FISHERIES AND MARINE SERVICE

Translation Series No. 3883

Comparative investigations on the determination of total protein and albumin in the serum of man, monkey, dog and rat

by A. Metz and A. Schütze

Original title: Vergleichende Untersuchungen zur Bestimmung von Gesamtprotein und Albumin im Serum von Mensch, Affe, Hund und Ratte

From: Z. Klin, Chem. Klin. Biochem. 13(9): 423-426, 1975

Translated by the Translation Bureau (KMF)
Multilingual Services Division
Department of the Secretary of State of Canada

Department of the Environment
Fisheries and Marine Service
Halifax Laboratory
Halifax, N.S.

1976

9 pages typescript

DEPARTMENT OF THE SECRETARY OF STATE
TRANSLATION BUREAU

MULTILINGUAL SERVICES DIVISION

CANADA

SECRÉTARIAT D'ÉTAT
BUREAU DES TRADUCTIONS

DIVISION DES SERVICES MULTILINGUES

TRANSLATED FROM – TRADUCTION DE	INTO – EN
German	English

AUTHOR – AUTEUR

A. Metz and Annerose Schütze

TITLE IN ENGLISH – TITRE ANGLAIS

Comparative investigations on the determination of total protein and albumin in the serum of man, monkey, dog and rat.

TITLE IN FOREIGN LANGUAGE (TRANSLITERATE FOREIGN CHARACTERS)
TITRE EN LANGUE ÉTRANGÈRE (TRANSCRIRE EN CARACTÈRES ROMAINS)

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REFERENCE IN FOREIGN LANGUAGE (NAME OF BOOK OR PUBLICATION) IN FULL. TRANSLITERATE FOREIGN CHARACTERS.
RÉFÉRENCE EN LANGUE ÉTRANGÈRE (NOM DU LIVRE OU PUBLICATION), AU COMPLET, TRANSCRIRE EN CARACTÈRES ROMAINS.

Z. Klin. Chem. Klin. Biochem.

REFERENCE IN ENGLISH – RÉFÉRENCE EN ANGLAIS

Journal of Clinical Chemistry and Clinical Biochemistry

PUBLISHER – ÉDITEUR	DATE OF PUBLICATION / DATE DE PUBLICATION			PAGE NUMBERS IN ORIGINAL / NUMÉROS DES PAGES DANS L'ORIGINAL
not shown	YEAR / ANNÉE	VOLUME	ISSUE NO. / NUMÉRO	423 - 426
PLACE OF PUBLICATION / LIEU DE PUBLICATION				NUMBER OF TYPED PAGES / NOMBRE DE PAGES DACTYLOGRAPHIÉES
not shown	1975	13	9	9

REQUESTING DEPARTMENT / MINISTÈRE-CLIENT: Environment

TRANSLATION BUREAU NO. / NOTRE DOSSIER N°: 1101528

BRANCH OR DIVISION / DIRECTION OU DIVISION: Fisheries & Marine

TRANSLATOR (INITIALS) / TRADUCTEUR (INITIALES): KMF

PERSON REQUESTING / DEMANDÉ PAR: Allan T. Reid

NOV 2 9 1976

YOUR NUMBER / VOTRE DOSSIER N°:

DATE OF REQUEST / DATE DE LA DEMANDE: October 19, 1976

UNEDITED TRANSLATION
For information only
TRADUCTION NON REVISÉE
Information seulement

SOS-200-10-6 (REV. 2/68)
7530-21-029-5333

Z. Klin. Chem. Klin. Biochem. 13 (1975) pp. 423 - 426

Comparative investigations on the determination of total protein and albumin in the serum of man, monkey, dog and rat.

A. Metz and Annerose Schütze
Medical-Biological Research Sandoz AG,
Basle, Switzerland

Summary: In the present study, serum albumin was determined colorimetrically in three animal species and in man with the help of four currently used dye-reagents (protein binding reagents). The results were compared with those obtained from corresponding electrophoretic and biuret determinations, using Versatol as a standard-control throughout. Using 2-(4-hydroxyazobenzene)-benzoic acid and bromocresol purple as reagent for the albumin determinations, species-specific differences from the electrophoretic results were found. No such differences occurred with bromocresol green, except in rats.

There was no significant difference in albumin concentrations between man, monkeys and rats. Total protein concentration was only similar in man and monkeys.

