEREOISOMERS

	k'	
	Nucleosil C_{18}	*Partisil 5 SCX*
[D-Tyr²]-Oxytocin	1.78	12.20
[D-Tyr²DGln⁴]-Oxytocin	2.30	10.44
[D-Gln⁴]-Oxytocin	2.75	13.40
Oxytocin	3.43	15.60

It was possible to obtain separations similar to the ion-pair reversed-phase systems on LiChrosorb Si 60 (mobile phase 8.3 m*M* H_3PO_4 adjusted to pH 7.0 with NH_3; 5% v/v acetonitrile), but with reduced resolution of the peaks. Since problems with column stability and variation between different silica packings were experienced, this separation was not investigated further.

Cation exchange

In view of the above results and published work[2-4], it seemed worthwhile to examine bonded ion-exchange packings in the hope of obtaining more favourable

selectivity. On Partisil 5 SCX, oxytocin was eluted before ergometrine, and many of the contaminating peaks appeared close to the solvent front. No fluorescent peak was co-eluted with oxytocin. Treatment of the formulation with thermolysin showed that no other peak was co-eluted with oxytocin (Fig. 3). A similar separation was obtained on Partisil 10 SCX, but not on other cation exchangers.

The three diastereoisomers were separated from oxytocin on Partisil 5 SCX (Table I), both separately and when incorporated in a formulation.

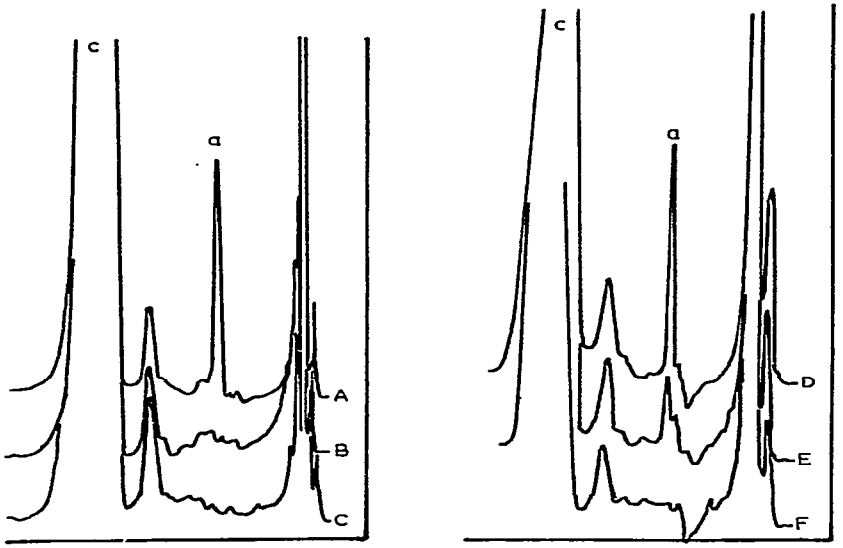

Fig. 3. Removal of the peptide by digestion with thermolysin. Stationary phase: Partisil 5 SCX (50 × 4.6 mm; mobile phase: 8.3 m*M* H_3PO_4–NH_3 (pH 5.0); 12% (v/v) acetonitrile; flow-rate 1 ml min^{-1}. A, 800 ng of oxytocin + 4 μg of ergometrine; B, 800 ng of oxytocin + 4 μg of ergometrine treated with thermolysin for 1.25 h; C, 4 μg of ergometrine treated with thermolysin overnight; D, 80 μl of formulation; E, 80 μl of formulation treated with thermolysin for 1.25 h; F, 80 μl of formulation treated with thermolysin overnight.

Reproducibility and linearity of response

Examination of the chromatograms in Figs. 4 and 5 shows that any suitable internal standard for the ion-pair reversed-phase system must emerge after ergometrine, but might be eluted between oxytocin and ergometrine in the cation-exchange system. Fortunately, ephedrine exhibited just this behaviour, and was therefore a suitable internal standard for either system. The coefficient of variation of five or more replicate injections of oxytocin on Partisil 5 SCX (50-mm column, septum injection) was 2.5% (peak-height measurements, with ephedrine as internal standard). For the ion-pair system (Nucleosil C_{18} column, 37% acetonitrile), reproducibility was better for oxytocin without an internal standard, and the coefficient of variation was 2.9% for peak-height and 2.5% for peak-area measurements. The coefficient of variation for ergometrine with ephedrine as internal standard using peak-height measurement was 1.0% for the ion-pair system and 0.8% for the cation-exchange (100-mm) column.

