



Validation and stability analysis of a modified lactate dehydrogenase (LDH) test method to be employed for an *in vitro* viable skin model



I. Bauhammer^{*}, M. Sacha, E. Haltner

Across Barriers GmbH, Science Park 1, 66123, Saarbrücken, Germany

ARTICLE INFO

Keywords:

Biochemistry

Biotechnology

ABSTRACT

In view of increasing numbers of dermatological disorders, transdermal drug delivery along with *in vitro* research is becoming increasingly popular. Therefore, qualified *in vitro* skin models are required. The objective of this study was the optimization and validation of a modified lactate dehydrogenase (LDH) release assay during the establishment of an *in vitro* viable human skin model, employable for a variety of skin associated disorders. Firstly, the most suitable LDH isoform for the study was determined. Subsequently, a stability study was conducted to investigate the best storage conditions of the LDH enzyme. Finally, the test system was validated in terms of linear range, range limits and system suitability. The results indicate LDH-5 as most suitable isoform due to its predominance in skin. The stability samples stored at -20°C in the presence of polyethylene glycol (PEG) as cryoprotector displayed the targeted recovery of $100\% \pm 15\%$ until the end of the four-week study in contrast to other investigated conditions. A six-point calibration without PEG and a seven-point calibration with PEG including evaluation of system suitability and quantification limits were established with both correlation coefficients r^2 above 0.99 and all deviations below 15%. Concluding from those results, this method can be considered valid and useful for its employment in viability determination of viable *in vitro* skin models.

1. Introduction

Dermal health is one of the greatest concerns in both the human and the veterinary field, as skin related disorders, especially allergies, but also infections are rising worldwide [1, 2, 3]. Increasing resistances can make conventional therapy very time consuming and often ineffective [4, 5, 6]. Therefore, new therapeutic options are required.

Because of the complicated and expensive pharmacological development process, *in vitro* research became more and more popular over the last years [7, 8]. *In vitro* research also addresses the controversial issue of animal testing. Social acceptance of animal experimentation declined over the last years due to ethical reasons [9, 10] and resulted in new scientific principles [11] as well as international legislation strengthening animal rights (e.g. Declaration of Bologna in 1999 etc.). Furthermore, the outcome of these tests may, in many cases, not be transferable to humans [12, 13]. Thus, *in vitro* research was gradually becoming more important, as it can be a solution to many of those problems, leading to the development of different skin models. The main aim of this research was the establishment of an *in vitro* viable skin model for the investigation of cutaneous diseases and as basis for the development of new drug formulations [14]. It may also be suitable for other applications, e.g. gene

therapy [15]. While for most of those possible applications, the evaluation of percutaneous absorption with non-viable skin may be sufficient, for the investigation of skin diseases and inflammatory processes, a more complex approach is necessary. Usage of viable skin can be challenging, as it has to be maintained viable for a certain time [14]. However, considerable metabolic activity was shown in viable skin [16]. This metabolic enzymes strongly influences the uptake and efficacy of drugs and leads to a better simulation of *in vivo* conditions [16].

Therefore, an *in vitro* human skin model was established. Therefore, human (or animal) full skin is freshly obtained from cosmetic surgery. The still viable tissue is cleaned and cut into several specimen. These are cultivated at $37^{\circ}\text{C}/5\% \text{CO}_2$ with the stratum corneum at the air-liquid-interface for two weeks. Every day, the cultivation medium is changed and samples taken which are then analysed with a viability marker. In this way, as first step, the development of skin viability under different conditions can be monitored and optimized. A second step could involve the application of different (e.g. anti-inflammatory) nanoformulations and the evaluation of their effects on the tissue.

For the determination of skin viability, a suitable method had to be chosen. Measurement of TEWL (transepidermal water loss) showed a low correlation to skin barrier integrity *in vitro* and was described as

^{*} Corresponding author.

E-mail address: I.Bauhammer@acrossbarriers.de (I. Bauhammer).

Table 1
Timetable and conditions for stability study.

