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Research Article

EFFECT OF AMINO ACID ADDITION AS STABILIZERS ON TOTAL PROTEIN CONTENT OF INTRAVENOUS THERAPEUTIC HUMAN NORMAL IMMUNOGLOBULIN

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ABSTRACT

Objectives: This study was conducted to show the variation in total protein content of therapeutic immunoglobulin batches after the addition of free amino acids.

Methods: A total of 10 different batches of therapeutic human normal immunoglobulin from five different manufacturers were taken. The total protein content in these samples was determined by both Kjeldahl and Biuret methods. The total protein in these samples was also determined by both methods after the addition of 0.3 M glycine, 0.3 M arginine, and 0.3 M histidine separately.

Results: In neat samples, both methods quantified similar total protein content. However, both methods showed different protein contents where amino acids such as glycine, arginine, or histidine have been added. The total protein content by the Kjeldahl method in the samples added with glycine, arginine, or histidine was found higher as compared to the Biuret method.

Conclusion: Our study suggests that the manufacturers of therapeutic immunoglobulin should replace amino acid stabilizers with nonnitrogenous stabilizer because the Kjeldahl method recommended in various pharmacopeias is based on the detection of total nitrogen. Therefore, the addition of any nitrogenous stabilizer will be quantified as protein and results may be out of specification when minimum and maximum limits are prescribed in the pharmacopeia.

Keywords: Human immunoglobulin, Quality control, Protein estimation, Kjeldahl, Biuret.

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INTRODUCTION

Therapeutic human immunoglobulin is a plasma-derived blood product. It mainly contains immunoglobulin G (IgG) against various pathogens. The therapeutic use of human intravenous immunoglobulin (IVIg) for the treatment of primary and secondary immunodeficiency was started in the 1950s [1]. Various manufacturing processes such as chromatographic techniques as well as chemical treatments are used to prepare human immunoglobulin from human plasma. At least 1000 plasma donations are pooled to produce therapeutic human normal immunoglobulin. Therefore, selection of donors, plasma fractionation, and viral inactivation are crucial steps to maintain quality, safety, and tolerance. The manufacturing process as well as composition of immunoglobulin preparations depend on the route of administration and application [2,3]. The concentrations of immunoglobulin preparations for intravenous use are adjusted at a protein concentration of 160 g/L or 50–120 g/L. IVIg preparations must exhibit Fc functions of native immunoglobulin and must have defined proportions of subclasses of IgG [3]. IVIg preparations are manufactured in lyophilized form or as a stabilized solution with stabilizers such as albumin, amino acids, sugars, and nicotinamide [2,4]. IVIg is given at replacement levels to immune-deficient individuals (0.04 g/kg body weight), whereas large dosages are given to patients with autoimmune and inflammatory disorders (1–2 g/kg body weight) [5]. A ready-to-use liquid formulation is sought from both an economical and user-friendliness standpoint. However, in a liquid formulation, many proteins are vulnerable to both chemical and physical deterioration (such as oxidation and deamination) as well as aggregation and precipitation [6,7]. Immunoglobulin, in particular, has a propensity to agglomerate, dimerize, fragment, or denature. Aggregates can cause serious negative effects, including anaphylactic shock if such solutions are intravenously administered. IgG dimers are not known to cause anaphylactic shock, but it has been found that high dimer IgG preparations are less well tolerated when administered intravenously and may cause unfavorable side effects such as fever, nausea, and occasionally lowered blood pressure. Dimer formation is less of an issue when lyophilizing IgG preparations quickly after they are prepared [8]. Earlier, the IVIg formulation was stabilized using carbohydrates such as sucrose and maltose. Numerous amino acids have been utilized recently in innovative formulations to stabilize immunoglobulin molecules and prevent dimerization [9]. The researchers found that by adjusting the final preparation's pH to 4.2–5.4 and adding a basic or nonpolar amino acid as a stabilizing agent, along with stabilizers added to the solution at a final concentration of at least 0.2 M, it is possible to stabilize liquid protein preparations to an unexpected degree. With one or more stabilizers selected from the group of nonpolar and basic amino acids, the present invention therefore produces a protein composition with enhanced stability. Histidine, arginine, lysine, ornithine, and isoleucine are a few examples of nonpolar and basic amino acids that can be utilized to achieve the objectives of the current invention. Valine, methionine, glycine, and proline are a few nonpolar amino acid examples. It has been shown that the addition of excipient (arginine, glycine, histidine, alanine, lysine, or proline) improves the long-span solidity of biomolecules of protein, to provide the therapy, maintenance, and avoidance of diseased condition [10]. Arginine is among the most common amino acids that are used in therapeutic preparations, as it can reduce the viscosity of heavy and reconstituted dilutions and has a good impact on the solubility of proteins. However, arginine decreases the temperature at which the collapse occurs, necessitating cautious drying cycles. Arginine is also capable of lowering the formation of the aggregates by 2–2.5 times [7].

