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Characterisation of the rat lung lavage model as biological assay for testing the activity of surfactant preparations

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Abstract

The present report describes how pharmacological assays may be validated and sets a basis for a discussion on the validation of biological test systems. The note for guidance on the validation of analytical procedures published by the European agency for the evaluation of medicinal products was adapted to the validation of a pharmacological test system. The presently described rat lung lavage test (RLL-test) is an animal model that has great similarities to the pathophysiology of the acute respiratory distress syndrome of humans. In this RLL-test, the activity of surfactants can be tested in a standardised fashion. The usefulness of the point estimator and the corresponding confidence intervals (CI) as a statistical test procedure for equivalence was demonstrated. A validation can be based on the above mentioned guidance but should be adjusted to pharmacological needs. Based on the presented experiences, it can be concluded that a specific guideline for validation of pharmacological or biological tests is desirable.

Keywords: Validation; Biological assay; Pharmacological assay; Point estimator

1. Introduction

For the development and approval of a new surfactant preparation, a biological functional test was developed and validated. The importance of validated bioanalytical methods to investigate potentially active new drugs in the pharmaceutical development has been discussed by Braggio et al. [1]. The authors described a sequential design for validation of methods for pharmacokinetic analysis or other methods which serve for analysis of new drugs. There is further literature available that deals with this problem [2–4]. Information on the validation of pharmacological or biological test systems for drug discovery or development is scarce. Since there are only few published reports on the validation of biological tests, mostly deal-

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ing with bioanalytical methods, and due to the lack of specific guidelines for the problem, this paper describes a procedure for validation of such an assay. As a guideline, we used the note for guidance on validation of analytical procedures [5] and the parent guideline [6]. The latter deals with the definitions of terms and the first describes the methodology. Both guidelines were not originally intended for pharmacological assays. Some requirements may yet be interpreted for use as they fit partially to the validation of pharmacological test systems. The guideline was adapted to the validation of our pharmacological test system and a validation plan was written, accordingly. This report demonstrates how such assays may be validated and sets a basis for discussion on validation of biological test systems. Furthermore, this article intends to describe how in the case of biological active drugs, pharmacological assays can be used for bioanalytical analysis, i.e. to show that the integrity of such drugs can be characterised in appropriate assays.

The presently described rat lung lavage test (RLL-test) is an animal model that has great similarities to the pathophysiology of the acute respiratory distress syndrome [7,8] in humans. In several pilot studies, it was shown that treatment with surfactants is an effective treatment strategy in patients who suffer from ARDS [9–11]. In the RLL-test, the activity of surfactants can be tested in a standardised fashion [12]. The specific aims of this report are to describe the validation of this RLL-test as an assay for the determination of: (1) the activity/potency of lung surfactant factor preparations; and (2) the biological integrity of such surfactant preparations.

2. Methods

2.1. Animals and experimental methods

Male Sprague Dawley rats with a body weight (b.w.) of 230–295 g were used. Further specifications regarding animal numbers will be mentioned in the appropriate sections. The animals were supplied by HARLAN Winkelmann, D-33176 Borchen, Germany.

2.1.1. Anaesthesia and preparation

The anaesthetic and surgical methods were the same as previously described [7,13]. Briefly, after the introduction of inhalational anaesthesia, a catheter was placed into one carotid artery. Thereafter, the animals received an intraperitoneal (i.p.) injection of pentobarbitone (stock solution: 60 mg ml^{-1}; 1 ml kg^{-1} b.w.). After tracheotomy, a tube was secured into the trachea of each animal. The animals received an i.m. injection of pancuronium bromide (1 ml kg^{-1} b.w., concentration of the solution 2 mg ml^{-1}) and ventilation was started using a Servo Ventilator (900°C, SIEMENS-ELEMA, Solna, Sweden). The tracheal tubes of six animals were connected to a distributor and animals were ventilated simultaneously at a respiratory rate of 30 breaths min^{-1}, a fraction of inspired oxygen (FiO_2) of 1.0, an inspiration: expiration ratio of 1:2 and a peak inspiratory pressure (PIP) of 15 cm H_2O which included a positive end-expiratory pressure (PEEP) of 2 cm H_2O. The pressures which were generated by the ventilator were monitored continuously with a pressure transducer which was integrated in the respiratory circuit connected to a recorder (linearrecorder Mark VII WR3101, Watanabe, Herrsching, Germany) which contained an integrated amplifier. Additional pentobarbitone (i.p., 0.25 ml kg^{-1} of the stock solution) and pancuronium bromide (i.m., 1 ml kg^{-1} b.w.) were given as needed.

2.1.2. Experimental design

The reported variable is the partial arterial oxygen pressure (PaO_2) at 180 min after the last lavage (which is equivalent to 120 min after treatment). This time was chosen because in preliminary experiments, some surfactants showed decreasing activity towards this time and a good quality surfactant should show stable activity. Blood gas analysis was performed with a blood gas analyser (Radiometer Copenhagen ABL 500, Radiometer Deutschland GmbH, Willich, Germany). This blood gas analyser was checked weekly for quality of the analysis by quality checks provided by the supplier. After the determination of pre-treatment values, only animals with PaO_2 values of more than 480 mm Hg were

included in the experiments. Peak inspiration pressure (PIP) was raised to 28 cm H_2O and PEEP to 8 cm H_2O and the animals were subjected to multiple lung lavage (6–8 times) with 1 ml per 30 g b.w. of isotonic saline solution. Only those animals which had PaO_2 values between 50 and 110 mm Hg were included in the study. Blood gases were determined at 5, 30, and 60 min after the last lavage. The surfactants were instilled 1 h after the last lavage as described [7,13]. Untreated controls received sham treatment with air. Subsequently, 30, 60, 90, 120 and 150 min after surfactant instillation (equivalent to 90, 120, 150, 180 and 210 min after the last lavage) blood gases were determined. During the whole experimental period, the PIP and PEEP was kept constant at 28 and 8 cm H_2O, respectively.

