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Laser Nephelometric Measurement of Seven Serum Proteins Compared with Radial Immunodiffusion

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Summary: Quantification of immunoglobulins IgG, IgA, IgM, C_3 complement, siderophilin, α_2-macroglobulin and haptoglobin in serum by a Behring laser nephelometer coupled with a data processing apparatus was compared with values obtained by the radial immunodiffusion method of *Mancini*. The precision was not improved by the use of the laser nephelometer as compared to *Mancini* radial immunodiffusion, but within run precision was better than that among runs for both methods. The analytical recovery studies for laser nephelometry showed a close concordance between the theoretical and measured values for all proteins. The two techniques were also compared by means of correlation studies.

Lasernephelometrische Bestimmung von sieben Serumproteinen im Vergleich zur radialen Immunodiffusion

Zusammenfassung: Die Konzentrationen der Immunoglobuline IgG, IgA und IgM, der C_3 Komplement-Komponente, von Siderophilin, α_2-Makroglobulin und Haptoglobulin wurden mit Hilfe eines an einen Computer angeschlossenen Behring-Lasernephelometers bestimmt und mit den Ergebnissen der radialen Immundiffusion nach *Mancini* verglichen. Die lasernephelometrische Messung verbessert die Präzision nicht. In beiden Verfahren ist jedoch die Präzision in der Serie besser als die zwischen den Serien. Die Wiederfindungsteste beweisen, daß für alle lasernephelometrischen Bestimmungen eine sehr gute Übereinstimmung zwischen den erwarteten und den gemessenen Werten besteht. Die beiden Verfahren wurden auch durch Korrelationsteste geprüft.

Introduction

In quantifying serum proteins, laser nephelometry is becoming more and more routinely used instead of radial immunodiffusion. This is due to the fact that the latter is slow and laborious, and cannot be performed automatically, whereas laser nephelometry allows easy automatic handling of sera, so that large numbers of specimens can be analysed at relatively low cost with satisfactory statistical precision. From this standpoint, *Alexander* (1), in a quite recent paper, compares the two methods for quantifying IgG, IgA, IgM, C_3 and C_4, and provides a list of references on the subject.

The present study compares the laser nephelometry quantification of the serum proteins IgG, IgA, IgM, C_3 complement, siderophilin, α_2-macroglobulin and haptoglobin with values obtained by the radial immunodiffusion method of *Mancini* (2).

Materials and Methods

Instrumentation

Laser nephelometrie analyses were made with a Behring Laser Nephelometer. The data processing apparatus was a 9815A Hewlett-Packard, and the dilutor was a Hamilton digital dilutor.

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All three instruments were supplied by Hoechst-Behring (F-75008 Paris).

Reagents

Hoechst-Behring supplied Tri-partigen plates for IgG, IgA and IgM, and M-partigen plates for C_3, siderophilin, α_2-macroglobulin and haptoglobin. Hoechst-Behring radial immunodiffusion standards were also used, except for the C_3 standard, which was bought from Hyland (Laboratoires Travenol, Plaisir, France). Laser nephelometric analyses were performed with laser standard serum as a compound reference, and nephelometric laser-grade antisera from Behring. However, the laser standard serum was retitrated by radial immunodiffusion on C_3 M-partigen plates, and compared with the Hyland complement C_3 reference serum. Consequently, it was possible to compare, on the basis of a common reference, C_3 values obtained by the two methods.

Procedures

Radial immunodiffusion was carried out according to the method of *Mancini*. The measurements of the diameters of the precipitin rings were made by means of a Desaga Measuring Projector Multiskop (C. Desage GmbH, D-6900 Heidelberg 1).

Laser nephelometry was performed according to the Behring method, strictly following the manufacturer's recommendations listed in the table of instructions for the use of the Behring Nephelometer. For IgG, it should be pointed out that the 10 µl serum sample was used, so that the final dilution was 1 : 101. The incubation time for IgM was 45 min, whereas that for all the other proteins tested was 30 min. The reading time chosen was 15 s.

