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APPLICATION OF PREPARATIVE REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY TO THE ISOLATION OF INSULIN-LIKE GROWTH FACTOR II FROM HUMAN SERUM

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SUMMARY

Substantially purified insulin-like growth factor II (IGF-II) was prepared from human serum. Initial enrichment using ion-exchange chromatography on DEAE Sephadex A50, followed by gel permeation chromatography on Sephadex G-75 in 1% formic acid produced material suitable for application to a preparative reversed-phase high-performance liquid chromatographic (HPLC) column containing LiChroprep RP-18. The latter step gave about 90-fold purification with a recovery of about 70% IGF-II bio-activity. Finally, a small reversed-phase HPLC column achieved a 17-fold purification with similar yield of activity. Overall, the four steps gave IGF-II of about 90% purity in yield of 12%.

INTRODUCTION

The insulin-like growth factors (IGFs) or somatomedins are a family of polypeptides which circulate in serum. These compounds are of great interest because of their ability to stimulate the in vitro incorporation of [^{35}S]sulphate into cartilage [1] and their insulin-like activity [2], which is not suppressible by anti-insulin antisera. Initially, the sulphation activity led to the concept that these factors were the mediators of the action of growth hormone on skeletal growth. Later, this concept was broadened when it was found that they possessed other types of biosynthetic activity and the more encompassing term 'somatomedins' was introduced to describe them [3]. Then, in 1976, Rinderknecht and Humbel [4] isolated two compounds from serum with non-suppressible insulin-like activity (NSILA) both of which were later shown [5,6] to share a remarkable degree of homology with insulin itself and hence were named IGF-I and IGF-II.

Several molecular species of NSILA differing in molecular size and isoelectric

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point appear to be present in serum. IGF-I and IGF-II of molecular masses 7649 and 7471, respectively, appear to be bound to high-molecular-mass carrier proteins in serum. In its unbound form, IGF-I has a p*I* of 8.2 while IGF-II has a p*I* of 6.5. Somatomedin C, a basic peptide [7], is identical to IGF-I [8,9] while somatomedin A [10] is a neutral form which has been shown to be a deamidated form of IGF-I [11]. An acidic form of NSILA with p*I* 4.8 has also been reported [12], which, like IGF-I and IGF-II, is bound to carrier proteins in serum.

Acid-stable NSILA (NSILA-P) is a high-molecular-mass form known to be different from the IGFs [2]. One important difference is that it remains in a high-molecular-mass form despite repeated treatments with acid, unlike the IGF–carrier protein complexes. This difference can be made use of in separation of the IGFs from NSILA-P.

Most of the methods used to prepare IGFs are based on extraction of serum or Cohn fraction IV-1 with a mixture of ethanol and hydrochloric acid, but where this is done, low yields are often obtained. Reversed-phase high-performance liquid chromatography (HPLC) has also been utilized in the latter stages of purification of IGF-I and IGF-II after chromatofocusing [13] or isoelectric focusing [14].

The present paper seeks to make use of other types of extraction procedures for preparing enriched IGF-II fractions from serum, coupled with the use of preparative and small-scale HPLC for subsequent purification. HPLC has been shown to be an important tool for the separation of insulin-related peptides prepared by solid-phase synthetic procedures [15] and there appear to be few problems with resolution of polypeptides of molecular mass less than 10 000 on octadecylsilane columns [16]. Volatile acids such as trifluoroacetic acid (TFA) [17] may be used to facilitate the chromatographic process and allow easy removal prior to the bio-assay procedure.