Our institute is a Central Drugs Laboratory in India for quality testing of imported as well as indigenously manufactured batches of therapeutic

human immunoglobulin. The quality testing of these immunoglobulin batches is done as per the monograph given in Indian Pharmacopoeia (IP) which is at par with European Pharmacopoeia. As per IP, the determination of total protein content is one of the quality testing parameters for these plasma-derived products. A few manufacturers add amino acids, namely glycine, arginine, and histidine as a stabilizer in their therapeutic human normal immunoglobulin, which may increase the total protein content because the compendial method recommended for the determination of protein content is based on total nitrogen estimation. Therefore, this work was planned to study the effect of amino acids, namely glycine, arginine, and histidine on the total protein content of therapeutic human normal immunoglobulin by the Kjeldahl method and Biuret method.

METHODS

Samples

Samples from 10 batches of therapeutic human normal immunoglobulin liquid formulations were taken for this study (Table 1). To avoid conflict and biasness, the names of manufacturers and batch numbers of samples have been coded.

Addition of amino acids

Four sets of all 10 immunoglobulin samples were prepared. The first set comprised neat immunoglobulin samples. To the second set of samples, 0.3 M glycine which is equivalent to 2.25%, to the third set 0.3 M arginine which is equivalent to 5.22%, and to the fourth set 0.3 M histidine which is equivalent to 4.65% was added.

Total protein determination

The total protein content in all four sets of immunoglobulin samples was determined by Biuret [11] and Kjeldahl method [12,13].

RESULTS AND DISCUSSION

We carried out 18 tests for quality evaluation of therapeutic human normal immunoglobulin (intravenous) based on IP. IP recommends the Kjeldahl method for the determination of total protein content in these plasma-derived products [14]. The total protein content of all four sets of samples was determined by both Kjeldahl [12-14] and Biuret methods [11] for comparison. A total of 10 performances were done on this set of samples by the Biuret method and the averages of all these 10 performances were taken for analysis (Table 2). Similarly, two performances in two replicates were done on this set of samples by the Kjeldahl method and averages of these performances were used for the analysis (Table 2). Since the Kjeldahl method is IP recommended method, therefore number of performances were less than the Biuret method.

In sample set-1, both methods (Biuret and Kjeldahl) showed similar results except for batch no. C1 and C2. Kjeldahl method reported higher protein in batch no. C1 and C2 than Biuret method. In sample set-2 (added with 0.3 M glycine), the Biuret method did not show any significant effect in protein content due to the addition of glycine but the Kjeldahl method showed higher protein content upon addition of glycine. Similarly, in sample set-3 (added with 0.3 M arginine), Biuret method did not show any significant change in protein content due to the addition of arginine but the Kjeldahl method detected higher protein content. However, in sample set-4 (added with 0.3 M histidine), both methods (Biuret and Kjeldahl) reported higher protein content but the protein content reported by the Kjeldahl method was much higher than the protein content reported by the Biuret method (Table 1).

IP is followed for the quality testing of batches of therapeutic human normal immunoglobulin to be marketed in India. The protein content in these batches should be a minimum of 90% and a maximum of 110% of their stated label claim (i.e., 4.5% to 5.5% for 5% label claim and 9% to 11% for 10% label claim) and this criterion is same in European as well as in British Pharmacopoeia. These pharmacopeias also recommend the Kjeldahl method for the determination of total protein in these products. The Kjeldahl method estimates the total nitrogen present in a sample which is multiplied by a factor of 6.25 (proteins contain 16% nitrogen) to calculate total protein [15]. In neat samples (sample set-1),