2.2. Test substances and administration regimen

bLES (bovine lipid extract surfactant, (BLES Biochemicals, London ON, Canada; batch no. 960219) is a phospholipid-fraction from cow lungs obtained by lavage. Each vial contains 5 ml of a suspension that is ready to use. The PL profile was determined by Yu et al. [14] using gas liquid chromatography. According to Yu et al. [14], the PL profile of bLES® was: $79 \pm 1.6\%$ phosphatidylcholine, $1.5 \pm 0.4\%$ lyso-bis-phosphatidic acid, $11.3 \pm 0.5\%$ phosphatidylglycerol, $1.8 \pm 0.3\%$ phosphatidylinositol, $3.5 \pm 0.5\%$ phosphatidylethanolamine and $2.6 \pm 0.5\%$ sphingomyelin. The protein content was determined by the Lowry procedure and, therefore, only the total protein content can be cited. It was $0.97 \pm 0.07\%$ related to the total amount of PL. Due to the used method, no specifications regarding the amount of surfactant protein B or C could be given. The total PL amount is 27 mg PL per ml. bLES was chosen as a reference surfactant because it was shown to be a surfactant with superior activity to, e.g. Survanta [15] and other surfactants [16], and bLES was shown to be an active surfactant in a pilot study with children who suffered from ARDS [11]. Furthermore, bLES resulted in excellent outcome in this pilot study [11].

rSP-C surfactant (BYK Gulden, Konstanz, Germany; batch no. EB 454) contains 2% recombinant surfactant protein C (= rSP-C, w/w related to PL) associated with two PL (dipalmitoylphosphatidylcholine [= DPPC] and palmitoyloleoylphosphatidylglycerol [= POPG]) at a ratio of 70:30 plus 5% (w/w related to PL) palmitic acid (PA).

rSP-C surfactant (0.5) (BYK Gulden, Konstanz, Germany; batch no. EB 572) contains 0.5% recombinant surfactant protein C (= rSP-C, w/w related to PL) associated with the same PL and PA content as the 2% rSP-C containing rSP-C surfactant.

2.2.1. Dosage

Due to the complex PL profile of bLES, all doses are given with respect to the total amount of PL. The surfactant preparations were given intratracheally (i.t.) using doses of 12.5, 25, 50, 100 and 200 mg PL per kg b.w. of rSP-C surfactant and doses of 25, 50, and 100 mg PL per kg b.w. of bLES. The preparations were diluted with 0.9% NaCl-solution to achieve concentrations of 6.25, 12.5 and 25 mg PL per 1.2 ml. Controls did not receive any instillation and were ventilated, following the same scheme as the treated animals during the whole experimental period.

2.2.2. Mode and volume of administration

All surfactants were instilled i.t. as bolus as previously described [7,13]. The standard application volume is 1.2 ml per 250 g animal. In the case of testing, the influence of application volumes, two different volumes were used (half the standard volume and twice the standard volume).

2.3. Statistical analysis

Before starting the experiments, a validation plan was written in accordance with the note for guidance on validation of analytical procedures [5]. The RLL-test was performed following a written test procedure and standard operation procedures (SOP).

2.3.1. Evaluation of activity and linearity

It should be noted that deviating from the cited guidance [5] in the following, we refer to a more general definition of linearity, i.e. that a test substance should display dose dependence. Monotone dose dependency was tested by the Jonckheere–Terpstra test ($\alpha = 5\%$). In the case of a significant test result, many-to-one comparisons were conducted according to the sequentially rejecting Jonckheere-Terpstra test [17]. The different application volumes were tested for differences by the Kruskal–Wallis test.

2.3.2. Evaluation of similarity

Similar activity between the two highest doses of rSP-C surfactant (50 and 100 mg PL per kg b.w.) and bLES (50 and 100 mg PL per kg b.w.) was assessed using the lower limits of the one-sided nonparametric 95% confidence intervals (CI) for the differences of the population medians for rSP-C surfactant and bLES. No relevant inferiority, i.e. at least equivalence, was concluded if the corresponding lower limit was not smaller than a biologically relevant difference (-15% of the median of bLES). Similar activity was assumed if for both comparisons at least equivalence was shown. Due to the intersection–union principle [18] no α-adjustment was needed.

2.3.3. Distinction of two rSP-C concentrations

The statistical test of the null hypothesis of no difference was performed by the one-sided Wilcoxon test at the 5% level.

2.3.4. Robustness and repeatability

The hypothesis of no relevant influence of: (1) administration volume; (2) days on which the assay is performed; (3) supply of animals at different times; and (4) technicians was assessed by the lower and upper limits of the two-sided nonparametric 90% CI [19]. A biologically relevant difference was assumed beyond $\pm 20\%$ of the lowest median of the groups that were compared.

2.3.5. System suitability testing

The statistical test of the null hypothesis of no difference was performed by the Kruskal-Wallis test at level 5%.

3. Results

3.1. General remarks

Due to a priori determined inclusion and exclusion criteria, the intended number of animals was not always reached. Furthermore, due to mortality, not all treated animals could be included for statistical analysis at the time 120 min after treatment. The results are presented according to the different sections of the note for guidance on validation of analytical procedures [5] which was used as the basis of the validation plan.