EXPERIMENTAL

Preliminary enrichment of IGF-II

Normal human serum (70 ml) obtained from a pool of about 250 adult volunteers was chromatographed on a 60×3.5 cm column of DEAE Sephadex A50 (Pharmacia, Uppsala, Sweden) using Tris–HCl buffer (0.05 *M*, pH 8.1) as recently described [18]. Material eluted in buffer containing 0.15 *M* sodium chloride (fraction C) contained the major part of the immunoreactive IGF-II [18] and almost all of the bioactivity (NSILA) as indicated by an assay based on the incorporation of [^{14}C]glucose into [^{14}C]lipid by isolated rat adipocytes in the presence of excess anti-insulin antiserum [19]. Specific activity of fraction C and all subsequent fractions was used as a measure of their IGF-II content.

Dialysed and lyophilised fraction C (0.5 g) was then dissolved in 1% (v/v) formic acid (5 ml) and adjusted to pH 2.5 with 20% (v/v) formic acid. After 2 h this IGF-II enriched material was applied to a 90×2.5 cm column of Sephadex G-75 (Pharmacia) in 1% (v/v) formic acid and eluted at a rate of 20 ml/h using the latter as the mobile phase. Material eluted between K_{av} 0.65 and 0.90 was pooled, lyophilised and assayed for NSILA as before.

Large-scale (preparative) HPLC

Reversed-phase HPLC was performed using a Waters Assoc. (Milford, MA, U.S.A.) liquid chromatograph, consisting of two M6000-A pumps and an M660 solvent programmer, an M440 UV detector and a U6K universal injection system. The 50×1 cm column was packed with LiChroprep RP-18 (Merck, Darmstadt, F.R.G.) and operated at a flow-rate of 5.5 ml/min. The flow-rate was optimised by measuring peak width with acenaphthene as the retained component and uracil as the indicator of dead volume.

Distilled water and HPLC-grade acetonitrile (Hi Per Solv, BDH, Poole, U.K.) were filtered through F.P. Vericel membrane filters, pore size 0.45 μm (Gelman Sciences, Ann Arbor, U.S.A.) and degassed under vacuum prior to use. Mobile phase A was 0.1% (v/v) TFA in water (Fluka, Buchs, Switzerland) containing 10% (v/v) acetonitrile. Mobile phase B was 0.1% (v/v) TFA in water, containing 50% (v/v) acetonitrile.

Samples of enriched IGF-II (20 mg) were dissolved in 200 μl of mobile phase A and injected. After injection, a solvent program was produced by pumping in mobile phase A through the column for 12 min and then a linear gradient from 0 to 100% B was applied over 90 min. The acetonitrile content was then increased stepwise to 80% and finally to 100%. A constant flow-rate of 5.5 ml/min was maintained throughout this program. Detection of peaks was by UV at 230 nm. Protein in fractions from HPLC was estimated by the method of Lowry et al. [20]. A sample of pure IGF-II (courtesy of Dr. G. Enberg, Karolinska Hospital, Stockholm, Sweden) was used as standard to check the point of elution and recovery. Fractions were assayed for NSILA after lyophilisation.

Small-scale HPLC

Reversed-phase HPLC was also carried out on the active material from the large-scale column. The equipment used was as described above, except that the column was a Regis C_{18} type (Regis, Morton Grove, IL, U.S.A.), 30×0.46 cm, operated at a flow-rate of 1 ml/min. Mobile phase A was again 0.1% (v/v) TFA in water containing 10% (v/v) acetonitrile. Mobile phase B was 0.1% (v/v) TFA in water, containing 60% (v/v) acetonitrile.

Samples of crude IGF-II (0.1 mg protein) were dissolved in 100 μl of mobile phase A and injected. A linear gradient from 0 to 100% B was applied over 45 min. Detection of peaks was by UV at 220 nm. Fractions were lyophilized and assayed for NSILA as before. Calculations of overall yield and specific bio-activity were performed on the most active fraction (C3).

