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Reference ranges for serum protein fractions as determined by capillary zone electrophoresis

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SUMMARY

We performed capillary zone electrophoresis (CZE) on serum specimens from 129 healthy Japanese volunteers, ages 7 to 90 years. Our goal was to estimate the reference ranges for the five major serum protein fractions: albumin, α_1 -globulin, α_2 -globulin, β -globulin, γ -globulin and for the five minor fractions: in which prealbumin, hemopexin, α_1 -acid glycoprotein, α_1 -antitrypsin or transferrin predominated. Results by CZE for the above ten fractions correlated well ($p < 0.001$) with the assays by CAE or immunonephelometry for the same protein fraction. Owing to the Gaussian distribution of the data, we can state the reference ranges as the mean ± 2 SD. For α_1 -acid glycoprotein, α_1 -antitrypsin and transferrin fractions, reference values were estimated only in terms of percent composition. Our reference values (concentrations in parenthesis) for CZE were albumin, 52.7 to 67.1% (3.9–5.2 g/dl); α_1 -globulin, 3.6 to 6.8% (0.27–0.52 g/dl); α_2 -globulin, 4.8 to 9.0% (0.36–0.69 g/dl); β -globulin, 8.2 to 13.5% (0.59–1.06 g/dl); and γ -globulin, 10.7 to 22.5% (0.73–1.81 g/dl); prealbumin, 0.1 to 0.9% (10.7–66.4 mg/dl); hemopexin fraction, 1.1 to 2.7% (76–212 mg/dl); α_1 -acid glycoprotein fraction, 1.3 to 3.4%; α_1 -antitrypsin fraction, 2.0 to 3.8%, and transferrin fraction, 3.7 to 6.1%.

Key words: capillary zone electrophoresis, cellulose acetate electrophoresis, immunonephelometry, method comparison study, reference ranges, serum proteins.

INTRODUCTION

Cellulose acetate electrophoresis (CAE) can separate serum proteins into five bands: albumin, α_1 -globulin, α_2 -globulin, β -globulin, and γ -globulin. Over the past thirty years in Japan, CAE has been the most commonly used procedure in clinical laboratories. Recently, an automated device for the separation of serum proteins based on capillary zone electrophoresis (CZE) was developed by Beckman Coulter Instruments (Brea, CA).¹⁾ With their CZE instrument, automating sampling, electrophoretic migration, and quantitation of the separated proteins are possible. We evaluated the

analytical performance of the Beckman CZE 2000 system and found good results in accuracy, reproducibility, sensitivity, and correlation with CAE for the five primary protein bands.²⁾ CZE also resolves the bands for prealbumin, α_1 -acid glycoprotein, α_1 -antitrypsin, hemopexin, transferrin, and complement fractions.

However, we reported electrophoretic separation in terms of percent composition of each band and have not evaluated the results in terms of absolute concentration from the total quantity of serum protein, since the CZE results in percent composition for albumin were, on average, 10% lower than CAE, and α_1 -globulin and β -globulin were 93% and 15% higher than by CAE,

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Abbreviations: CZE, capillary zone electrophoresis; CAE, cellulose acetate electrophoresis.

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respectively, regardless of the high correlation coefficient between CZE and CAE (>0.8) for each fraction.²⁾ Because absolute concentration of serum proteins should be independent of analytical method, there were errors if the concentrations were different between CZE and CAE. Here, we compared the quantitated values for serum proteins between CZE and CAE or a nephelometric procedure to evaluate whether CZE results could be reported by quantitated values in g/dl. The final objects of this study was to estimate the reference values for serum protein fractions (i. e., prealbumin, α_1 -acid glycoprotein, α_1 -antitrypsin, hemopexin, transferrin, and complement as well as the traditional five fractions) of the Japanese population by gender and age with the use of the Beckman CZE 2000 system.

MATERIALS AND METHODS

Specimens

Blood was collected in plain evacuated tubes from 150 hospitalized patients who had various pathologies. We also collected blood in plain tubes from 49 men ages 7 to 90 y, and from 80 women ages 10 to 83 y, who were in apparent good health and showed no chemical or hematological abnormalities. Of the 150 patients, some had protein abnormalities. We compared CZE and CAE results in 31 patients and also compared the CZE and nephelometry values in 119 patients. The latter individuals who were in apparent good health were used to estimate the reference ranges of the serum protein fractions by CZE. We used the mean ± 2 SD in all cases to estimate the reference ranges.

