Journal of Chromatography, 362 (1986) 443–449
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 754

Note

Large scale high-performance liquid chromatography of urogastrone produced by recombinant DNA technology

STEPHEN J. BREWER*
Searle R&D, Lane End Road, High Wycombe, Buckinghamshire (U.K.)
CHARLES H. DICKERSON
CJB Developments Ltd., Airport Service Road, Portsmouth, Hampshire (U.K.)
and
JONATHAN EWBANK and ANTHONY FALLON
Searle R&D, Lane End Road, High Wycombe, Buckinghamshire (U.K.)
(First received March 27th, 1986; revised manuscript received April 22nd, 1986)

The developments in genetic engineering have made possible the production of potentially therapeutically useful proteins, *e.g.* insulin and growth hormone[1,2]. Before clinical administration, however, such preparations must fulfil criteria of purity hitherto unprecedented in protein chemistry. This means that for the development of a commercially viable production process, the purification achieved during each step of the process must be optimised for both recovery and purification factor. Because of the complexity of covalent, ionic and hydrophobic interactions involved in maintaining the structure of biologically active proteins, more sophisticated methods of purification are required than those used in the preparation of conventional pharmaceuticals.

The introduction of high-performance resins for protein purification (TSK-PW from Toya Soda and Mono S from Pharmacia) has complemented the advances in genetic engineering and reports are now appearing in which analytical quantities of proteins have been prepared. In some cases milligram preparations are possible using analytical columns.

The object of this work was to assess the use of large-scale HPLC as a final step in the purification of recombinant β-urogastrone. β-Urogastrone is a polypeptide hormone isolated in small quantities in human urine[3]. It is a potent inhibitor of gastric acid secretion and also promotes epidermal cell proliferation. The protein may be of use for ulcer therapy and the healing of wound injuries. Using genetic engineering techniques, the protein has been produced in *E. coli* with a N-terminal expression fusion and a C-terminal polyarginine purification fusion[4,5]. β-Urogastrone is recovered following limited trypsin digestion. The product, however, is contaminated with digestion products caused by an ambiguous trypsin cleavage site at Lys(13), Lys(14) to produce Lys(14)-urogastrone, and a small amount of over-digested material (Fig. 1).

0021-9673/86/$03.50

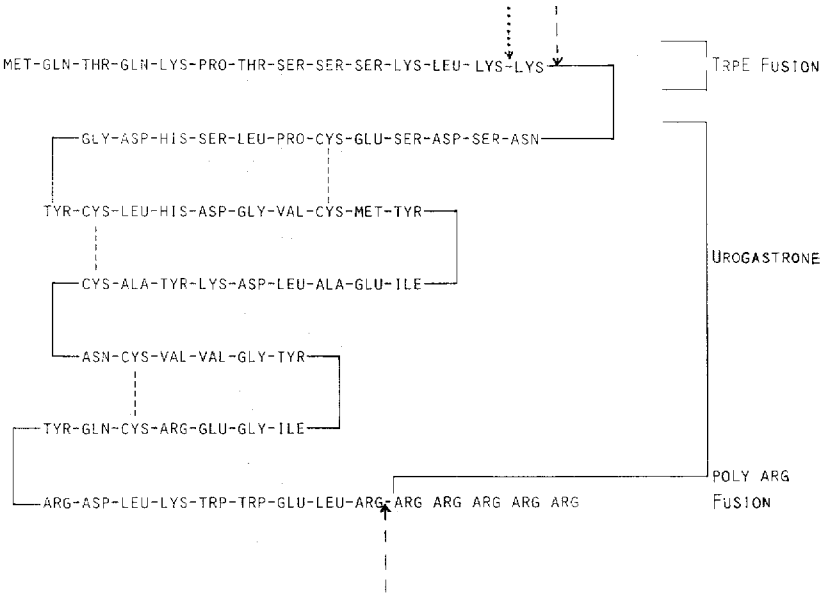

Fig. 1. Trypsin cleavage of an N-terminal expression fusion and a C-terminal purification fusion from urogastrone. The desired trypsin cleavage sites to obtain β-urogastrone from trypE-fused polyarginine tailed β-urogastrone are shown (– – – →). An additional cleavage site yielding a major contaminant, Lys-urogastrone, is also indicated (·····→).