C_{18} Sep-Pak experiments

The results of these experiments were important from the point of view of obtaining information on the removal of the major biologically active contaminants NSILA-P and IGF-I. When isoelectric focusing is used to separate the IGF-I from IGF-II, C_{18} Sep-Pak cartridges are useful for removing the contaminating ampholytes. It was also important to determine whether the reversed-phase chromatography was able to remove any NSILA-P not removed by acidic gel permeation chromatography (GPC). In order to determine whether the high-molecular-

TABLE I

RESULTS OF IEC, GPC AND HPLC OF SERUM SHOWING SPECIFIC ACTIVITY AND PURIFICATION AT EACH STEP

Material	Specific activity (mU insulin equiv. per mg protein)	Fold purification
Serum	0.0023 (14 mU per 6 g protein)	1
Fraction C (IEC of serum)	0.018	8
Fraction C1 (active fraction from GPC)	0.21	91
Fraction C2 (active fraction from preparative HPLC)	19	8261
Fraction C3 (active fraction from final HPLC step)	320* (1.7 mU per 5.3 μg)	139 133

*Overall recovery of activity was 12%.

mass acid-stable NSILA co-eluted with the IGF-II during HPLC, some preliminary experiments were conducted with C_{18} Sep-Pak cartridges (Waters Assoc.). Serum (0.3 ml) was mixed with 0.1% (v/v) TFA in water (5 ml) and allowed to stand at 4 °C for 2 h. The solution was passed through a C_{18} Sep-Pak and fractions were successively eluted with 90:10, 60:40 and 40:60 (v/v) water–acetonitrile mixtures (5 ml), all containing 0.1% TFA and chilled to 4 °C. Fractions were then lyophilized and assayed for NSILA.

The same experiment was repeated with an acid-stable high-molecular-mass fraction obtained by chromatography of acidified serum on Sephadex G-75 in 1% (v/v) formic acid. Material of K_{av} 0–0.4 ($M_r > 15\,000$) was dialysed and lyophilised. A sample of this material (30 mg) was dissolved in 0.1% (v/v) TFA (3 ml), applied to a cartridge and the fractions obtained by elution with solutions of different acetonitrile content were lyophilised and assayed as before. Another sample (25 mg) of the acid-stable high-molecular-mass material was dissolved in mobile phase A (200 μl) and applied to the preparative HPLC column to obtain further information about its behaviour.

Pure IGF-II (1 μg) dissolved in 0.1% TFA (3 ml) was also applied to a Sep-Pak cartridge and fractions were eluted and assayed as before. Likewise, this material (1 μg) was applied to the preparative HPLC column to test the point of elution and the recovery of NSILA.

RESULTS

The results of ion-exchange chromatography (IEC), GPC and HPLC of serum (Table I) show the recovery of NSILA (mainly IGF-II) in yield of 12% with an overall fold-purification of 139 133. The elution profile for GPC is shown in Fig. 1. Peaks of bioactivity were obtained at K_{av} 0.15 (NSILA-P) and K_{av} 0.78 (IGF-II). The latter bioactivity was obtained from fractions between the two protein peaks.