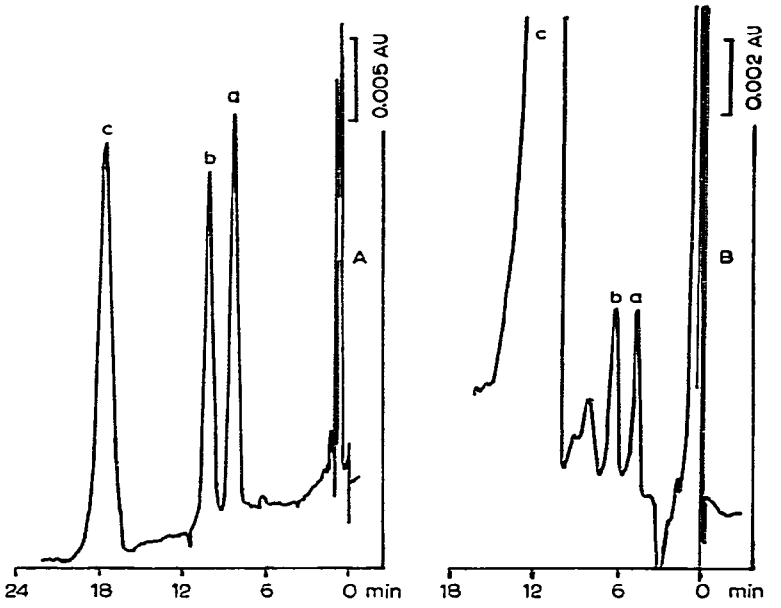

Fig. 4. Chromatograms obtained by ion-pair reversed-phase HPLC. Support: Nucleosil C_{18}. Mobile phase: as in Fig. 1, except with 35% acetonitrile for Fig. 4A and 37% (v/v) of acetonitrile for Fig. 4B. Flow-rates 2.5 ml min^{-1} for Fig. 4A and 1 ml min^{-1} for Fig. 4B. a = Oxytocin; b = ephedrine; c = ergometrine.

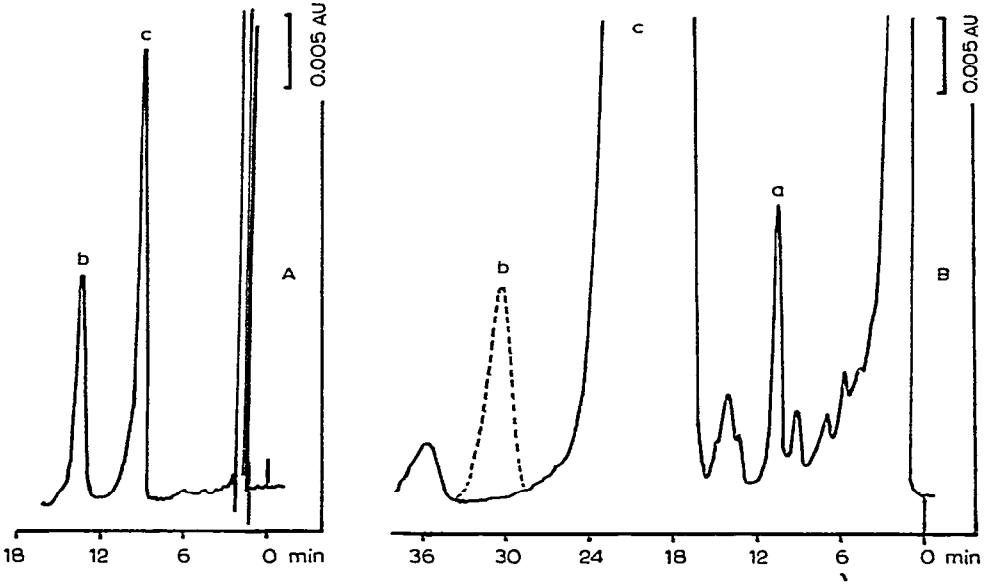

Fig. 5. Chromatograms obtained by cation-exchange HPLC. Stationary and mobile phases as in Fig. 3. Fig. 5A, standards (1 ml min^{-1}); Fig. 5B, formulation (2 ml min^{-1}). Symbols as in Fig. 4.

Injections of oxytocin (50–1000 ng) containing ergometrine at the concentration in the formulation were made by septum on to Partisil 5 SCX (50-mm column; ephedrine as internal standard) and by valve on to Nucleosil C_{18} (37% acetonitrile; no internal standard). For both systems, variation in peak height with oxytocin concentration displayed a linear relationship and the best fit passed through the origin. Likewise, the variation in peak height with ergometrine concentration was linear for both procedures (1.0–7.5 μg, Partisil 5 SCX, 100-mm column; Nucleosil C_{18}, 37% acetonitrile). Extrapolation of the line did not pass through the origin for the ion-pair system, and, with increase in concentration of ergometrine, a corresponding decrease in the peak height of the standard and increase in height of an unidentified peak was observed. Use of an alternative standard (chlorocresol) gave a non-linear response. Injection of ergometrine at lower quantities (160–1200 mg) gave a variation in peak height that was both linear and passed through the origin.