Introduction

Today, two possible methods are available to us for the routine determination of serum albumin, namely electrophoresis and calorimetric techniques. The latter make use of the fact that azo dyes such as 2-(4-hydroxyazobenzene)-benzoic acid, bromocresol-green, bromocresol-purple, methyl orange, methyl red and Spectu AB-2 (1) form albumin-azo dye complexes with albumin in accordance with its binding capacity for these substances; these complexes can then be determined by photometric analysis. Of these dyes, 2-(4-hydroxyazobenzene)-benzoic acid (2-5) has, in the past, enjoyed the widest application as albumin reagent. This may be one of the reasons why its disadvantages, too, are well known: poor correlation with other methods, interferences with bilirubin, fatty acids and drugs such

as salicylates, sulfonamides and Penicillin (5-7), as well as "species-specific differences" (8-9). All these facts were reason for various authors - particularly in recent times - to recommend the bromocresol-green method (1,7,10,11,12) and the determination with bromocresol-purple (13).

As far as we know, the literature does not contain any data on the routine applicability of these latter methods with laboratory animals. It is therefore the aim of the present study to close the existing gap, to test the interchangeable use of animal and human serum for reasons of quality control, to demonstrate species-specific differences and to find the method which is most suitable for the animal laboratory. In the background loom also the problems of using one and the same standard for various species and with multiple analytical techniques. Possible interference with drugs will be pointed out in this study but has not been examined in practical experiments.

A comparative study with various species examined the four best known methods of albumin determination: electrophoresis, the 2-(4-hydroxyazobenzene)-benzoic acid method (with and without the addition of detergents), the bromocresol-green and bromocresol-purple method and the protein determination with the biuret reagent. In the following text we use the abbreviations which are customary in the literature and in routine work in place of the fully spelled out terms: HABA for 2-(4-hydroxyazobenzene)-benzoic acid, BCG for bromocresol-green and BCP for bromocresol- (424)
purple.

Material and Methods

Equipment

Blood centrifuge (MSE); Technicon auto-analyzer system with built-in Eppendorf photometer (single-channel system); microphoresis system (cellogen as support); 4201 W + W recorder with integrator (Dr. Vaudaux, Basle, Switzerland); M4 QII Zeiss Scanner.

Species	Totalprotein	Albumin (g/l)					
	(g/l)	Elektro-phorese	HABA (ohne Detergenz)	HABA with Brij)	HABA W. Levor IV)	Bromkresol-green ⊕	Bromkresol-purple
Rat	64 ± 4	46 ± 3	20 ± 2 ***	14 ± 2 ***	26 ± 3 ***	32 ± 3 ***	11 ± 2 ***
Dog	58 ± 2	31 ± 3	17 ± 2 ***	15 ± 2 ***	31 ± 4 –	32 ± 2 –	8 ± 1 ***
Monkey	74 ± 3	47 ± 4	27 ± 2 ***	37 ± 4 ***	42 ± 2 **	47 ± 3 –	38 ± 4 ***
Man	71 ± 3	44 ± 4	40 ± 3 *	45 ± 3 –	45 ± 3 –	46 ± 5 –	53 ± 7 **

Table 1

Comparison of methods between electrophoresis and photometric albumin determinations with HABA, BCG and BCP reagents in man, monkey, dog and rat (mean values of 5 ♂ and 5 ♀ per species ± standard deviation)

1) HABA (without detergent)
*: Significance of differences between photometric albumin determination and electrophoresis (same species)

⊕: Addition of detergent according to instructions

Reagents

HABA dye (6 mmol/litre) T21-0179-07 (Technicon Geneva); formaldehyde (3999, 370 g/kg[1]); glacial acetic acid (90063); sodium acetate (6267); sodium hydroxide (6498); potassium iodide (5043); potassium-sodium tartrate (8087); copper sulfate (2791) bromocresol-green (8121); bromocresol-purple (3025); Brij-35 (Atlas Chemicals, Essen); methanol (6009); 5,5-diethyl-barbituric acid (Na salt)(6318); Ponceau S (Fluka 81460); barbital (276); Levor IV (Technicon Geneva).

Methods

The total protein was determined with biuret reagent (14), albumin with HABA-(4) (with or without the detergents Brij and Levor IV), with bromocresol-green (12) and with bromocresol-purple reagent (13) with the autoanalyzer according to data in the literature. For the albumin determination the electrophoretic separation of serum albumin was carried out on cellogel at 230 V. For man, monkey and dog the running time lasted 21 minutes; for

1) Analytical reagents by Merck Co. Darmstadt were used, unless otherwise indicated.

the rat this had to be extended to 30 minutes to obtain better separation. The buffer solution used for electrophoresis contained 7.28 mmol diethyl-barbituric acid and 49.5 mmol sodium-diethyl barbiturate (pH 8.6) per litre; dye solution (Ponceau S), decolorizing and transparent solutions were prepared according to the instructions provided by Chemetron Milano Co. (for cellogel). All statistical calculations were carried out with the t-test according to Student.

