

Scientific Notes

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Agarose as an Alternative Supporting Medium for Protein Composition Determination in Therapeutic Immunoglobulins

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SUMMARY

For therapeutical human immunoglobulins the European Pharmacopoeia requires the determination of the protein composition by zone electrophoresis. The current compendial method employs cellulose acetate gel strips as supporting medium for the electrophoresis. In the present study the use of agarose gels as an alternative is investigated.

The agarose supported method proved to be highly accurate with a relative deviation of $\leq 1.57\%$ and showed adequate specificity since all serum protein fractions are well resolved from the gammaglobulin fraction. With relative standard deviations of 0.3 % for the repeatability and 1.2 % for the intermediate precision the method proved to be sufficiently precise. Good linearity was demonstrated by a linear correlation coefficient (r^2) of 0.995. The limit of quantification was determined to be 1.0 % and the working range for the method was set at 1 % to 100 %.

Thus agarose gel supported zone electrophoresis can be implemented as an alternative method for the current compendial test where cellulose acetate gels are used.

KEYWORDS

Human immunoglobulin, protein composition, zone electrophoresis, agarose gels.

1. INTRODUCTION

Human therapeutical immunoglobulins are derived from human plasma by separating the immunoglobulins from other plasma proteins resulting in an almost pure gammaglobulin fraction. The requirements to the quality of human immunoglobulin preparations are laid down in the European Pharmacopoeia monographs on Human Normal Immunoglobulin [1] and Human Normal Immunoglobulin for Intravenous Administration [2]. One major aspect of quality is the purity which is assessed by the determination of protein composition. The test suggested by the above mentioned monographs is zone electrophoresis using strips of suitable cellulose acetate as the supporting medium. For the purpose of routine analysis a modification of this method using agarose strips as a support is much more convenient because of the shorter analysis time (Electrophoresis time: 25 min compared to 35 min) and the higher throughput of samples (10 samples compared to 4 samples per gel). In the following study the use of agarose was compared to the compendial method in order to assess the possibility to implement agarose gels as alternative supporting medium for the determination of protein composition.

2. MATERIALS AND METHODS

2.1. Compendial method

The compendial method was used as a reference method exactly following the instructions of the Ph. Eur. monograph for human immunoglobulins for intravenous administration [2].

2.2. Agarose supported method

The agarose supported method was performed by using a commercially available serum protein electrophoresis kit according to the manufacturer's instructions (Paragon Serum Protein Electrophoresis Kit, P/N 655900 from

Beckman Coulter Inc., Fullerton CA, USA). Prior to loading onto the agarose gel, samples are diluted with the supplied barbital buffer to 10 g/L.

2.3. Samples and Reference material

Various therapeutical human immunoglobulins for intravenous administration (IGIV) and therapeutical human albumins and mixtures thereof were used as samples. All of these therapeutics are currently licensed in the EU.

Human Immunoglobulin For Electrophoresis BRP Batch 02 was used as a reference preparation. This reference preparation is supplied by the European Department for the Quality of Medicines (EDQM) and consists of a lyophilised human immunoglobulin preparation with a purity of 75.7-80.9% gammaglobulin. In the following this reference preparation is abbreviated as IG-BRP.

A commercially available serum protein standard for clinical chemistry (Precinorm[®] Protein from Roche Diagnostics GmbH, Mannheim, Germany) was used to determine the specificity of the method. It is a lyophilised serum preparation containing all serum protein fractions in specified amounts.

SPE-01 is an in-house quality control standard which was derived from a licensed human immunoglobulin spiked with a significant amount of human albumin as artificial impurity. The preparation is lyophilised and was reconstituted daily before use.

2.4. Test conception, statistical analyses, and acceptance criteria

2.4.1. Validation strategy

According to the ICH guideline Validation of Analytical Methods / Definitions and Terminology [3] the determination of protein composition of immunoglobulins is considered to

be a quantitative test for impurities, requiring the validation of accuracy, specificity, repeatability, intermediate precision, limit of quantitation, linearity, and range.

2.4.2. Accuracy

The accuracy was determined by analysing the above mentioned biological reference preparation IG-BRP. The reference preparation was analysed 9 times and the resulting data were compared to raw data obtained in our laboratory during a collaborative study of EDQM [4] to determine the gammaglobulin content of this reference preparation. In this collaborative study the original compendial method was used for the determination of purity.

