Critical differences of clinical chemical components in blood from laboratory rabbits

by *A. L. Jensen*[1], *E. Wedø*[2] & *M. Bantz*[1]

[1] Central Laboratory, Department of Clinical Studies,
The Royal Veterinary and Agricultural University, Bülowsvej 13, DK-1870 Frederiksberg C, Denmark.
[2] Animal Department, State Serum Institute, Artillerivej 5, DK-2300 Copenhagen S, Denmark.

Introduction

Clinical chemical components are frequently used to assist in diagnosis and monitoring of diseases in animals. The value of a clinical chemical component is traditionally compared to a corresponding population-based reference interval that is derived from an observed distribution of measurements of the clinical chemical component in an appropriate group of animals and contains the central 95 % of the distribution (*Solberg* 1983). Then, if the value is outside the corresponding reference interval, the animal is classified as abnormal indicating an unusual or pathological situation. However, as the scope of the analysis in most cases is to observe whether the current measurement is part of a trend toward another physiological state in the particular animal, it may be suggested that the animal can serve as its own reference by comparing analytical results from samples obtained serially at intervals appropriate to the experimental influence or to the expected rate of development of disease or improvement.

For that purpose, the critical difference (d_k) has been developed as a tool to follow the course of a clinical chemical component in an individual in consecutive measurements (*Stamm* 1982, *Costongs et al.* 1985). The critical difference is based on the following considerations. The standard deviation of the difference between two independent, random variables with the same expected value and the same standard deviation, σ, is $\sqrt{2}\ \sigma$. The probability that the difference between the two variables is less than or equal to $2 \cdot \sqrt{2} \cdot \sigma$ is about 95 % and if the difference between two consequtive results is greater than $2 \cdot \sqrt{2} \cdot \sigma$, then the results can be regarded as being different (*Stamm* 1982). The implication of this is, that if two consecutive results differ less than the critical difference ($2 \cdot \sqrt{2} \cdot \sigma$), then, using a probability of 95 %, the difference may be ascribed to random variation. Otherwise, if two consecutive results differ by at least the critical difference, then other causes such as disease, experimental procedures or therapy, may be responsible for the difference.

On many occations, the interest may primarily be on changes in analytical results occurring within an interval of two or three days or a week in order to follow the progress or course of disease or the response to treatment. The present study was therefore conducted to establish the critical differences for some routinely used clinical chemical components in blood from rabbits on a day-to day basis.

Materials and methods

Animals

Twenty-five outbred albino-rabbits (*Oryctolagus cuniculus,* Ssc:CPH) originating from the conventional breeding colony of the State Serum Institute were used for the monitoring of clinical-chemical components.

The 13 female and 12 male rabbits, 14–19 weeks old and weighing 2.5–3.0 kilograms were housed individually or in pairs in wire-mesh cages in the breeding colony during the period of blood sampling. Temperature in the animal room was regulated at 17 ± 2°C and relative humidity 60–70 %. All animals were clinically healthy at the beginning and during the study. From weaning to the

test period the rabbits were fed a commercial pelleted diet (Rabbit Full-diet, 085-3, Slangerup Foderstofforening) *ad libitum* and given free access to drinking-water.
Experimental procedures often include the withhold of feed for a short period and diseased or experimentally affected animals frequently present various degrees of inappetence. To approximate these situations more closely feed but not water was withheld for 12 hours before blood sampling.

Sample collection

Once daily between 09.00 and 11.00 a.m., blood samples for clinical chemical analysis were collected from each rabbit by puncture of the marginal ear vein. At each venepuncture a total of 5 ml of blood was collected in vacutainers containing sterile sodium fluoride with sodium heparin and in vacutainers containing clot activator.
Blood collection took place in the breeding colony and care was taken not to excite the rabbits during venepuncture in order to reduce any effect on the clinical chemical components investigated caused by handling, transport and adaption to new environment.
Serum and plasma for clinical chemical analyses were obtained by centrifugation (750 g, 5 min), divided in several portions and stored at –78°C for no longer than 10 days.

