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Liquid chromatographic control of the identity, purity and "potency" of biomolecules used as drugs*

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Abstract: The use of high-performance liquid chromatography (HPLC) in the control of rDNA-derived human insulin and human growth hormone is described. Powerful identity tests based upon reversed-phase HPLC separation of enzymatic digests have been developed. Size exclusion and reversed-phase assays are used to control higher molecular weight materials and monomeric derivatives, respectively, for both proteins. Finally, HPLC is used to control the relevant protein content, which in concert with other information controls the biopotency of the protein preparations.

Keywords: *Insulin; human growth hormone; reversed-phase chromatography; protein potency.*

Introduction

The new era of biotechnology presents unique challenges to the quality control laboratory. The establishment of the identity of a protein drug and the determination of the purity and potency of highly purified recombinant DNA-derived proteins requires new technology relative to traditional drugs and protein preparations. For these purposes, reversed-phase and size exclusion HPLC have proven to be particularly powerful tools.

Potency assignment is a particular challenge with proteins. Only an *in vivo* assay can in the strictest sense determine potency, because of the possibility of conformational issues which are not detected by physico-chemical techniques. However, it is possible to design and validate manufacturing processes which consistently produce properly folded material. Then an HPLC method can be designed which selectively determines only the active forms of the protein. Once a conversion factor (U/mg) for material from this process has been experimentally determined, the potency of pharmaceutical preparations can be calculated from the HPLC content assay. Since this is not a direct potency control a limited biological assay can be used as a "bioidentity" check. Our experience with HPLC of human insulin and growth hormone is summarised herein.

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Characterisation of insulin

As part of the proof of structure of a new protein product, a whole series of techniques are utilised. Once the structure is established, a more limited set of techniques is applied to each production batch to verify that it is identical to the others. Of these identity methods, the comparison of a chromatographic separation (or fingerprint) of a proteolytic enzyme digest of a production batch, versus the analytical reference standard digested and chromatographed in parallel, is particularly powerful. This is demonstrated by the fact that the *Staphylococcus aureus* protease fragments of bovine, porcine and human insulins are easily distinguished (Fig. 1).

For purity determinations, reversed-phase HPLC (RP-HPLC) is a very powerful technique. The resolving power of RP-HPLC for proteins of molecular weight <50 kDa can be demonstrated by the assays developed for the determination of monomeric related substances in human insulin [1] and human growth hormone. For example, human insulin can be separated from bovine and porcine insulins and their principal degradation products, A21-desamido insulins, under isocratic conditions using a 300Å Vydac C-18 column and an 75% 0.2 M sulphate buffer (pH 2.3)/25% CH_3CN as the eluent (Fig. 2). A gradual increase in the percentage of CH_3CN in the eluent can be added, causing the elution of other forms of insulin if present.

Alternative RP-HPLC assays may be used as necessary for other substances closely related to human insulin. For example, desthreonine (B30) can be a by-product of the preparation of human insulin from human proinsulin. The chromatographic resolution of desthreonine (B30) insulin from human insulin was achieved by using a Zorbax C8 (150 Å) column eluted with 69% 0.1 M phosphate buffer, pH 3.5/31% CH_3CN, containing 0.58% of the ion-pairing agent pentane sulphonic acid (sodium salt).

Size exclusion chromatography (HPSEC) is used to monitor the covalent insulin dimer contents of insulin crystals and formulations. HPSEC offers several advantages over gel filtration chromatography (GFC). HPSEC requires less sample, gives better resolution, can be automated, and is faster than GFC. Covalent insulin dimers can be separated from the monomers using a Zorbax GF-250 column with a mobile phase of 69% 0.4 M

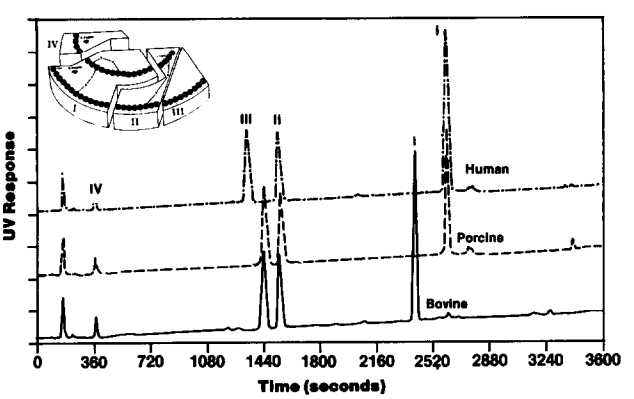

Figure 1
HPLC "fingerprint" following *S. aureus* digestion of three insulins of commercial interest. HPLC conditions: Zorbax column C8 150 Å (25 cm); gradient elution beginning with 13% CH_3CN in 0.25 M sulphate buffer, pH 2.0, increasing the CH_3CN to 30% linearly over 60 min; flow rate: 1 ml min^{-1} and detection at 214 nm; column temperature: 40°C.