Stability	Week 1				Week 2			Week 3		Week 4
	T0/day0	T1/day1	T2/day3	T3/day6	T4/day8	T5/day10	T6/day14	T7/day20	T8/day27	
Refrigerator + PEG	RT	4 °C	4 °C	4 °C	4 °C	4 °C	4 °C	4 °C	4 °C	4 °C
Freezer + PEG	RT	−20 °C	−20 °C	−20 °C	−20 °C	−20 °C	−20 °C	−20 °C	−20 °C	−20 °C
Refrigerator − PEG	RT	4 °C	4 °C	4 °C	4 °C	4 °C	4 °C	4 °C	4 °C	4 °C
Freezer − PEG	RT	−20 °C	−20 °C	−20 °C	−20 °C	−20 °C	−20 °C	−20 °C	−20 °C	−20 °C

TP = time point, RT = room temperature (22–25 °C).

unreliable in the literature [17]. As the viability of the same skin tissue had to be assessed continuously at different time points, a non-destructive method was required which excludes the popular WST or MTT tests acting destructive on tissue [18, 19]. Hence, the non-destructive LDH release assay was selected as viability marker (Roche, 2016). This assay was previously modified for tissue usage instead of cell culture systems. It is also simple to use, provides fast results and does not need expensive equipment. In order to be able to use this assay in the above-mentioned way, and to ensure reliable results, this test method has to be validated. Therefore, one aim of this particular study was to conduct a partial validation, including the following parameters: Linear range, range limits and system suitability. A stability trial was another part of this study, as the issue of LDH stability in various conditions is controversially discussed in the literature [20, 21, 22, 23] and no conclusion could be drawn. A literature review about nomenclature and specific properties of LDH isotypes was also included in this study.

2. Materials & methods

2.1. Basic research on LDH nomenclature and isotype distribution

In order to be able to perform any study with an enzyme, information about its specific properties has to be collected, i.e. temperature and pH optimum [24], substrates, storage conditions etc. The potential existence of several serotypes should be clarified as well as their major differences in catalytic activity. Therefore, intensive literature research was conducted about LDH isoenzymes and their features.

2.2. Determination of LDH stability in different storage conditions

For this experiment, recombinant LDH from rabbit muscle with a specific activity of 844 U/mg protein was diluted in Dulbecco's modified Eagle's medium (DMEM) solution (Sigma Aldrich Chemie GmbH, Taufkirchen, Germany). Sodium bicarbonate (Sigma Aldrich Chemie GmbH, Taufkirchen, Germany), glucose (VWR International GmbH, Darmstadt, Germany) and gentamycin sulfate (Merck Millipore, Darmstadt, Germany) were added to the solution. PEG 400 (VWR International GmbH, Darmstadt, Germany) was selected as cryoprotector [25]. For LDH measurement, the Cytotoxicity Detection Kit ^{PLUS} (Roche Diagnostics GmbH, Mannheim, Germany) was used. LDH catalyzes the conversion of pyruvate to lactate and reverse by reducing/oxidizing the co-factor NADH/NAD⁺. This enzymatic reaction indirectly produces a red formazan salt, which can be quantified by photometry [26].

The stability study was conducted over four weeks with measurement time points directly after preparation (T0) and on days 1, 3, 6, 8, 10, 14, 20 and 27 (Table 1) with a high (0.438 U/mL), medium (0.135 U/mL)

and low (0.034 U/mL) concentration. Each of them were measured in six-fold determination, with PEG and without, and stored at 4 °C and −20 °C. Two stock solutions were prepared, from which the samples were diluted to the corresponding concentration levels. Fifteen percent of PEG were added to one of the stock solutions. The same amount of DMEM was added to the stock solution without PEG to exclude any measurement interferences due to concentration and volume. Blank samples (only medium) were carried with and without PEG to evaluate the influence of PEG on the absorbance. For the measurement, 100 µL of sample per well were pipetted into a generic 96-well plate (Nunc Delta Surface from Thermo Fisher Scientific, Karlsruhe, Germany), using piston-stroke pipettes (Eppendorf GmbH, Wesseling, Germany). 100 µL of reaction mixture (kit) were added and the plate incubated for 30 min. at 150 rpm on a horizontal shaker. After adding 75 µL stop solution (normally the enzymatic reaction would continue as long as substrate is available. To ensure that each plate is incubated exactly 30 min, the reaction is terminated with the stop solution), the plate was gently shaken by hand for ca. 10 seconds and then measured by photometry in a plate reader (Microplate reader Wallac 1420 Victor 2). All the numerical results (expressed as LDH activity in absorbance units) were divided by 2.75 during evaluation. This dilution factor is caused by the addition of reaction mix and stop solution (100 µL sample + 100 µL reaction mix + 75 µL stop solution = 275 µL. Hence, the sample is diluted in the ratio 1:2.75 which has to be considered in the evaluation, in order not to underestimate the measured activity). Tubes from Eppendorf and Corning (Corning Life Sciences, Kaiserslautern, Germany) were used for sampling and measurement. All the measurements were performed in triplicate.