Table 1: List of human normal immunoglobulin samples

Serial number	Sample name (%)	Manufacturer	Batch number
1	Human normal immunoglobulin for intravenous use (5)	A	A1
2	Human normal immunoglobulin for intravenous use (5)	A	A2
3	Human normal immunoglobulin for intravenous use (5)	B	B1
4	Human normal immunoglobulin for intravenous use (5)	B	B2
5	Human normal immunoglobulin for intravenous use (10)	C	C1
6	Human normal immunoglobulin for intravenous use (10)	C	C2
7	Human normal immunoglobulin for intravenous use (5)	D	D1
8	Human normal immunoglobulin for intravenous use (5)	D	D2
9	Human normal immunoglobulin for intravenous use (5)	E	E1
10	Human normal immunoglobulin for intravenous use (5)	E	E2

Table 2: Protein (in percentage) determined by Biuret and Kjeldahl method in all four sets of samples

Batch number	Set-1		Set-2		Set-3		Set-4	
	Neat		+0.3 M glycine		+0.3 M arginine		+0.3 M histidine	
	Biuret	Kjeldahl	Biuret	Kjeldahl	Biuret	Kjeldahl	Biuret	Kjeldahl
A1	4.59	4.92	4.76	7.09	4.78	13.78	5.77	11.48
A2	4.65	4.94	4.68	7.48	4.72	14.13	5.97	11.80
B1	4.87	4.81	4.81	7.52	4.91	13.98	6.04	12.11
B2	4.83	4.87	4.96	7.66	4.94	12.78	6.23	12.12
C1	10.24	16.06	9.86	15.69	10.14	23.34	13.56	22.98
C2	10.15	13.60	10.03	15.16	10.13	20.74	13.56	21.56
D1	4.98	5.04	4.82	6.74	5.01	13.43	5.74	12.02
D2	4.82	4.91	4.91	7.02	5.03	13.14	5.87	12.13
E1	5.07	4.76	5.01	7.15	5.03	13.46	6.12	11.73
E1	5.17	4.87	5.01	7.46	5.04	13.95	6.31	13.09

protein content obtained by both methods (Biuret and Kjeldahl) was similar except for batch no. C1 and C2 and it was higher by the Kjeldahl method because it was noticed that the manufacturer had added 0.3 M glycine in these batches. Furthermore, the Biuret method did not show any significant effect on protein content in sample set-2 due to the addition of glycine externally, but the Kjeldahl method showed higher protein content upon the addition of glycine. The 0.3 M glycine which is equivalent to 2.25% of glycine in a sample should therefore show additional 2.25% protein content in set-2 samples by the Kjeldahl method. Similarly, 0.3 M arginine in sample set-3 and 0.3 M histidine in sample set-4 should report an additional 5.22% and 4.65% protein, respectively, by the Kjeldahl method. However, the amount of protein contributed does not correspond to the quantity of amino acid added to the samples (Fig. 1).

The biuret method did not correlate with the Kjeldahl method in samples containing additional glycine or arginine or histidine because it does not detect amino acids having less than two peptide bonds [14]. However, histidine is detected by the Biuret method up to some extent (Fig. 2).

Since the Kjeldahl method is based on the estimation of total nitrogen, it also quantitates the nitrogen which may not be part of the product and may be present in free form as an amino acid stabilizer in the therapeutic immunoglobulin solution. Therefore, it may be said that the addition of amino acids such as glycine, arginine, and histidine effects the total protein content of therapeutic immunoglobulin if determined by the Kjeldahl method. However, if the Biuret method is used, an insignificant effect is reported in the case of glycine and arginine, but

