

Special Article - Hematology

Analytical Validation Study of Hematological Parameters under Good Laboratory Practice Regulations in Different Laboratory Animal Species

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Abstract

Background: In the research field, the Good Laboratory Practice (GLP) is a quality system of management controls for research laboratories and organizations to ensure the uniformity, consistency, reliability, reproducibility, quality, and integrity of pre-clinical safety tests.

Objectives: In this paper we aim to contribute with methodological results data to help the researcher to establish the validation of the analytical methods used within the research.

Methods: The procedure was performed to validate each hematological parameter (WBC, RBC, HB, HCT, MCV, MCH, MCHC, and PLT) in the following laboratory animal species: pig, sheep, dog and rabbit. One single animal of each species was randomly chosen to obtain a 4ml blood sample using EDTA as anticoagulant. In order to carry out the validation of the analytical methods, the repeatability (performing six measurements of each parameter by a single analyst) and the intermediate precision (by performing two measurements of each parameter by three different analysts) of all the parameters mentioned was calculated. The obtained results were statistically analyzed; the mean, the standard deviation and the coefficient of variation were calculated.

Results: Coefficients of variation below 5% were obtained for all studied parameters in all species, except for the platelets in rabbits, which showed a coefficient of variation of 5.85% in the repeatability study and 7.91% in the intermediate precision study.

Conclusions: The results obtained during the verification are acceptable, so the analytical method have been developed with an adequate precision. Those study results ensure the quality and integrity of the measurements obtained in our laboratory, which is necessary in the performance of preclinical studies.

Keywords: Analytical methods validation; GLP; Veterinary hematology

Introduction

The Good Laboratory Practice (GLP) regulations were used in a regulatory context for first time in New Zealand in 1972, where the Testing Laboratory Act came out to specify the conditions for planning, performing and recording studies in order to ensure the reliability of the results. Later, the US Food and Drug Administration (FDA), followed by the Environmental Protection Agency (EPA), developed their GLP regulations covering chemical safety and efficacy testing [1-3].

During 1979 and 1980, an international group of experts established a special program on the control of chemicals, and created the "OECD Principles of Good Laboratory Practice", this document was developed based on scientific practice and experience from various national and international sources [4].

The purpose of these rules is to ensure the quality and integrity of all data obtained during a specific study, and its compliance is required for all non-clinical safety research on pharmaceutical products [4,5],

testing under GLP regulations is mandatory when the studies are performed as a requirement for a marketing authorization [6].

Therefore, compliance with GLP regulations is a requirement that clinical laboratories should meet to increase the use of standardized practices and procedures, optimize management operations, and enhance the obtaining of reproducible and reliable results [7,8].

To properly use and interpret laboratory analysis, it is necessary a validation of the techniques before being routinely used at the laboratory [8,9]. Thus, through a validation process, the reliability of the method and the expected results obtained within pre-established conditions are verified [10].

The procedure to carry out the validation of analytical methods should contain the following sections [11,12]:

- Development of a Protocol or Experimental Plan.
- Validation of the method, done through the determination of the precision. Precision is a parameter that represents the degree

Table 1: Hematological laboratory references values for different animal species.

	Units	SWINE	SHEEP	DOG	RABBIT
WBC	(10 ⁹ /μl)	11–22	4–12	6–17	5–12
RBC	(10 ⁶ /μl)	5–8	9–15	5.5–8.5	4–8
HB	(g/dl)	10–16	9–15	12–19	10–15
HCT	(%)	30–50	27–45	37–55	33–48
MCV	(fl)	50–68	28–40	60–77	55–75
MCH	(pg)	17–21	8–12	12–20	18–23
MCHC	(g/dl)	30–34	30–34	32–36	28–37
Plt	(10 ⁹ /μl)	300–700	300–700	200–500	250–600

Feldman, BF. et al. [16]. Schalm’s Veterinary Hematology. 5th edition.
Kahn CM. Manual Merck Veterinaria. 6th edition. [30].

of dispersion among a series of measurements obtained from a homogeneous sample under preset conditions and depending on the factors that are modified, two types precision can be obtained: repeatability and intermediate precision.

- Development of a final report including the verification of the equipment used, and also the primary results and statistics for each parameter. The discussion of the results and the conclusions of validation must also be included.