Analytical procedures

Clinical-chemical analysis included alanine aminotransferase (E.C. 2.6.1.2. ALAT), aspartate aminotransferase (E.C. 2.6.1.1. ASAT), alkaline phoshatase (E.C. 3.1.3.1. ALP), amylase (E.C. 3.2.1.1.), urea, creatinine, serum $protein_{Total}$, albumin, glucose, and lactate. The clinical chemical components were measured using analytical methods applied to the Cobas Fara®(Roche) centrifugal analyzer.
On the day of analysis of a clinical component, specimens from all five days of each rabbit were thawed at room temperature, each mixed thoroughly, and analysed in a single working day using the same lots of quality control materials, calibrators, and reagents. All samples were analysed in duplicate.

Statistical analysis

The entire set of results of each clinical chemical component was evaluated for approximate normally of distribution using the Shapiro-Wilk statistic in the PROC UNIVARIATE NORMAL-procesure in SAS®.
Regression analysis assisted by the PROC REG procedure in SAS® was used to observe whether a change in the clinical components occurred with time.
The critical difference (d_k) of each clinical chemical component was calculated as previously described (*Jensen et al.* 1992) using nested analysis of variance to divide the total variance into the component of variance between rabbits (S^2_{Inter}), the component of variance for days within rabbits (S^2_{Intra}, and the component of variance for measurements (S^2_{Anal}). Because the variance in the present study predominantly depends on S^2_{Inter}, S^2_{Intra}, and S^2_{Anal}, the component of variance due to other factors (S^2_{Others}) being minimized, the standard deviation for one rabbit is calculated from the S^2_{Intra} and S^2_{Anal}. Thus, the critical difference can be calculated in absolute value as

$$d_k = 2 \cdot \sqrt{2\,(S^2_{Intra} + S^2_{Anal})}$$

For each clinical chemical component, the F-test described by *Snedecor & Cochran* (1967) was used to observe whether the clinical chemical components varied from day to day (i.e. S^2_{Intra} greater than 0) and from rabbit to rabbit (i.e. S^2_{Inter} greater than 0). Briefly, the F value for variation from day to day was calculated as the mean square of S^2_{Intra} divided by the mean square of S^2_{Anal}, and the F value for variation between rabbits was calculated as the mean square of S^2_{Inter} divided by the mean square of S^2_{Intra}.
To calculate the reference interval for each clinical chemical component, the data from

the first sample on the first day of the study were used. The reference interval would be calculated as either mean value ± 2 standard deviations if the data were normally distributed or as the 5^{th} to 95^{th} percentile interval if the data were not normally distributed.

Results

The Shapiro-Wilk statistic indicated, that all raw data were distributed in a Gaussian manner. The regression analysis showed no significant changes in the clinical chemical components with time.

The mean value of each clinical chemical component for each rabbit was in general within the respective reference intervals displayed in Table 2. For each clinical chemical component, the mean value and the variation around the mean value in the individual rabbit differed considerably between rabbits, as exemplified in Figure 1 for alanine aminotransferase. Further, as also exemplified in Figure 1, the variation of the results around the mean value for each clinical chemical component in each rabbit was in general smaller than the dispersion of the corresponding reference interval.