The evaluation of the raw data was performed using an equivalence procedure based on the Shuirmann test procedure [5]. In a first step the confidence limits of the two-sided 95 %-Confidence interval of the difference of the means ($CI_{diff,U}$ and $CI_{diff,L}$) were calculated by the formula:

$$CI_{diff,L} = (\bar{X}_1 - \bar{X}_2) - S_{diff} \times t_{(df,5\%)}$$

and

$$CI_{diff,U} = (\bar{X}_1 - \bar{X}_2) + S_{diff} \times t_{(df,5\%)}$$

with:

$\bar{X}_1 - \bar{X}_2$: difference of the means of method 1 and 2

df : degrees of freedom ($n_1 + n_2 - 2$)

$t_{(df,5\%)}$: critical value of the t-distribution

S_{diff} : standard error of the difference of the means

$$S_{diff} = \sqrt{\frac{\sum_{j=1}^{n_1} (X_{1,j} - \bar{X}_1)^2 + \sum_{j=1}^{n_2} (X_{2,j} - \bar{X}_2)^2}{n_1 + n_2 - 2}} \times \left(\frac{1}{n_1} + \frac{1}{n_2} \right)$$

These limits were then compared to predefined acceptance limits. The acceptance limits were derived from the EDQM acceptance criteria of the reference preparation for gammaglobulins which is 75.7-80.9 % corresponding to a range of 5.4 %. Thus the lower acceptance limit ($L_{acc,L}$) was set to -2.7 % and the upper limit ($L_{acc,U}$) was set to +2.7 % resulting in the same range of 5.4 % around a difference of the means of 0. The methods are considered equivalent if $CI_{diff,L} \geq L_{acc,L}$ and $CI_{diff,U} \leq L_{acc,U}$.

2.5. Specificity

The specificity of an electrophoretic test method largely depends on the difference of the electrophoretic mobility of the possible analytes and matrix compounds. In immunoglobulin preparations all serum protein fractions may occur. Thus the specificity of the method can easily be demonstrated by running a commercially available serum protein control used in clinical chemistry (Precinorm® Protein). All serum protein fractions must be resolved and detected.

Due to the manufacturing and formulation of immunoglobulin preparations no interfering substances are to be expected. Since excipients like carbohydrates, glycine, or sodium chloride are not stained by the protein staining procedure, they do not interfere with the quantification of the proteins.

2.6. Precision

2.6.1. Repeatability

To determine the repeatability, the in-house quality control standard SPE-01 was analysed nine times on the same day. Experimental quantification data were used to determine mean, standard deviation, and relative standard deviation of the gammaglobulin fraction.

2.6.2. Intermediate Precision

The SPE-01 in-house quality control standard was used for the determination of the intermediate precision. 6 gels were run by 2 different operators on 6 different days using 2 different gel lots. Experimental quantification data were used to determine mean, standard deviation, and relative standard deviation of the gammaglobulin fraction.

2.7. Linearity

For the investigation of the linearity a licensed 5 % human albumin solution was spiked with increasing amounts of a 5 % human immunoglobulin solution (5 % IGIV) in a way that the total protein concentration remained at 50 g/L. After quantification the relative gammaglobulin content measured was plotted against the applied amount of gammaglobulin. For the evaluation of the linearity the correlation coefficient, the y-intercept and the slope of the regression line was calculated and displayed within figure 1. The acceptance limit for the correlation coefficient was set to more than 0.98.

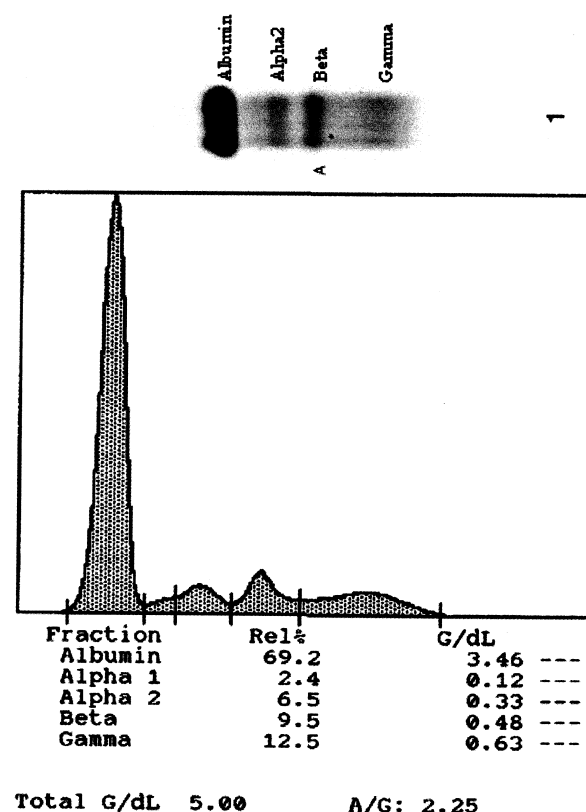


Figure 1 – Specificity of serum protein electrophoresis using an agarose gel as a support

2.8. Limit of Quantification (LOQ)

For the investigation of the LOQ an immunoglobulin preparation with an impurity of 1 % was created by the addition of a defined amount of 5 % human albumin to a 5 % IGIV.