2.3. Method validation

To ensure the accuracy and reproducibility of the developed test method, the modified LDH release test was subjected to a partial validation including linearity (calibration curve), system suitability test (SST) and measurement range limits (ULOQ = upper limit of quantification, LLOQ = lower limit of quantification), with acceptance criteria loosely based on some of the Food and Drug Administration guideline for bio-analytics [27].

The establishment of calibration curves for enzyme assays can be challenging, therefore a three-step approach was carried out as following. For the first preliminary experiments a four-point (partial) linearity was sufficient while in another step for the planned main study a six-point (full) linearity was targeted. As in the course of the stability study the usage of PEG was evaluated, a third linearity containing PEG was needed, targeting seven points. For all of them the preparation principle was the same, only for the last one 15 % of PEG were added to the stock solution.

Known concentrations of the re-suspended LDH enzyme were prepared as calibration standards and measured according to the same method described in the stability section. For each measurement series an array of six blanks was carried, and mean blank absorbance subtracted from the received absorbance values of the standards. The LLOQ was calculated based on the mean blank absorbance plus standard deviation of the blanks multiplied by 3 in accordance with the guideline. The highest standard was defined as ULOQ. The SST was performed using an LDH activity level in a medium-high range of the calibration in six fold

Table 2
Description and comparison of LDH nomenclature.

LDH Isotypes	Isotype 1	Isotype 2	Isotype 3	Isotype 4	Isotype 5
Synonym names	LDH-1	LDH-2	LDH-3	LDH-4	LDH-5
	H4	H3M	H2M2	HM3	M4
	HHHH	HHHM	HHMM	HMMM	MMMM
	B4	B3A	B2A2	BA3	A4
	BBBB	BBBA	BBAA	BAAA	AAAA

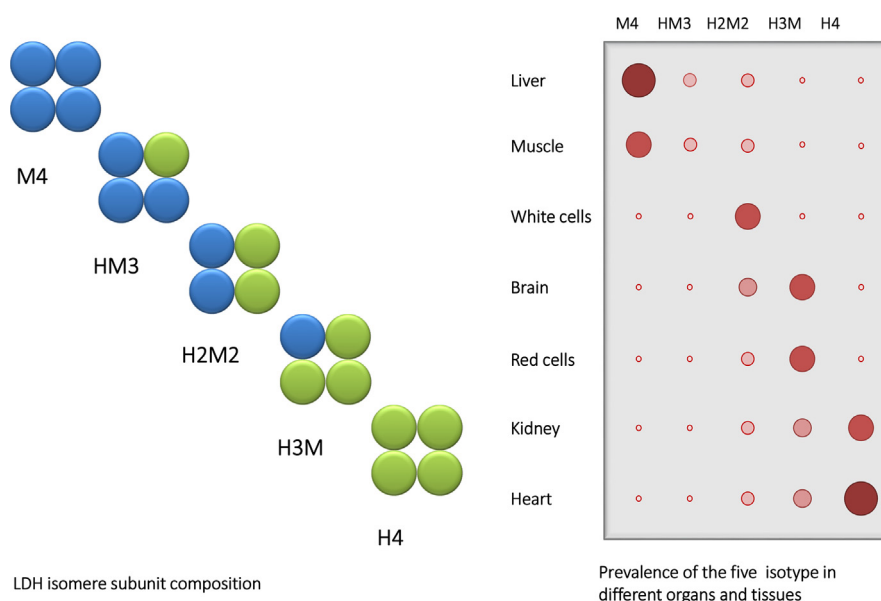


Fig. 1. LDH tetramers and isotype distribution in the human body. Reproduced and modified according to <https://www.slideshare.net/obanbrahma/enzymes-32065056> 08.01.19.

determination. Based on the guideline, $\leq 15\%$ RSD (relative standard deviation) for the calculated activity of LDH was set as acceptance criterion for each validation parameter except LLOQ, where $\leq 20\%$ marked the limit of acceptance. All the measurements were performed in triplicate. The mean of the three values was reported.

Data evaluation: The obtained absorbance values with blank subtraction were plotted against the corresponding theoretical concentrations. The coefficient of correlation (r^2) and the back-calculated calibration standard activities were reported.