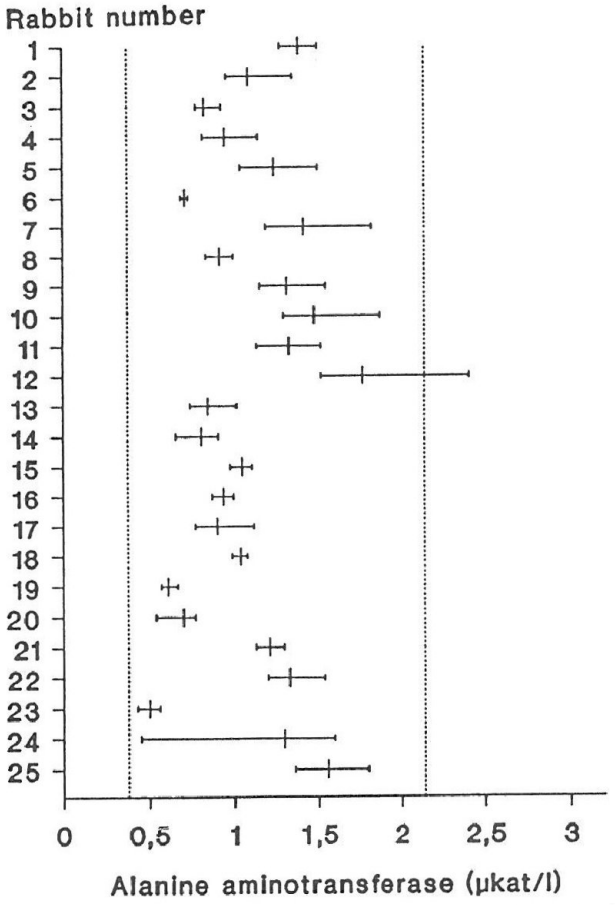

Figure 1. Parametric mean values and absolute ranges for the alanine aminotransferase activity measured daily for 5 consecutive days in 25 clinically healthy rabbits. The dotted lines represent the reference interval (0.38–2.14 µkat/l) obtained from Table 2.

Table 1 summarizes the component of variance between rabbits (S^2_{Inter}), the component of variance for days within rabbits (S^2_{Intra}), the component of variance for measurements (S^2_{Anal}), the overall mean value ($\bar{x}$), and the critical difference as an absolute value (d_k) for each of the components examined in the present study.

All clinical chemical components varied from day to day and also from rabbit to rabbit since S^2_{Inter} as well as S^2_{Intra} were significantly greater than 0.

As all data were normally distributed, the reference interval in Table 2 for each clinical chemical component is given as mean value ± 2 standard deviations.

Discussion

The most common method in veterinary medicine of assessing results from clinical chemical analyses is perhaps to compare the analytical results to corresponding population based reference intervals. If the variation around the mean value of a clinical chemical component of each animal is smaller than the dispersion of the corresponding reference interval, as illustrated in Figure 1, then it is possible that a single animal with a disease affecting the clinical chemical component could have an analytical result outside its own reference interval, but within the corresponding population based reference interval at the time of collecting the blood sample. Thus, in these situations comparing the analytical result to the corresponding population based reference interval

Table 1. The critical difference in absolute values (d_k) calculated from the component of variance between rabbits (S^2_{Inter}), the component of variance for days between rabbits (S^2_{Intra}), the component of variance for measurements (S^2_{Anal}), as well at the overall mean value ($\bar{x}$) for 11 clinical chemical components in the adult rabbit.

Component	Unit	S^2_{Inter}	S^2_{Intra}	S^2_{Anal}	$\bar{x}$	d_k
Alanine aminotransferase	(μkat/l)	0.098	0.029	0.0002	1.09	0.48
Aspartate aminotransferase	(μkat/l)	0.003	0.005	0.0002	0.33	0.20
Alkaline phosphatase	(μkat/l)	2.023	0.652	0.0176	5.02	2.32
Amylase	(μkat/l)	2.715	1.371	0.2253	12.50	3.57
Urea	(mmol/l)	0.951	3.838	0.2068	6.20	5.69
Creatinine	(μmol/l)	132.509	59.635	27.668	112	26
Albumin	(g/l)	0.664	3.496	1.902	38.9	6.57
Serum $protein_{Total}$	(g/l)	6.067	22.896	1.4571	63.2	14.0
$Cholesterol_{Total}$	(mmol/l)	0.230	0.039	0.0051	1.34	0.59
Glucose	(mmol/l)	0.009	0.543	0.0077	6.44	2.10
Lactate	(mmol/l)	0.181	2.592	0.0512	3.31	4.60

may not be sufficient. As an aid to identify analytical results outside an animals own reference interval, the critical difference calculated in the present study could be used as the critical difference may help to judge whether the difference between two consecutive analytical results with a certain probability (95 %) may be ascribed to natural variation or not. However, it could be argued that the critical differences calculated in this study are not directly applicable to rabbits with diseases as the calculations were based on clinically healthy rabbits. No definite answer to this point can be obtained from the present study, but, in human medicine, it has been reported, that, in nonacute pathological processes where new homeostatic steadystates are reached, biological varia-

Table 2. Reference intervals for 11 clinical chemical components in the adult rabbit.