3.2. Activity

To assess the feasibility of the RLL-test as an assay for the determination of the efficacy of surfactants, the commercially available bovine derived surfactant preparation bLES was used. The effects of bLES were compared to a surfactant preparation (rSP-C surfactant, batch EB 454, BYK Gulden, Konstanz, Germany) containing recombinant surfactant protein C (rSP-C; content of rSP-C: 2%) and PL. The results are shown in Table 1. They represent the PaO_2 values at 120 min after surfactant treatment. The RLL-test can be accepted as suitable to determine the activity of surfactant, if bLES could be shown to be an active surfactant. The intended reference rSP-C surfactant was used to show that this surfactant preparation is equally active to the commercially available bovine derived surfactant bLES. For this purpose, both surfactant preparations were tested with an intended number of $N = 12$ animals per dose at three dose levels (25, 50 and 100 mg PL per kg b.w.). This investigation was done to show the equivalent biological activity of rSP-C surfactant in comparison to bLES (bovine natural surfactant). In addition, an untreated control group was used to show that ventilation only, without surfactant treatment, has no influence on the reduced PaO_2 after lavage in this assay. In the case of equal activity between rSP-C surfactant and bLES, all further validation steps could be performed with the rSP-C surfactant (same batch EB 454) as an internal reference.

Both surfactant preparations were able to improve the PaO_2 values compared to untreated controls (Table 1). All doses of bLES led to higher PaO_2 values than untreated controls resulting in a clear proof of activity. Therefore, the assay is able to determine the activity of surfactant preparations. To test for no relevant inferiority, the lower limits of the one-sided nonparametric 95% CI for the differences of the medians for rSP-C surfactant and bLES were compared. No relevant inferiority, i.e. at least equivalence, can be concluded if the corresponding lower limit is not smaller by a biologically relevant difference which is assumed to be 15% of the lowest median of the two groups treated with bLES (50 and 100 mg PL per kg). This difference between bLES as reference and rSP-C surfactant was $\sim -4\%$ with respect to the dose of 100 mg PL per kg. With regard to the dose of 50 mg PL per kg, this difference was $\sim -12\%$. Therefore, similar activity was assumed because for both comparisons at least equivalence was shown. Further investigations were performed with rSP-C surfactant as an internal reference.

Tests were performed to check whether different application volumes have an influence on the activity of surfactants. For this purpose, two doses of rSP-C surfactant (50 and 100 mg PL per kg b.w.) were compared using two different application volumes. The volumes were half and twice the standard application volume of 1.2 ml per 250 g animal. It was intended to use $N = 12$ animals per dose and concentration. The results obtained with the standard volume were taken from the above described comparison to bLES. At a dose of 50 mg PL per kg b.w., three different concentrations of 6.25, 12.5 and 25 mg/1.2 ml were compared. In the case of the dose of 100 mg PL per kg b.w., the concentrations were 12.5, 25 and 50 mg per 1.2 ml. The results are given in Table 2.

At a dose of 100 mg PL per kg, there were no differences detectable between the PaO_2 values after all three application volumes ($P = 0.329$, Kruskal–Wallis test). However, the high application volume resulted in a larger range and a greater SD than the low and standard volumes. For this dose of PL, all three comparisons were within a $\pm 20\%$ range of the lowest median (Table 2) of the three compared groups (Fig. 1). This shows that a dose of 100 mg PL is not sensitive against different application volumes; when the medians and ranges are compared, a difference appears making the high volume less appropriate for further tests.

At a dose of 50 mg PL per kg, the high application volume led to higher PaO_2 values than both low volumes. The median after administering the high-volume was statistically significantly higher than the median values following the administration of the low and standard volume with

Table 1
PaO_2 values at 120 min after treatment with bLES or rSP-C surfactant[a]

Dose (mg kg^{-1})	bLES			rSP-C surfactant		
	Median (range)	Mean ± SD	N	Median (range)	Mean ± SD	N
12.5	n.t.	n.t.	—	142 (73; 363)	198 ± 114	12
25	412 (76; 532)	373 ± 138	12	359 (116; 525)	318 ± 120	12
50	466 (276; 557)	441 ± 88	10	440 (321; 526)	443 ± 58	11
100	458 (411; 551)	467 ± 43	10	496 (406; 535)	480 ± 43	11
200	n.t.	n.t.	—	493 (378; 586)	491 ± 49	12

PaO_2 values at 120 min of untreated controls. The PaO_2 values are given as median and range as well as mean ± SD

	Median (range)	Mean ± SD	N
Controls	65 (48; 101)	69 ± 18	11

[a] The doses are given in mg phospholipids per kg b.w. PaO_2 values are given for each dose as median and range as well as mean ± SD; n.t., not tested.

Table 2
PaO_2 values at 120 min after treatment with rSP-C surfactant at different application volumes[a]

Dose (mg kg^{-1})	Application volume (ml)	Median (range)	Mean ± SD	N
50	0.6	420 (293; 497)	409 ± 56	12
50	1.2	440 (321; 526)	443 ± 58	11
50	2.4	520 (235; 561)	494 ± 85	12
100	0.6	486 (347; 534)	479 ± 49	12
100	1.2	496 (406; 535)	480 ± 43	11
100	2.4	505 (33; 534)	470 ± 139	12

[a] The results for the volume of 1.2 ml are taken from Table 1 for both doses. The doses are given in mg phospholipids per kg b.w. PaO_2 values are given for each dose as median and range as well as mean ± SD.

$P \leq 0.05$ (Kruskal–Wallis test). Between low and standard volume values, no differences were detectable. PaO_2 values based on the high-volume had a larger range and a greater SD than PaO_2 values based on both low volumes. This effect was also obvious when looking at the lower and upper 90% CI (Fig. 1). Only the comparison for the two low-volume values was within the ± 20% range according to the procedure described above. The comparison for the 0.6 versus the 2.4 ml per animal volume was clearly above this range. The comparison for the 1.2 versus the 2.4 ml per animal volume was also above this range but only with the upper confidence limit (24%). Therefore, it can be concluded that the 50 mg kg^{-1} dose is sensitive to high application volumes, i.e. low concentrations of this dose may lead to a larger range of values and may interfere with the reproducibility.

