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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF ^{125}I-LABELLED PROTEINS WITH ON-LINE DETECTION

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SUMMARY

Microgram amounts of ^{125}I-labelled proteins were chromatographed using hydrophilic molecular exclusion columns (Waters μBondapak–protein I125). A sodium iodide crystal was used as an on-line detector. Inherent hydrophobic properties of the column were overcome with a mobile phase containing 0.36 mol/l pyridinium formate–*n*-propanol (75:25), yielding recoveries in the range 90–95% of injected material. The resolution increased with increasing column length. Two columns (30 × 0.8 cm) in series seem to be optimal. Compared with Sephadex chromatography of microgram amounts, high-performance liquid chromatography is superior with regard to resolution and operating time.

INTRODUCTION

^{125}I-labelled proteins are generally purified by gel filtration, *e.g.*, crude reaction mixtures containing labelled protein, chloramine-T, salts, etc.[1], are applied to appropriate Sephadex columns. In recent years, however, hydrophilic molecular exclusion columns for high-performance liquid chromatography (HPLC) have become available for the separation of proteins with molecular weights in the range 2000–80,000 daltons. These columns were expected to yield high resolutions and improved speed of chromatography. The aim of this study was to establish conditions for the HPLC of microgram amounts of ^{125}I-labelled proteins and to compare the results with those obtained by Sephadex chromatography.

EXPERIMENTAL

A Waters liquid chromatograph was used (Fig. 1).

Purified samples were collected at the Waters 6000A pump and evaluated by immunological methods. For the γ-detector (Fig. 2) a PTFE capillary was inserted in steel tubing, and passed through a drilled 2-in. sodium iodide crystal (Laboratorium Prof. Berthold). The radioactivity was recorded with a photomultiplier and rate meter.

Between 5 and 20 μg of proteins were labelled with ^{125}I according to Hunter and Greenwood[1]. The crude mixtures were injected directly and chromatographed.

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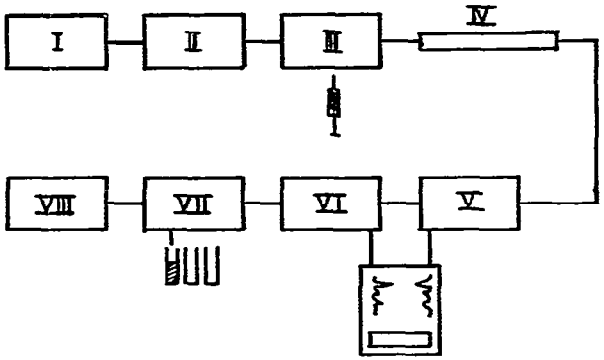

Fig. 1. Flow diagram of the chromatograph. I, Mobile phase; II, Waters 6000A pump; III, Waters U6K injection system; IV, Waters μBondapak–protein 1125 column; V, γ-detector; VI, Waters 440 UV detector; VII, Waters 6000A sample collector; VIII, waste.

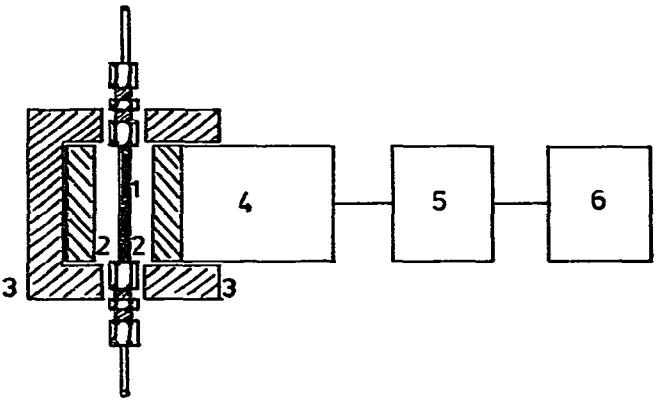

Fig. 2. γ-Detector. 1, PTFE capillary; 2, 2-in. sodium iodide crystal (Laboratorium Prof. Berthold); 3, lead shielding; 4, photomultiplier; 5, rate meter (Laboratorium Prof. Berthold); 6, recorder.