Preparation of Blood

Blood was taken according to the peculiarity of the species (5♂ and 5♀): in man (healthy specimens 20 - 40 years) from the Vena cubitalis, in monkeys (Indian Rhesus monkeys, approx. 2-3 years old) from the Vena saphena magna, in dogs (beagles, 6-7 months old) from the Vena brachialis and in SPF rats (OFA Sandoz, 8 weeks old) retro-orbitally. The serum was obtained by centrifuging at 4,000 g for 5 minutes.

Standard and Control Serum

As standard or control serum we used Versatol from the same lot (Cosmopharm Zurich) which also provides the basis for all calculations of protein and albumin values. The results were determined with the aid of standard curves (four standards with linearly increasing concentration). Double determinations and "absolute measurements" in the form of extinction measurements were carried out.

Results

The photometric results of the albumin determination in four species which are based on Versatol as standard, are compared in Table 1 with the results obtained with electrophoresis and total protein determinations. In man, only the detergent-containing HABA and BCG reagents yield the same albumin values as electrophoresis under the standard conditions selected for these experiments. In dogs, the results obtained with the HABA method (in the presence of Levor IV) and the BCG method are in agreement with the electrophoretic results; in monkeys only the BCG reagent

yielded similar serum concentrations as electrophoresis. All photometric determinations in the rat yielded values which were not in agreement. (Remarkable in this animal is the above-mentioned lower rate of migration with the electrophoretic separation of albumin under conditions comparable to those used with other species). The determination with the BCP reagent showed values which deviated completely from electrophoretic values in all examined species.

Apart from the autoanalyzer methods and in order to preclude the possibility of relative differences (e.g. relative to the standard), we carried out manual measurements with increasing amounts of serum (10,20,30,40,50,60,70,80,90 µl/5 ml reagent solution) and measured the extinction for 10 animals per species. (425) The absorption curves (Fig. 1) show the same species-specific differences as the results based on Versatol. At the same time, this diagram also shows the linearity range, the recovery rate and the reproducibility of the method for the manual as well as for the mechanized analysis.

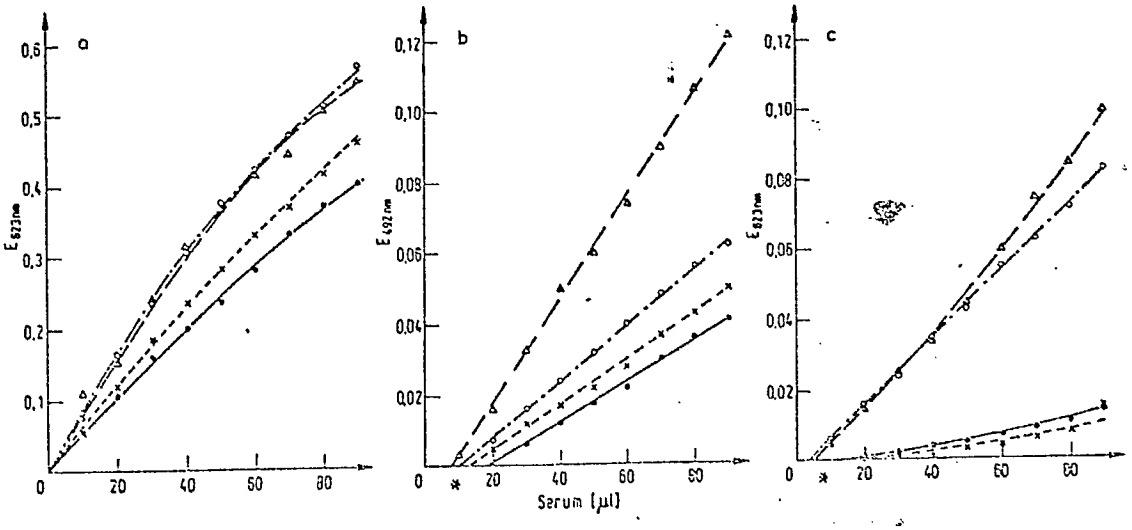

Fig. 1

Dependence of extinction on the albumin concentration (expressed in µl serum/5 ml test solution: 10, 20, 30, 40,50,60,70,80,90 µl/5 ml). The measurements were carried out in a serum pool of 10 individuals/species.

a) Bromocresol-green method
b) 2-(4-hydroxyazobenzene)-benzoic acid method
c) Bromocresol-purple method
●—● dog x—x rat ○—○ monkey Δ—Δ man
*Limit of accurate readability

n	Albumin (g/l)		Quotient Eph/BCG
	Elektrophorese* $\bar{x} \pm s$	BCG* $\bar{x} \pm s$	$\bar{F} \pm \Delta F$
30	44,4 ± 3,3	31,4 ± 2,9	1.42 ± 0,13
20	48.6 ± 3,7	31,2 ± 1,8	1.56 ± 0,15
20	49,1 ± 3,9	32,1 ± 2,0	1,54 ± 0,13
20	46,1 ± 4,6	31,4 ± 1,6	1,48 ± 0,17
10	45,6 ± 2,7	34,4 ± 2,5	1,33 ± 0,11
100	46,7 ± 4,1	31.8 ± 2,4	1.47 ± 0,16