EXPERIMENTAL

Urogastrone digest

The purification of polyArg-urogastrone with an N-terminal expression fusion by chromatography on SP-Sephadex has been described[6]. The fusion protein (5 g) was precipitated from Tris–urea buffer by 50% (w/v) ammonium sulphate and dialysed against 10 m*M* acetic acid. The protein was digested at a protein concentration of 9 mg/ml with 28 mg of trypsin (N-tosyl-L-phenylalanine chloromethyl ketone treated, Worthington) for 8 min at 37°C maintaining a pH value of 8.0 by the addition of 0.1 *M* sodium hydroxide. The digestion was stopped by the addition of 50 m*M* sodium acetate–50 m*M* sodium chloride and lowering the pH to 3.6 with acetic acid. The protein concentration in the digest was 2 mg/ml.

Preparative HPLC

A schematic representation of the preparative HPLC system is shown in Fig. 2. All equipment was supplied by Anachem (Luton, U.K.). A Gilson 303 system was employed with 100 ml/min pump heads. By activating valve 1, the sample was applied to the column through pump A. Deactivation of valve 1 allowed pumps A and B to be programmed to produce the desired salt gradient from the A and B buffers and elute the protein. Activating valve 2 allowed sodium hydroxide to clean the column before re-equilibration. The whole system was fully automatic and programmable.

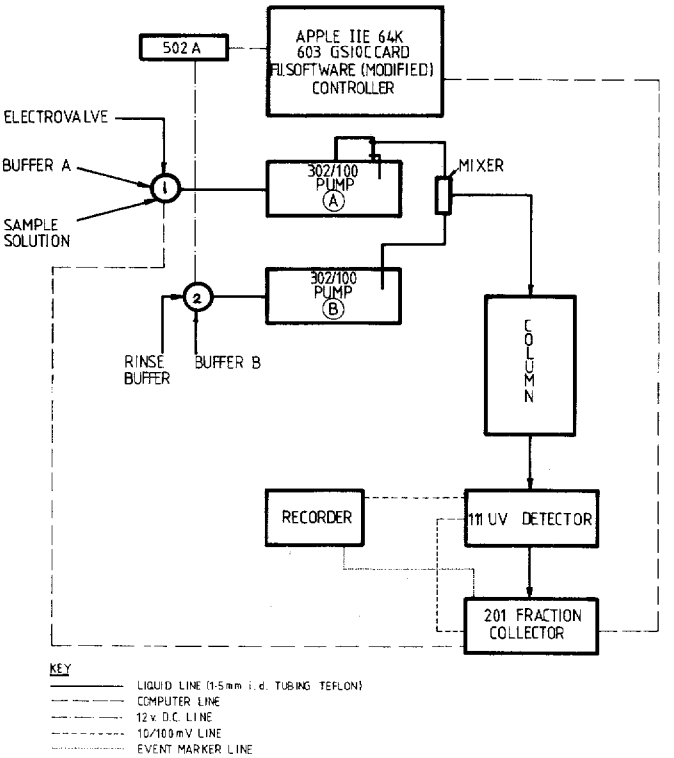

Fig. 2. The preparative HPLC system. Preparative cation-exchange HPLC columns were equilibrated at 20% B buffer with valves 1 and 2 open to buffers A and B, respectively. Valve 1 was activated to load the column with urogastrone digest, then deactivated and the column washed with 20% buffer B. β-Urogastrone was eluted from the column by 32% buffer B and the peak fraction collected. The column was then flushed with 80% B buffer, then cleaned by switching valve B to pump 0.1 *M* sodium hydroxide onto the column. If required, the cycle was repeated automatically and the column re-equilibrated in 20% B buffer.

HPLC columns and buffers

Chromatography columns were supplied by Bio-Rad (Luton, U.K.) packed with a hydrophilic ion-exchange resin, TSK SP-5PW. The column sizes were: 75 × 7.5 mm (3.3 ml); 150 × 21.5 mm (54 ml) and 200 × 55 mm (475 ml). Buffer flow-rates were 1 ml/min; 9 ml/min and 45 ml/min, respectively. All reagents were analytical grade quality. The A buffer was 50 m*M* sodium acetate–50 m*M* sodium chloride, pH 3.6, the B buffer was 500 m*M* sodium acetate–500 m*M* sodium chloride, pH 3.6 and the rinse buffer was 0.1 *M* sodium hydroxide in water.

Development of separation

A conventional HPLC analytical system on a 75 × 7.5 mm column was used to establish suitable gradient elution conditions. This gradient separation was scaled up on a 150 × 21.5 mm column on the preparative HPLC system. Further development allowed a single step in buffer B concentration to elute purified β-urogastrone.