Electrophoresis

CZE was performed in a Beckman Paragon CZE 2000 system according to the system instructions and manufacturer's reagents.^{1,2)} We used a fused silica capillary of a 20 μ m inner diameter and 20 cm length. The 150 mmol/l, pH 10 borate electrophoresis buffer was also from Beckman. After filling the capillary with buffer, about 60 nl of the 21-fold diluted serum was injected into the capillary, and 9000 volts for 4.3 minutes was applied to the capillary thermostatted at 24°C. The high voltage within the capillary generates an electroosmotic flow and an electrophoretic migration of the charged components in the buffer solution. Absorbances of the effluent were made at 214 nm; the absorbance was proportional to the concentration of peptide bonds. Since it was proposed by this manufacturer that the Paragon CZE can identify prealbumin, α_1 -acid glycoprotein, α_1 -antitrypsin, hemopexin, transferrin and complement (C3 and C4), we confirmed their migration times by adding various pure proteins purchased from Sigma Chemical Co. and Dade Behring (Marburg, Germany). Thus, we defined them as fractions in which prealbumin, α_1 -acid glycoprotein, α_1 -antitrypsin, hemopexin, transferrin and complement predominated,

respectively.

CAE was performed on an Olympus AES 630 system (Olympus Co., Ltd.) according to the system procedure and reagents. We used Sartorius cellulose acetate membranes in a pH 8.6 barbital buffer, ionic strength 0.06, and Ponceau S staining; this method is based on the standard procedure of the Japanese Electrophoresis Society.³⁾

The concentrations of the five principal bands and the minor fractions were calculated from the total protein concentration measured by the biuret method in a Hitachi 7250 analyzer and reagents from Dia-Iatron Co. The serum albumin concentrations were also determined by the bromocresol green (BCG) method in the same instrument and source of reagents. We also assayed the concentrations of prealbumin, α_1 -acid glycoprotein, α_1 -antitrypsin, hemopexin, transferrin, and complement fractions by determining the total protein concentration and percent composition of the serum proteins by CZE. We also measured the protein fractions by immunonephelometric procedures using a BNA nephelometer⁴⁾ (Dade Behring) and an Immage nephelometric instrument⁵⁾ (Beckman Coulter Instruments). All assays were performed within two hours of blood collection.

RESULTS

1. Comparison of CZE and CAE results

The five principal protein bands found in the sera of hospitalized patients were measured by CZE and CAE. The least squares regression equations and other statistics are shown in Table 1. In confirmation of our earlier report,²⁾ the results for albumin by CZE were, on average, 10% lower than CAE, and α_1 -globulin and β -globulin were 95% and 13% higher than by CAE, respectively; $p < 0.001$. When albumin by CZE was compared with that by the BCG method, the results agreed with those by the BCG method: CZE = 1.12 (BCG) - 0.4, $r = 0.953$, Sy. x = 0.2 g/dl ($n = 30$); and the results by CZE were, on average, only 1% higher than those by the BCG method.

2. Comparison with nephelometry

The Paragon CZE system automatically separates serum proteins into the usual five bands; it can also separate prealbumin, α_1 -acid glycoprotein, α_1 -antitrypsin, hemopexin, transferrin, and complement fractions (Fig. 1).

Preliminarily, analytical values of prealbumin, α_1 -acid glycoprotein, α_1 -antitrypsin, transferrin, complement (C3 and C4) were compared between the BNA and Immage system. Since good correlation were obtained for all values from both analyzers: BNA = 0.97 (Image) - 1, $r = 0.964$, Sy. x = 2 mg/dl for prealbumin; BNA = 1.14 (Image) - 2, $r = 0.982$, Sy. x = 4 mg/dl for α_1 -acid glycoprotein; BNA = 0.86 (Image) + 12, $r =$