The coefficients of variation for five repeated dilutions for the cation-exchange system were 1.1% for ergometrine (100-mm column) and 3.7% for oxytocin (50-mm column, septum injection). The coefficient of variation on Partisil 5 SCX (100-mm column) between vials from the same batch was 1.6% for ergometrine (mean: 109.7% of the labelled dose) and 3.0% for oxytocin (mean: 105.1% of the labelled dose).

Both separation procedures are sufficiently reproducible and sensitive to determine the active ingredients quantitatively. The separation on Partisil 5 SCX was good, and use of a valve injector might improve the reproducibility for measurements of the peptide. This packing is not, as yet, generally available, but, in principle, Partisil 10 SCX should give similar results. The ion-pair separation is a preferable alternative to the cation-exchange system, since adequate separations were obtained on several commercial packings. A disadvantage experienced with the reversed-phase system was that the relative elution position of oxytocin changed with alteration of pH. The presence of Et_3N will make the pH of the mobile phase sensitive to fluctuation in temperature. In this study, the ambient temperature varied by at least 4°C; consequently, improvement in the reproducibility of the oxytocin peak might be obtained by thermostatic control of the column.

We believe that, subject to appropriate assessments, one or other of the procedures described should be a suitable replacement for the assay methods at present official in the British Pharmacopoeia.

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REFERENCES

1 *British Pharmacopoeia*, 1980, p. 606.
2 *British Pharmacopoeia*, 1980, Appendix XIVC, A142.
3 K. Krummen, K. Maxl and F. Nachtmann, *Pharm. Technol. Int.*, October (1979) p. 37.
4 K. Krummen and R. W. Frei, *J. Chromatogr.*, 132 (1977) 429.
5 B. Larsen, V. Viswanatha, S. Y. Chang and V. J. Hruby, *J. Chromatogr. Sci.*, 16 (1978) 207.
6 D. L. Sondack, *J. Chromatogr.*, 166 (1978) 615.

7 P. H. Corran and D. H. Calam, in A. Frigerio and L. Renoz (Editors), *Recent Developments in Chromatography and Electrophoresis*, Elsevier, Amsterdam, Oxford, New York, 1979, p. 341.
8 H. Bethke, B. Delz and K. Stich, *J. Chromatogr.*, 123 (1976) 193.
9 P. Cuatrecasas and C. B. Anfinsen, *Methods Enzymol.*, 22 (1971) 345.
10 J. Porath, *Methods Enzymol.*, 34 (1974) 13.
11 J. H. Knox and J. Jurand, *J. Chromatogr.*, 125 (1976) 89.
12 S. P. Sood, L. E. Sartori, D. O. Wittmer and W. G. Haney, *Anal. Chem.*, 48 (1976) 796.
13 T. R. Koziol, J. T. Jacob and R. G. Achari, *J. Pharm. Sci.*, 68 (1979) 1135.
14 E. Tomlinson, T. M. Jefferies and C. M. Riley, *J. Chromatogr.*, 159 (1978) 315.
15 R. A. Pask-Hughes, P. H. Corran and D. H. Calam, *Anal. Proc. (London)*, 18 (1981) 247.
16 C. Horvath, W. Melander, I. Molnar and P. Molnar, *Anal. Chem.*, 49 (1977) 2295.
17 R. S. Deelder, H. A. J. Linssen, A. P. Konijnendijk and J. L. M. van de Venne, *J. Chromatogr.*, 185 (1979) 241.
18 R. A. Barford and B. J. Sliwinski, *J. Chromatogr.*, 171 (1979) 445.
19 S. A. Lacks, S. S. Springhorn and A. L. Rosenthal, *Anal. Biochem.*, 100 (1979) 357.
20 M. J. O'Hare, E. C. Nice and M. Capp, *J. Chromatogr.*, 198 (1980) 23.
21 W. Melander, J. Stoveken and Cs. Horváth, *J. Chromatogr.*, 199 (1980) 35.
22 A. Sokolowski and K.-G. Wahlund, *J. Chromatogr.*, 189 (1980) 299.
23 B. Larsen, B. L. Fox, M. F. Burke and V. J. Hruby, *Int. J. Peptide Protein Res.*, 13 (1979) 12.
24 B. B. Wheals, *J. Chromatogr.*, 187 (1980) 65.