- It is necessary for veterinary laboratories to consider the quality procedures and policy as part of the methodological development, and include such activities as an integral part in the production of test results [13,14]. Quality controls in hematology include three important aspects: the calibration of automatic instruments, the monitoring of the accuracy and precision of instruments and procedures, and the verification of the result’s reliability [8,15].

The importance of performing veterinary hematology tests in compliance with GLP regulations is due to the essential role of those tests in the diagnosis of animal diseases and the monitoring of the health status during research studies [16].

The objective of this paper is to verify and document the validity of the analytical method in hematologic parameters, to ensure the quality and integrity of the data obtained at the MISCJU Laboratory during the implementation of GLP regulations in order to meet the degree of analytical accuracy required, ensuring that the results obtained for the studies are reliable, repeatable and auditable.

The main aim of this study is to describe the methodology for the validation of these analytical techniques and present the results in order to serve as a guideline to other research centers.

Materials and Methods

Healthy animals housed at the animal housing facilities of the Minimally Invasive Surgery Center Jesús Usón (MISCJU) were randomly selected for this study, one animal of the following species were used: Large White pig, Merina sheep, Beagle dog and New Zealand rabbit.

In the pig blood sampling was performed in the cranial cava vein, in sheep the sample was obtained from the external jugular vein, in dog blood sampling was performed in the cephalic vein and in the rabbit the sample was collected from ear marginal vein.

Table 2: Repeatability study results in swine.

	SWINE							
	WBC (10 ⁹ /μl)	RBC (10 ⁶ /μl)	HB (g/dl)	HCT (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)	PLT (10 ⁹ /μl)
1	12.8	5.82	9.7	30.4	52.2	16.7	31.9	526
2	12.9	5.9	9.8	30.8	52.2	16.6	31.8	537
3	12.9	5.9	9.7	30.8	52.2	16.4	31.5	550
4	12.4	5.79	9.7	30.4	52.5	16.8	31.9	550
5	12	5.89	9.7	30.7	52.1	16.5	31.6	545
6	12.7	5.81	9.5	30.1	51.8	16.4	31.6	521
Mean	12.61	5.85	9.68	30.53	52.17	16.57	31.72	537.93
SD	0.36	0.05	0.1	0.28	0.23	0.16	0.17	12.42
CV	2.85%	0.85%	1.03%	0.92%	0.44%	0.97%	0.54%	2.31%

In all animals 4 ml of blood were collected, all samples were collected using EDTA tubes. In all species, except the rabbit, a vacuum tube with a needle system (Vacutainer, Becton Dickinson, New Jersey, U.S.A.) was used for a direct blood extraction.

Once the sample was obtained, it was directly lead to the laboratory and homogenized in the roll and tilt (Nahita 683, Auxilab S.L., Navarra, Spain) during 5 minutes, after that the analysis were performed using an hematology analyzer (MEK-6318, Nihon Kohden iberica S.L., Madrid, Spain), following parameters were measured: White Blood Cells (WBC), Red Blood Cells (RBC), Hemoglobin (HB), Hematocrit (HCT), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC) and Platelets (PLT). The repeatability and intermediate precision of the method were measured as validation parameters.

The analytical validation study carried out is a partial validation, specifically a minor validation or verification, due to the fact that these are standardized analytical methods. To assess the repeatability of the method, six repetitions of the same sample were analyzed in the same conditions (same analyzer, same reagents and same material). Intermediate precision was calculated using also one single sample of each animal species, making in this case two repetitions by three different analysts, all in the same conditions (same analyzer, same reagents and same material). After completing the determinations, statistical analysis of the data was performed using statistical software (SPSS 15.0 statistical package for Windows, SPSS Inc, Chicago, III).

The mean and the Standard Deviation (SD) of each hematological parameter studied (WBC, RBC, HB, HCT, MCV, MCH, MCHC, and PLT) were calculated using the data obtained from each animal species (pig, sheep, dog and rabbit) in each validation parameter (6 measurements for the repeatability of the method and 6 repetitions for the intermediate precision). The dispersion have been expressed in percentage as the Coefficient of Variation (CV) using the following formula: $CV (\%) = (SD/mean) \times 100$. In both parameters (repeatability and intermediate precision) was established a limit of acceptance below or equal to a 5% coefficient of variation ($CV \leq 5\%$).

