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Determination of CSF Proteins by a Simple and Rapid Immunonephelometric Method

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Summary. A simple, fast and reliable immunonephelometric (I-NEPH) method has been worked out for the determination of CSF proteins. A good quality and low price I-NEPH apparatus was used. The results were obtained from regression lines, constructed from various parts of the calibration curve, calculated by using a simple pocket calculator. Ultrasound was found to be a simple and effective cleaning technique for nephelometry. The method was used for determining concentrations of albumin, IgG, IgA, IgM and the ratio of the light kappa and lambda chains in CSF and serum from 15 control cases, 11 MS patients, and three patients with plasma cell dyscrasias. The I-NEPH method was found to be a valuable complement to high separation techniques including especially isoelectric focusing used for CSF examinations, e.g., for evaluating influence of serum protein composition and degree of barrier damage. An increased kappa-lambda ratio was observed in some of the patients with MS in accordance with previous investigations but was normal in four of the nine MS cases where the ratio was examined.

Key words: CSF and serum proteins – Immunonephelometry – Multiple sclerosis.

Zusammenfassung. Ein einfaches, schnelles und zuverlässiges immunonephelometrisches (I-NEPH) Verfahren ist für die Bestimmung der CSF-Proteine ausgearbeitet worden. Ein billiger Nephelometer von guter Qualität wurde gebraucht. Die Ergebnisse wurden aus Regressionslinien erhalten, die mittels eines einfachen Taschenrechners aus verschiedenen Teilen der Standardkurve ausgerechnet wurden. Ultraschall erwies sich als ein einfaches und wirksames Reinigungsverfahren für Nephelometerröhrchen. Das Verfahren wurde für die Konzentrationsbestimmungen von Albumin, IgG, IgA, IgM und des kappa : lambda-Verhältnisses der Immunoglobulinen von Liquor und Serum verwendet. Es wurden Kontroll-Patienten (15), MS-Patienten (11) und Patienten mit Plasmazell-Dyskrasien (3) untersucht. Das I-NEPH-Verfahren erwies sich als ein wertvolles Komplement zu den Feintrennungsmethoden,

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insbesondere für die isoelektrische Fokussierung des Liquors. Es erlaubt Rückschlüsse auf den Einfluß der Serumproteinzusammensetzung und das Ausmaß der Schädigung der Blut-Gehirn-Schranke. Ein erhöhtes kappa : lambda-Verhältnis wurde in einigen der MS-Patienten beobachtet. Bei vier von den neun untersuchten Patienten war das Verhältnis aber normal.

Introduction

Examinations of the CSF proteins are very important in many neurological diseases. High-separation methods for proteins have been used at our laboratory for several years, including isoelectric focusing (IEF), and later isotachophoresis (ITP). The laboratory has a wide experience with thin-layer IEF of CSF and serum proteins, performed on more than 4000 patients with mainly neurological disorders or normal cases. A computed densitometric evaluation of the IEF findings is now being developed. However, a rapid and reliable method for quantitative determination of the main proteins in CSF and serum seems to be very valuable, e.g. when visual evaluation of IEF findings is found doubtful regarding the influence of serum proteins, especially the gammaglobulins, on the CSF protein patterns, and the degree of barrier damage. Modern nephelometric equipments obviously meet these demands, i.e. quantitative electrophoresis can be substituted by immunonephelometric (I-NEPH) analysis. It seems astonishing that the I-NEPH, applied to CSF proteins in some reports [6, 12, 14], in spite of the simplicity, high sensitivity and reproducibility performed with modern equipment, has obviously neither been used more extensively, as judged from reports in the last few years, nor been introduced generally as a nice clinical routine examination for CSF proteins.

One probable reason is the earlier difficulties to get sufficient amounts of high affinity, monospecific antibodies at reasonable cost. Moreover, improved equipment and conditions for the immunoreaction are now available [13].