Table 1. Linear regression statistics.^a

Protein	Number of sera	Method on Y-axis	Method on X-axis	Slope	Y-intercept	Correlation coefficient	Sy. x
Albumin	31	CZE	CAE	1.05	-0.6	0.983	0.1 ^b
α_1 -Globulin	31	CZE	CAE	1.52	0.1	0.877	0.1 ^b
α_2 -Globulin	31	CZE	CAE	1.27	-0.1	0.904	0.1 ^b
β -Globulin	31	CZE	CAE	1.09	-0	0.912	0.1 ^b
γ -Globulin	31	CZE	CAE	1.06	-0	0.992	0.1 ^b
Prealbumin	112	CZE	Nephelometry	1.42	-2	0.850	7 ^c
α_1 -Acid glycoprotein	119	CZE	Nephelometry	0.52	122	0.567	40 ^c
α_1 -Antitrypsin	105	CZE	Nephelometry	1.56	35	0.794	45 ^c
Hemopexin	58	CZE	Nephelometry	0.41	73	0.660	15 ^c
Transferrin	112	CZE	Nephelometry	0.91	148	0.691	50 ^c
Complement 3 (C3)	113	CZE	Nephelometry	1.08	193	0.237	87 ^c
Complement 4 (C4)	113	CZE	Nephelometry	0.60	287	0.055	89 ^c
C3+C4	113	CZE	Nephelometry	0.72	212	0.205	87 ^c

^a, Data from hospitalized patients; CZE, capillary zone electrophoresis; CAE, cellulose acetate electrophoresis; ^b, g/dl; ^c, mg/dl.

Fig. 1. A typical electropherogram of proteins in normal serum by the Paragon 2000 capillary zone electrophoresis (CZE) system.

Peak 1 is probably prealbumin; 2, albumin; 3, consisted mainly of α_1 -acid glycoprotein; 4, consisted mainly of α_1 -antitrypsin; 5, α_2 -globulin; 6, consisted mainly of hemopexin; 7, consisted mainly of transferrin; 8, consisted mainly of complement; and 9, γ -globulin. With the Paragon CZE system, peaks 3 and 4 are indicated as α_1 -globulin, and peaks 6, 7 and 8 are β -globulin.

0.976, Sy. x=5 mg/dl for α_1 -antitrypsin; BNA=0.88 (Immage)+14, $r=0.984$, Sy. x=9 mg/dl for transferrin; BNA=0.87 (Immage)+14, $r=0.969$, Sy. x=5 mg/dl for C3; and BNA=1.32 (Immage)-1, $r=0.990$, Sy. x=1 mg/dl for C4, CZE results were compared with nephelometry data with no distinction between BNA and Immage. However, hemopexin by CZE was exclusively compared with BNA, as Immage did not measure hemopexin.

Thus, the concentrations of the above CZE fractions were calculated from their percent composition and the total protein by the biuret method. Then, analytical values for prealbumin, α_1 -acid glycoprotein, α_1 -antitrypsin, transferrin, and complement (C3 and C4)

fractions were compared to the immunonephelometric method. The regression data are summarized in Table 1. The CZE- and nephelometry-derived concentrations are in Table 1. CZE results for prealbumin, α_1 -acid glycoprotein, α_1 -antitrypsin, hemopexin, and transferrin fractions correlated significantly with the nephelometric results ($p<0.001$), but the CZE results for α_1 -acid glycoprotein, α_1 -antitrypsin, and transferrin fractions were, on average, 31%, 81%, and 53% higher, in this order, than the values found by nephelometry. The results for prealbumin and hemopexin fractions agreed well with those by the nephelometric method (Fig. 2, $p<0.001$). In 112 sera analyzed, five showed no detectable prealbumin fraction by the CZE procedure. We did not find a linear relations between CZE and the nephelometric procedure for C3, C4, and the sum of C3 and C4. All correlation coefficients were about 0.2, even though serum specimens were analyzed by BNA and Immage within two hours after blood was collected.

3. Findings in normal Japanese adults

We determined the serum protein fractions in 129 healthy volunteers by the Paragon CZE 2000 system (See Table 2 and Table 3). We found no statistically significant gender differences for α_1 -globulin, α_2 -globulin, β -globulin, prealbumin, α_1 -acid glycoprotein, α_1 -antitrypsin, hemopexin, and transferrin fractions. Men, ages 20 to 49 y, had significantly higher concentrations of prealbumin and albumin fractions than women of the same age group. Women, ages 30 to 49 y had significantly higher concentrations of γ -globulin than men of the same age group.