RESULTS

The automated HPLC system incorporating a sodium hydroxide rinse was tested using the 150 × 22.5 mm column. Separations were very reproducible and purified β-urogastrone could be collected in a single fraction (Fig. 3). Automation allowed the product from multiple runs to be collected as a pooled product. As many as 20 sequential runs have been achieved using this system with no deterioration in the separation.

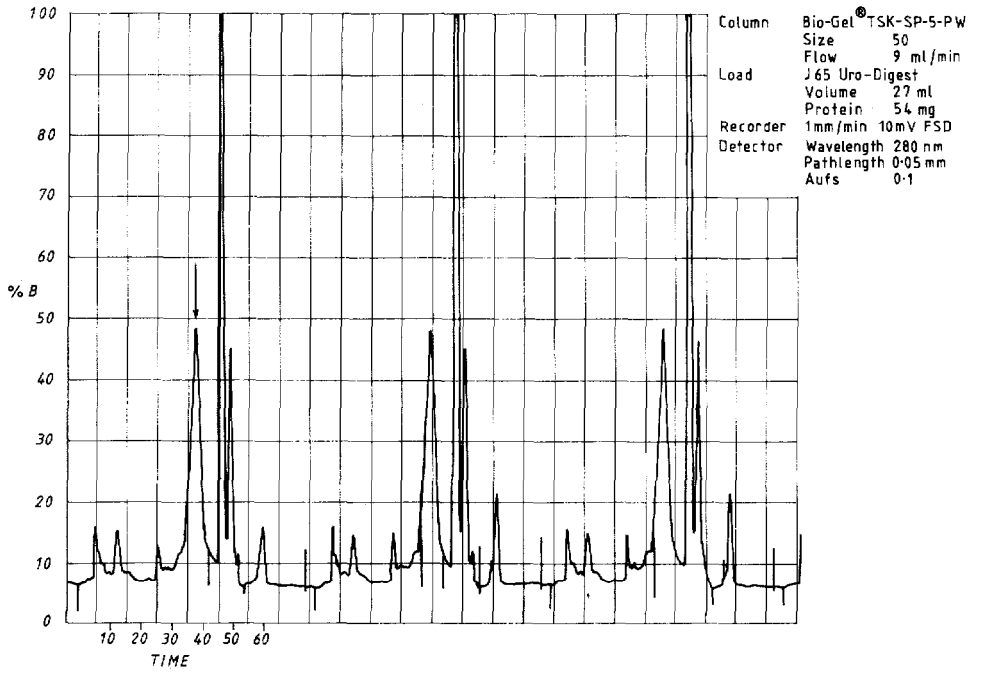

Fig. 3. Reproducibility of β-urogastrone HPLC system. β-Urogastrone digest (54 mg) was purified on a semipreparative column (150 × 21.5 mm) at a flow-rate of 9 ml/min using the automated preparative HPLC system described in Fig. 2. Buffer volumes were: equilibration, 180 ml; load, 27 ml; wash, 180 ml; eluate, 180 ml; 80% B buffer, 45 ml; sodium hydroxide, 36 ml. The separation was repeated automatically five times and the UV trace obtained for runs 3–5 are shown. Purified β-urogastrone (arrow) pooled from all 5 runs (SS pool) were analysed by PAGE as described in Fig. 5.

A further scale-up to a 475-ml column was examined. Separations obtained from sequential runs using increased loadings up to a maximum of 1.5 g of urogastrone digest were studied (Fig. 4). For column loads of up to 1 g, similar separation profiles to those obtained with a 54-ml column were obtained. When 1.5 g of protein were applied, however, an assymetric peak shape was observed. Polyacrylamide gel electrophoresis (PAGE) indicated that only a single species was present in all the purified fractions (Fig. 5).