The aim of the present study was to examine the fitness for use of a modern, not very expensive (less than 5000 dollars) I-NEPH apparatus for CSF protein determinations, as judged from analyses of albumin (alb.), immunoglobulins (IgG, IgA, IgM) and kappa : lambda light chain ratios. CSF and serum from control subjects and patients with MS were studied.

Material

The CSF and serum samples were collected from patients treated in the Department of Neurology, Karolinska Hospital, Stockholm. The patients consisted of 15 subjects where repeated examinations had not revealed any reasonable protein abnormalities in the CSF, 11 patients with verified MS and three patients with plasma cell dyscrasias.

The CSF was obtained by lumbar puncture in a standardized way (fasting, forenoon), and serum samples were collected at the same time. The samples were stored no more than a few days at +4° C, and were otherwise frozen at −23° C, before testing. Determination of the total protein concentration, IEF, and, in some cases, quantitative electrophoresis were performed as previously described [16].

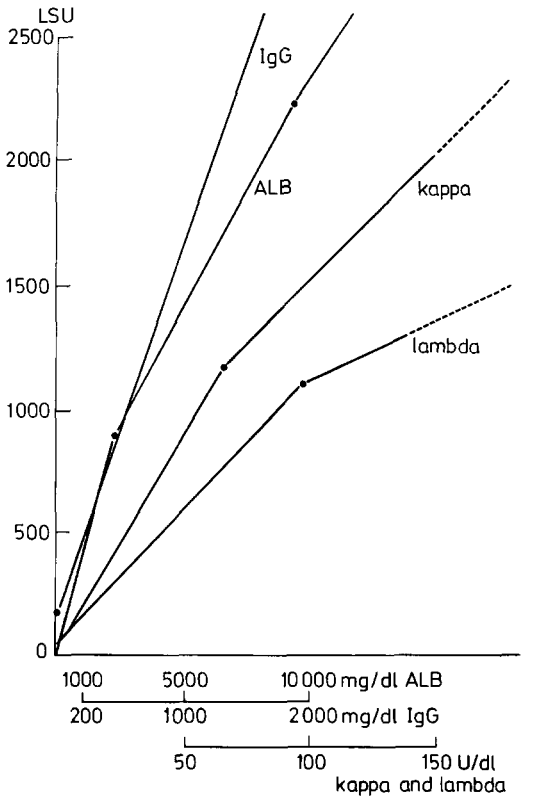

Fig. 1. Regression lines used to determine concentrations of albumin, IgG and immunoglobulin light chains kappa and lambda

Method

The I-NEPH was performed with a Kallestad LSA 290 apparatus, measuring dispersed light at 90° to incident light of 610 nm wavelength. Physiological saline was used unfiltered with no adverse results; other reagents were filtered through Millipore membrane filters at a pore size of 0.25 nm. All dilutions and liquid transfers were performed with Finnipette adjustable micropipettes. Mixing and immunoreaction were performed in borosilicate glass tubes which were sufficiently cleaned with bichromatic sulphuric acid for 16 h, or—even better—by ultrasound in a detergent bath for 15 min. Both procedures included five pre-, and five post-washings in distilled water. F-test revealed variance ratios in favor of ultrasound cleaning ($P<0.001$) according to a test where 50 tubes were treated and tested with diluent by either method during 3 consecutive days.

The immunoreagents were from Kallestad: goat antiserum to human serum albumin lot N 209 H 012, human IgG lot N 204 L 052, and LSA 290 diluent 10 X, giving a working solution 4% in polyethylene glycol molar weight approximately 6000, which was found necessary in order to shorten analysis time [9]. Rabbit immunoglobulins against human Ig-kappa chains 10-9K2 lot 097, Ig-lambda chains 10-9L2 lot 068A, were obtained from Dako Immunoglobulins Ltd Denmark. All antibody solutions were diluted 1:80 instead of 1:100, the latter ratio recommended by Kallestad. The reason was that higher concentration gave calibration curves which could be treated more like straight lines.