Both men and women had their highest levels for β -globulin at ages 30 to 49 y in men or 50 to 69 y in women; for hemopexin, this occurred at ages 30 to 49 y in men. Serum concentrations of α_1 -globulin and α_1 -acid glycoprotein in both men and women tended to be lowest in the younger age range of 7 to 19 y. However, in

Fig. 2. Comparison of CZE results for fractions consisted mainly of prealbumin (left) and hemopexin (right) with those by nephelometry in terms of absolute concentration.
(●) by BNA and (○) by Immage nephelometer analyzers, but regression equations were drawn with no distinction between BNA and Immage.

Table 2. Serum protein fractions by gender and ages in terms of percent composition.

Age, y	Traditional five fractions (%)					Minor fractions (%) consisted mainly of				
	Albumin	α_1 -Globulin	α_2 -Globulin	β -Globulin	γ -Globulin	Prealbumin	α_1 -Acid glycoprotein	α_1 -Antitrypsin	Hemopexin	Transferrin
7-19										
Men (n=11)	61.8±3.1	4.6±0.5 ^d	7.6±1.2	10.2±0.6	15.4±3.1	0.5±0.1	1.8±0.3 ^d	2.8±0.3	2.0±0.5	4.8±0.3
Women (n=9)	62.2±2.6	4.8±0.2 ^d	7.6±0.6	10.1±0.5	15.0±3.0	0.5±0.5	2.0±0.3 ^d	2.8±0.2	1.8±0.3	5.0±0.4
20-29										
Men (n=9)	63.1±4.2 ^{a,c}	5.3±0.5	6.3±1.0	9.9±1.1	14.7±3.5	0.6±0.1	2.4±0.4	2.9±0.3	1.8±0.5	4.4±0.4
Women (n=17)	59.8±3.8	5.1±1.2	6.8±1.1	10.7±1.7	17.1±2.0	0.5±0.2	2.1±0.5	3.0±0.8	1.6±0.4	4.8±0.9
30-49										
Men (n=14)	61.6±3.5	5.5±0.8	6.3±1.2	11.2±1.0 ^c	14.6±2.7	0.7±0.2 ^{a,c}	2.7±0.7	2.8±0.4	2.2±0.3 ^c	4.8±0.5
Women (n=34)	58.8±2.8	5.1±0.7	6.7±0.9	11.0±1.3	18.0±2.2 ^{b,c}	0.4±0.2	2.3±0.4	2.8±0.4	1.9±0.4	5.1±0.6
50-69										
Men (n=12)	58.4±2.8	5.6±0.9	7.2±1.0	10.8±0.8	17.3±3.2	0.6±0.2	2.6±0.6	3.0±0.5	1.9±0.4	4.9±0.5
Women (n=17)	58.4±3.0	5.2±0.5	6.9±1.0	11.8±1.5 ^c	17.3±1.6	0.5±0.2	2.4±0.3	2.8±0.3	1.9±0.4	5.2±0.7
70-90										
Men (n=3)	57.0±8.3	5.2±0.2	7.1±1.1	10.2±0.5	19.9±6.7	0.5±0.1	2.2±0.2	3.0±0.3	1.7±0.2	4.7±0.2
Women (n=3)	60.4±2.3	6.3±1.2	8.3±1.0	11.1±1.5	13.3±1.5	0.6±0.3	2.7±0.9	3.7±0.5	2.0±0.6	5.0±0.5
All ages										
Men (n=49)	60.9±4.1	5.3±0.8	6.9±1.2	10.6±1.0	15.8±3.6	0.6±0.2	2.4±0.6	2.9±0.4	2.0±0.4	4.8±0.4
Women (n=80)	59.3±3.2	5.2±0.8	6.9±1.0	11.0±1.5	17.1±2.4	0.5±0.2	2.3±0.4	2.9±0.5	1.8±0.4	5.1±0.7
Both sexes (n=129)	59.9±3.6	5.2±0.8	6.9±1.1	10.9±1.3	16.6±2.9	0.5±0.2	2.3±0.5	2.9±0.5	1.9±0.4	4.9±0.6
Reference range										
Both sexes	52.7-67.1	3.6-6.8	4.8-9.0	8.2-13.5	10.7-22.5	0.1-0.9	1.3-3.4	2.0-3.8	1.1-2.7	3.7-6.1