Specimens

The blood samples were collected from patients (Hôpital du Bocage, Dijon). However, turbid and grossly lipaemic sera were discarded; in addition, sera containing monoclonal immunoglobulin detected by immunoelectrophoresis were eliminated. Since the study was carried out over a 3-week period, all serum samples were preserved with 1 g/l sodium azide and kept at 4 °C.

Statistical methods

The study of precision was made through "within run" and "between runs" sample analyses. For both methods, low-content and high-content samples were examined and the precision expressed in terms of the coefficient of variation (CV %). The sample sizes for the "within run" and "between runs" studies were 10 and 20, respectively, but the pooled serum for radial immunodiffusion was in most cases different from that used for laser nephelometry. This could explain differences observed for some proteins between the two methods.

Laser nephelometric recovery studies were performed as follows. For each protein, a low-content pooled sample as well as a high-content pooled sample were prepared. Each of these was analysed seven to ten times by laser nephelometry. The two means were taken as reference points from which a scale of four intermediate theoretical equally spaced values was constructed by mixing the low and high sera in 4 : 1, 3 : 2, 2 : 3 and 1 : 4 proportions. Each of the four intermediate mixtures was analyzed three times by laser nephelometry. This procedure, already utilized by *Adlercreutz* et al. (3), was chosen for the determination of recovery, because the pure proteins are not commercially available. Two linear regression analyses were carried out on each of the seven sets of data : one according to the general linear regression model, and one on the model through the origin. The null hypothesis H_0 : intercept $\alpha = 0$ versus the alternative hypothesis H_1 : intercept $\alpha \neq 0$ was tested by F. Also, the coefficient of determination, r^2 (4), was given on the basis of the ANOVA performed on the chosen model. The final linear regression equation was chosen by testing whether the true slope β was equal to, or different from, 1.

The two quantifying methods were compared by linear regression analyses of laser nephelometry (y) on radial immunodiffusion (x), the latter being considered as the reference standard, and the significance of the intercept was tested. In each ANOVA, the number of ordered pairs was 120, except for the comparison of siderophilin quantification, when only 100 samples were used. The data were collected from routinely tested patients. Contrary to the recovery tests, both variables were random, so that the correlation coefficient could be rightfully used as a measure of the closeness of association of the two variates. Furthermore, a 95% confidence interval for each true correlation coefficient, ϱ, was calculated. Also, prediction equations are given for laser nephelometry on radial immunodiffusion. Finally, the equation of the orthogonal regression line is provided for each protein (5).

Results

Table 1 presents the precision studies. Analytical recovery studies for laser nephelometry and the comparison between the two quantifying methods are shown in tables 2 and 3, respectively.

Precision

Coefficients of variation are meaningful in comparing samples on the bases of their respective variabilities, only when both mean and variance are known in each sample. The comparison between two samples on the basis of their CV % is especially easy when the means are approximately equal. In such a case, a difference in CV % can be fully attributed to a difference in standard deviation, and therefore in variability. This is why it is important that "within run" and "between runs" trials for a given protein be performed on the same serum pool. However, since laser nephelometry and radial immunodiffusion quantifications for a given protein were not always made on the same pool of sera, horizontal comparisons in table 1 are somewhat more hazardous than those made vertically.

Analytical Recovery

Analytical recovery studies for laser nephelometry are summarized in table 2 following the outline presented in the section on Statistical Methods.

Comparison between methods

For each protein, table 3 presents the results gathered in accordance with the design proposed in the section on Statistical Methods.