3. RESULTS

3.1. Accuracy

The raw data of the accuracy study are presented in Table 1. All values were within the acceptance limits of the reference preparation and had a similar standard deviation.

Statistical analysis by the above mentioned equivalence test resulted in a lower confidence limit of the difference ($CI_{diff,L}$) of 0.86, which is well above the acceptance limit of -2.7, and an upper confidence limit of the difference ($CI_{diff,U}$) of 1.61, which is well under the upper acceptance limit of +2.7. Both equivalence criteria ($CI_{diff,L} \geq L_{acc,L}$ and $CI_{diff,U} \leq L_{acc,U}$)

were met. Thus the new method is considered equivalent to the reference method.

To get a value for the relative deviation of the accuracy (in per cent) of the new method compared to the reference method the following equation was used:

$$\left| \frac{(\bar{X}_r - \bar{X}_{EP})}{\bar{X}_{EP}} \right| \times 100 = \left| \frac{(76.97 - 78.20)}{78.20} \right| \times 100 = 1.57\%$$

with:

\bar{X}_r : mean of the determination using the agarose supported method

\bar{X}_{EP} : mean of the determinations using the compendial method

3.2. Specificity

Figure 1 illustrates that all human serum fractions were well resolved electrophoretically. Quantification showed that each protein fraction occurred in amounts which were well within the specified ranges (Table 2).

3.3. Precision

3.3.1. Repeatability

The determination of the repeatability revealed consistent results with a low relative standard deviation of 0.3 %. The mean of 9 determinations was 93.8 % with a range from 93.5 % to 94.2 %.

3.3.2. Intermediate Precision

The results for the determination of the intermediate precision are summarised in Table 3. The data showed a high precision with a relative standard deviation of 1.2 %.

3.3.3. Evaluation

Both the repeatability and the intermediate precision of the agarose supported method for the determination of purity of immunoglobulins were found to be adequate.

Table 1 – Summary data on the determination of the accuracy

| Determination number | Y-Globulin-Content (%) | |
|--|--|--------------------------------|
| | Compendial method (Day 1 of coll. Study) | Agarose supported method (SPE) |
| 1 | 77.8 | 76.6 |
| 2 | 77.7 | 77.1 |
| 3 | 77.9 | 77.2 |
| 4 | 78.4 | 76.7 |
| 5 | 78.5 | 77.0 |
| 6 | 79.0 | 76.7 |
| 7 | 78.2 | 77.3 |
| 8 | 78.5 | 76.2 |
| 9 | 77.8 | 77.9 |
| Mean | 78.20 | 76.97 |
| Standard deviation | 0.44 | 0.49 |
| Lower Limit of Confidence Interval ($CI_{diff,L}$) | | 0.86 |
| Lower Acceptance Limit ($L_{acc,L}$) | | -2.7 |
| Upper limit of Confidence Interval ($CI_{diff,U}$) | | 1.61 |
| Upper Acceptance Limit ($L_{acc,U}$) | | +2.7 |

Table 2 – Specificity: amounts of serum protein fractions in a commercially available control standard

| Serum fraction | Range (%) | Observed values (%) |
|---------------------|-----------|---------------------|
| Albumin | 60.4-73.6 | 69.2 |
| α 1-Globulin | 1.6-3.4 | 2.4 |
| α 2-Globulin | 5.1-8.7 | 6.5 |
| β -Globulin | 6.9-11.1 | 9.5 |
| γ -Globulin | 11.0-18.2 | 12.5 |

Table 3 – Summary data on the determination of intermediate precision

| Gel number | Operator | Gel Lot | Date (2003) | Gammaglobulin (%) |
|---------------------------------|----------|---------|-------------|-------------------|
| 1 | 1 | M210091 | 13.05 | 93.6 |
| 2 | 1 | M210091 | 14.05 | 93.2 |
| 3 | 1 | M210091 | 15.05 | 92.9 |
| 4 | 1 | M210091 | 20.05 | 93.2 |
| 5 | 2 | M210090 | 21.05 | 93.0 |
| 6 | 1 | M210090 | 05.06 | 95.7 |
| Mean | | | | 93.6 |
| Standard Deviation | | | | 1.1 |
| Relative Standard Deviation [%] | | | | 1.2 |