3.3. Linearity

As mentioned above, deviating from the cited guidance [5] in the following, we refer to a more general definition of linearity, i.e. that a test substance should display dose dependence. Therefore, tests for dose-dependence were performed using the results mentioned in Section 3.1 and testing two additional doses of rSP-C surfactant to get a total of five doses (Table 1). rSP-C surfactant and bLES exhibited increasing monotone dose-dependence. The monotone increase for rSP-C surfactant was highly significant ($P \leq 0.001$, Jonckheere Terpstra Test) when using all five doses. The three doses of bLES exhibited also a monotone increasing dose dependence, but the P-value was slightly above the significance level of 0.05 (Jonckheere Terpstra test).

In addition to the dose response curve, a batch containing only 0.5% rSP-C (EB 572) was tested with an intended animal number of $N = 12$ animals at a dose of 50 and 100 mg PL per kg b.w. to show the ability of the RLL-test to discriminate between two different rSP-C concentrations. These results are presented in Table 3. Based on both doses of PL, the amount of 2% rSP-C resulted in higher PaO_2 values than the low concentration of 0.5%. This difference was statistically significant for comparisons based on both doses ($P \leq 0.05$, Wilcoxon test, one-sided). Thus, the RLL-test proved its ability to differentiate between different amounts of rSP-C at equal doses of PL.

3.4. Precision (repeatability)

For repeatability tests, six determinations with $N = 12$ animals at a dose of 50 mg PL per kg b.w. were planned. Each of the two technicians performed one complete determination on the same day and repeated this procedure on another 2 days. These results are presented in Table 4.

The repeatability was excellent for technician A when comparing all 3 days (Fig. 2). All CI's were in the range ± 20% of the lowest median of the three groups (Days 1, 2 and 3). The point estimator (expressed as a percentage, Fig. 2, Panel B) was around the ideal value of 0%. The results of technician B (Fig. 2) also showed excellent repeatability. On all 3 days, the 90% CI for the

comparisons of the different days were in the range of ± 20% of the lowest median of the three groups (Days 1, 2 and 3). Again, the point estimator (expressed as a percentage, Fig. 2, Panel B) was around the ideal value of 0%.

Furthermore, the comparison of the results between both technicians showed an excellent equivalence between both technicians (Fig. 3) with respect to all 3 days. The point estimator (expressed as a percentage, Fig. 3, Panel B) was around the ideal value of 0%. The 90% CI of all 3 days for the comparison of technician A versus B was within the ± 20% range of the lowest median of the three groups (Days 1, 2 and 3).

3.5. Robustness

The robustness of the RLL-test was shown by the reliability of the assay with respect to deliberate variations in the method parameters. These parameters are: (1) days on which the assay is performed (Section 3.3); (2) influence of techni-

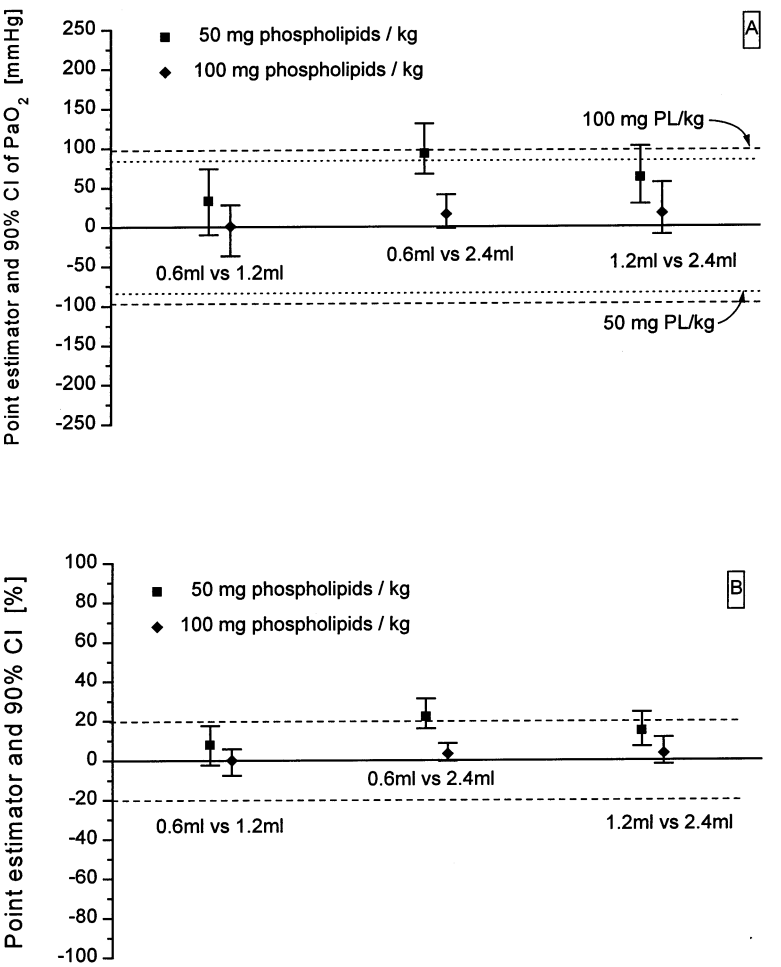

Fig. 1. Point estimators and two-sided 90% CI calculated for the comparison of the different application volumes based on each of the two doses (50 and 100 mg phospholipid per kg b.w.; $N = 11–12$ animals per dose and application volume). Panel A shows the comparison with respect to physiological units (partial arterial oxygen pressure (PaO_2), mm Hg). Values are given for the comparison of the different application volumes of 0.6 versus 1.2 ml per animal, 0.6 versus 2.4 ml per animal and 1.2 versus 2.4 ml per animal. The ranges are based on the ± 20% value of the lowest median for each of the dose. Panel B shows the corresponding values of Panel A in relative units (%), accordingly.