Results

All the hematological parameters evaluated have shown a

Table 3: Repeatability study results in sheep.

SHEEP								
	WBC	RBC	HB	HCT	MCV	MCH	MCHC	PLT
	(10 ⁹ /μl)	(10 ⁶ /μl)	(g/dl)	(%)	(fl)	(pg)	(g/dl)	(10 ³ /μl)
1	5.9	10.1	10.9	35.9	35.4	10.8	30.4	285
2	6.1	10	10.9	35.8	35.7	10.9	30.4	296
3	6.1	10.3	10.9	36.8	35.7	10.6	29.6	305
4	5.9	10.1	11	36.2	35.6	10.8	30.4	295
5	6.1	10	10.9	36	35.8	10.8	30.3	290
6	5.9	10.3	11	36.5	35.2	10.6	30.1	284
Mean	6	10.13	10.93	36.2	35.57	10.75	30.2	292.33
SD	0.11	0.14	0.05	0.38	0.23	0.12	0.32	7.88
CV	1.83%	1.38%	0.46%	1.05%	0.65%	1.12%	1.06%	2.70%

Table 4: Repeatability study results in dog.

DOG								
	WBC	RBC	HB	HCT	MCV	MCH	MCHC	PLT
	(10 ³ /μl)	(10 ⁶ /μl)	(g/dl)	(%)	(fl)	(pg)	(g/dl)	(10 ³ /μl)
1	8.3	8	19.3	52.3	65.4	24.1	36.9	339
2	8.2	7.98	19.2	52.4	65.7	24.1	36.6	338
3	8.6	8.02	19.4	52.4	65.3	24.2	37	340
4	8.3	8.02	19.1	52.4	65.3	23.8	36.5	335
5	8.3	7.88	19.3	51.7	65.6	24.5	37.3	340
6	8.6	8.13	19.2	53.2	65.4	23.6	36.1	346
Mean	8.38	8	19.25	52.4	65.45	24.05	36.73	339.63
SD	0.17	0.08	0.1	0.48	0.16	0.31	0.42	3.61
CV	2.03%	1.00%	0.52%	0.92%	0.24%	1.29%	1.14%	1.06%

Table 5: Repeatability study results in rabbit.

RABBIT								
	WBC	RBC	HB	HCT	MCV	MCH	MCHC	PLT
	(10 ⁹ /μl)	(10 ⁶ /μl)	(g/dl)	(%)	(fl)	(pg)	(g/dl)	(10 ³ /μl)
1	7.6	6.41	11.8	35.3	54.9	18.1	33.7	127
2	7.5	6.46	11.4	35.8	55.5	18.1	32.6	136
3	7.7	6.49	11.7	36	55.5	18	33.1	137
4	7.7	6.38	11.7	35.4	55.5	18.3	32.5	137
5	7.8	6.36	11.7	35	55.4	17.9	32.7	152
6	7.3	6.38	11.6	35	55.1	18.5	32.9	140
Mean	7.6	6.41	11.65	35.41	55.32	18.15	32.91	137.78
SD	0.18	0.05	0.14	0.41	0.26	0.22	0.44	8.08
CV	2.37%	0.78%	1.20%	1.16%	0.47%	1.21%	1.34%	5.86%

coefficient of variation below 5%, with the exception of the platelets value in the rabbit blood samples. In that case the coefficient of variation obtained was below 10% (5.86% CV in the repeatability study and 7.91% CV in the intermediate precision study).

All the obtained data are inside the normal range of reference values established in our laboratory (Table 1), except for the result in the rabbit platelets determination that was below the normal range.

Repeatability

Tables 2,3,4 and 5, show the primary data for the different hematological parameters for the different species studied: the mean, the standard deviation and the coefficient of variation obtained for the repeatability study.

The results obtained show that the analytical method for the hematological parameters evaluated is accurate in the preset conditions, being thus repetitive.

Regarding the coefficient of variation, white blood cells show a CV <3% in all species, while red blood cells, hemoglobin, hematocrit and erythrocyte indexes (MCV, MCH, MCHC) present a CV <2%, finally, the platelets present the highest coefficient of variation (especially in the rabbit study) reaching a CV of 5.86%, exceeding the limit of 5% CV that was established as acceptable in our laboratory.

Intermediate precision

Tables 6,7,8 and 9 show the data obtained in the hematological tests of each parameter for the different species studied: the mean, the standard deviation and the coefficient of variation obtained for the precision study.