^a, Significantly higher in men than women, $p<0.05$; ^b, Significantly higher in women than men, $p<0.001$; ^c, Significantly higher in ages, $p<0.05$; ^d, Significantly lower in ages, $p<0.05$.

women, the highest concentrations for γ -globulin was observed at ages 30 to 49 y; in men, the highest values for prealbumin occurred at ages 30 to 49 y and for albumin at 20 to 29 y. Finally, we estimated the reference values for the serum protein fractions in the Japanese population making no distinction between gender and ages, because the inter-individual variations were greater than the gender- and age-related differences. Because Gaussian distributions of the values were observed for the serum protein fractions, we

estimated the reference ranges using the mean ± 2 SD. We found the reference ranges determined by CZE to be 52.7 to 67.1% for albumin (3.9-5.2 g/dl), 3.6 to 6.8% for α_1 -globulin (0.27-0.52 g/dl), 4.8 to 9.0% for α_2 -globulin (0.36-0.69 g/dl), 8.2 to 13.5% for β -globulin (0.59-1.56 g/dl), 10.7 to 22.5% for γ -globulin (0.73-1.81 g/dl), 0.1 to 0.9% for prealbumin (10.7-66.4 mg/dl), 1.3 to 3.4% for α_1 -acid glycoprotein fraction, 2.0 to 3.8% for α_1 -antitrypsin fraction, 1.1 to 2.7% for hemopexin fraction (76-212 mg/dl), and 3.7 to 6.1% for

Table 3. Serum protein fractions by gender and ages in terms of absolute concentration.

Age, y	Traditional five fractions (g/dl)					Minor fractions (mg/dl) consisted mainly of	
	Albumin	α_1 -Globulin	α_2 -Globulin	β -Globulin	γ -Globulin	Prealbumin	Hemopexin
7-19							
Men (n=11)	4.7±0.3	0.35±0.04 ^d	0.57±0.09	0.77±0.08	1.18±0.29	34.4±8.7	157±45
Women (n=9)	4.6±0.3	0.36±0.02 ^d	0.56±0.05	0.75±0.06	1.12±0.24	35.0±12.7	132±26
20-29							
Men (n=9)	4.9±0.3 ^{a,c}	0.41±0.05	0.49±0.08	0.77±0.10	1.15±0.33	44.2±11.4	139±46
Women (n=17)	4.4±0.3	0.38±0.09	0.51±0.08	0.80±0.15	1.27±0.20	37.7±6.6	120±28
30-49							
Men (n=14)	4.6±0.3	0.41±0.06	0.47±0.09	0.84±0.09 ^c	1.10±0.24	51.5±17.9 ^{a,c}	165±26 ^c
Women (n=34)	4.5±0.3	0.40±0.06	0.52±0.07	0.86±0.13	1.40±0.22 ^{b,c}	32.5±12.8	150±33 ^c
50-69							
Men (n=12)	4.5±0.3	0.43±0.07	0.56±0.07	0.83±0.05	1.34±0.28	45.6±17.6	143±28
Women (n=17)	4.4±0.3	0.40±0.05	0.52±0.08	0.90±0.13 ^c	1.32±0.15	37.1±11.8	143±30
70-90							
Men (n=3)	4.2±0.3	0.39±0.04	0.54±0.13	0.76±0.04	1.15±0.63	39.4±6.1	129±13
Women (n=3)	4.2±0.2	0.44±0.10	0.58±0.09	0.77±0.08	0.92±0.08	39.7±21.0	141±38
All ages							
Men (n=49)	4.6±0.3	0.40±0.06	0.52±0.09	0.81±0.09	1.21±0.32	44.1±15.4	151±36
Women (n=80)	4.5±0.3	0.39±0.06	0.52±0.08	0.84±0.13	1.30±0.23	35.1±11.8	140±32
Both sexes (n=129)	4.5±0.3	0.39±0.06	0.52±0.08	0.83±0.12	1.27±0.27	38.5±13.9	144±34
Reference range							
Both sexes	3.9-5.2	0.27-0.52	0.36-0.69	0.59-1.06	0.73-1.81	10.7-66.4	76-212

^a, Significantly higher in men than women, *p*<0.001 ; ^b, Significantly higher in women than men, *p*<0.001 ; ^c, Significantly higher in ages, *p*<0.05 ; ^d, Significantly lower in ages, *p*<0.05.

transferrin fraction.