3. Results

3.1. Basic research on LDH nomenclature and isotype distribution

The different isoforms of LDH enzyme are listed in Table 2. Five naming systems are used in parallel. One refers to the different isotypes as LDH-1/2/3/4/5. The others depend on the prevalence and distribution of LDH subunits H and M with A = M and B = H. H4/H3M/H2M2/M4 corresponds to B4/B3A/B2A2/BA3/A4, while each of them can be also written as HHHH, HHHM, HHMM, or BBBB, BBBA etc.

There are five main isotypes of the LDH enzyme, composed of two tetramere subunits (Fig. 1) and commonly named as LDH-1 to LDH-5. They differ in catalytic activity, mostly depending on the amount of each subunit. There are two “pure” forms, HHHH (H4) and MMMM (M4) and three hybrids HHHM (H3M), HHMM (H2M2), HMMM (HM3). M-rich isoforms have a higher activity in the presence of high pyruvate concentration, hence being predominant in tissues with considerable anaerobic metabolism, e.g. skeletal muscle or liver (reduction of pyruvate). The activity of H-rich isoforms is inhibited by high pyruvate concentrations and they are predominant in tissues with aerobic metabolism, e.g. heart (oxidation of pyruvate) [28, 29]. Following the same principle, H units can also be termed B units and M units as A [30].

The predominant LDH form in skin is LDH-5, followed by LDH-4 [30, 31] and LDH-3. Isoforms LDH-1 and LDH-2 cannot be found in the epidermis and the percentage of subunit M and the ratio of LDH-5 to LDH-4 is significantly higher than in the dermis, where all five isoenzymes can be detected [32]. It was essential to compare the main LDH isoform of the tested LDH enzyme with the main isoform of the used tissue, as, if they do not match, the obtained results would not be representative. LDH-5 is the main LDH type in the skin. In the recombinant enzyme LDH-5 was also the main isotype, in a similar ratio (based on the information obtained from

supplier). Therefore, the results of all the experiments with the recombinant LDH, especially concerning storage conditions, can be regarded as comparable to skin and therefore valid.

3.2. Stability of LDH

The outcome of the stability test is summarized in Figs. 2 and 3. Fig. 2 shows for all three concentrations that only storage with PEG at -20°C kept the recovery above 85% until the end of the four weeks. The samples without PEG at both temperatures (4°C , -20°C) show a sharp drop of recovery to around 50% (low samples) or 20% (medium and high samples) on the second measurement time point after 24 h of storage. From there, the recovery either decreased further to around 7% (high samples) or stayed in this range (low and medium samples). The samples with PEG stored at 4°C also exhibited a marked decrease, ranging from approx. 25% recovery (low samples) to 12% (high samples). This decrease was less pronounced than for no-PEG samples and their level was reached after 6 days of storage on the fourth sampling time point.

The twelve possible conditions resulting from the experimental setting were then plotted into one graph to facilitate the evaluation of the different influences and detect potential codependencies of certain factors, as seen in Fig. 3 where the stability of LDH at the end point of the study (day 27) is shown. There, the samples with low concentration and without PEG displayed a recovery of $48.95\% \pm 0.001\%$ (4°C) and $50.04\% \pm 0.001\%$ (-20°C). The medium samples without PEG maintained a recovery of $22.67\% \pm 0.02\%$ (4°C) and $17.29\% \pm 0.01\%$ (-20°C) while the high samples showed recoveries of $6.99\% \pm 0.02\%$ (4°C) and $8.01\% \pm 0.001\%$ (-20°C).

All the samples with PEG showed more differences between storage temperatures. At 4°C the PEG samples range from $5.76\% \pm 0.002\%$ (high) to $8.66\% \pm 0.001\%$ (medium) and $24.6\% \pm 0.001\%$ (low). At -20°C the recovery of PEG samples for all three concentration levels was finally within the favorable recovery range of $100\% \pm 15\%$ ($85.1\% \pm 0.02\%$ low, $102.97\% \pm 0.03\%$ medium, $98.70\% \pm 0.22\%$ high concentration).

Residual standard deviation (RSD) of all the stability results was $<15\%$.