Table 3
PaO_2 values at 120 min after treatment with rSP-C surfactant that contains either 0.5 or 2.0% rSP-C[a]

Dose (mg kg^{-1})	rSP-C surfactant (0.5% rSP-C)			rSP-C surfactant (2.0% rSP-C)		
	Median (range)	Mean ± SD	N	Median (range)	Mean ± SD	N
50	381 (71; 480)	338 ± 145	10	440 (321; 526)	443 ± 58	11
100	424 (66; 513)	336 ± 177	10	496 (406; 535)	480 ± 43	11

[a] The doses are given in mg phospholipids per kg b.w. PaO_2 values are given for each dose as median and range as well as mean ± SD.

cians (Section 3.3); and (3) supply of animals at different times. These parameters were tested at a standard dose of 50 mg PL per kg b.w. of the above mentioned batch EB 454 of rSP-C surfactant. The influence of the two technicians was investigated during the repeatability tests, by conducting per technician $N = 12$ animals per day and by repeating this procedure two times (Table 4). Also, with this last investigation, the influence of days on which the assay was performed was investigated.

The influence of the supply of animals at different times was investigated by testing three different animal supplies over 3 weeks with an intended number of $N = 12$ animals. The results are presented in Table 5. Based on the lower and upper 90% CI, there were no differences between the animal supply of week 1 and 2 (Fig. 4). The lower and upper confidence limits were -9.1 and 5.4%, respectively. For the comparison of the supply of week 1 and 3, the lower and upper confidence limits were 2.2 and 14.5%, respectively. These limits were 3.0 and 16.8%, respectively for the comparison of week 2 with week 3. Based on these results, a good equivalence for the three deliveries can be concluded because the differences of all 3 weeks were in the range ± 20% of the lowest median of the three groups (Weeks 1, 2 and 3; Table 5).

3.6. System suitability testing (biological integrity)

Evaluation of this part was done by investigating the above mentioned standard batch EB 454 of rSP-C surfactant after ultrasound inactivation. The purpose of this investigation was to show that despite all surfactant components are still included in the surfactant, the surfactant was inactive or less active after sonification. This procedure reflects that with this assay the correct biophysical conformation (biological integrity) of surfactant can be corroborated. The test was performed at a standard dose of 50 mg PL per kg b.w. and an intended animal number of $N = 12$ animals. The results are presented in Table 6 and are compared to the results of the 50 mg kg^{-1} dose achieved during the tests for activity (Section 3.1.) and to the untreated controls. The rSP-C surfactant was resuspended and the suspension was treated for 10 min with ultrasound (sonifier 250, Branson; the following settings were used: output control: 4, duty cycle: continuous). After sonification, the activity of the rSP-C surfactant vanished. The difference to the active rSP-C surfactant was highly significant ($P \leq 0.001$, Kruskal Wallis test). Furthermore, there was no statistically significant difference to untreated controls.

4. Discussion

To our knowledge, this is the first report on the validation of a pharmacological test system. The presently described validation can serve as a practical example for validation of biological assays. The note for guidance on validation of analytical procedures [5] was adapted for this validation. The specific experience with this note will be discussed in the following. Therefore, each part of the note for guidance will be used to assess the practicability of each step for validation of biological/pharmacological test systems.

4.1. Activity

Based on the investigations with bLES, it was concluded that this RLL-test is able to characterise the activity of surfactant preparations. Furthermore, the tests of similarity with the rSP-C surfactant (Batch No. EB 454) showed equivalence to the standard surfactant bLES. Due to this equivalence, all further validation steps were performed with rSP-C surfactant as an internal reference standard.

To investigate the sensitivity of this test against the use of different application volumes, three application volumes were chosen and the influence was tested for two different doses (50 and 100 mg PL per kg b.w.). The dose of 50 mg PL per kg was more sensitive against different application volumes than the dose of 100 mg PL (Fig. 1). Therefore, tests with a dose of 50 mg PL should be performed with an application volume of 1.2 or 0.6 ml per animal. With regard to this dose, it can be concluded that volumes above 1.2 ml per animal may lead to irregular results because the response after the high volume led to PaO_2 values comparable to the values of the higher dose. The dose of 100 mg PL per kg was not sensitive to the use of different application volumes. Therefore, the testing of this dose does not require a specific application volume. However it is recommended to use the standard application volume of 1.2 ml per animal in order to minimise variation.

The activity of a new drug in an assay should be shown by either comparing it to a clinically used effective treatment or by the clear superiority of the new drug above sham treatment if no proven treatment is available. During tests for activity, factors that may influence the test results should be studied. In the case of surfactant treatment, the influence of different application volumes on the results was tested because the distribution of surfactant is supposed to be dependent on the application volume [20] and, therefore, the activity of surfactants may differ in the RLL-test.

4.2. Specificity

The presently validated RLL-test was only intended to be used for the determination of the activity, potency and biological integrity of surfactants. As the above mentioned guideline states: '… investigation of specificity should be conducted during the validation of identification tests, the determination of impurities and the assay', there were no specificity tests conducted for the validation of the RLL-test with regard to the meaning of this statement because: (1) the RLL-test does not discriminate between surfactant protein containing surfactant preparations of different composition and equal activity; (2) the RLL-test is not capable to identify impurities; and (3) the identification and purity of the ingredients of the surfactant is performed by more subtle and specific chemical or/and biochemical analysis, that will show the content of the PL, the rSP-C and impurities in the rSP-C surfactant.

However, in addition to chemical/biochemical tests, the specificity of a pharmacological test with regard to the activity of a new drug or surfactant is important. Therefore, we would propose to use a more general definition of specificity. This

Table 4
PaO_2 values at 120 min after treatment with rSP-C surfactant on different days[a]

Dose (mg kg^{-1})	Day	Technician A			Technician B		
		Median (range)	Mean ± SD	N	Median (range)	Mean ± SD	N
50	1	493 (455; 558)	497 ± 26	11	523 (464; 539)	515 ± 22	10
50	2	522 (452; 558)	522 ± 34	10	511 (469; 552)	512 ± 26	10
50	3	483 (409; 549)	481 ± 46	11	479 (445; 520)	484 ± 25	11

[a] The results show the comparisons of technician A and B and the influence of the day on which the experiments were performed. The dose is given in mg phospholipids per kg b.w. PaO_2 values are given for each dose as median and range as well as mean ± SD.