Fig. 2 shows that most of the bioactivity (IGF-II) was obtained at 41–43%

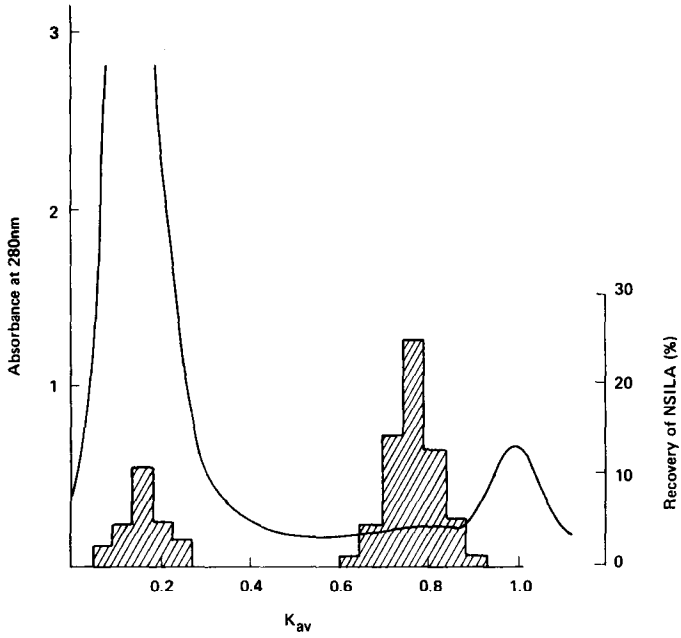

Fig. 1. GPC of active fraction from IEC of serum on DEAE Sephadex (fraction C). Column: Sephadex G-75, 90×2.5 cm; solvent: 1% (v/v) formic acid, aqueous; flow-rate: 20 ml/h; fractions: 5 ml; ▨ = active fractions (NSILA).

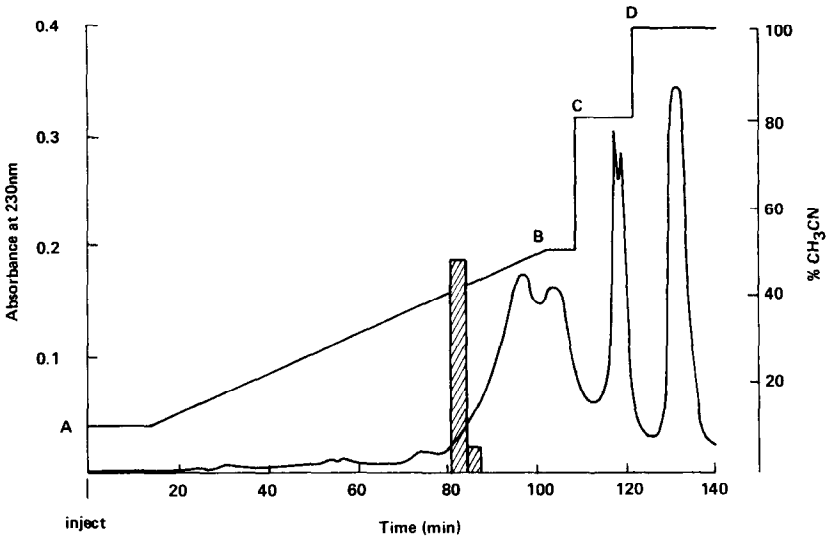

Fig. 2. Reversed-phase HPLC of IGF-II-enriched fraction obtained after IEC and GPC of serum (fraction C1). Column: LiChroprep RP-18, 50×1 cm; mobile phases: (A) 0.1% TFA containing 10% acetonitrile, (B) 0.1% TFA containing 50% acetonitrile, (C) 0.1% TFA containing 80% acetonitrile, (D) 0.1% TFA in acetonitrile; flow-rate: 5.5 ml/min; fractions: 15 ml; ▨ = active fractions (NSILA).

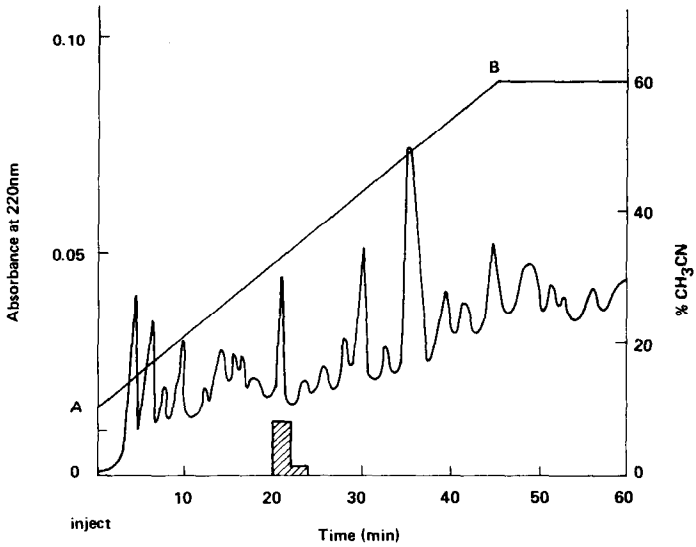

Fig. 3. Reversed-phase HPLC of fraction C2 from IEC, GPC and preparative HPLC of serum. Column: Regis C_{18}, 30×0.46 cm; mobile phases: (A) 0.1% TFA containing 10% acetonitrile, (B) 0.1% TFA containing 60% acetonitrile; flow-rate: 1 ml/min; fractions: 2 ml; ▨ = active fractions (NSILA).

