

# Comparative Study of Rabbit Pyrogen Test and Human whole Blood Assay on Human Serum Albumin

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## Summary

*A comparative study of rabbit pyrogen test and human whole blood assay was performed on released preparations of human serum albumin. In addition, the samples were spiked with 5 IU/ml (in whole blood 0.5 IU/ml too) and 10 IU endotoxin/ml. The unspiked samples were negative in both assays.*

*The human whole blood test resulted in the same level of security for the products as the rabbit pyrogen test did. Both, the borderline 5 IU/kg and the 10 IU/kg-Spike partially lead to results of the rabbit test which would cause further testing with additional animals. In contrast, the human whole blood assay resulted in a 100% detection for the 5 IU/ml and 10 IU/ml-Spike. We designed a study protocol for a minimised number of test animals and were able to show the general usefulness of the human whole blood assay.*

**Zusammenfassung:** Vergleichsstudie von Kaninchen-Pyrogentest und humanem *in vitro* Pyrogentest am Beispiel von Albuminpräparaten

*In einer Vergleichsstudie wurden Albuminpräparate verschiedener Hersteller im Kaninchen-Pyrogentest und im humanen Vollbluttest miteinander verglichen. Zusätzlich wurden die Produkte mit 5 IU (Vollblut zusätzlich 0.5 IU) bzw. 10 IU LPS/ml dotiert. Die undotierten Chargen waren sämtlich negativ.*

*Der Vollbluttest ergab für die getesteten Albuminpräparate die gleiche Sicherheit wie der Kaninchen-Pyrogentest. Sowohl bei der grenzwertigen 5 IU/kg als auch bei der 10 IU/kg-Dotierung kam es im Kaninchen-Pyrogentest teilweise zu Ergebnissen, welche eine Wiederholungsprüfung mit weiteren Versuchstieren notwendig gemacht hätte. Im Gegensatz zum Kaninchen-Pyrogentest lieferte der Vollbluttest eindeutige Ergebnisse für 5 IU sowie 10 IU/ml. Mit dem auf einen möglichst geringen Tierversuchsausgleich Versuch konnte die grundsätzliche Eignung des Vollbluttests als sichere Alternative zum Kaninchen-Pyrogentest bestätigt werden.*

**Keywords:** comparative study, human whole blood assay, rabbit pyrogen test, albumin

## 1 Introduction

The screening for pyrogenic contaminations (pyrogens) is a crucial step in the quality control of pharmaceuticals. Since its introduction in 1942 (United States Pharmacopoeia), the rabbit pyrogen test became the golden standard of pyrogen testing and has established a high level of security in pharmaceutical products. The sensitivity of man and rabbit for pyrogens is comparable.

After intravenous injection of the test substance into a group of three rabbits, the individual changes in body temperatures are recorded over a defined period of time. In general, the decision whether a substance passes, fails or may be re-tested is based on the total of temperature rises in a group of animals. The rabbit pyrogen test detects any pyrogen which

causes a temperature rise (in rabbits). Rare pyrogenic reactions of patients under treatment with rabbit-tested pharmaceuticals and experimental data (e.g. with human immunoglobulin preparations) indicate species-specific differences between man and rabbit.

Endotoxin of Gram-negative bacteria is the most famous pyrogen. The Limulus-Amebocyte-Lysate test (LAL) was developed and established as an *in vitro* alternative to the rabbit pyrogen test.

The LAL is highly specific for endotoxin, but fails to detect non-endotoxin pyrogens. The specific detection of endotoxin by LAL may be performed in a qualitative or quantitative manner, depending on the LAL-method chosen. The development of quantitative LAL-assays made it possible to define and to control endotoxin threshold-limits for pharma-

ceuticals (depending on the application route (e.g. subcutaneous, intravenous, intrathecal...)). Although the LAL has replaced the rabbit pyrogen test in most cases, it has to be pointed out that the LAL is not a pyrogen test. The LAL is an endotoxin test. The national and international Pharmacopoeias clearly distinguish between pyrogen tests and endotoxin tests. In comparison to the rabbit pyrogen test, the LAL-readout reveals no information on the biological impact of a positively tested substance.

Many biologicals like blood products interfere with the LAL, and still have to be tested with the rabbit pyrogen test. Because most of these biologicals are immunogenic, test animals can only be used once (in general, re-use of test rabbits after a defined safety period is in agreement with different pharma-

copeias). Therefore users of the rabbit pyrogen test are in a dilemma: due to the high quality in pharmaceutical production pyrogenic contaminations are rare, nevertheless the obligatory rabbit pyrogen test causes a high consumption of animals.

