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Analysis of non-covalent aggregation of synthetic hPTH (1–34) by size-exclusion chromatography and the importance of suppression of non-specific interactions for a precise quantitation

Marika Kamberi*, Paul Chung, Richard DeVas, Lily Li, Zengji Li, Xiaoyan Ma (Sharon), Steven Fields, Christopher M. Riley

Department of Analytical Sciences, ALZA Corporation, Mountain View 94039-7210, 1501 California Avenue, Palo Alto, CA 94304, USA

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Abstract

There are few methods available for the rapid and precise quantitation of non-covalent aggregation. Size-exclusion chromatography (SEC), a traditional approach, used to measure the non-covalent aggregation can easily disrupt the weak forces holding an aggregate together. Under the conditions described in this paper the disaggregation of non-covalent aggregate of the synthetic human parathyroid hormone hPTH (1–34) due to hydrophobic/electrostatic interactions with the size-exclusion chromatography column packing was completely suppressed. This report details the effectiveness of adding salts and organic solvents in the mobile phase to overcome non-specific interactions that disrupt the aggregate during the SEC process and may aid in the understanding precise quantitation of non-covalent aggregation.

Keywords: SEC; PTH; Non-covalent aggregation

1. Introduction

Aggregation is a significant problem in the pharmaceutical development of proteins, since it can have several detrimental effects including loss of activity, altered half-life, and increased immunogenicity [1]. Aggregation may be induced by several stress conditions such as heating, freezing, or agitation [2–4]. It may be either covalent (i.e., aggregates formed via covalent bonds such as disulfide bonds, ester or amide linkages, etc.), or non-covalent, occurring via hydrophobic interaction or charge–charge complexation. Because of the pharmaceutical consequences of protein aggregation, methods to assess the degree of aggregation are required for the development of a safe and efficacious protein drug product.

Quantitation of aggregation is often difficult and compounded by the multiple forms in which the aggregates may be found. A number of methods have been successfully employed to monitor and/or quantitate covalent aggregation. These methods include size-exclusion chromatography (SEC), reversed-phase chromatography, gel electrophoresis, viscometry, dynamic light scattering, low angel and multi-angle laser light scattering, and ultracentrifugation [5–9].

However, non-covalent soluble aggregates such as dimers and higher ordered species formed as a consequence of much weaker intermolecular interactions (e.g., hydrophobic, electrostatic, and hydrogen bonding) are much more difficult to quantitate. Several authors have reported the difficulties of using chromatography for such aggregate quantitation [5,10,11]. Interactions with the SEC column itself may disrupt the aggregation state [12,13].

* Corresponding author. Tel.: +1 650 564 2217; fax: +1 650 564 2074.
E-mail addresses: mkamberi@alzus.jnj.com, kmarika55@hotmail.com (M. Kamberi).

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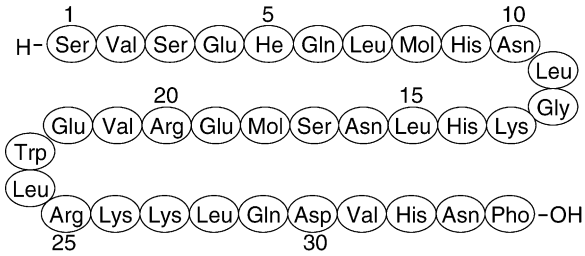

Fig. 1. Chemical structures of hPTH (1–34).

This study was intended to elucidate the chromatographic conditions under which the non-specific interactions of solutes with the column materials are suppressed, and to demonstrate the successful use of the SEC for the separation and quantitation of the non-covalent aggregate from the non-aggregated form of the human parathyroid hormone hPTH (1–34) (Fig. 1), which is a potential therapeutic agent for the treatment of osteoporosis in post-menopausal women [14,15].

2. Experimentals

2.1. Materials

hPTH (1–34) was provided by Bachem Inc. (Torrance, CA, USA) as a highly purified, lyophilized powder. All other chemicals were of analytical reagent grade. Sodium chloride, hydrochloride acid, and acetonitrile were obtained from Mallinckrodt Baker, Inc. (Phillipsburg, NJ, USA). Trifluoroacetic acid (TFA) was purchased from Pierce Technology Co. (Boston, MA, USA). Sucrose, Tween20, and EDTA were purchased from Sigma Chemical Co. (St. Louis, MD, USA).

