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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY IN THE QUALITY CONTROL OF IMMUNOGLOBULIN PREPARATIONS DURING PRODUCTION AND STORAGE

HANNU SUOMELA* and JAAKKO-JUHANI HIMBERG
Finnish Red Cross Blood Transfusion Service, Helsinki (Finland)
and
TAPANI KURONEN
National Public Health Institute, Helsinki (Finland)

SUMMARY

High-performance liquid chromatography (HPLC) has been used in the routine quality control of immunoglobulin preparations to measure the contents of aggregates, dimers, monomers and degradation products. The correlation coefficients between TSK 3000 SW, Ultrogel AcA 34 and Sephacryl S-300 chromatography were calculated and a reasonable correlation was found. The decrease in the potency of anti-tetanus immunoglobulin at room temperature was accompanied by an increase in degradation products. The speed and high sensitivity of HPLC make it suitable for the detection of aggregates in intravenous immunoglobulin preparations.

INTRODUCTION

High-performance liquid chromatography (HPLC) is a useful method for the analysis of molecular forms of immunoglobulin G (IgG)[1]. Human IgG for therapeutic use consists of several molecular species. Aggregates consisting of polymers and oligomers are formed during the Cohn ethanol fractionation method. The relative amount of aggregates depends on the production conditions and is low when the production methods are adequate. Aggregates are also regarded as being responsible for spontaneous complement activation, which prevents the intravenous use of these preparations. Products for intravenous use are permitted to contain only traces of aggregates.

Degradation products of IgG include components of several molecular sizes. Degradation is measured in order to study the stability of IgG preparations during their self-life. HPLC is much faster than conventional column chromatography with soft gels, and is therefore especially suitable for quality control and production monitoring. This study was carried out in order to compare a TSK-G 3000 SW column in HPLC, Ultrogel AcA 34 and Sephacryl S-300 column chromatography.

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EXPERIMENTAL

Immunoglobulin preparations for intramuscular (i.m.) use were made by Cohn fractionation. The age of samples varied between 0.5 and 12 years. Old samples were used, because they contain degraded IgGs, which are lacking in fresh preparations. Immunoglobulins for intravenous (i.v.) use were obtained from several manufacturers. The freeze-dried products were dissolved immediately before the assays.

Tetanus antibodies were assayed by the mouse toxin-neutralization test at the toxin level of $L + /10$ (ref. 2). The assay was calibrated with the international tetanus antitoxin standard, obtained from the WHO. Plasmin and plasminogen were assayed with chromogenic substrate S-2251 (Kabi)[3].

The HPLC analysis was performed on a TSK-3000 SW column (600 × 7.5 mm I.D.). Phosphate-buffered saline (0.15 *M*, pH 7.0) containing 0.1% sodium azide was used as a solvent. The column was tested according to the instructions given by the manufacturer and found to have about 42,000 theoretical plates per metre. The flow-rate was kept at 0.75 ml/min and the inlet pressure was 30–35 bar.

The HPLC instrument used was a Hewlett-Packard 1084B, equipped with an automatic injection system and sample changer, together with a Model 1040 A variable-wavelength fast scanning UV detector. The chromatograms were monitored at 280 nm with a reference wavelength of 550 nm.

IgG solutions were diluted with phosphate-buffered saline to a protein concentration of 4 mg/ml. After dilution, the samples were filtered through 0.22- or 0.45-μm cellulose acetate filters. The sample volume injected was 150 μl (600 μg of protein).

Ultrogel ACA 34 and Sephacryl S-300 columns (90 × 2.5 cm I.D.) were used with phosphate-buffered saline (pH 7.0). A 2-ml volume of 4% protein solution was applied as a sample. A descending flow of 20 ml/h was maintained by a peristaltic pump. The absorbance at 280 nm was detected with a Pharmacia UV-1 detector and recorded with a Pharmacia or LKB recorder. For the quantification the chromatogram was photocopied and peaks were cut out and weighed.

RESULTS AND DISCUSSION

Comparison between TSK 3000 SW, Ultrogel AcA 34 and Sephacryl S-300

Twenty lots, mainly aged immunoglobulins, were chromatographed in TSK 3000 SW, Ultrogel AcA 34 and Sephacryl S-300 columns. The elution profiles of one sample in the three gels is illustrated in Fig. 1. The sample contains polymers, oligomers, dimers, monomers and degradation products.

None of the gel media separates oligomers as a distinct peak. In practice, oligomers are usually calculated as aggregates, together with the first void volume peak, which represents polymers.