The human whole blood assay was developed as a real *in vitro* alternative to the rabbit pyrogen test. The basic idea was to mimic the fever reaction. In general, the detection of exogenous pyrogens (e.g. endotoxin) by blood cells causes them to release endogenous pyrogens like IL-1 $\beta$ , IL-6 and TNF $\alpha$ . These cytokines affect the thermal regulation centre in the brain and increase the body temperature by changing its set point. The test principle is based on the detection of IL-1 $\beta$  (ELISA), which was released by human blood cells after contact with pyrogens. Like the rabbit pyrogen test the human whole blood assay reveals the biological impact of pyrogenic contaminations. The predictability of the human whole blood assay is high. It is a physiological test system without species-specific differences to man.

Comparative studies between animal experiments and new test methods are necessary for the replacement of animal experiments. The general usefulness of the human whole blood assay was shown in a first project supported by the BMBF (German ministry of education and research: "Evaluation and pre-validation of a whole blood model for the replacement of the rabbit pyrogen test", 1.7.97 – 30.6.00). The study on albumin presented here was part of this first project. Earlier data on different materials were already published in *ALTEX* 15 Suppl. 98 (Fischer, M. et al., 1998; Hartung, T. et al., 1998). Meanwhile, validation of the human whole blood assay is performed within a follow-up BMBF-project ("Replacement of the rabbit pyrogen test by a whole blood assay", 1.10.00 – 30.9.03), by testing product groups like factor VIII. In parallel, an EU-project, ("Comparison and validation of nivel pyrogen tests based on the human fever reaction", 1.1.00 – 31.12.02) was started in which the human whole blood assay as well as other alternative *in vitro* pyrogen tests are compared in a Europe wide double-blinded study.

## 2 Animals and methods

29 batches of human albumin preparations (5%, 20%, 25%; with one exception three batches of each product; all available on the German market) of five companies were tested in parallel in the rabbit pyrogen test and in the human whole blood assay. The pure products were tested in the PEI as routine batch release. The tests with spiked albumin preparations were done as officially approved animal experiment. Chinchilla bastards (Charles River, Kisslegg, SPF-animals) were used for the rabbit pyrogen test. The albumin preparations were additionally spiked with 5 IU and 10 IU/ml endotoxin (Control Standard endotoxin *E. coli* O113, Pyroquant, Walldorf; 1 IU = 100 pg). The spiked preparations were applied with 1 ml/kg body weight (resulting in doses of 5 IU and 10 IU/kg). The 5 IU/kg dose (1ml containing 5 IU per kg) is at the detection limit of the most sensitive rabbits (defined as dose at which 50% of animals show a defined rise in temperature). Depending on the preparation (and its monographs in the different pharmacopoeias) a maximal volume of 10 ml/kg body weight can be applied. Taken into account the detection limit of 5 IU/kg, under optimal test conditions (injection of 10 ml test solution per kg) the most sensitive rabbits can detect 0.5 IU endotoxin/ml (0.5 IU per ml in 10 ml results in a dose of 5 IU/kg). The detection limit depends on the endotoxin used and the rabbit species. For the whole blood assay, the 5 IU/ml-samples were additionally pre-diluted 1:10 to achieve the rabbit-test limit concentration of 0.5 IU/ml. Therefore, 0 IU/kg (batch release), 5 IU/kg and 10 IU/kg were tested in the rabbit pyrogen test, whereas 0 IU/ml, 0.5 IU/ml, 5 IU/ml and 10 IU/ml were tested in the human whole blood assay. The human whole blood assay was performed as described previously (Hartung, T. and Wendel, A., 1995).

100  $\mu$ l heparinised blood of two healthy donors (two exceptions: one experiment one donor, one experiment 5 donors) was added to 1000  $\mu$ l clinical saline and 100  $\mu$ l sample, and incubated at 37°C for 20 h. After incubation, samples were mixed and centrifuged, IL-1 $\beta$

in the supernatant was detected by ELISA. The test kit used was the Pyrocheck-Kit (Milenia Biotech), which is now distributed in a modified version by Charles River Endosafe (Kisslegg, Germany) as "*in vitro* pyrogen test (IPT)". The test result was only evaluable if the blood of the given donor was capable to detect the 50 pg/ml endotoxin-control (resembling the sensitivity of the most sensitive rabbits). Samples were classified "positive" if the resulting OD in the ELISA exceeded the individual cut-off of the given donor.