2.2. Size-exclusion chromatography

SEC was performed with an Agilent 1100 series HPLC system (Agilent Technologies, Inc., Palo Alto, CA, USA) provided with a binary pump, a thermostatted autosampler, a thermostatted column compartment, and a multiple wavelength diode array detector (DAD)/ultraviolet (UV) detector. Data were collected and analyzed using Turbochrom Client Server Software, version 6.2 (Perkin-Elmer, Inc., Boston, MA, USA). A TSK-GEL G2000SWXL 300 mm × 7.8 mm (MW range, 5000–100,000 Da) column (Tosoh Bioscience LLC, Montgomeryville, PA, USA) was chosen for the present studies based on preliminary investigations of the chromatographic properties of several SEC columns under various mobile phase conditions. The optimal mobile phase composition consisted of 0.1% aqueous TFA in 0.2 M sodium chloride/acetonitrile (80:20, v/v). The detector was set at 215 nm and the flow rate was typically 0.5 ml/min. The injection volume was 40 μl.

2.3. Light scattering

The molecular weight of the monomer and the aggregated product were determined by SEC-light scattering (SEC-LC) using on-line Mini DAWN LS and OptiLab DSP refractive index detectors (Wyatt Technology, Santa Barbara, CA, USA). The construction and principles of this system have been described in detail elsewhere [9].

2.4. Sample preparation

The hPTH (1–34) was typically prepared at a concentration of 2 mg/ml. Since we were interested in the aggregation state under the formulation conditions, the formulation excipient (20% sucrose, 12.7% hydrochloric acid, 0.2% Tween20, and 0.03% EDTA) was used as the sample diluent for all of our studies. Samples were incubated for 3–6 months at 40 °C.

3. Results and discussion

3.1. Monitoring of the effects of the mobile phase composition on the disruption of hPTH (1–34) non-covalent aggregation

Since the aggregation of polypeptides into oligomers causes an increase in their size, such interactions can be measured by SEC. Separation of non-covalent aggregation by a mechanism based solely on species size (ideal SEC) occurs only when there is no interaction between the solute and the column matrix. Although high performance SEC columns are designed to minimize non-specific interactions, most modern SEC columns are weakly anionic (negatively charged) and slightly hydrophobic, resulting in deviation from ideal size-exclusion behavior [16,17]. Under such non-ideal SEC conditions, the non-specific interactions between the matrix and polypeptides, which may disrupt the non-covalent aggregation, may be pronounced. The non-specific interactions may be minimized by manipulation of the mobile phase. Indeed, it has been pointed out that if the conformational character of a peptide–protein mixture in a particular mobile phase is uncertain and ideal size-exclusion behavior is desired, SEC should always be carried out under highly denaturing conditions [18]. The use of denaturants has many drawbacks when one works with proteins; however, these properties may actually be advantageous when studying peptides, since the molecular weight range in which separation occurs is reduced in denaturing solvent because all polypeptides acquire a random coil structure, increasing their size.

Several researchers have noticed the advantages of 0.1% TFA for chromatography of proteins in terms of protein solubility, low absorbance of UV light, volatility, suppression of ionization of silanol groups, and demonstrated that the use of 0.1% TFA as the mobile phase in SEC is particularly applicable to the separation of peptides of low molecular weight [19,20]. Colin et al. showed that non-specific interaction of

Table 1
Percentage aggregation measured as a function of the SEC mobile phase composition

Mobile phase (composition)	Percentage aggregation (S.D.)	Percentage difference to A
A (0.1% aq. TFA)	3.14 (0.08)	
B (0.1% aq. TFA + 5% ACN + 50 mM NaCl)	5.14 (0.13)	63.69
C (0.1% aq. TFA + 10% ACN + 50 mM NaCl)	5.50 (0.13)	75.16
D (0.1% aq. TFA + 10% ACN + 100 mM NaCl)	5.34 (0.11)	70.06
E (0.1% aq. TFA + 10% ACN + 200 mM NaCl)	5.08 (0.12)	61.78
F (0.1% aq. TFA + 20% ACN + 200 mM NaCl)	5.78 (0.08)	84.08
G (0.1% aq. TFA + 30% ACN + 200 mM NaCl)	5.34 (0.08)	70.06
H (0.1% aq. TFA + 40% ACN + 400 mM NaCl)	4.77 (0.09)	51.95

aq. TFA, aqueous trifluoroacetic acid; ACN, acetonitrile; NaCl, sodium chloride. Column TSK-G2000-SW, 7.5 mm × 300 mm; flow-rate, 0.5 ml/min; temperature, 25 °C; detector wavelength, 215 nm. Values represent mean ± S.D. for six samples.