The standard solutions for determination of standard curves for albumin and IgG were included in the Kallestad kits. Dilutions of standard human serum ORDT 08/03 batch 1003U, Behringwerke, GFR, were used as standards in kappa and lambda chain determinations.

Serum samples and calibration solutions were generally diluted 1:100 (20 µl to 2 ml) with physiological saline; CSF samples were used undiluted. Sample volumes were 25 µl for IgG, kappa and lambda. However, for albumin 30 µl of a further dilution 1:10 was used (it is serum 1:1000, CSF 1:10), because the recommended 3 µl:s of the samples was difficult to pipet reproducibly.

All calculations were performed on a Texas Instruments-57 (TI-57) pocket calculator, programmed with the three program sets: linear regression; one straight line, and crossing coordinates to another; and Student's *t*-test for two means.

When measuring the light scattering units (LSU:s), the measuring button was depressed shortly 3 to 5 times, and the majority figure was noted. When the measuring button was depressed longer than 5 s, the values were irreproducibly decreasing. The immunocomplex formation was allowed to proceed for 1 h, although 45—50 min was sufficient. Reaction times longer than 3 h gave irreproducible results.

LSU:s from the standards were plotted versus mg/dl or Behring Units/dl (for Ig light chains). Most standard curves were approximated to a straight line with a correlation coefficient exceeding 0.96. On evaluating the test LSU:s, two or three lines were used, one each for the upper and lower part, and very often an intermediate one for the very straight calibration sequence ($r > 0.99$), where most samples were collected (Fig. 1). When readings were out of calibration range, they were rejected and redetermined after a further dilution, if possible.

The reliability of the Kallestad dilutions was checked against Kallestad Quantitrol, included in their kit and also against Behring standard serum, both adequately diluted. These tests were performed on albumin, transferrin, IgG, IgA and IgM. Three curves were drawn for each protein. The relevant regression lines were graphically found to be practically overlapping, confirming the reliability of the method used.

Results

The I-NEPH results are summarized in Table 1. The standard deviation values from the calculator were multiplied with n/(n-1), thus the s-values for the Student's *t*-test are presented. The deviation % is (standard deviation × 100)/

Table 1a. Results from normal subjects

	Mean value	Standard deviation	Deviation %	*s*	*n*
Albumin (alb.) CSF	17.81 mg/dl	3.131	17.58	3.355	15
Albumin serum	4864	827.2	17.01	886.3	15
IgG CSF	1.544	0.2651	17.18	0.2840	15
IgG serum	957.9	141.2	14.74	151.3	15
Kappa/lambda CSF	0.9402 U/dl	0.3924	41.74	0.4316	11
Kappa/lambda serum	1.081	0.3796	35.13	0.4142	12
IgG/alb. CSF	0.0905	0.01474	16.29	0.01579	15
IgG/alb. serum	0.2043	0.03410	16.69	0.03654	15
Laurell's ratio	0.4612	0.07569	16.41	0.08110	15

Table 1b. Results from patients with MS

	Mean value	Standard deviation	Deviation %	s	n
Alb. CSF	23.47 mg/dl	8.819	37.57	9.707	11
Alb. serum	4197	811.1	19.3	892.0	11
IgG CSF	5.744	2.528	44.01	2.809	10
IgG serum	979.2	444.0	45.34	448.3	11
Kappa/lambda CSF	2.430	1.567	64.48	1.763	9
Kappa/lambda serum	1.069	0.2311	21.62	0.2568	10
IgG/alb. CSF	0.2328	0.0730	31.36	0.08110	10
IgG/alb. serum	0.2211	0.0743	33.59	0.08170	11
Laurell's ratio	1.100	0.4070	37.02	0.4522	10