Component	Unit	Mean value	Standard deviation	Reference interval
Alanine aminotransferase	(μkat/l)	1.26	0.44	0.38– 2.14
Aspartate aminotransferase	(μkat/l)	0.38	0.08	0.22– 0.54
Alkaline phosphatase	(μkat/l)	6.16	1.90	2.36– 9.96
Amylase	(μkat/l)	12.89	1.84	9.21–16.57
Urea	(mmol/l)	9.13	2.53	4.07–14.19
Creatinine	(μmol/l)	108	11	86–130
Albumin	(g/l)	42.2	2.5	37.2–47.2
Serum $protein_{Total}$	(g/l)	66.8	3.3	60.2–73.4
$Cholesterol_{Total}$	(mmol/l)	1.31	0.52	0.27– 2.35
Glucose	(mmol/l)	5.56	0.72	4.12– 7.00
Lactate	(mmol/l)	4.35	2.06	0.23– 8.47

tion around the new mean values are of the same magnitude as in healthy persons (*Fraser & Hearne* 1982, *Pascoe et al.* 1984). Other reports have, however, indicated that the intra-individual variation is greater in patients with chronic diseases compared to healthy persons (*Hölzel* 1987 a, 1987 b, 1987 c). Evidently, there may be a problem when the first measurement as well as the second may be affected by the disease status of the dog. Moreover, when a clinical chemical component is measured for the first time, the only way it can be assessed is for most cases by comparing the analytical result to the corresponding population based reference interval.

Thus, the most appropriate use of the critical difference seems to be in conjunction with the corresponding population based reference intervals as those listed in Table 2.

In the present study, the critical differences for eleven clinical chemical components in the rabbit were calculated in absolute values from the component of variance between rabbits (S^2_{Inter}), the component of variance for days within rabbits (S^2_{Intra}), and the component of variance for measurements (S^2_{Anal}). For the clinical chemical components investigated in the present study, and as illustrated in Figure 1, the intra-individual variations around the mean value of the individual rabbit were of different magnitudes. Thus, the critical differences calculated in the present study serves only as guidelines, as the critical differences may be an underestimation for some rabbits and an overestimation for other rabbits. It may also be argued that the critical differences calculated in the present study are in fact too low as they have been calculated from the S^2_{Intra} and S^2_{Anal}, leaving the component of variance due to other factors (S^2_{Others}) out of account. However, efforts have been made in the present study, i.e. standardization of the sample collecting procedure and handling of serum and plasma samples, to minimize S^2_{Others} to a degree where it, although no proof has been given, may be neglected. Further, to minimize the S^2_{Anal}, each clinical chemical component in all samples from the entire study was analysed in the same run and therefore, no between-day variance contributes to the total variance as would probably be the case in practical settings.

Seemingly, the critical differences calculated in the present study may be used as guidelines to evaluate the difference between two consecutive analytical results. However, analytical results should not be assessed by the critical differences alone, but should also be compared to the corresponding reference intervals.