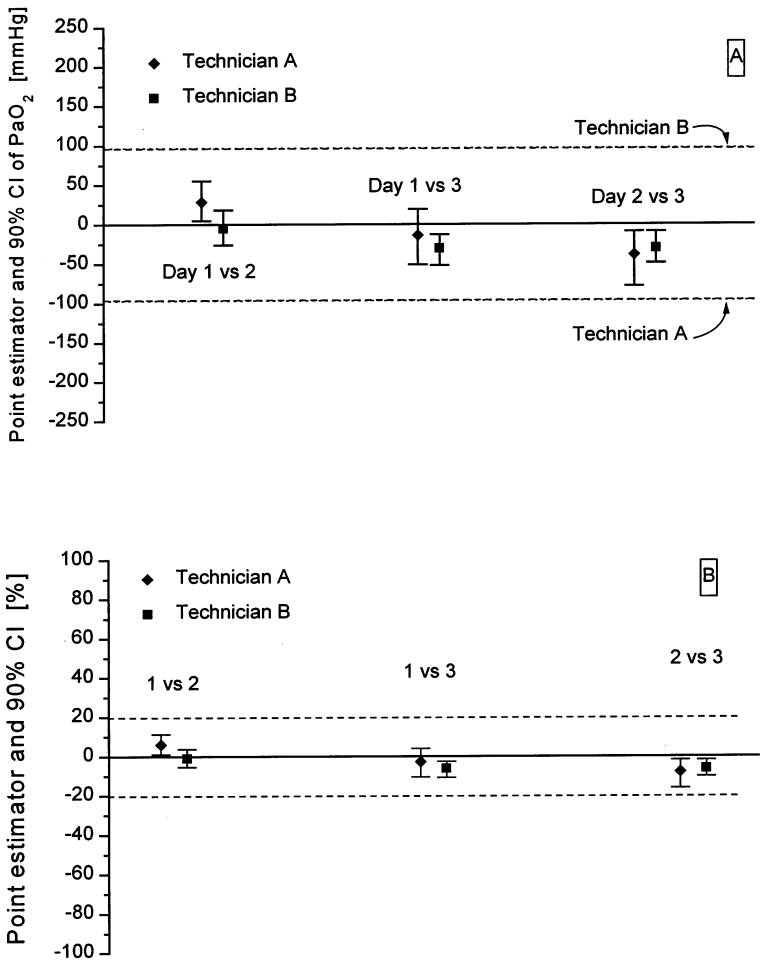

Fig. 2. Point estimators and two-sided 90% CI calculated for the comparison of the experiments investigating the influence of days on which the assay is performed using a dose of 50 mg phospholipids per kg b.w. ($N = 10–11$ animals per day and technician). The comparison is shown for the two technicians: Technician A (solid diamonds) and Technician B (solid squares). Panel A shows the comparison with respect to physiological units (partial arterial oxygen pressure (PaO_2), (mm Hg)). The ranges are based on the $\pm 20\%$ value of the lowest median of 3 test days for each technician. Panel B shows the values of Panel A in relative units (%), accordingly.

should be based on the similarity of a pharmacological assay with the human disease. Only a test with great similarity to human diseases can give reliable information about the usefulness of a drug in clinical trials. In the case of rSP-C surfactant a reliable assessment was possible because of the great similarity of the RLL-test with human disease [7,8] and because of the possibility of direct comparison to a drug that showed beneficial effects in humans with ARDS [11]. This is not always the case in drug discovery, but if not both prerequisites (test system with close relation to human disease and comparison to approved or clinically effective treatment) can be fulfilled, the test system for characterising new drugs should at least be closely related to the human disease.

4.3. Linearity

The linearity of this assay was assessed by the dose response curve of five doses of rSP-C surfactant (Table 1) and by comparing two different

concentrations of rSP-C (Table 3) for two doses of PL (50 and 100 mg PL per kg b.w.). There was a clear dose-dependence based on the five administered doses (Table 1). In addition, the assay was able to discriminate between two different concentrations of rSP-C (Table 3) based on the two tested doses of phospholipids.

Linearity measured as dose response characteristics is a typical and specific attribute of any pharmacological test system in contrast to, e.g. concentration/extinction curves in usual analytical tests. It is an essential prerequisite of such tests.

4.4. Range

The specification limits are only needed if a pharmacological/biological assay shall serve as a bioanalytical assay, e.g. to investigate bioequivalence of different batches of the same drug. In most cases, this can be shown by other bioanalytical methods.