TABLE II

RESULTS OF EXPERIMENTS WITH ACID-STABLE HIGH-MOLECULAR-MASS PREPARATION (30 mg) APPLIED TO SEP-PAK CARTRIDGES

Material	Insulin equivalents (mU)		
	Experiment 1	Experiment 2	Mean
Applied preparation	0.37	0.45	0.41
Unbound fraction	0.28	0.33	0.31
Eluted with 10% acetonitrile	0.02	0.03	0.03
Eluted with 40% acetonitrile	Not detected	Not detected	–
Eluted with 60% acetonitrile	Not detected	Not detected	–

TABLE III

RESULTS OF EXPERIMENTS WITH PURE IGF-II (1 μg) APPLIED TO SEP-PAK CARTRIDGES

Material	Insulin equivalents (mU)		
	Experiment 1	Experiment 2	Mean
Applied IGF-II	0.30	0.36	0.33
Unbound fraction	Not detected	Not detected	–
Eluted with 10% acetonitrile	Not detected	Not detected	–
Eluted with 40% acetonitrile	0.36	0.28	0.32
Eluted with 60% acetonitrile	0.05	0.02	0.04

acetonitrile before the major protein peaks, but it did not correspond to any protein peak.

Fig. 3 shows that bioactivity (IGF-II) is associated with a major peak of absorbance corresponding to a fraction eluted between 32 and 34% acetonitrile.

Experiments in which serum was loaded onto Sep-Pak cartridges showed that of NSILA applied, about half was recovered in the unbound fraction (0% acetonitrile) and half in the fraction eluted with 40% acetonitrile. Confirmation of the presence of the acid-stable high-molecular-mass NSILA (NSILA-P) in the unbound fraction was obtained using the high-molecular-mass material from the acid Sephadex G-75 chromatography of serum. Most of this latter material was recovered in the unbound fraction (Table II). Experiments with pure IGF-II showed that most of the NSILA applied was recovered in the fraction eluted with 40% acetonitrile (Table III).

Both these results were confirmed on the preparative HPLC column. The acid-stable high-molecular-mass material was eluted with mobile phase A (0.1% TFA, 10% acetonitrile) with a recovery of 50% in a broad peak. When the pure IGF-II sample was applied, activity was eluted at 40–42% acetonitrile with a recovery of 72% (data not shown).

DISCUSSION

The procedure used for the preparation of IGF-II from human serum reported here relies on several chromatographic methods. These methods are complementary in that they rely on different principles of separation. The methods give an efficient separation which can be made use of for the laboratory-scale preparation of small amounts of this growth factor. Overall, the process allowed 139 133-fold purification from serum in four steps with a recovery of 12%. The protocol offers the advantage of increased yields of IGF-II over processes based on acid–ethanol extraction [13]. It seems likely that after acid–ethanol extraction, IGF-II is not completely precipitated by the chilled acetone or acetone–ethanol mixture. The present protocol also avoids the use of expensive, large sectional columns [13] or simple, large columns [14] by placing GPC as the second step in the process.