3.4. Linearity

The purity of immunoglobulins are tested by the evaluation of relative peak areas in the densitograms. For the investigation of the linearity a licensed 5 % human albumin solution was spiked with increasing amounts of a 5 % human immunoglobulin solution (5 % IGIV) in a way that the total protein concentration remained at 5 %. After quantification, the relative gammaglobulin content measured was plotted against the applied amount of gammaglobulin (Figure 2). For evaluation of the linearity the correlation coefficient, the y-intercept and the slope of the regression line was calculated and displayed within Figure 2. The correlation coefficient was found to be better than 0.99, indicating acceptable linearity of the test method.

3.5. Limit of Quantification (LOQ)

In a review of all immunoglobulin preparations currently licensed in Germany it was found that the most restrictive specification limit for the purity was not less than 98 % immunoglobulin. This means that the impurity content must not exceed 2 %. Thus a limit of quantification of 1 % would be sufficient for the evaluation of the purity of all currently licensed immunoglobulins.

For the investigation of the LOQ an immunoglobulin preparation was spiked with human albumin to give an impurity of 1 %. The mean of 9 determinations was 1.0 % with a range from 0.9-1.0 % with regard to the albumin content. The standard deviation was found to be 0.1 % with regard to the albumin content indicating that 1 % is an acceptable LOQ.

3.6. Range

From the results of the studies of the linearity and the determination of the LOQ a range from 1 % to the maximum of 100 % was derived.

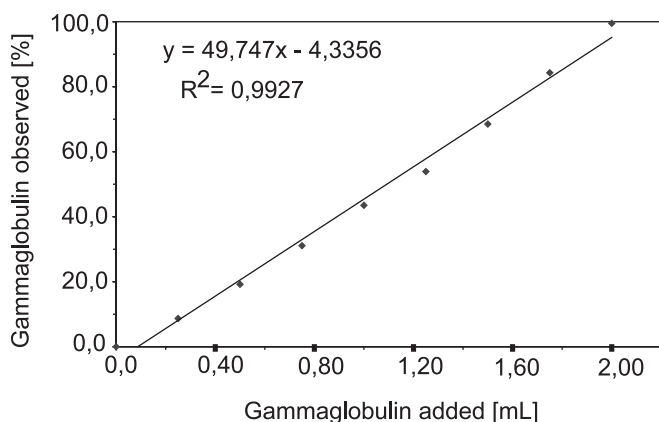


Figure 2 – Linearity of serum protein electrophoresis using an agarose gel as supporting medium

4. DISCUSSION

In the European Pharmacopoeia the determination of the protein composition by zone electrophoresis is required for all human immunoglobulins (IGIM and IGIV). The test method described in the relevant monographs [1,2] is based on the use of cellulose acetate gel strips as supporting medium. The test is derived from routine procedures applied in clinical chemistry to determine the relative amounts of serum proteins. In clinical laboratories, however, agarose is now widely distributed as an alternative supporting medium for the serum protein electrophoresis. In the present study it was shown that the determination of protein composition using agarose gels as a support is equivalent to the compendial method with respect to the accuracy and specificity. Regarding precision, linearity, limit of detection, and range the method proved to be adequate for the evaluation of purity as an important quality parameter for therapeutical immunoglobulin preparations. The main advantages of agarose as a support are the relatively easy handling of the gels, the shorter analysis times and the higher sample throughput possible with those gels.

In our opinion agarose gels are exceptionally suited

for the determination of protein composition by zone electrophoresis. Considering the more convenient and easier handling we think that the agarose supported electrophoresis should be discussed as an alternative to the currently used method and we suggest the implementation of the described method into the immunoglobulin monographs. For analysis of the protein composition of human albumin solutions a similar method is used exploiting cellulose acetate gel instead of agarose. Though not investigated here, we expect, that for these preparations agarose gels can also be used successfully as alternative supporting medium.

5. ABBREVIATIONS

BRP: Biological Reference Preparation; CI: Confidence interval; CV: Coefficient of variation; EDQM: European directorate for the Quality of Medicines; IG-BRP: Human immunoglobulin for electrophoresis BRP Batch 02; IGIV: Human immunoglobulin for intravenous administration; RSD: Relative standard deviation; SD: Standard deviation.

6. REFERENCES

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