the peptides with the TSK SW packing were minimal in 0.1% aqueous TFA [20]. At low pH, ionization of silanol groups is suppressed and it would be expected that electrostatic effects would be reduced. However, it should be noted that hydrophobic and/or electrostatic characteristics may be more pronounced with one size-exclusion column than with another [17] and, as seen in Figs. 2 and 3 and Table 1, the use of a simple volatile mobile phase may be insufficient to suppress these interactions. Electrostatic effects between non-covalent aggregation and the column matrix may be minimized by the addition of salts to the eluent. Fig. 2 demonstrates the effect of increasing concentrations of sodium chloride in 0.1% aqueous TFA on the measured hPTH (1–34) aggregate. As the ionic strength of the mobile phase increased to 100 mM, the electrostatic effects were gradually minimized. Salts suppress non-specific ionic interactions of peptides with a SEC column matrix by competing with the matrix for interaction with the charged groups on the peptides [21].

Increasing the sodium chloride to over 100 mM did not improve the chromatographic profile and disrupted the aggregation, possibly due to the promotion of hydrophobic interactions at this high salt level. These results support the view that high-ionic-strength solvents should generally be avoided in SEC [17].

Several researchers have demonstrated the utility of adding volatile organic solvents, such as acetonitrile or trifluoroethanol, to 0.1% aqueous TFA for effective separations of peptides and proteins [21,22]. Apart from their UV transparency, these organic modifiers decrease non-specific hydrophobic interaction of peptides with the SEC matrix and increase the overall solubility of proteins. Fig. 3 demonstrates the effect of increasing acetonitrile concentration in 0.1% aqueous TFA on the disruption of hPTH (1–34) non-covalent aggregation.

As the concentration of the organic modifier increased from 0 to10%, the disruption rate of the aggregation decreased, presumably as the hydrophobic interactions were overcome, and than proceeded to increase again. The results suggest that further increases in acetonitrile concentration could promote ionic interactions of the peptide with the column material. The effect of combination of salt and acetonitrile in the eluent on the disruption of the aggregate hPTH (1–34) was also studied. Different concentrations of sodium chloride and acetonitrile were added together to the 0.1% aqueous TFA to determine the minimum effective levels required to inhibit both the ionic and hydrophobic interactions (Table 1). The results showed that the measured percentage hPTH (1–34) aggregate for the same sample increased when

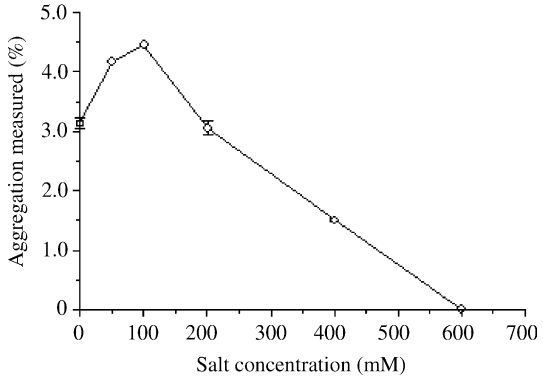

Fig. 2. Effect of increasing sodium chloride concentration in the mobile phase on the percentage aggregation measured. Column TSK-G2000-SW, 7.5 mm × 300 mm; mobile phase, 0.1% aqueous TFA (pH 2.0), containing 0 mM, 50 mM, 100 mM, 200 mM, 400 mM, and 600 mM sodium chloride; flow-rate, 0.5 ml/min; temperature, 25 °C; detector wavelength, 215 nm. Values represent mean ± S.D. for six samples.

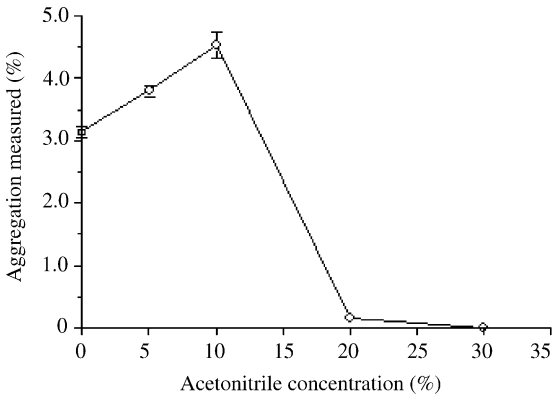

Fig. 3. Effect of increasing acetonitrile concentration in the mobile phase on the percentage aggregation measured. Column TSK-G2000-SW, 7.5 mm × 300 mm; mobile phase, 0.1% aqueous TFA (pH 2.0), containing 0%, 5%, 10%, 20%, and 30% acetonitrile; flow-rate, 0.5 ml/min; temperature, 25 °C; detector wavelength, 215 nm. Values represent mean ± S.D. for six samples.