DISCUSSION

In a comparing assay results for the five common protein fractions, we found that the results for albumin by CZE were, on average, 10% lower than by CAE ; α_1 -globulin and β -globulin were 95% and 13% higher with CZE as compared with CAE, respectively. This difference is the expected finding, because albumin binds more dye per g of protein than the globulins. This phenomenon does not occur in CZE assays. The only assumption with CZE is that the concentration of peptide residues is directly related to the concentration of the protein. The expected, and our finding, is that albumin gives higher values by CAE and the globulins lower values as compared to CZE. Because the CZE results for albumin agreed well with those of the established BCG method, CZE should give more accurate values for serum albumin concentrations than CAE. The reasons for the higher α_1 -globulin results are probably due to α_1 -acid glycoprotein included in the α_1 -globulin fraction. The higher sialic acid content of α_1 -acid glycoprotein has been reported to interfere with the binding of Ponceau S dye used to quantitate the protein fraction by CAE.^{6,7)} We considered that the CZE analysis for α_1 -globulin was more promising than CAE. Moreover, CZE results for β -globulin were 13% higher than CAE, but the bias (ca. 0.9 g/dl) would have no clinical significance in diagnosing hypo- or hyper-

globulinemia. Therefore, we were able to establish the prospective reference values for five traditional bands by CZE both in terms of percent composition and absolute concentrations. Our reference range, especially α_1 -globulin, was much like those reported by McManamon et al.⁸⁾ and Harper.⁹⁾ McManamon reported the reference value for α_1 -globulin as 0.1 to 0.4 g/dl ; and Harper reported that the relative reference value for α_1 -globulin as to 5.3% by the Tiselius moving boundary electrophoresis in which the electrophoretic patterns are measured optically. Although Tiselius electrophoresis is a classical technique, we dared to cite this relative reference value, because the CZE separates and monitors individual zones in a manner similar to that involved in the Tiselius moving boundary system, and neither method involves dye binding. Recently, Kurosu et al.¹⁰⁾ also analyzed serum proteins by CZE with 50 μ m $\times$ 50 cm untreated fused silica and by CAE ; he reported that the CZE results in patients and normal sera for α_1 -globulin were, on average, 34% and 97% higher than CAE, respectively.

In the comparison of the minor fractions assayed by CZE and nephelometry, results by CZE for α_1 -acid glycoprotein, α_1 -antitrypsin, and transferrin fractions were significantly higher (*p*<0.001) than those obtained by the nephelometric procedure. Because CZE results for prealbumin and hemopexin fraction agreed well with those by the nephelometric method (*p*<0.001), we could estimate reference values for prealbumin and hemopex-

in fraction both in terms of percent composition and the concentration in mg/dl. Overestimation for α_1 -acid glycoprotein, α_1 -antitrypsin, and transferrin fractions by CZE occurs, because other proteins are migrating together and appearing as α_1 - and β -globulin fractions, e. g., α_1 -lipoprotein, α_1 -microglobulin and hormone-binding globulins on α_1 -fraction; and β -lipoprotein, β_2 -microglobulin, properdin and complements other than C3 and C4 migrating with β -globulin fraction. With CZE, we could not separate α_1 -globulin and β -globulin into more than two or three fractions, respectively (See Fig. 1). We therefore estimated reference values for α_1 -acid glycoprotein, α_1 -antitrypsin, and transferrin fractions by CZE only in terms of percent composition. The CZE results for these three protein fractions (α_1 -acid glycoprotein, α_1 -antitrypsin, and transferrin) were significantly correlated with the findings by the nephelometric procedure ($p < 0.001$). Unfortunately, we did not use the CZE results for complement fraction neither in terms of absolute concentration nor in terms of percent composition.

In conclusion, we have good estimates of the reference values for serum protein fractions in the Japanese population by CZE. We present data here for the five principal bands (albumin, α_1 -globulin, α_2 -globulin, β -globulin and γ -globulin) and minor protein fractions (prealbumin and hemopexin) both in terms of percent composition and g/dl (or mg/dl) concentration. For α_1 -acid glycoprotein, α_1 -antitrypsin and transferrin fractions, our data are in terms of percent composition, because the quantitative CZE results for these three proteins were erroneously higher than the results by the nephelometric procedure. However, reference values for the age group under 7 y requires further investigation.

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