Table 2. Student's t-test of MS compared to the controls

	t	n	Significance
Kappa/lambda CSF	2.720	11 + 9	$0.01 < P^{+} < 0.02$
Kappa/lambda serum	0.07700	10 + 12	$P > 0.2$
Albumin CSF	3.355	11 + 15	$0.001 < P^{++} < 0.01$
IgG CSF	5.809	10 + 15	$0.001 > P^{+++}$
IgG serum	0.1598	11 + 15	$0.2 < P < 0.5$
IgG/albumin CSF	6.676	10 + 15	$0.001 > P^{+++}$
IgG/albumin serum	0.6976	10 + 15	$0.1 < P < 0.5$
Laurell's ratio	5.3980	10 + 15	$0.001 > P^{+++}$

Table 3. Kappa/lambda ratio in CSF for MS_1 and MS_2 compared with the controls ("N")

	Mean value	s	n	t	Significance
MS_1	4.26	1.295	5	9.290	$0.001 > P^{+++}$
MS_2	0.89	0.1762	4	0.2146	$0.2 < P < 0.5$
("N")	0.94	0.4316	11		

mean value. The calculated significance of the above values is given in Table 2.

The MS cases were divided into two groups: five with statistically significant elevated kappa/lambda ratios in CSF (MS_1), and four with normal ratios (MS_2). The figures are presented in Table 3.

In Table 1a all mean values agreed well with figures reported for other methods [1, 2, 10, 11]. However, the mean value (1.5 mg/dl) for CSF-IgG was somewhat lower in our cases than the figures (2—3 mg/dl) generally given in the literature.

Table 4. Plasma cell dyscrasias compared to the controls

	Mean value mg/dl	s	n	t ($N_{tot} = 18$)	Significance
Albumin CSF	57.57	29.06	3	5.223	$0.001 \gg P^{+++}$
Albumin serum	3631	1794	3	1.217	$0.2 > P > 0.5$
IgG CSF	57.28	39.31	3	6.336	$0.001 \gg P^{+++}$
IgG serum	1533	1030	3	1.9900	$0.05 > P > 0.1$
IgG/alb. CSF	0.9423	0.3908	3	9.594	$0.001 \gg P^{+++}$
IgG/alb. serum	0.5772	0.7251	3	2.224	$0.02 > P^{+} > 0.05$
Laurell's ratio	3.321	6.797	3	1.873	$0.05 > P > 0.1$

Table 5. Complete results of the MS patients

Case		Albumin mg/dl	IgG mg/dl	Kappa U/dl	Lambda U/dl	Kappa: lambda	IgG: ALB	Laurell's ratio
1	CSF	44.03	12.01	177.6	61.63	2.882	0.2728	0.6374
	serum	5187	2220	107.1	152.7	0.701	0.4280	
2	CSF	27.83	5.935	73.76	21.81	3.383	0.2133	1.451
	serum	5352	786.7	156.5	116.3	1.346	0.1470	
3	CSF	25.28	—	—	—	—		
	serum	4333	769.5	—	—	—	0.1776	
4	CSF	12.92	5.743	81.84	15.42	5.308	0.1788	1.000
	serum	3938	704.3	77.30	53.41	1.447	0.1788	
5	CSF	20.12	4.471	0.3782	0.4851	0.7796	0.2223	1.176
	serum	4719	1459	161.6	183.4	0.8813	0.1891	
6	CSF	25.48	5.428	0.5128	0.4608	1.113	0.2130	1.161
	serum	4909	900.3	86.37	72.73	1.188	0.1834	
7	CSF	33.23	4.397	0.3622	0.4514	0.8024	0.1323	0.6176
	serum	3306	708.3	52.88	69.55	0.7603	0.2142	
8	CSF	18.71	3.081	0.2858	0.3280	0.8713	0.1647	0.7304
	serum	3144	709.0	57.08	60.85	0.9380	0.2255	
9	CSF	16.81	5.822	0.01320	—	—	0.3463	1.198
	serum	3239	936.3	89.80	76.93	1.167	0.2891	
10	CSF	21.09	7.877	1.127	0.2681	4.204	0.3735	2.051
	serum	4789	871.8	86.86	77.21	1.125	0.1821	
11	CSF	12.69	2.676	0.3249	0.1288	2.523	0.2108	0.9723
	serum	3255	705.6	61.11	53.84	1.135	0.2168	

Increased Laurell's ratio (= IgG/alb.$_{CSF}$: IgG/alb.$_{serum}$) [5] was used as measure of intrathecal IgG synthesis.