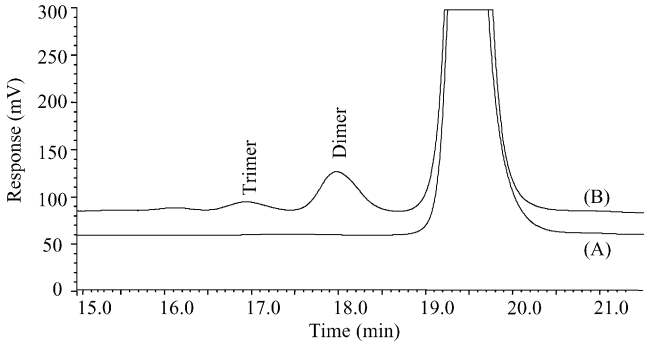

Fig. 4. Separation of hPTH (1–34) monomer and oligomers. hPTH (1–34) standard (A); partly aggregated hPTH (1–34) sample (B). The mobile phase used for the analysis was 0.1% aqueous TFA in 0.2 M sodium chloride/acetonitrile (80:20, v/v). Column TSK-G2000-SW, 7.5 mm × 300 mm; flow-rate, 0.5 ml/min; temperature, 25 °C; detector wavelength, 215 nm.

both the sodium chloride and acetonitrile were added to the 0.1% aqueous TFA, with the maximal level and an excellent elution profile obtained when 0.1% aqueous TFA in 0.2 M sodium chloride/acetonitrile (80:20, v/v) was used as the mobile phase (Table 1 and Fig. 4). While the optimal concentration of sodium chloride along was at 100 mM (Fig. 2) and that of acetonitrile along was 10% (Fig. 3), in the combination, the optimal concentrations of sodium chloride and acetonitrile were 200 mM and 20%, respectively (Table 1). The changes in optimal concentrations of sodium chloride and acetonitrile clearly suggested that there may be a counter-balancing effect occurring.

3.2. SEC analyses of SEC fractions

To confirm that the mobile phase consisting of 0.1% aqueous TFA in 0.2 M sodium chloride/acetonitrile (80:20, v/v) was the most appropriate one for the separation of hPTH (1–34) aggregate from the non-aggregate form on the TSK-G2000-SW column, the SEC analyses of SEC fractions was performed. A partly hPTH (1–34) aggregated sample was injected onto the SEC system, eluted with this mobile phase and fractionated over a 7-min period until all of the peaks had completely eluted. These fractions were desalted and concentrated to the initial concentration by filtration with the highly selective OMEGA ultrafiltration membrane using MICROSEP microconcentrators (Pall Filtration Co, MA, USA); they were then rechromatographed in different eluents, and recovery of the aggregated product was calculated (Table 2).

Table 2
Recovery of hPTH (1–34) aggregation after rechromatography

Mobile phase (composition)	Percentage aggregation		Recovery (%)
	Control	Rechromatographed	
A (0.1% TFA)		9.1	55.2
B (0.1% TFA + 100 mM NaCl)		12.9	78.2
C (0.1% TFA + 10% CAN)		13.1	79.4
D (0.1% TFA + 10% ACN + 100 mM NaCl)		15.4	93.3
E (0.1% TFA + 20% ACN + 200 mM NaCl)	16.5	16.0	97.0
F (0.1% TFA + 40% ACN + 400 mM NaCl)		13.6	82.4

TFA, trifluoroacetic acid; ACN, acetonitrile; NaCl, sodium chloride. Column TSK-G2000-SW, 7.5 mm × 300 mm; flow-rate, 0.5 ml/min; temperature, 25 °C; detector wavelength, 215 nm.

Table 3
Accuracy of molecular masses of monomer and aggregated product of hPTH (1–34) determined by light scattering

	Mass from structure (Da)	Light scattering (Da)	Apparent error (%)
Monomer	4117	4185	1.6
Dimer	8234	7671	6.8
Trimer	12351	11810	4.4

The observations summarized in Table 2 show the importance of mobile phase composition for accurate quantitation of hPTH (1–34) aggregate. It is obvious that the ideal size-exclusion behavior is never obtained on the TSK-GEL G2000SWXL column with the 0.1% aqueous TFA mobile phase or with the addition of only salt or acetonitrile to the 0.1% aqueous TFA; therefore, these conditions were found inappropriate for the quantitation of hPTH (1–34) aggregate. However, no disruption or promotion of the hPTH (1–34) aggregated product was found during passage through the column when 0.1% aqueous TFA in 0.2 M sodium chloride/acetonitrile (80:20, v/v) was used as the mobile phase. The extent of the aggregation after rechromatography was consistently the same as that observed after the first pass through the column (Table 2). This means that, at the above established chromatographic conditions, the SEC system itself is not disrupting the aggregation.