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Summary

The purpose of the present study was to calculate the critical difference between two analytical results for 11 clinical chemical components in the adult rabbit. The critical difference can be used to judge whether the difference between two consecutive analytical results from the same animal is due to natural variation or not. From 25 adult rabbits, blood samples were collected once daily after a 12 hour fasting period for 5 consecutive days, and the total variance of the analytical results of each clinical chemical component was divided into the component of variance between rabbits (S^2_{Inter}), the component of variance for days within rabbits (S^2_{Intra}), and the component of variance for measurements (S^2_{Anal}) using nested analysis of variance. The critical difference was then calculated from S^2_{Intra} and S^2_{Anal} as 0.48 µkat/l for alanine aminotransferase (ALAT), 0.20 µkat/l for aspartate aminotransferase (ASAT), 2.32 µkat/l for alkaline phosphatase (AP), 3.57 µkat/l for amylase, 5.69 mmol/l for urea, 26 µmol/l for creatinine, 6.6 g/l for albumin, 14.0 g/l for serum $protein_{Total}$, 2.10 mmol/l for glucose, and 0.59 mmol/l for $cholesterol_{Total}$, and 4.60 mmol/l for lactate. These critical differences may be used as guidelines to evaluate the difference between two consecutive analytical results of the above clinical chemical components. However, the analytical results should not be assessed by the critical differences alone, but should also be compared to the corresponding population based reference intervals.

Sammendrag
Formålet med nærværende undersøgelse var at beregne den kritiske differens mellem to analytiske resultater for 11 klinisk-kemiske parametre hos voksne kaniner. Ved bestemmelse af den kritiske differens bliver det muligt med rimelig sikkerhed at afgøre, om en ændring af den målte kliniskkemiske parameters værdi enten kan skyldes tilfældigheder eller ej. Der udtoges blodprøver fra 25 voksne kaniner en gang dagligt efter en 12 timers fasteperiode 5 dage i træk.
Ved hjælp af hierakisk variansanalyse beregnedes den inter-individuelle, den intra-individuelle og den analytiske variation, og på basis af dette beregnedes den kritiske differens for de analyserede klinisk-kemiske parametre som følger: 0,48 µkat/l for alanine aminotransferase (ALAT), 0,20 µkat/l for aspartate aminotransferase (ASAT), 2,32 µkat/l for basisk phosphatase (BASP), 3,57 µkat/l for amylase, 5,69 mmol/l for carbamid, 26 µmol/l for creatinin, 6,6 g/l for albumin, 14,0 g/l for serum $protein_{Total}$, 2,10 mmol/l for glucose, 0,59 mmol/l for $cholesterol_{Total}$, og 4,60 mmol/l for laktat. Det må dog anbefales, ikke at basere vurderingen af konsekutive analyseresultater på den kritiske differens alene, men tillige vurdere analyseresultaterne i forhold til de respektive reference intervaller.

Yhteenveto / K. Pelkonen
Tutkimuksen tarkoitus oli löytää kahden mittaustuloksen välisen eron nk. kriittinen suruus. Aikuisista kaneista tutkittiin 11 mittauskohdetta. Kriittisen eron avulla on kohtuullisella varmuudella mahdollista päätellä, johtuuko kahden mittaustuloksen välinen ero sattumasta vai että arvot todella ovat erisuuria. Tutkimusta varten otettiin viitenä perättäisenä päivänä 25 täysikasvuisesta kanista verinäytteet alna 12 tunnin paaston jälkeen.
Hierarkkisen varianssianalyysin avulla laskettiin yksilöidenvälinen, yksilönsisäinen ja analyyttinen vaihtelu ja näiden perusteella määritettiin kriittiset erot seuraaville kliiniskemiallisille muuttujille: ALAT 0,48 µkat/l; ASAT 0,20 µkat/l; AFOS 2,32 µkat/l; amylaasi 3,57 µkat/l; karbamidi 5,69 mmol/l; kreatiniini 26 µmol/l; albumiini 6,6 g/l; seerumin kokonaisproteiini 14,0 g/l; glukoosi 2,10 mmol/l; kokonaiskolesteroli 0,59 mmol/l; laktaatti 4,60 mmol/l.
Kirjoittajat huomauttavat ettei perättäisten mittaustulosten arviointia kuitenkaan sasi kokonaan perustaa krittiselle erolle, vaan niitä tulisi myös vertailla vastaaviin viitearvoihin.

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