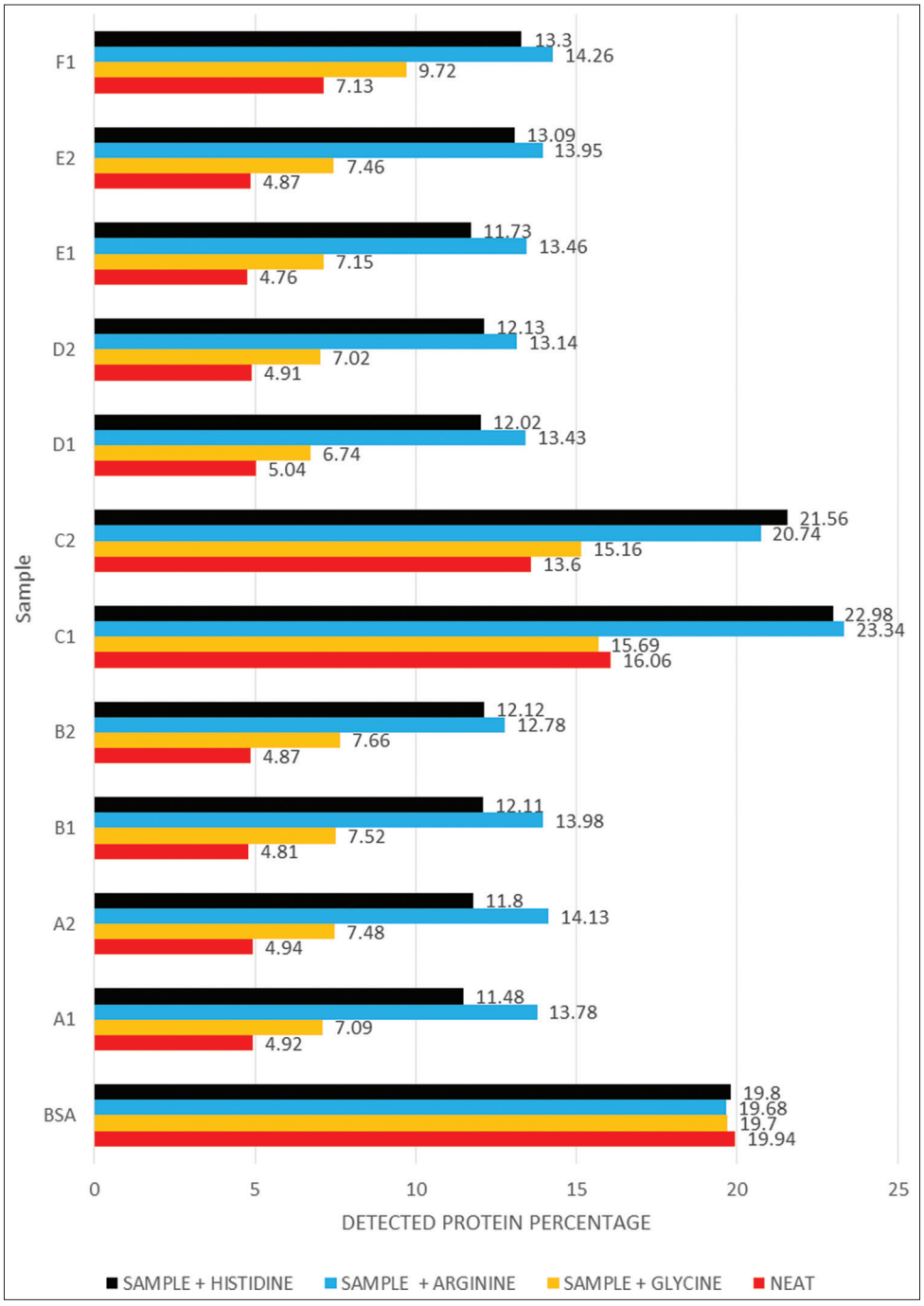

Fig. 1: Protein content determined by Kjeldahl method in sample (Neat), Sample+Glycine, Sample+Arginine, Sample+Histidine, Internal control-BSA