In Table 2, all serum values differed insignificantly between MS and the controls. The CSF values, as well as Laurell's ratio, differed significantly between the two groups.

In Table 4 three patients with plasma cell dyscrasias are compared with normal cases. IgG and albumin are significantly (on the 0.1% level) elevated in CSF, as are IgG/albumin. IgG/albumin in serum was elevated, but less significantly (on the 5% level). Laurell's ratio was insignificantly elevated.

IgA and IgM were studied in a limited number of patients: IgA was determined in serum of five control subjects, ranging from 51—281 mg/dl, mean value was 125 mg/dl. In four MS patients the serum values were 188, 356, 177 and 90 mg/dl. IgA in CSF was above the lower calibration limit in three of five control subjects, range: 0.08—0.12 mg/dl, mean value 0.11 mg/dl. IgA values in CSF from MS patients were elevated: 0.416, 0.629, 0.127 and 0.335 mg/dl mean value 0.386.

All IgM determinations in CSF were far below calibration standard (0.114 mg/dl), except one MS case (0.34 mg/dl). In serum from five control subjects, IgM ranges from 2.5—88 mg/dl, mean value 50.31 mg/dl were found. In serum from three MS patients the IgM values were 361, 248, and 89.5 mg/dl.

Discussion

Savory et al. [14] and Ritchie et al. [12] found that autoanalyzer based I-NEPH determinations of CSF-IgG and different CSF proteins respectively, correlated well with various agarose gel immunoprecipitation methods. The major traditional gel immunoprecipitation methods, electroimmunoassay (EIA) and radial immunoprecipitation, have a major disadvantage compared to I-NEPH in the time required (8—24 h), and the laborious discrete treatment steps. However, more antibody are usually needed (2—4 times) in using I-NEPH than in EIA.

Schliep and Felgenhauer [15] compared EIA and the recently developed laser I-NEPH in CSF investigations, the latter replacing the former. The laser I-NEPH requires special nephelometer grade antibody solutions to perform optimally, and is furthermore expensive. Since our laboratory has no autoanalyzer equipment, and a limited frequency of samples are examined, manual I-NEPH equipment, easy to operate, with developmental possibilities, including printing computer with programs and dispensing unit, at moderate cost, was our choice.

The I-NEPH is characterized by its easy handling, reliability with good reproducibility and especially by the short time in which the results are obtained. Thus, the 1-NEPH results are tabulated in 4 h after the test is initiated on for instance 200 samples.

According to Lizana [8] the minimum detection limit in I-NEPH is as low as 20—40 ng per nephelometer tube.

The LSU values from 60—120 min after mixing were practically identical. After 3 h the higher values were decreasing (precipitation?) while the lower levels were slowly increasing, or unchanged. Initially, antigen titers were obtained manually from a calibration curve which was found impractical: slow, inaccurate

and laborius. Contrary to this procedure, the use of a simple pocket calculator (TI-57) programmable only to 50 steps, costing less than 200 dollars, was found very effective.

The increased kappa : lambda ratio described by Link and Zettervall [7] and confirmed by several others [3, 4], in CSF from MS patients was observed in five of the nine MS cases examined. However, it is interesting, that in four cases, e.g. about half of the MS cases, normal kappa : lambda ratios were found. This finding is now further studied, as well as a better I-NEPH procedure for determination of IgA and IgM, which may hopefully also be suitable for IgD and IgE, recently determined in CSF by a radioimmunoassay technique by Nerenberg et al. [11].

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