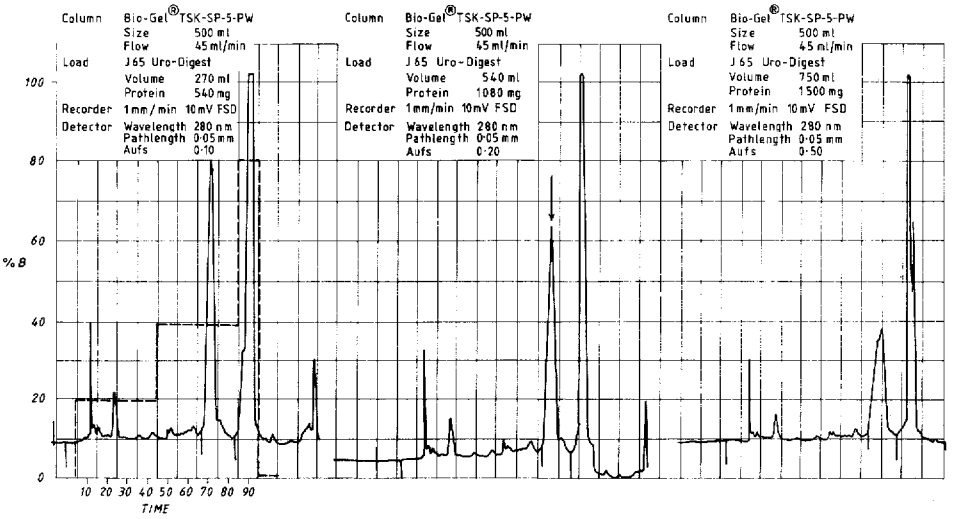

Fig. 4. Effect of protein load on preparative HPLC system. β-Urogastrone digest was purified on a preparative column (200 × 55 mm) at a flow-rate of 45 ml/min using the automated preparative HPLC system described in Fig. 2. Buffer volumes were; equilibration, 1800 ml; loads, 270 ml, 540 ml, 750 ml, respectively; wash, 1800 ml; eluate, 1800 ml; strip, 450 ml. The UV traces for 270 ml, 540 ml and 750 ml loads are shown. Purified β-urogastrone (arrow) from each eluate was collected (LS2, LS3 and LS4) and analysed by gel electrophoresis as described in Fig. 5.

DISCUSSION

Urogastrone has a very low solubility around its isoelectric point (p*I* 4.6). At higher pH values, the major contaminant, Lys-urogastrone, is eluted before β-urogastrone, causing contamination because of trailing peaks. These considerations dictated cation-exchange chromatography at one pH unit lower than the p*I* of urogastrone. The process developed from the analytical column was successfully scaled up one hundred and fifty fold. This was achieved without accepting the general philosophy of process HPLC which dictates the compromise between quantity and quality of the product[8].

The large column was operated at the maximum flow-rate recommended by the manufacturer, which resulted in a decreased linear flow-rate (2.5–1.9 cm/min). However, the profiles at all stages were almost identical and the products had similar purity as judged by PAGE analysis. The total amount of active β-urogastrone from a single run was in the order of 0.5 g, but no attempt has yet been made to overload the column. The loss of peak shape at the highest loading did not affect the purity as measured by PAGE analysis. Thus the poor peak shape may be due to the solubility of the protein being exceeded. The automation allowed multi-gram quantitaties of highly purified urogastrone to be prepared by sequential runs.

The level of production reported here is at the gram scale, which is sufficient to support clinical trial studies. Further scale-up may be possible by running the column in the overload situation and possibly by re-cycling[8]. The major disadvantage of the system is the cost of the resin. This is countered by the stability of these resins