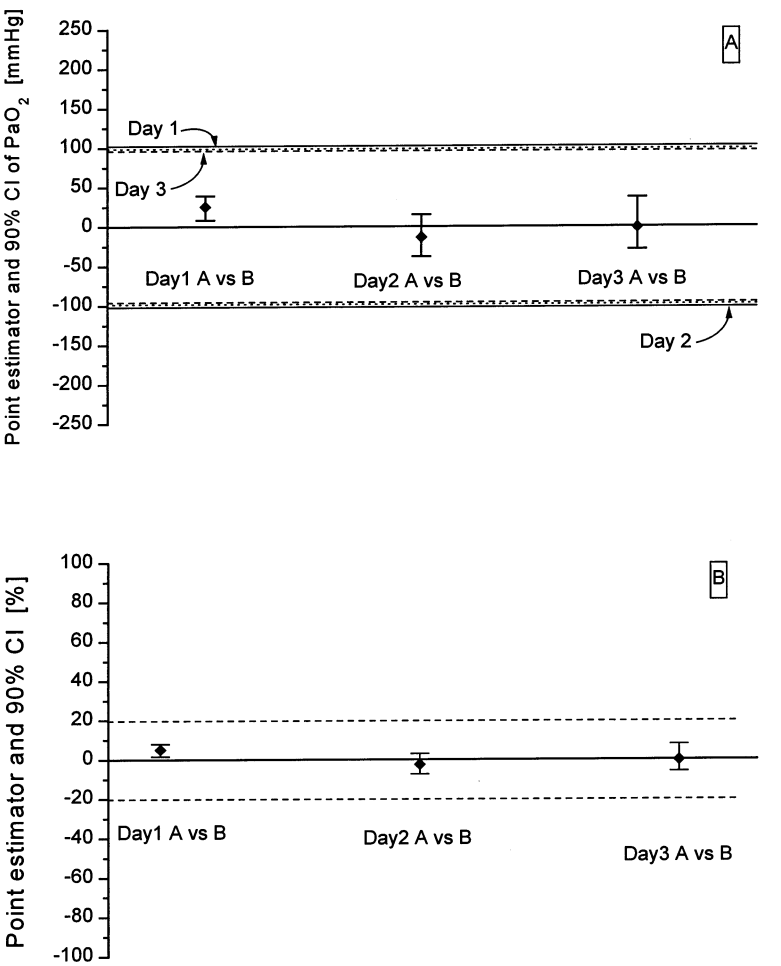

Fig. 3. Point estimators and two-sided 90% CI calculated for the comparison of the experiments investigating the influence of the technician who performs the assay using a dose of 50 mg phospholipids per kg b.w. ($N = 10–11$ animals per technician). The comparison is performed on the basis of the day on which the two technicians performed the experiments. Panel A shows the comparison with respect to physiological units (partial arterial oxygen pressure (PaO_2), mm Hg). The ranges are based on the $\pm 20\%$ value of the lowest median of the both technicians for each of 3 test days. Panel B shows the values of Panel A in relative units (%), accordingly.

Table 5
PaO_2 values at 120 min after treatment with rSP-C surfactant of rats delivered in 3 different weeks[a]

Week 1			Week 2			Week 3		
Median (range)	Mean ± SD	N	Median (range)	Mean ± SD	N	Median (range)	Mean ± SD	N
466 (322; 525)	458 ± 53	12	463 (253; 545)	448 ± 74	12	506 (438; 549)	502 ± 37	12

[a] The dose was 50 mg phospholipids per kg b.w. PaO_2 values are given for each dose as median and range as well as mean ± SD.

4.5. Accuracy

According to the guideline (point 4.1.2, section c), 'accuracy may be inferred once precision (point 3.6), linearity (point 3.3) and specificity (not necessary) have been established'. Only data from precision and linearity testing (dose dependence) are used to show the close relation between the value found and the accepted reference value. Dose dependence was demonstrated to be excellent (Table 1) and the precision was also established (see next point). It can be concluded that the assay has an adequate accuracy, when using $N = 12$ animals per test condition.

In the case of pharmacological tests, only precision and linearity can be reported when validation is performed in accordance with the note for guidance on validation of analytical procedures [5]. As mentioned, specificity needs to be defined as a specific close relation of an assay to pathomechanisms of human diseases.

4.6. Precision

4.6.1. Repeatability

It was shown that the repeatability is not dependent on the day on which the experiments are performed if $N = 12$ animals are used. This becomes obvious when looking at the results of the different days on which the test (Fig. 2) was performed. The influence of the day was not significant. A similar result was also obtained for the comparison between both technicians (Fig. 3).

4.6.2. Intermediate precision

Intermediate precision is related to within-laboratory variations. This point will be dealt with in Section 4.8, robustness.

4.6.3. Reproducibility

This is not applicable because there are no inter-laboratory trials intended as it is recommended by the note for guidance on validation of analytical procedures [5].

4.7. Detection limits and quantitation limits

Both points are beyond the scope of pharmacological or bioanalytical tests and more subtle and sensitive chemical/biochemical assays should be used in combination with bioanalytical tests. Therefore, we believe that both points are not applicable because pharmacological assays do not analyze impurities. In addition, those assays are only used for evaluation of the activity of new drugs and, therefore, no quantitation limits for impurities can be given.

4.8. Robustness

The robustness of this assay was shown by testing the influence of: (1) days on which the assay was performed; (2) influence of technicians; and (3) supply of animals at different times. These parameters were tested at a standard dose of 50 mg PL per kg b.w. of the above mentioned batch EB 454 of rSP-C surfactant. The influence of supply of animals at different times was investigated by testing three different animal supplies over 3 weeks with $N = 12$ animals. The results from investigating the influence of different supplies showed good reproducibility (Fig. 4 and Table 5). The minor influences of the weeks on which the animals were investigated (different supply of animals) are not significant.

The influence of the two technicians was investigated during the repeatability tests, by conduct-

ing per technician $N = 12$ animals per day and by repeating this procedure two times (Fig. 3). With this last investigation, the influence of days on which the assay is performed was investigated (Fig. 2). Both parameters did not have any influence. In conclusion, this assay is robust enough when using $N = 12$ animals to judge the quality/activity of a surfactant preparation.