Tab. 1. Precision study of the trials

	Radial immunodiffusion				Laser nephelometry			
	Low-content		High-content		Low-content		High-content	
	Mean (g/l)	CV (%)	Mean (g/l)	CV (%)	Mean (g/l)	CV (%)	Mean (g/l)	CV (%)
Within run (n = 10)								
IgG	7.50	5.5	16.67	3.0	8.95	3.3	18.60	4.5
IgA	0.84	5.9	3.13	4.4	0.87	4.5	3.53	2.3
IgM	0.81	2.6	2.35	1.8	0.51	21.6	2.43	5.9
C_3	1.55	5.7	1.85	5.9	1.57	7.1	2.34	7.6
Siderophilin	2.12	3.3	4.02	2.5	1.56	2.9	3.44	4.0
α_2-Macroglobulin	2.89	10.1	3.63	2.4	2.56	5.2	2.88	3.6
Haptaglobin	1.88	2.8	4.30	4.9	1.16	2.6	3.04	3.7
Between runs (n = 20)								
IgG	8.35	6.7	16.47	4.2	9.02	8.9	17.49	10.3
IgA	0.80	7.6	3.07	7.3	0.86	6.4	3.61	8.8
IgM	0.80	11.3	2.42	6.8	0.72	29.0	2.54	14.5
C_3	1.46	7.0	1.75	6.9	1.57	9.4	2.41	9.2
Siderophilin	1.85	9.6	3.85	9.3	1.68	15.3	3.53	14.4
α_2-Macroglobulin	2.73	11.9	3.74	6.2	2.57	12.5	3.21	14.2
Haptoglobin	1.86	5.9	4.14	15.9	1.16	2.5	3.04	6.0

Tab. 2. Analytical recovery studies for Laser Nephelometry

Proteins	Range investigated (g/l)	Significance F test for $H_0 : \alpha = 0$ *vs* $H_1 : \alpha \neq 0$	Chosen linear equation	Coefficient of determination r^2	Significance t test for $H_0 : \beta = 1$ *vs* $H_1 : \beta \neq 1$	Final linear regression equation
IgG	8.95–18.60	0.04 ns	$\hat{y}_i = 1.0026\ x_i$	0.9970	0.26 ns	$\hat{y}_i = x_i$
IgA	0.87– 3.54	1.26 ns	$\hat{y}_i = 0.9841\ x_i$	0.9989	2.69	$\hat{y}_i = 0.9841\ x_i$
IgM	0.51– 2.43	0.04 ns	$\hat{y}_i = 0.9900\ x_i$	0.9957	0.85 ns	$\hat{y}_i = x_i$
C_3	1.18– 2.94	0.07 ns	$\hat{y}_i = 0.9916\ x_i$	0.9986	1.20 ns	$\hat{y}_i = x_i$
Siderophilin	1.31– 2.47	0.99 ns	$\hat{y} = 0.9882\ x_i$	0.9982	1.49 ns	$\hat{y}_i = x_i$
α_2-Macroglobulin	2.32– 3.48	0.08 ns	$\hat{y} = 0.9803\ x_i$	0.9980	2.41	$\hat{y}_i = 0.9803\ x_i$
Haptoglobin	1.30– 3.46	0.10 ns	$\hat{y}_i = 1.0200\ x_i$	0.9981	2.32	$\hat{y}_i = 1.0200\ x_i$

y_i = Value of the protein measured by laser nephelometry
x_i = Protein content theoretical value
ns: Not significant; unmarked t values are significant at P (type I error) < 0.05

Tab. 3. Comparison of radial immunodiffusion (RID) and laser nephelometry (LN) by regression and correlation studies

Proteins	Range investigated (g/l)		Linear regression of y on x	r	$I_{0.95}$ for ϱ	Orthogonal regression line
	y(LN)	x(RID)				
IgG	3.20–26.84	3.20–23.20	$\hat{y}_i = 1.1616\ x_i$	0.9568	0.9385–0.9697	$\hat{y}_i = -0.4537 + 1.2060\ x_i$
IgA	0.54– 6.56	0.80– 5.12	$\hat{y}_i = -0.3516 + 1.2957\ x_i$	0.9736	0.9623–0.9816	$\hat{y}_i = -0.4582 + 1.3409\ x_i$
IgM	0.32– 2.72	0.26– 2.38	$\hat{y} = 1.0853\ x_i$	0.9770	0.9671–0.9839	$\hat{y}_i = -0.0276 + 1.1093\ x_i$
C_3	0.71– 2.62	0.78– 2.48	$\hat{y}_i = 1.0763\ x_i$	0.9614	0.9450–0.9730	$\hat{y}_i = -0.0682 + 1.1242\ x_i$
Siderophilin	0.54– 2.80	0.60– 2.70	$\hat{y}_i = 0.9954\ x_i$	0.9911	0.9868–0.9940	$\hat{y}_i = -0.0321 + 1.0117\ x_i$
α_2-Macroglobulin	0.40– 3.48	0.96– 3.40	$\hat{y}_i = -0.6112 + 1.0660\ x_i$	0.9650	0.9501–0.9755	$\hat{y}_i = -0.6953 + 1.1085\ x_i$
Haptoglobin	0.53– 5.72	0.60– 5.64	$\hat{y}_i = 0.9192\ x_i$	0.9472	0.9250–0.9630	$\hat{y}_i = -0.0933 + 0.9589\ x_i$