By mass, $F(ab)_2$ (MW 108,000) is the dominant fraction of degradation products[1]. When the degradation products consist of less than 6–7% of total protein, HPLC does not separate them as a distinct peak from IgG monomer. Ultrogel AcA 34 and Sephacryl S-300 separate IgG monomer (MW 150,000) and $F(ab)_2$ more clearly also when $F(ab)_2$ is present in low concentration (Fig. 1).

The samples in this study were selected so as to represent a wide range of

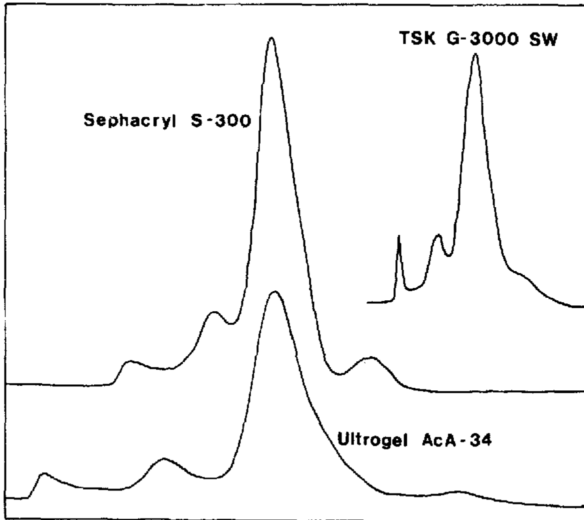

Fig. 1. Elution profiles of the sample in Ultrogel AcA 34, Sephacryl S-300 and TSK-3000 SW gels. Polymers = first peak; oligomers = area between the first and second peaks; dimers = second peak; monomers = main peak; degradation products = last peak.

different molecular forms of IgG. Thus, polymers ranged from 0 to 7%, dimers from 3 to 22%, monomers from 22 to 93% and degradation products from 0 to 60%. When the results for a given sample in all three gels are compared, large variations may appear, especially when the relative content of the fraction is low. However, the wide range of different IgG forms makes the correlation coefficients between the runs reasonable, as can be seen in Table I.

TABLE I

CORRELATIONS BETWEEN TSK 3000 SW, ULTROGEL AcA 34 AND SEPHACRYL S-300 GEL CHROMATOGRAPHY

Correlation coefficients and equations are derived from twenty immunoglobulin lots, chromatographed in the three gels.

Species	*Correlation coefficient*	*Equation*
Polymers + oligomers:		
HPLC/AcA-34	0.883	$x = 0.83y + 0.83$
HPLC/S-300	0.799	$x = 0.84y + 0.06$
AcA/S-300	0.778	$x = 0.89y - 0.45$
Dimers:		
HPLC/AcA	0.929	$x = 0.90y + 0.83$
HPLC/S-300	0.953	$x = 1.21y - 4.49$
AcA/S-300	0.818	$x = 0.99y - 0.92$
Monomers:		
HPLC/AcA	0.960	$x = 0.99y + 0.73$
HPLC/S-300	0.982	$x = 1.18y - 9.92$
AcA/S-300	0.961	$x = 1.09y - 4.35$
Degradation products:		
HPLC/AcA	0.988	$x = 1.00y - 0.37$
HPLC/S-300	0.994	$x = 1.15y - 1.75$
AcA/S-300	0.996	$x = 1.14y - 1.25$

Stability study of anti-tetanus IgG

Three lots of anti-tetanus γ-globulin were studied every sixth month. Storage was either in a cold-room (about +4°C) or at room temperature (about 23°C). HPLC was used to detect the degradation and biological potency assay in mice was used to detect the biological potency.

All three lots studied maintained their full biological activity when stored in the cold-room. Similarly, no degradation products could be measured by HPLC in these samples. However, at room temperature, a slight decrease in anti-tetanus activity could be demonstrated. Small increases in the degradation band in HPLC could also be noticed (Table I).

Although only three lots were studied, one may conclude that roughly half of the observed decrease in activity was observed as an increase in degradation products. The polymer and dimer contents were not changed.

The plasmin and plasminogen contents in the three lots were determined and were as follows: Lot 79274, plasmin 0.003 IU/ml and plasminogen 0.054 IU/ml; Lot 80280, plasmin 0.015 IU/ml and plasminogen 0.215 IU/ml; and Lot 81294, plasmin 0.011 IU/ml and plasminogen 0.084 IU/ml. As can be seen in Table II, the decrease in biological activity and degradation of IgG correspond approximately with the measured proteolytic activity.