The method presents a good precision, since all the coefficients of variation are below 5% with the exception of the platelets measurements in rabbits that presented a CV of 7.91%.

Regarding the coefficient of variation, white blood cells show in all species a CV<2%, with the exception of precision study in sheep, in which the CV obtained is 4.91%. Red blood cells, hemoglobin, hematocrit and erythrocyte indices (MCV, MCH, MCHC) present a CV<2% in pigs, sheep and dog, but in the case of the rabbit the CV was below 3%. Platelets, as in the repeatability study, are the parameter with the highest coefficient of variation in the four species studied, especially in the rabbit, which exceeds the established limit of 5% coefficient of variation, obtaining a result of 7.91%.

Discussion

Pharmacological research studies using experimental animals are essential to the research and application of possible therapeutic strategies for both, prevention and treatment of diseases. These studies allow measuring the efficacy and safety of a pharmaceutical product [17,18].

Table 6: Intermediate Precision study results in swine.

SWINE								
	WBC	RBC	HB	HCT	MCV	MCH	MCHC	PLT
	(10 ⁹ /μl)	(10 ⁶ /μl)	(g/dl)	(%)	(fl)	(pg)	(g/dl)	(10 ³ /μl)
Analyst 1	12.9	5.86	9.7	30.5	52	16.6	31.8	526
	13.3	5.84	9.7	30.4	52.1	16.6	31.9	551
Analyst 2	13.2	5.86	9.7	30.6	52.2	16.6	31.7	531
	12.9	5.72	9.7	30	52.4	17	32.3	530
Analyst 3	13.1	5.78	9.7	30.2	52.2	16.8	32.1	560
	13	5.81	9.7	30.7	52.8	16.7	31.6	555
Mean	13.06	5.81	9.7	30.4	52.28	16.72	31.9	541.83
SD	0.16	0.05	0	0.26	0.29	0.16	0.26	14.8
CV	1.23%	0.86%	0	0.85%	0.55%	0.96%	0.82%	2.73%

Table 7: Intermediate Precision study results in sheep.

SHEEP								
	WBC	RBC	HB	HCT	MCV	MCH	MCHC	PLT
	(10 ³ /μl)	(10 ⁶ /μl)	(g/dl)	(%)	(fl)	(pg)	(g/dl)	(10 ³ /μl)
Analyst 1	4.9	10.2	10.7	36	35.3	10.5	29.7	294
	4.8	10.2	10.4	36.1	35.2	10.1	28.8	299
Analyst 2	5	9.8	10.6	34.7	35.3	10.8	30.5	289
	5.1	10.1	10.6	35.8	35.2	10.4	29.6	303
Analyst 3	5.4	10.3	10.7	36.6	35.5	10.4	29.2	285
	5.4	10.4	10.9	36.7	35.3	10.5	29.7	281
Mean	5.09	10.17	10.65	35.97	35.3	10.45	29.57	291.63
SD	0.25	0.2	0.16	0.72	0.11	0.23	0.57	8.4
CV	4.91%	1.97%	1.50%	2.00%	0.31%	2.20%	1.93%	2.88%

Table 8: Intermediate Precision study results in dog.

DOG								
	WBC	RBC	HB	HCT	MCV	MCH	MCHC	PLT
	(10 ³ /μl)	(10 ⁶ /μl)	(g/dl)	(%)	(fl)	(pg)	(g/dl)	(10 ³ /μl)
Analyst 1	8.6	8.13	19.2	53.3	65.6	23.6	36	365
	8.5	7.95	19.3	52.2	65.7	24.3	37	330
Analyst 2	8.6	8.09	19.2	52.5	64.9	23.7	36.6	341
	8.5	8.04	19.2	52.9	65.8	23.9	36.3	344
Analyst 3	8.3	8.07	19.3	53.1	65.8	23.9	36.3	345
	8.6	7.97	19.2	52.3	65.6	24.1	36.7	349
Mean	8.52	8.04	19.23	52.72	65.57	23.92	36.48	345.67
SD	0.12	0.07	0.05	0.45	0.34	0.26	0.35	11.45
CV	1.41%	0.87%	0.26%	0.85%	0.52%	1.09%	0.96%	3.31%

This objective is achieved by using previously validated analytical techniques in these studies, because the validation is a prerequisite for Good Laboratory Practice (GLP) compliance [10,18], being a condition that clinical laboratories must meet to increase the use of standardized practices, optimize management operations, and enhance the obtaining of reproducible and reliable results, while ensuring safety [7,8].