RESULTS

Recovery

In order to assess the hydrophobic properties of the column, five consecutive injections of 20 μg of hPL* in 100 μl of mobile phase were made. As Fig. 3 (I–V) demonstrates, the recovery of injected material increases with apparent saturation of the column, thus indicating the occurrence of hydrophobic interactions between the column and the protein.

The recovery could be further increased by co-chromatographing bovine serum albumin (BSA) (VI) or pre-saturation of the column with BSA. This was found, however, only for a limited number of proteins, including hPL and hFSH. The recovery of other labelled proteins, including hTSH and hLH, was unsatisfactory.

* Abbreviations: hPL = human placental lactogen; hFSH = human follicle-stimulating hormone; hTSH = human thyrotrophin; hLH = human luteinising hormone.

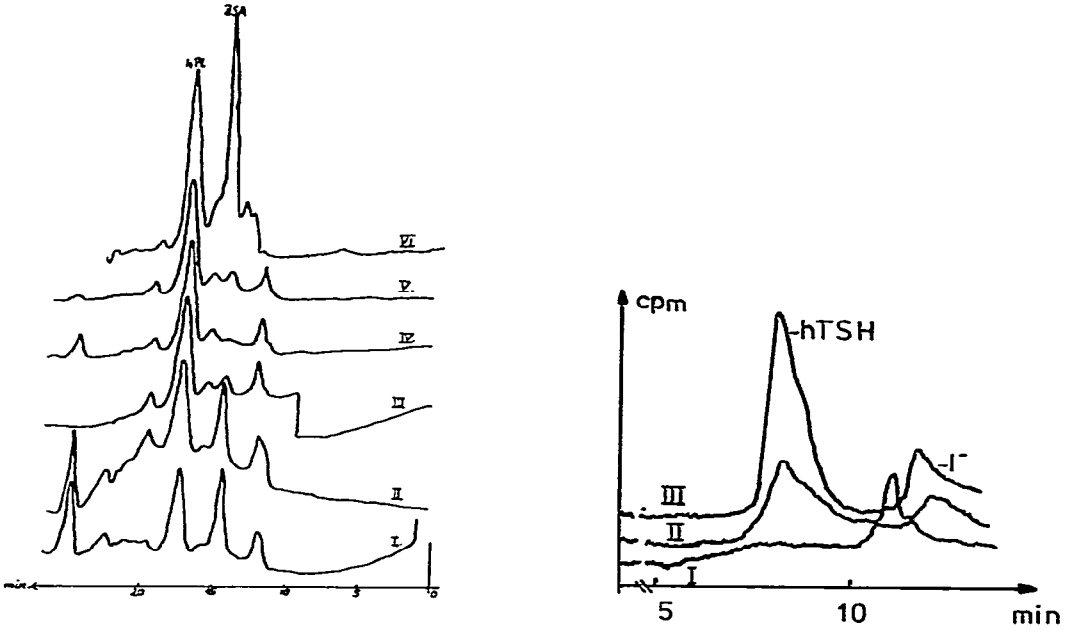

Fig. 3. HPLC of hPL. Columns, two 30 × 0.8 cm I.D. μBondapak–protein 1125; mobile phase, 0.05 mol/l phosphate-buffered saline (pH 7.2); flow-rate, 1.0 ml/min; detection, UV (254 nm), 0.01 a.u.f.s. I–V = consecutive injections of 20 μg of hPL in 100 μl of mobile phase each; VI = injection of 20 μg of hPL and 50 μg of BSA in 100 μl mobile phase.

Fig. 4. HPLC of [^{125}I]hTSH using different mobile phases. Column, 30 × 0.8 cm I.D. μBondapak–protein 1125; flow-rate, 1.0 ml/min; detection, γ-detector; injection, 8 μg of [^{125}I]hTSH. Mobile phase: I, 0.01 mol/l phosphate-buffered saline (pH 7.2); II, 8% acetic acid–*n*-propanol (95:5); III, 0.36 mol/l pyridinium formate (pH 3.0)–*n*-propanol (75:25).

Hydrophobic properties were overcome with the use of mobile phases containing organic solvents (Fig. 4).

Thus, on going from buffer to acetic acid–*n*-propanol the recovery increased, although there was still tailing of peaks and equilibration of the column took a long time. With pyridinium formate–*n*-propanol the recovery was 90–95% of injected material, with acceptable separation. Changes in the *n*-propanol content, *e.g.*, 22% instead of 25%, altered the chromatograms significantly. Using this mobile phase, UV detection at 254 nm is impossible owing to the pyridinium content.