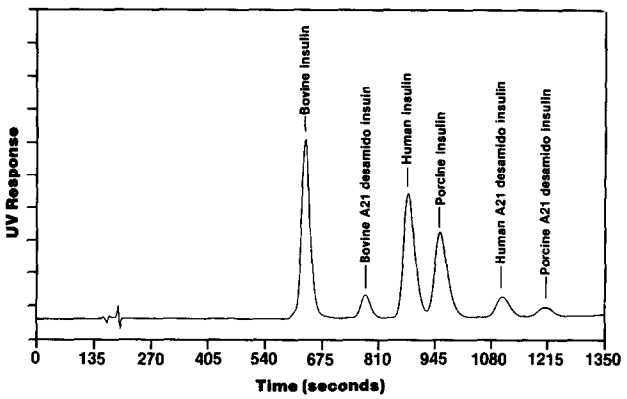

Figure 2
Reversed-phase HPLC separation of insulins and their A21 desamido derivatives. Conditions: Vydac 300 Å C18 column (25 cm); eluent: 0.2 M sulphate buffer, pH 2.3/CH_3CN (75:25); flow rate: 1 ml min^{-1}; detection at 214 nm; column temperature: 40°C.

NH_4HCO_3/31% CH_3CN. The method is equally applicable to bovine, porcine, and human insulins, and all of their formulations [2].

Historically, the potency of insulin has been determined by a rabbit biological assay [3]. RP-HPLC is an appropriate modern replacement in the case of human insulin, because of its specificity (see Fig. 2) and because of the high purity of human insulin products. A reversed-phase method for the determination of biosynthetic human insulin "potency" has been developed and validated by comparison to the rabbit bioassay. The results, summarised in Table 1, showed no statistically significant difference between the two assays. RP-HPLC clearly is more precise, and it also offers specificity as compared to the rabbit bioassay. In our experience, human insulin can be manufactured with even greater precision than that of this relatively precise HPLC assay.

Characterisation of hGH

HPLC has been extensively used in the isolation and characterisation of related substances present in hGH. Size exclusion HPLC was used to isolate hGH dimer [4]. This substance was found to be primarily non-covalent and to have essentially no biological activity, as determined by the traditional hypophysectomised rat bioassay. Reversed-phase HPLC has been used extensively to monitor monomeric chemically modified forms of hGH [5]. The predominant related substances determined by reversed-phase HPLC include desamido (Asn-149) hGH and (Met-14) hGH sulphoxide.

Table 1
Comparison of HPLC and USP rabbit bioassay potency of human insulin crystals (N = 11)

	Average potency (U/mg)	S.D.	Range
Biopotency	28.8	1.0	27.2–30.5
HPLC potency	28.5	0.3	28.2–29.2

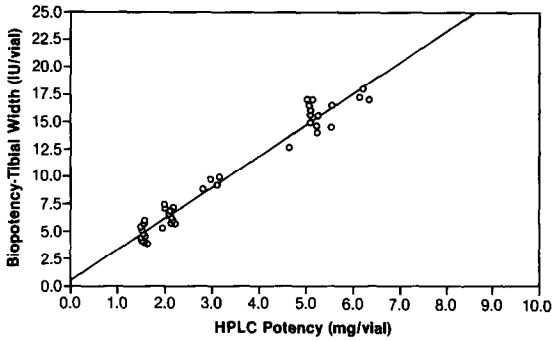

Figure 3
Correlation of HPLC and rat tibial width. Graph contains data on 41 lots of hGH. For conditions see reference 7.

Both of these derivatives have been fully characterised and were found to have biopotencies indistinguishable from unmodified hGH [6]. Reversed-phase HPLC was also employed in conjunction with enzymatic digestion with trypsin to locate the exact site of modification of these derivatives [6]. As with insulin, RP-HPLC is now routinely used to control monomeric derivatives and HPSEC to control the dimer content of hGH preparations.

Based on the observations described above regarding biopotencies of the predominant hGH related substances, HPSEC was selected as the most appropriate technique for chromatographic determination of hGH "potency". A method based on size exclusion HPLC was developed and extensively validated, including an exhaustive comparison to the traditional hypophysectomised rat bioassay, using both the body weight gain (see Fig. 3) and tibial cartilage width methods [7]. In every case the HPLC assay correlated well with the bioassay and, in fact, the HPLC assay correlated better with either of the bioassays than the bioassays correlated with each other (due to imprecision in the bioassays). In addition, the HPLC assay was much more rapid and precise and less labour-intensive than the rat bioassay.

Conclusion

It is clear that HPLC can be utilised to control the identity, purity, and "potency" of therapeutic proteins. Each protein has unique characteristics however, and the origin, purity and biochemistry of individual proteins will dictate different approaches to these assays.

References

[1] H. W. Smith *et al.*, *J. Liquid Chrom.* **8**, 419–439 (1985).
[2] L. M. Atkins, R. Chance, G. Dorulla, J. Masengale, H. Smith and D. Weisman, unpublished results.
[3] *USP XXI* U.S. Pharmacopeial Convention (1985).
[4] G. W. Becker *et al.*, *Biotechnology and Applied Biochemistry* **9**, 478–487 (1987).
[5] R. M. Riggin *et al.*, *Anal. Biochem.* **167**, 199–209 (1987).
[6] G. W. Becker, P. M. Tackitt, W. W. Bromer, D. S. LeFeber and R. M. Riggin, *Biotech. Appl. Biochem.* (in press).
[7] R. M. Riggin *et al.*, *J. Chromatography* **435**, 307–318 (1988).

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