Discussion

From the CV % (tab. 1), it can be said that for both laser nephelometry and radial immunodiffusion, the within run precision is somewhat better than that among runs. Except for a few values, our CV % data seem to be in fairly good agreement with *Alexander*'s results (1). It should be kept in mind, however, that we present quantifications made over a wide range

of values for both methods, whereas *Alexander* made analyses on normal sera. *Deaton* at al. (6) quantified IgG, IgA, IgM and C_3, with a Hyland Laser Nephelometer, for sera ranging from low up to high pathological values. Their CV % values are somewhat better than ours, but the sample sizes used by these authors were 20 and 55 for "within run" and "between runs", respectively. This may explain part of the discrepancies. It should be pointed out that our low-content laser nephelometric values for IgM yield abnormally high CV % for both "within run" and "between runs" assays (21.6 and 29.0).

Buffone & Lewis (7) studied a pediatric population for C_3 in order to establish immunochemical reference limits. Their "within run" (n = 20) and "between runs" (n = 26) CV % were 3.9 and 4.5, respectively (Hyland Laser Nephelometer). These values are about half as high as those we obtained with the Behring apparatus; however, our "within run" sample size was only 10. *Buffone*'s radial immunodiffusion "between runs" CV % was equal to 4.9 while ours is about 7.0 for both low and high content sera. *Walsh* et al. (8) carried out a comparative study of "PDQ"-Hyland laser nephelometer, Behring laser nephelometer and radial immunodiffusion in measuring IgG. For a sample size equal to 10 on a pool of normal sera, the "within run" means (and CV %) were 8.19 (2.07), 11.25 (3.78) and 12.55 (2.23) for Hyland, Behring and radial immunodiffusion, respectively, and comparable to our values (Behring and radial immunodiffusion). This also roughly holds for the "between runs" values obtained on 20 days. As for the additional three proteins, i.e., siderophilin, α_2-macroglobulin and haptoglobin, the "within run" CV % appear to be fairly satisfactory, whereas the "between runs" CV % are rather high for both Behring laser nephelometry and radial immunodiffusion.

To summarize the discussion on precision, table 1 shows that the use of a Behring Laser Nephelometer does not seem to improve the precision of the results as compared to *Mancini*'s method. However, as pointed out in the Introduction, laser nephelometry offers the possibility of an automated analytical method which can be operated under satisfactory conditions.

According to our study, the proportion of recovery (tab. 2) is practically equal to 1.00 for all seven proteins. This proportion is equal to the slope of the regression line in column 4, obtained after analysis of variance, or to that in column 7, chosen on the basis of an additional t test for $H_0 : \beta = 1$. The practically admissible figures are 1.00 for IgG, IgM, C_3 and siderophilin, 0.98 for IgA and α_2-macroglobulin, and 1.02 (slight "over-recovery") for haptoglobin. Since, in addition, the r^2 values are all very close to 1, it can be concluded that there is a close agreement between the theoretical and laser nephelometric measured quantities.

The seven regression equations for the comparison of both methods are presented in table 3; except for siderophilin, all the slopes are statistically different from 1. Considering the regression of laser nephelometry on the radial immunodiffusion standard, all the slopes that are different from 1 are larger than 1, with the exception of haptoglobin for which b = 0.9192. Apart from IgA and α_2-macroglobulin, all intercepts were found to be statistically equal to zero.