Table 2

Comparative study with values from electrophoresis (Eph) and BCG in rats ($\sigma^{*}/♀ = 1:1$) for the determination of a mean quotient of error (F) or factor.

n: Number of animals
*: in both cases there is a normal distribution of values (calculated from 100 values of various animals)

$\bar{x} \pm s$: Mean value ± standard deviation;

$F \pm \Delta F$: Mean quotient of error ± standard deviation. The quotient Eph/BCG was calculated from the individual values of five different series examinations, indicating the mean quotients of error of the individual series as well as the value calculated from all individual values. The examinations were carried out on different days.

The results obtained with rats induced us to carry out further comparative studies between BCG and electrophoresis. It was our objective to investigate whether this difference in the results between electrophoresis and the BCG method could be reproduced also with larger, different collectives. The results of these experiments in Table 2 show that in rats the ratio of the results electrophoresis: BCG remains relatively constant (1.47 ± 0.16).

The species-specific differences in the total protein and albumin contents shown in Table 1 induced us to check these differences by statistical means. We tested the concentration differences between the various species with the aid of the t-test. The mean differences are listed in Table 3. There is thus no significant difference in the albumin content between the species man, monkey and rat. In the case of total protein, on the other hand, only man and monkey exhibit comparable concentrations.

Discussion

Today automation, standardization and quality control force the analyst to once more deal with basic methodological problems. This holds true all the more if one wishes to work with a uniform standard or control serum through the use of multiple analyzer systems. In place of the albumin standard specifically recommended for determinations in man (5,6,13) we have, for reasons mentioned earlier, tested the applicability of a human serum (Versatol) as standard for albumin determinations in different species. (426) Depending on the method used, its general applicability is limited by species-specific differences in protein bonding which occur in varying degrees. Here, the determination with bromocresol-green reagent emerges as the universally applicable method with the smallest differences between the species (with the exception of the rat); this has already been confirmed by similar results in pigs, cattle, horses, rabbits and sheep (1). In general it can be stated that albumin determination on the basis of protein dye complexes may become problematic in the evaluation of its results and more specific methods should therefore be used, particularly in doubtful cases (e.g. immunological determinations as long as the necessary conditions exist); for if the presence of several substances in the blood, similar to certain drugs, may result in a mutual shift in the protein bonding as well as in a mutual reinforcement of the protein bonding (15), similar possibilities for interference should be kept in mind also in the case of albumin determinations based on protein bonding. The differences in the results of the albumin values shown with and without the addition of detergents as indicated in Table 1 suggest similar possibilities of inhibition and reinforcement of protein bonding in the case of albumin determination with HABA. It is also possible that they originate from substance- or detergent-dependent changes in the secondary structure of the albumin molecule. However, whether and to what degree the addition of substances or certain detergents can generally eliminate substance- and species-dependent bonding interferences

must remain the objective of further, more extensive studies. The differences in the total protein and albumin contents and the differences in protein bonding in the relatively young but sexually mature animals used in these experiments are in agreement with observations made by us over many years in routine determinations.

* Speziesvergleich	Total-Protein (g/l)	Albumin (g/l)	
		Elektrophorese	BCG
	$\Delta\bar{X}$	$\Delta\bar{X}$	$\Delta\bar{X}$
1. Mensch–Ratte	+ 7	- 2	- 1⊛
Signifikanz	***	–	–
2. Mensch–Hund	+13	+13	+14
Signifikanz	***	***	***
3. Mensch–Affe	- 3	- 3	- 1
Signifikanz	–	–	–
4. Affe–Ratte	+10	- 1	0⊛
Signifikanz	***	–	–
5. Affe–Hund	+16	+16	+15
Signifikanz	***	***	***
6. Hund–Ratte	- 6	-15	-15⊛
Signifikanz	***	***	***

Table 3

Statistical investigations into inter-species differences of total protein and albumin in man, monkey, dog and rat (t-test)

* Comparison of species

1. Man - rat
2. Man - dog
3. Man - monkey
4. Monkey - rat
5. Monkey - dog
6. Dog - rat

$\Delta\bar{X}$: Species differences in the protein and albumin concentration

⊛: in rats, calculated by using the quotient of error.

Significance of species differences:

*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

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AUTHOR'S ADDRESS: Dr. A. Metz,
Sandoz AG.,
Pharm. Dept.
CH-4002 Basle
Switzerland