Since our laboratory performs preclinical studies and some of them require the investigation of the hematologic status of the animals, it was essential the application of this quality system (GLP) as well as the need to verify that the results are within the required accuracy, providing security and ensuring that all the followed steps are reproducible and auditable, because many of the decisions made are based on the information that these results provide.

Regarding the obtained data, all results were within the reference range established in the laboratory, except for the platelets in rabbit, which were below the range. This situation may be produced, as mentioned by some authors, because a labored extraction can promote clot formation [19] causing a false thrombocytopenia [20], since venipuncture in rabbits can sometimes be difficult. In addition, blood should preferably be taken directly with the vacuum tube (Vacutainer, Becton Dickinson, New Jersey, U.S.A.) because this method reduces platelet clumping and clot formation. Extraction

Table 9: Intermediate Precision study results in rabbit.

RABBIT								
	WBC	RBC	HB	HCT	MCV	MCH	MCHC	PLT
	(10 ³ /μl)	(10 ⁶ /μl)	(g/dl)	(%)	(fl)	(pg)	(g/dl)	(10 ³ /μl)
Analyst 1	7.6	6.61	11.5	36.2	54.8	17.4	31.8	161
	7.5	6.26	11.6	34.6	55.3	18.5	33.5	146
Analyst 2	7.5	6.38	11.7	36.1	56.6	18.3	32.4	134
	7.7	6.31	11.7	35	55.5	18.5	33.4	136
Analyst 3	7.6	6.31	11.6	34.7	55	18.4	33.4	159
	7.5	6.31	11.7	34.5	54.7	18.5	33.9	141
Mean	7.57	6.36	11.63	35.17	55.31	18.26	33.05	145.43
SD	0.08	0.13	0.08	0.77	0.7	0.43	0.79	11.51
CV	1.06%	2.04%	0.69%	2.19%	1.27%	2.35%	2.39%	7.91%

without the use of this device significantly reduces the platelet count [19], unfortunately that kind of vacuum devices are not used in rabbits. The argument is that the sample collection in rabbits must be carried out gently and gradually, because the rabbits have small and fragile blood vessels that can easily break or collapse with the vacuum pressure. Therefore, in agreement with results reported in previous studies that point out thrombocytopenia as one of the main causes increasing the coefficient of variation in animals [21], we have obtained coefficients of variation above 5% for this species. Specifically in rabbits, some studies state coefficients of variation below 3% for all hematologic parameters [22], which is in agreement with the results obtained for this species in our study, just with the exemption of platelets.

We have also found other studies [23-25] carried out in different animal species that obtain coefficients of variation above 5% in the platelet count, the same that happens in our study in the case of the rabbits.

The Coefficient of Variation (CV) obtained for all the other hematological parameters, meet the acceptance criteria established (CV≤5%) in our laboratory, and are in accordance with those established by other authors [16,26-28].

Automatic equipment establishes coefficients of variation between 3% and 5% in the case of erythrocytes and leukocytes [16] and in our results the CV is below 3% in both cases and in all tested species. Regarding the hematocrit [16,27,28], coefficients of variation between 1% and 2% have been stated, and our work results for all parameters are inside this range with the exception of the precision study in the rabbit, that obtain a CV of 2.19%.

One study carried out [29] with the same hematology equipment (MEK-6318, Nihon Kohden iberica S.L., Madrid, Spain) but using human blood, established maximum coefficients of variation for red blood cells, hemoglobin, hematocrit, white blood cells and platelets of 3.2%, 3.8%, 3.6%, 9.3%, and 10.8% respectively. Our study the results are significantly lower, obtaining values more acceptable, repetitive and precise.

The implementation of the “Good Laboratory Practice” has improved the analytical quality of our laboratory, obtaining values of coefficient of variation in compliance with acceptable criteria.

The simplicity of the procedure allows the establishment of these parameters, precision and repeatability, in laboratories requiring quality studies. The degree of dispersion and the degree of concordance of our hematological results are good and reproducible, which is of a great value providing accuracy in preclinical studies results.

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