## 3 Results

All pure albumin preparations were negative in both assays (Tab. 1), resembling the high quality of pharmaceutical production. The spike of 5 IU/kg was clearly found by the rabbit pyrogen test in five batches, 23 batches resulted in a temperature rise which would have allowed repetitive testing (each batch three animals = 23 x 3), one batch was tested negative. The same samples (5 IU/ml; test volume 100  $\mu$ l resulting in 0.5 IU per assay) were all tested positive in the human whole blood assay (29 batches positive). For the 1:10 pre-dilution of the 5 IU/ml spike (resulting in 0.05 IU per assay, only performed in the whole blood assay), 18 batches were positive, 11 were negative. The dose of 10 IU/kg was detected by the rabbit pyrogen test in 21 batches, 8 would have allowed repetition (8 x 3 animals), no batch was tested negative. All batches spiked with 10 IU/ml were found positive in the human whole blood assay.

## 4 Discussion

The rabbit pyrogen test has remarkable merits in drug safety. Nevertheless, in the sense of animal protection it should be replaced as far as possible by an appropriate *in vitro* method which guarantees the same level of product safety. New therapeutic strategies like genetically engineered human cells enforce the development of new pyrogen tests, as they can not be tested in the rabbit pyrogen test or the LAL-test. The LAL-assay

**Tab. 1: Comparison of rabbit pyrogen test and human whole blood assay**

endotoxin		rabbit pyrogen test			human whole blood assay	
rabbit IU per kg	human whole blood assay IU per assay	positive > 2.65°C	repetition > 1.15°C ≤ 2.65°C	negative ≤ 1.15°C	positive	negative
0	0	0	0	29	0	29
	0.05	not done	not done	not done	18	11
5	0.5	5	23	1	29	0
10	1	21	8	0	29	0

29 different batches of albumin were tested pure and spiked with 5 IU/ml and 10 IU/ml (the 5 IU/ml samples were additionally prediluted 1:10 for the *in vitro* pyrogen test, to achieve a test solution at the sensitivity limit of the rabbit pyrogen test of 0.5 IU/ml).

is a highly specific endotoxin test, therefore it is not a replacement for the rabbit pyrogen test. Within the last years it has become obvious that there are several non-endotoxin pyrogens (Lipoteichoic acids, lipoproteins, bacterial DNA etc.), which further emphasise the need for an *in vitro* pyrogen test. Nevertheless, we employed only LPS (Control Standard Endotoxin) in this study, as it is the only available and accepted pyrogenic standard material. In the meantime, we have access to purified pyrogens from Gram-positive bacteria and are evaluating the human whole blood assay with these substances.

In this study, the rabbit pyrogen test and the human whole blood assay were compared on albumin preparations. The animal experiments were inevitable, as we had to compare the sensitivity of both test systems with defined samples. A wide comparison of marketed albumin preparations like in this study would not have been possible with the rare pyrogenic batches which are found during quality control. Albumin belongs to those substances which still are tested in the rabbit pyrogen test. Human albumin is immunogenic, repetitive tests have to be done with new animals, which causes further animal consumption.

Comparing the two test systems, one has to consider the volume to inject according to the regulations. The endo-

toxin concentrations used were 5 IU/ml and 10 IU/ml, respectively. The rabbits received 1 ml per kg body weight, in the blood assay 100 µl each were applied. According to the European Pharmacopoeia, low concentrated therapeutical albumin (3.5 – 5%) has to be applied in a volume of 10 ml per kg body weight. As a consequence, in these cases for example 5 IU endotoxin per ml (concentration in the drug) in the rabbit pyrogen test have to be compared with 0.5 IU endotoxin per ml (5 IU in 10 ml = 0.5 IU per ml) in the human whole blood assay. Testing albumin spiked with 0.5 IU endotoxin per ml in the human whole blood assay (0.05 IU per assay) most of our experiments were positive but we saw uncertain and negative results too. Therefore, the sensitivity of the new test system is comparable to the rabbit's sensitivity assuming even worst case conditions. If necessary, the sensitivity of the test can be improved by increasing the sample volume.

On the other hand, most of the drugs to be tested in the rabbit pyrogen test have to be injected in smaller volumes. Therefore, in practice the new test would produce more sensitive results in most of the cases. An interesting example represents coagulation factor VIII, which we are currently testing in a similar study. In order to obtain a comparable sensitivity of both tests, one would have to inject 10

ml of factor VIII per kg body weight (approximately 30 ml per rabbit, 90 ml per test). Taking into account the market price of factor VIII (1000 IU per container) the drug value would be around 4,500 per batch. The same safety level would be achieved using 100 µl in the human whole blood assay.

## References

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