Resolution

Influence of column length. In the process of labelling hTSH with ^{125}I and purification, aggregation or breakdown of molecules was sometimes observed, so the influence of column length on resolution with this system was investigated (Fig. 5).

With two 30-cm columns in series satisfactory resolution was obtained, but with one column the separation was not satisfactory. The consequent peak broadening and the increase in the time required for chromatography time were well within acceptable limits.

Influence of pH of mobile phase. In order to increase the resolution further and

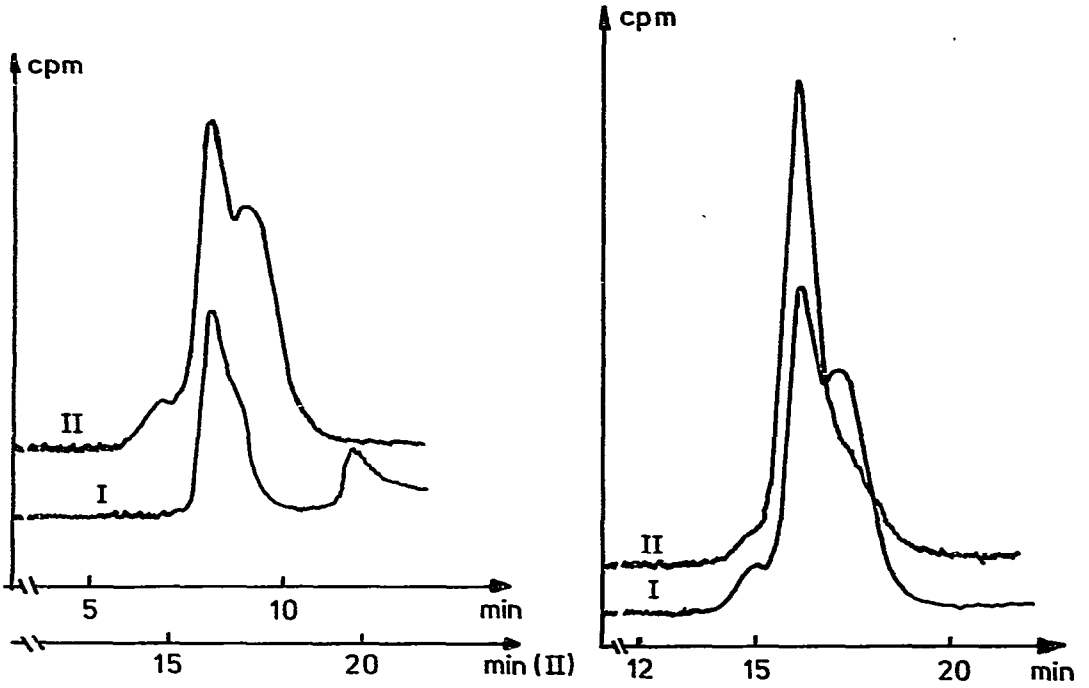

Fig. 5. Influence of column length on resolution. Columns: I, one μBondapak–protein I125 (30 × 0.8 cm I.D.); II, two μBondapak–protein I125 in series (30 × 0.8 cm I.D.). Flow-rate, 1.0 ml/min; detection, γ-detector; mobile phase, 0.36 mol/l pyridinium formate (pH 3.0)–*n*-propanol (75:25); injection, 8 μg of [^{125}I]hTSH.

Fig. 6. Influence of pH on resolution. Columns, two 30 × 0.8 cm I.D. μBondapak–protein I125; flow-rate, 1.0 ml/min; detection, γ-detector; injection, 8 μg of [^{125}I]hTSH. Mobile phase: I, 0.36 mol/l pyridinium formate (pH 3.0)–*n*-propanol (75:25); II, 0.36 mol/l pyridinium formate (pH 5.0)–*n*-propanol (75:25).

at the same time to chromatograph under milder conditions with regard to proteins, the pH of the mobile phase was changed to more neutral conditions while keeping its composition constant (Fig. 6).