Previous results of radioimmunassay showed that the IGF-II-enriched fraction (fraction C) was contaminated to the extent of about 17% with IGF-I, whilst the IGF-I-enriched fraction was contaminated with about 24% IGF-II [18]. In addition, fraction C was shown to contain about 27% of the acid-stable high-molecular-mass fraction [18]. There are two ways of reducing the IGF-I impurities in fraction C, both of which are easier than HPLC. One way that has been reported [14] is to make use of the difference in isoelectric points by the use of isoelectric focusing. The other way, recently reported [18], is to make use of affinity chromatography on Con A Sepharose. When fraction C was thus chromatographed, most of the IGF-I complex was unbound whereas the IGF-II complex was bound and could be recovered by elution with α-methyl glucoside and α-methyl mannoside with less than 5% contamination by IGF-I [18].

When fraction C was acidified and chromatographed on Sephadex G-75, the complexes were disrupted and low-molecular-mass IGF-II (together with IGF-I

impurity) was recovered in the fraction K_{av} 0.65–0.90. The small amount of IGF-I impurity came from acidification of the IGF-I complex which was co-eluted with the IGF-II complex as part of fraction C. The bulk of the acid-stable high-molecular-mass material was removed in fractions up to K_{av} 0.65.

The Sephadex G-75 step is therefore a valuable step in the purification process and gives an eleven-fold purification from fraction C. It seems preferable to have the Sephadex G-75 step after the IEC step for two reasons. Firstly, the IEC step gives a very useful separation of the IGF-I and IGF-II complexes. Under these conditions there is no noticeable conversion of the IGFs to the acidic insulin-like activity as reported with S.P. Sephadex [21]. Secondly, there are limitations to GPC as a first step from serum because one normally cannot load more sample than about 2% of the total column volume for efficient separations. Thus, previously, 1 l of serum required a 50-l column of Sephadex G-75 for initial processing [14] whereas in the present process this amount of serum could be processed on an 8-l column of DEAE Sephadex A50. Separation of somatomedins (IGF-I and IGF-II) from somatomedin inhibitors in serum has been recently accomplished by size-exclusion HPLC on an analytical scale [22]. This focuses attention on the need to also consider such a step towards the end of processing. Alternatively, it has been reported [23] that Biogel P-10 has been used under acidic conditions, after Sephadex G-75, to remove the higher-molecular-mass inhibitors ($M_r \sim 16\,000$) from the lower-molecular-mass IGFs.

A most significant purification (approximately 90-fold) with good recovery of activity was achieved by the large-scale reversed-phase HPLC step. The use of a preparative HPLC column for the enrichment of a relatively crude IGF-II fraction represents an improvement in efficiency over previously reported methods. At this stage of purification the NSILA does not correspond to a discrete absorbance peak. Verification of the IGF-II-rich fraction can be made by bio-assay or radioimmunoassay. The point of elution (41–43% acetonitrile) corresponds to that of pure IGF-II when applied to the same column. There is obviously an efficient separation from much extraneous protein. It also appears that small amounts of contaminating acid-stable high-molecular-mass material (NSILA-P), not removed by the GPC step, would be removed at this stage. Evidence for this was obtained by the use of Sep-Pak cartridges, which may be used, if necessary, as a preliminary step for HPLC. Acid-stable high-molecular-weight material was not bound to the cartridge whereas IGF-II was practically all eluted with a mobile phase containing 40% acetonitrile. If isoelectric focusing is used to further purify the IGF-II from IGF-I, the Sep-Pak treatment is essential, as it is an effective means of removing contaminating ampholytes [14].

The final small-scale reversed-phase HPLC step achieved a seventeen-fold purification of IGF-II from crude material from the preparative column with good recovery of activity. IGF-II of specific activity 320 mU/mg was prepared, which is close to the value required for homogeneity (360 mU/mg). However, the amount of final product obtained was only sufficient for assay and a minimal ten-fold scale-up of the initial IEC and GPC steps would be required for it to be worthwhile as a preparative process.

Processes for the preparation of IGF-II from its natural source will probably

remain important for some years. At the present time, the main demand for IGF-II comes from investigators who are attempting to gain more knowledge of its mechanism of action and its role in growth processes. The process described here should help to meet part of that demand.

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