TABLE II

DECREASE IN ANTI-TETANUS ACTIVITY AND INCREASE IN DEGRADATION PRODUCTS IN ANTI-TETANUS IMMUNOGLOBULINS STORED AT ROOM TEMPERATURE

The same preparations were stable in the cold-room.

Months	*Lot No. 79274*		*Lot No. 80280*		*Lot No. 81294*	
	*Tetanus (%)**	*Degradation (%)***	*Tetanus (%)**	*Degradation (%)***	*Tetanus (%)**	*Degradation (%)***
6	nd***	nd	nd	nd	97	0
12	97	0	100	2	88	6
18	88	2	86	5	nd	nd
24	88	2	88	12	78	7.5
30	91	3	83	15	73	7
36	85	nd	nd	nd		
42	nd	3	74	15		

* Percentage potency left from time zero. Initially the preparation contained 165–175 IU/ml tetanus antitoxin.

** Percentage of degradation products measured by HPLC.

*** Not determined.

Detection of IgG aggregates in i.v. immunoglobulin preparations

Aggregated IgG causes harmful side effects in patients if immunoglobulin solution is infused intravenously. HPLC can be used in quality control to measure the amount of aggregates. Six different samples, representing six manufacturers of "unsplit" i.v. immunoglobulins, were studied. As demonstrated in Fig. 2, in two of the samples no aggregates can be demonstrated, whereas in four others traces of aggregates are present. The amount of dimers also varies greatly. Owing to the albumin

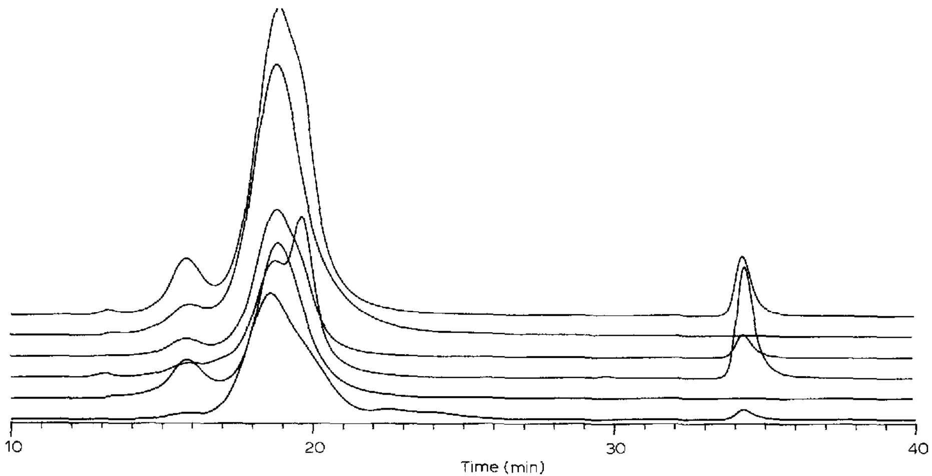

Fig. 2. Elution profiles of six immunoglobulin preparations for intravenous use on the TSK 3000 SW column. Each product is from a different manufacturer. Ten minutes are subtracted from beginning of the chromatograms. Void volume, retention time about 13 min indicates aggregates, dimers are eluted between 15 and 17 min. The main peak contains IgG monomers, including albumin as a shoulder to the right. Last peak, retention time 34–35 min, indicates acetyl tryptophanate.

used as a stabilizer, the IgG monomer peak is asymmetric. Under these circumstances albumin and IgG monomer cannot be resolved on the TSK 3000 SW column.

Only one of the samples contained detectable amounts of degraded protein. The last low-molecular-weight peak was identified as acetyl tryptophanate, which is used as a stabilizer of albumin.

In the development project and the quality control of intravenous immunoglobulins, HPLC is an excellent tool because of its speed and accuracy of detection.

REFERENCES

1 T. Tomono, T. Suzuki and E. Tokunaga, in M. T. W. Hearn, F. E. Regnier and C. T. Wehr (Editors), *High Performance Liquid Chromatography of Proteins and Peptides, Proceedings of the First International Symposium, November 16–17, 1981, Washington, DC,* Academic Press, New York, 1983,pp. 9–16.

2 J. P. Craig, in N. R. Rose and H. Friedman (Editors), *Manual of Clinical Immunology,* American Society for Microbiology, Washington, DC, 2nd ed., 1980, pp. 512–519.

3 P. Friberger and M. Knös, in M. F. Scully and V. V. Kakkar (Editors), *Chromogenic Peptide Substrates, Chemistry and Clinical Usage,* Churchill Livingstone, London, 1979, pp. 128–140.