Although the resolution remained unchanged the formation of by-products was reduced considerably. Thus, the use of pyridinium formate (pH 5.0)–*n*-propanol (75:25) as the mobile phase gave recoveries of 90–95% of injected material, acceptable separation of the product and by-products and reduced aggregation of proteins during chromatography.

Comparison of HPLC and gel filtration

To compare HPLC with gel filtration, labelled mixtures were chromatographed using HPLC (two 30 × 0.8 cm I.D. columns) and Sephadex G-75 (60 × 0.9 cm I.D. column), respectively. Fig. 7 shows the results obtained when 5 μg of [^{125}I]hLH were applied to each column.

HPLC gave much better separations of all products. In addition, the analysis time, including the time for column preparation and equilibration, was reduced by nearly 2 h (from 2–2.5 h to 30–45 min). As in HPLC all of the recovered material

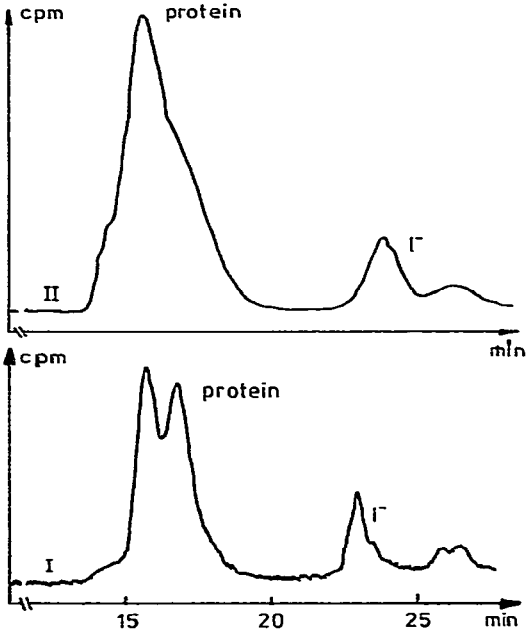

Fig. 7. Chromatography of [^{125}I]hLH using different techniques. Columns: I, two 30 cm × 0.8 cm I.D. μBondapak–protein I125 (HPLC); II, 60 × 0.9 cm I.D. Sephadex G-75 (gel filtration). Mobile phase: I, 0.36 mol/l pyridinium formate (pH 5.0)–*n*-propanol (75:25); II, 0.01 mol/l phosphate-buffered saline (pH 7.2). Detection, γ-detector.

retained its biological activity in immunological tests and behaved similarly to the material purified by gel filtration. HPLC of proteins using hydrophilic molecular exclusion columns and a pyridinium formate–*n*-propanol buffer is preferred for the purification of ^{125}I-labelled proteins. Results obtained so far are summarized in Table I, which compares the resolution, recovery and analysis times using HPLC and Sephadex chromatography.

TABLE I

COMPARISON OF HPLC AND SEPHADEX CHROMATOGRAPHY

Substance	*Resolution**		*Recovery (%)*		*Analysis time*	
	HPLC	*Sephadex*	*HPLC*	*Sephadex*	*HPLC*	*Sephadex*
[^{125}I]hTSH	+	0	95	95	45 min	2.5 h
[^{125}I]hFSH	+	0	93	95	45 min	2.5 h
[^{125}I]hLH	+	−	90	93	45 min	2.5 h
[^{125}I]hPL	+	0	95	95	45 min	2.5 h

* +, Good; 0, satisfactory; −, not satisfactory.

CONCLUSIONS

1. HPLC using hydrophilic molecular exclusion columns is a fast and convenient technique for purifying microgram amounts of ^{125}I-labelled proteins.

2. Inherent hydrophobic properties of the columns causing reduced recovery of injected material are overcome by a proper choice of the mobile phase; 0.36 mol/l pyridinium formate (pH 5.0)–*n*-propanol (75:25) is suitable.

3. The resolution is improved considerably by using two 30-cm columns in series instead of one. The consequent peak broadening and increased analysis time are within acceptable limits.

4. For the purification of microgram amounts of ^{125}I-labelled proteins, HPLC compares favourably with Sephadex chromatography with regard to resolution and analysis time. Thus it is possible to purify proteins in 30–45 min by HPLC compared with 2–2.5 h by Sephadex chromatography.

REFERENCE

1 W. M. Hunter and F. C. Greenwood, *Nature (London)*, 194 (1962) 495.