3.3. Confirmation that SEC is separating aggregates

The monomer and aggregate peaks were assayed by dynamic light scattering in order to confirm the peaks corresponding to the hPTH (1–34) aggregate and monomer, respectively. The early eluting peaks showed estimated molecular weights of 11.810 Da and 7.671 Da, corresponding to the trimer and dimer molar masses of the hPTH (1–34), respectively. The comparison of calculated and measured molecular masses by light scattering of the monomer and aggregated product is given in Table 3.

4. Conclusions

This paper has demonstrated that the key to success in the study of non-covalent protein aggregation by SEC depends on an understanding of hydrophobic and/or electrostatic interactions of the peptide with the SEC packing and on the manipulation of mobile phase conditions to suppress these interactions.

Under conditions that promote pure size-exclusion behavior, the SEC may represent a useful approach for screening

solution formulations for soluble non-covalent aggregates of peptides.

However, it should be noted that hydrophobic and/or electrostatic interactions may be more pronounced with one-size-exclusion column than with another, and that they can be affected by protein hydrophobicity, aggregate size, and the strength of non-covalent interactions. In order to suppress these interactions, the composition of the mobile phase should not be predicted apriori, and it has to be determined for each protein and column employed. If it can be shown that the aggregation:monomer ratio is not disrupted at certain established chromatographic conditions, the SEC can be used to accurately quantitate the non-covalent aggregation.

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References

[1] J.L. Cleland, M.F. Powell, S.J. Shire, Crit. Rev. Ther. Drug Carrier Syst. 10 (1993) 307.
[2] D.N. Brems, P.L. Brown, G.W. Becker, J. Biol. Chem. 265 (1990) 5504.
[3] P.L. Schwartz, M. Batt, Endocrinology 92 (1973) 1795.
[4] M. Hagenlocher, R. Pearlman, Pharm. Res. 6 (1989) S30.
[5] J. Brange, S. Havelund, P. Hougaard, Pharm. Res. 9 (1992) 727.
[6] G. Ralston, Introduction to Analytical Ultracentrifugation, Beckman Instruments, Fullerton, California, 1993.
[7] R.C. Cantor, R.R. Schimmel, Techniques for the study of biological structure and function. Part II, Biophysical Chemistry, WH Freeman, San Francisco, 1980.
[8] D.J. Andya, C.C. Hsu, J.S. Shire, AAPS Pharm. Sci. 5 (2003) 10.
[9] E.-F. Stogniew, K.R. William, J. Biomol. Tech. 10 (1999) 51.
[10] M. Draper, M. Savage, H.J. Collett, D. Attwood, C. Price, C. Booth, G.Q. Wang, Pharm. Res. 12 (1995) 1231.
[11] J.M. Kunitani, L.R. Cunico, J. Chromatogr. 443 (1988) 205.
[12] K.D. Clodfelter, A.M. Nussbaum, J. Reilly, J. Pharm. Biomed. Anal. 19 (1999) 763.
[13] K.D. Clodfelter, H.A. Pekar, M.D. Rebhun, A.K. Destrampe, A.H. Havel, R.S. Myers, L.M. Brader, Pharm. Res. 15 (1998) 254.
[14] J.T. Potts, F.R. Bringhurst, T. Gardella, S. Nussbaum, G. Segre, H. Kronenberg, in: H.R. Williams, D.J. Wilson, W.D. Foster (Eds.), Textbook of Endocrinology, WB Saunders Company, Philadelphia, PA, 1995, pp. 920–966.
[15] E. Seeman, J.A. Eisman, Med. J. Aust. 180 (2004) 298.
[16] W. Kopaciewiez, F.E. Regnier, Anal. Biochem. 133 (1983) 251.
[17] F.E. Regnier, Meth. Enzymol. 91 (1983) 137.
[18] G.B. Irvine, J. Biochem. Biophys. Meth. 56 (2003) 233.
[19] G.B. Irvine, S. Show, Anal. Biochem. 155 (1986) 141.
[20] S.M. Lau, A.K. Taneja, R.S. Hodges, J. Chromatogr. 317 (1984) 129.
[21] T.C. Mant, M.J. Parker, S.R. Hodges, J. Chromatogr. 397 (1987) 99.
[22] W.C. Mahoney, M.A. Hermodson, J. Biol. Chem. 255 (1980) 11199.