As compared to the results of *Alexander* (1), who studied Behring laser nephelometry versus radial immunodiffusion – the latter being performed by the technique of *Fahey & McKelvey* (9) – for quantifying IgG, IgA, IgM and C_3, our regression equations are somewhat better. This is mostly due to the fact that our equations, except that for IgA, do not have intercepts:

– for IgG, our relationship, $y = 1.1616x$, yields laser nephelometric values that are 16% higher than the corresponding radial immunodiffusion values; *Alexander*'s equation, $y = 1.427 + 1.054x$, provides laser nephelometric values that are about 50%, 17% and 11% higher than radial immunodiffusion values for very low- (3.2 g/l), medium- (12.0 g/l) and high-content (26.8 g/l) sera, respectively;

– for IgM, the comparison is as follows: our equation, $y = 1.0853\,x$, yields laser nephelometric values that are overestimated by 8.5% as compared to radial immunodiffusion, whereas *Alexander*'s $y = 0.017 + 1.114x$ provides 17-, 12- and 12%-overstimated values for 0.32, 1.60 and 2.70 g/l sera, respectively;

– for C_3, our overestimation, by $y = 1.0763x$, is 7.6%, while those of *Alexander*, by $y = 0.236 + 0.866x$, are 34%, 13% and 2% for 0.5, 0.9 and 1.5 g/l sera, respectively;

– for IgA, the situation is different since our equation also has an intercept; on the basis of $y = -0.3516 + 1.2957x$, our laser nephelometric/radial immunodiffusion differential percentages are – 35%, + 14% and + 24% as compared to those of *Alexander* ($y = 0.118 + 1.153x$), + 37%, + 21% and + 17%, for 0.54, 2.20 and 6.5 g/l sera, respectively.

It should be noted, however, that *Alexander*'s equations were established on the basis of normal sera, whereas ours were derived from a wide range of values: low, medium and high. This means that in reality, the comparison is fair only for medium values. Even so, our laser nephelometric/radial immunodiffusion relationships are better than those proposed by *Alexander*. This is most probably due to the fact that the *Mancini* technique is more accurate than that of *Fahey & McKelvey*.

Daigneuault & Lemieux (10) carried out the Behring laser nephelometry/*Mancini* radial immundiffusion comparison for IgG, IgA and IgM. Their results are excellent and our data suffer from the comparison, especially for IgA where our equation is not satisfactory.

Schmitz-Huebner et al. (11), comparing Behring laser nephelometry to *Mancini*'s radial immundiffusion for α_2-macroglobulin, propose a regression equation that is far better than ours, except for high-content sera. The slope of our equation is better than theirs, as it is closer to 1, but our total relationship is distorted by a heavy negative intercept.

Given the results obtained by studies on precision, analytical recovery and comparison of methods, it can be stated that on the whole, nephelometric quantification of a wide spectrum of proteins can replace the heavy radial immunodiffusion technique in laboratories involved in the analysis of large numbers of serum specimens.

Finally, it is fair to mention that other authors have already published similar studies. However, our paper is different for the following reasons:

– the authors mentioned have reported results for only a few proteins, or just a single protein (7, 8);

– the proteins analysed by the different authors were not all quantified by means of the same Laser Nephelometer (1, 10, 11: Behring; 6, 7: Hyland; 8: both Behring and Hyland, but for one protein only);

– radial immunodiffusion was performed by two different techniques (1, 8: *Fahey & McKelvey*; 6, 10, 11: *Mancini*; 7: both techniques, but for one protein only);

– none of the authors cited has reported on a comparative study of haptoglobin quantification by the two methods.

In contrast, we present compared quantifications of seven proteins, all carried out by the same technique of radial immunodiffusion (*Mancini*) on the one hand, and by the same Laser Nephelometer (Behring) on the other. Furthermore, all our results for each protein involve a wide range of low, medium and high values.

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