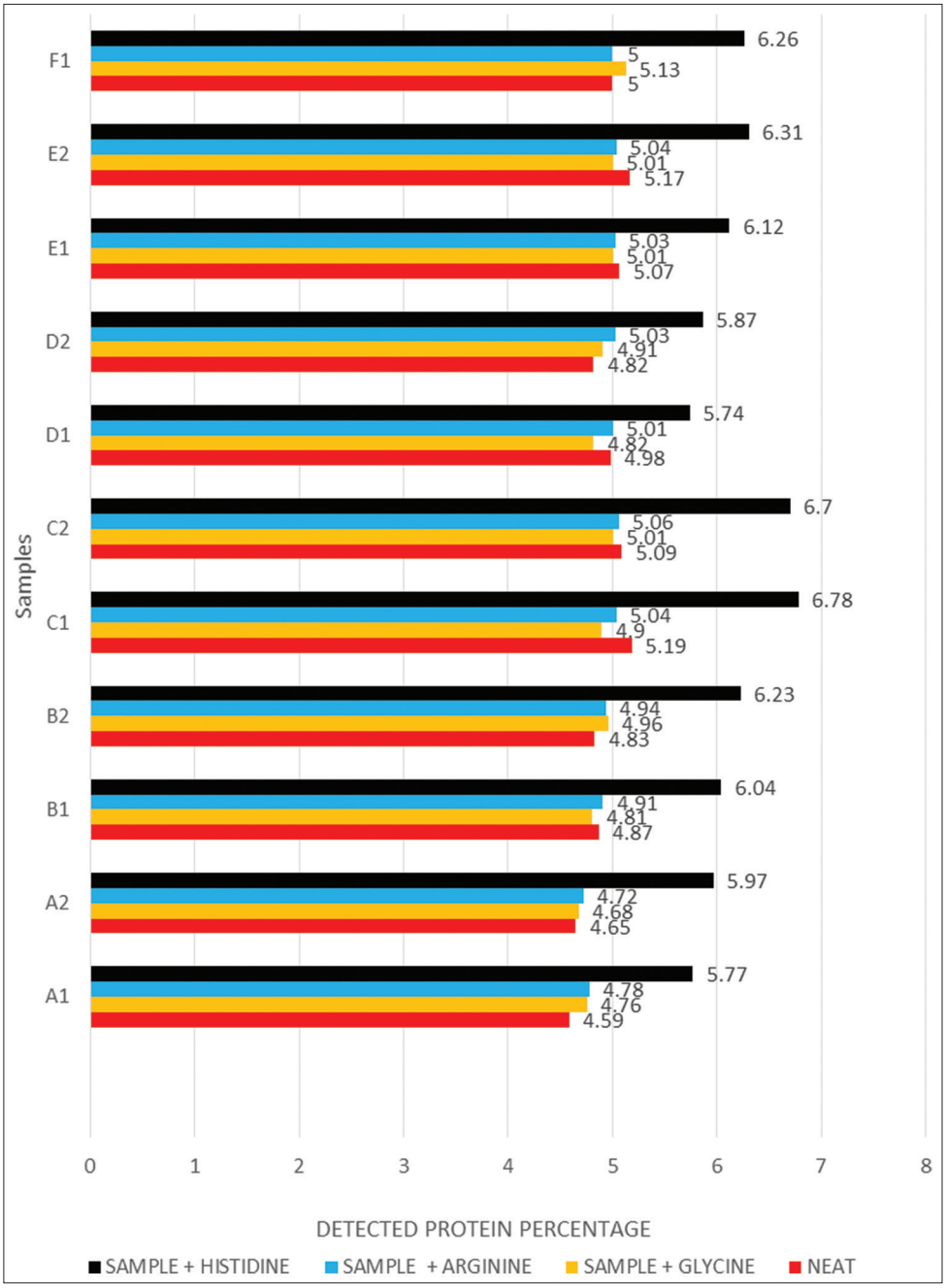

Fig. 2: Protein content determined by Biuret method in sample (Neat), Sample+Glycine, Sample+Arginine, Sample+Histidine

in the case of histidine, the Biuret method may also show significant variations. Most of the manufacturers of plasma-derived products have already moved to nonamino acid stabilizers. In view of this study, it is recommended that all manufacturers must avoid amino acids as a stabilizer in compliance with quality testing monograph of therapeutic human normal immunoglobulin given in IP, EP, and BP. If it is not implemented, the addition of amino acid increases the total protein content in the therapeutic immunoglobulin batches and which may lead to out-of-specification results by the Kjeldahl method which are otherwise would be within specifications. On the other hand, it may be advised that (1) the Biuret method may be used for such products or (2) blank-containing concentrations equivalent to amino acid stabilizers may be used in the Kjeldahl method. The Biuret method does not detect amino acids having less than two peptide bonds but it detects histidine partially therefore, the Biuret method may fail to show consistency in the results in many cases. In the case of use of a blank containing a concentration equivalent to the amino acid stabilizer, the results do not correspond to the quantity of added amino acid. Therefore, as per our experience, the use of such blank does not produce satisfactory results. In conclusion, therapeutic immunoglobulin-containing amino acid stabilizer should have different specifications in terms of protein content.

CONCLUSION

The manufacturers of therapeutic immunoglobulin should use nonnitrogenous stabilizer because the compendial method for estimation of total protein is based on the determination of total nitrogen. The free amino acids added as stabilizer will be reported in the total protein content in the therapeutic immunoglobulin samples and results may vary if the manufacturer has not mentioned these free amino acids as part of the total protein content.

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CONFLICTS OF INTEREST

There are no conflicts of interest by the authors.

AUTHORS' CONTRIBUTION

MKR: Conceptualized the study and wrote the original manuscript. MP and AS: Performed the experiments. TC and AK: Analyzed the data. MK: Supervised and reviewed the manuscript. All authors have read and approved the manuscript.

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