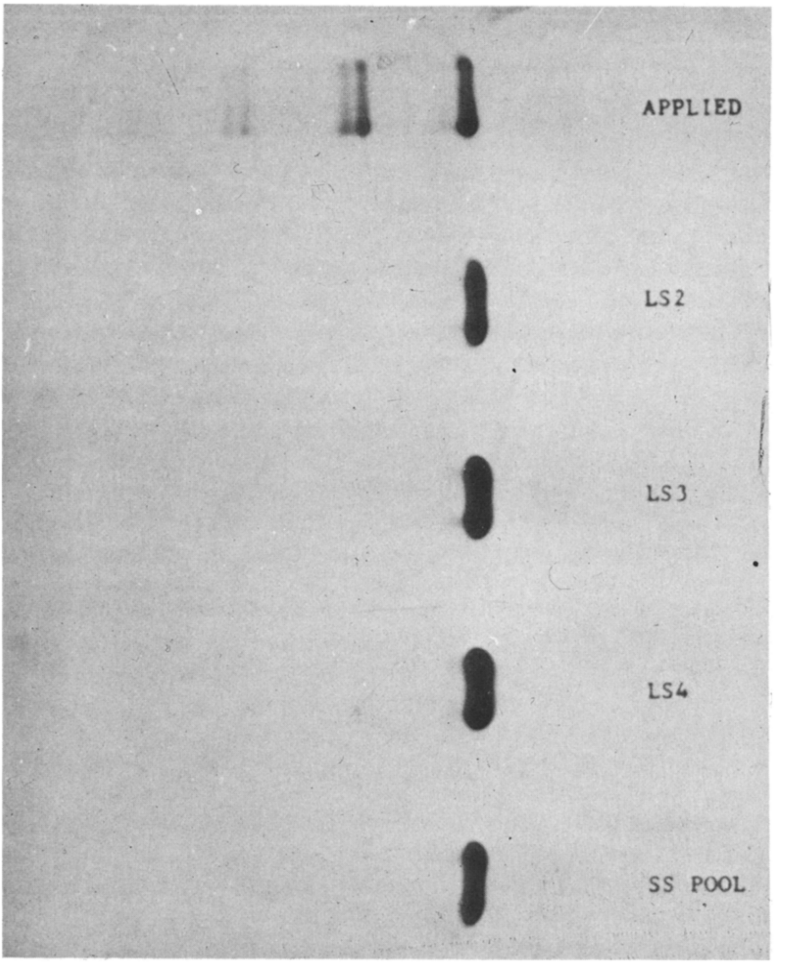

Fig. 5. PAGE analysis of HPLC purified β-urogastrone. β-Urogastrone samples were prepared as described in Figs. 3 and 4, desalted by dialysis against 10 m*M* acetic acid and freeze dried. Samples were resuspended in running buffer at 2.5 mg/ml and 20 μl were analysed by PAGE[7].

which allow many hundreds of separations to be achieved with little loss of resolution. For such a highly active pharmaceutical product these costs may not be prohibitive.

Large-scale HPLC supports based on silica have been used for a number of years mainly as normal phase or bonded reversed phase. The use of organic solvents for the purification of some proteins has had no deleterious effect[9] and even for large proteins[10]. However, the development of an ion-exchange resin[11,12] capable of operating at a wide range of pH values allows a separation to be performed in aqueous conditions compatible with maintaining the structure of the most complex proteins.

We have demonstrated that the resolution obtained with analytical separations need not be lost on scale-up. This was achieved without the need to sacrifice quality

of the final drug product. The high cost of the resin may not be intercipient where highly potent pharmacologically active proteins such as β-urogastrone are concerned. Therefore, large-scale HPLC may have a key role in the manufacture of recombinent proteins for clinical use.

REFERENCES

1 D. V. Goeddel, D. G. Klied, F. Bolivar, H. L. Heyneker, D. G. Yansura, R. Crea, T. Hirose, A. Kraszewski, K. Itakura and A. D. Riggs, *Proc. Nat. Acad. Sci. U.S.A.*, 76 (1979) 106.
2 D. V. Goeddel, H. L. Heyneker, T. Hozumi, R. Arentzen, K. Itakura, D. G. Yansura, M. J. Ross, G. Miozzari, R. Crea and P. H. Seeburg, *Nature (London)*, 281 (1979) 545.
3 H. Gregory and B. M. Preston, *Int. J. Pept. Protein Res.*, 9 (1977) 107.
4 S. J. Brewer and H. M. Sassenfeld, *Trends Biotechnol.*, 3 (1985) 119.
5 J. C. Smith, R. B. Derbyshire, E. Cook, L. Dunthorne, J. Viney, S. J. Brewer, H. M. Sassenfeld and L. D. Bell, *Gene*, 32 (1984) 321.
6 H. M. Sassenfeld and S. J. Brewer, *Bio/Technology*, 2 (1984) 76.
7 B. J. Davis, *Ann. N.Y. Acad. Sci.*, 121 (1964) 404.
8 J. L. Dwyer, *Bio/Technology*, 2 (1984) 957.
9 M. Rubinstein, M. S. Rubinstein, P. C. Familletti, R. S. Miller, A. A. Waldman and S. Prestka, *Proc. Natl. Acad. Sci. USA*, 76 (1979) 640.
10 R. V. Lewis, A. Fallon, S. Stein, K. D. Gibson and S. Udenfriend, *Anal. Biochem.*, 110 (1980) 318.
11 Y. Kato, K. Nakamura and T. Hashimoto, *J. Chromatogr.*, 245 (1982) 193.
12 Y. Kato, K. Komiya and T. Hashimoto, *J. Chromatogr.*, 246 (1982) 13.