The robustness of a pharmacological test system against deliberate variations is an important issue in the validation of such tests which are needed in the development of new drugs. It will provide information on the reliability of the specific test and the animal number that is needed for statistical calculations. Based on the required animal numbers, it is also possible to judge the potential effects of new drugs. It also gives information whether an assay is dependent on certain experimental conditions, e.g. certain supply of animals, or certain technicians. Tests which need high animal numbers may not be appropriate because their reliability may be inaccurate. Tests

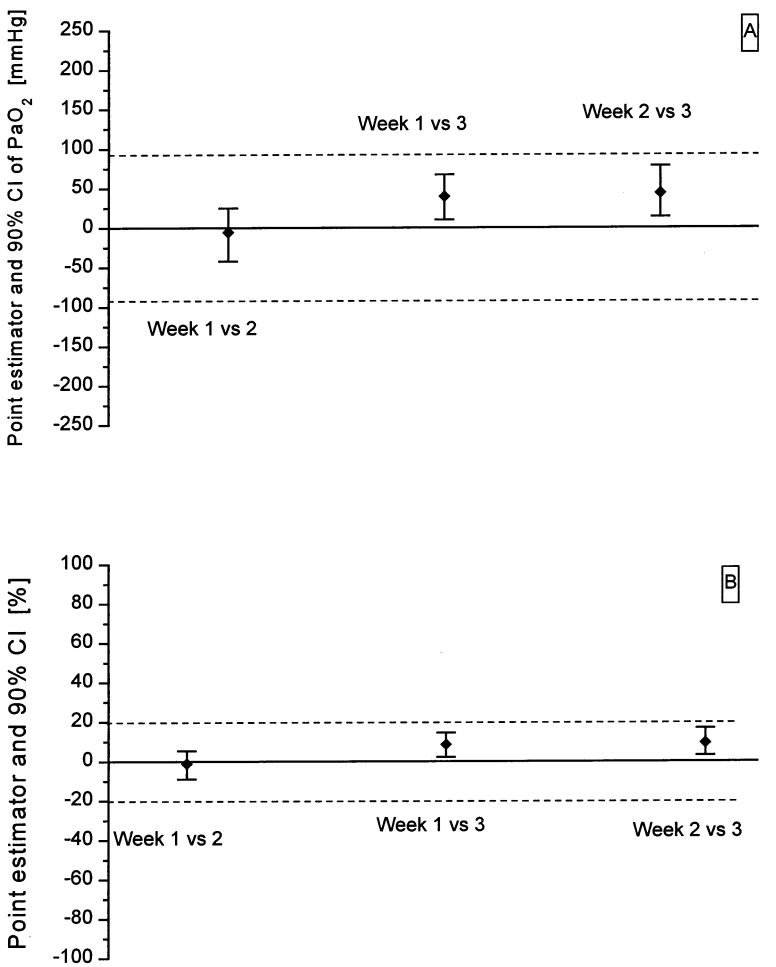

Fig. 4. Point estimators and two-sided 90% CI calculated for the comparison of the influence of the week of supply on the assay. The comparison is performed on the basis of the 3 different weeks on which the animals were delivered from the supplier and is based on a dose of 50 mg phospholipids per kg b.w. The comparisons are based on an animal number of $N = 10–12$ per group. Panel A shows the comparison with respect to physiological units (partial arterial oxygen pressure (PaO_2), mm Hg). The ranges are based on the $\pm 20\%$ value of the lowest median of all 3 weeks. Panel B shows the values of Panel A in relative units (%), accordingly.

Table 6
PaO_2 values at 120 min after treatment with sonified rSP-C surfactant[a]

	Dose (mg kg^{-1})	Median (range)	Mean ± SD	N
Controls	—	65 (48; 101)	69 ± 18	11
Inactivated	50	94 (52; 380)	150 ± 121	11
Normal	50	440 (321; 526)	443 ± 58	11

[a] The comparison shows the results of untreated controls, of ultrasound inactivated rSP-C surfactant and of not inactivated rSP-C surfactant (normal). The results of not inactivated rSP-C surfactant and of the untreated controls are taken from Table 1. The doses are given in mg phospholipids per kg b.w. PaO_2 values are given for each dose as median and range as well as mean ± SD.

which are dependent on the technician may have a minor predictive value because they may lead to irreproducible results and, therefore, possible false positive drug developments.

4.9. System suitability testing (biological integrity)

The evaluation of this part was also performed successfully. Although the contents of the tested surfactant were the same (no alterations in amount of rSP-C or PL), the activity of the surfactant vanished after sonification of the surfactant samples. From this test, it can be concluded that the assay is able to test the correct biological integrity of the surfactant.

Evaluation of this point may only be important when pharmacological tests are used for bioanalytical analysis. However, for this purpose special in vitro assays are more suitable because they are easier to use. Further advantages of these assays are the shorter test times and that the use of animals can be reduced.

5. Conclusion

The presently used RLL-test described in this validation report is able to determine: (1) the activity/potency of lung surfactant factor preparations; and (2) the biological integrity of such surfactant preparations. This test can also be used for the characterisation of the equivalence between different surfactant batches and or different surfactants.

From this report, it can be concluded that a specific guideline for the validation of pharmacological or biological tests is desirable. The note for guidance on validation of analytical procedures [5] and the parent guideline [6] as guidelines were useful to some extent. A validation can be based on these guidances but should be adjusted to pharmacological needs. There are some issues which are either redundant or not applicable for the validation of a pharmacological test. To our opinion the guideline should be adapted with respect to the point linearity. We suggest to use the determination of dose dependence as characterisation of a pharmacological test instead. Based on our experiences, a future guideline on validation of pharmacological tests should focus on activity, precision, robustness and biological integrity. Pharmacological tests will only be used to determine the activity, integrity and potency of new substances. The point detection limits and quantitation limits as defined by the guideline on validation of analytical methods [6] are not applicable and can be omitted. Furthermore, pharmacological assays are usually not suitable to detect impurities or degradation products [6].

Furthermore, with respect to specificity, we propose that it should be stated that at least one of the following prerequisites should be fulfilled by a validated test. The test system should have a close relation to human disease and, if there are data available, a comparison to approved or clinically effective treatment should be possible. The specificity can also be tested in comparison to sham treatment if no treatment is available. This statement also makes clear the difference between the specificity of bioanalytical assays and that of pharmacological or biological test systems.

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