3.3. Method validation

For the 6-point calibration without PEG (Table 3) a linear range from

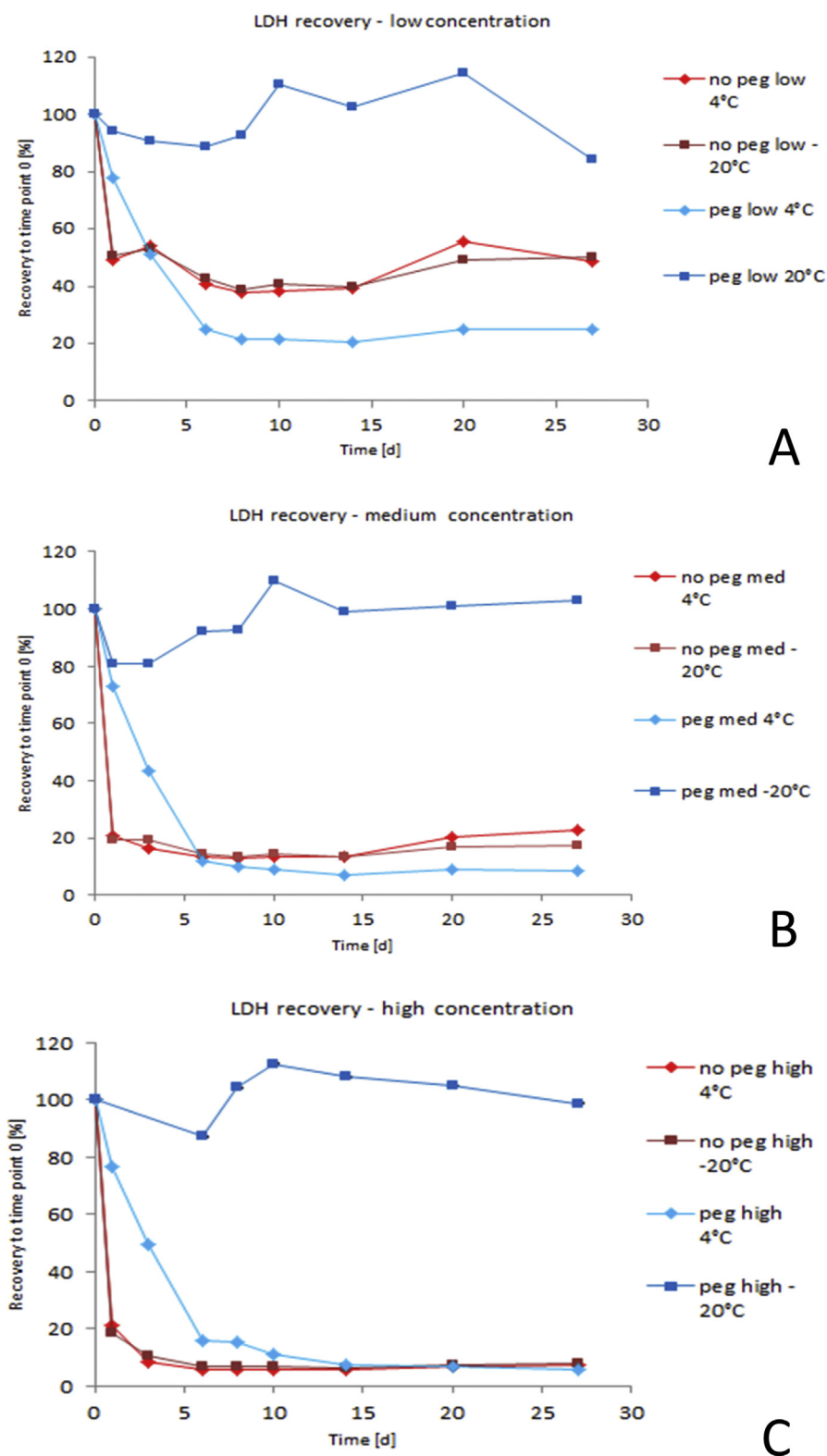


Fig. 2. Display of LDH recovery [%] over the course of 27 days. A: low concentration (0.034 U/mL), B: medium concentration (0.135 U/mL), C: high concentration (0.438 U/mL). All samples measured once directly after preparation (=T0), and on day 1/3/6/8/10/14/20/27 (n = 3).

0.020 U/mL to 0.409 U/mL (concentration in tubes, before dilution for measurement) was established with six calibration standards, each measured in triplicate. The determination coefficient r^2 was 0.9983 and hence higher than the acceptance value of ≥ 0.99 and the deviations

(RSD) ranging from 8.19 % to 14.92 % within the acceptance limits of FDA-guideline (deviations $\leq 15\%$, for LLOQ $\leq 20\%$). The ULOQ was set to 0.974 AU which corresponds to 0.409 U/mL and the LLOQ was set to 0.029 AU, corresponding to 0.020 U/mL accordingly. The values for AU

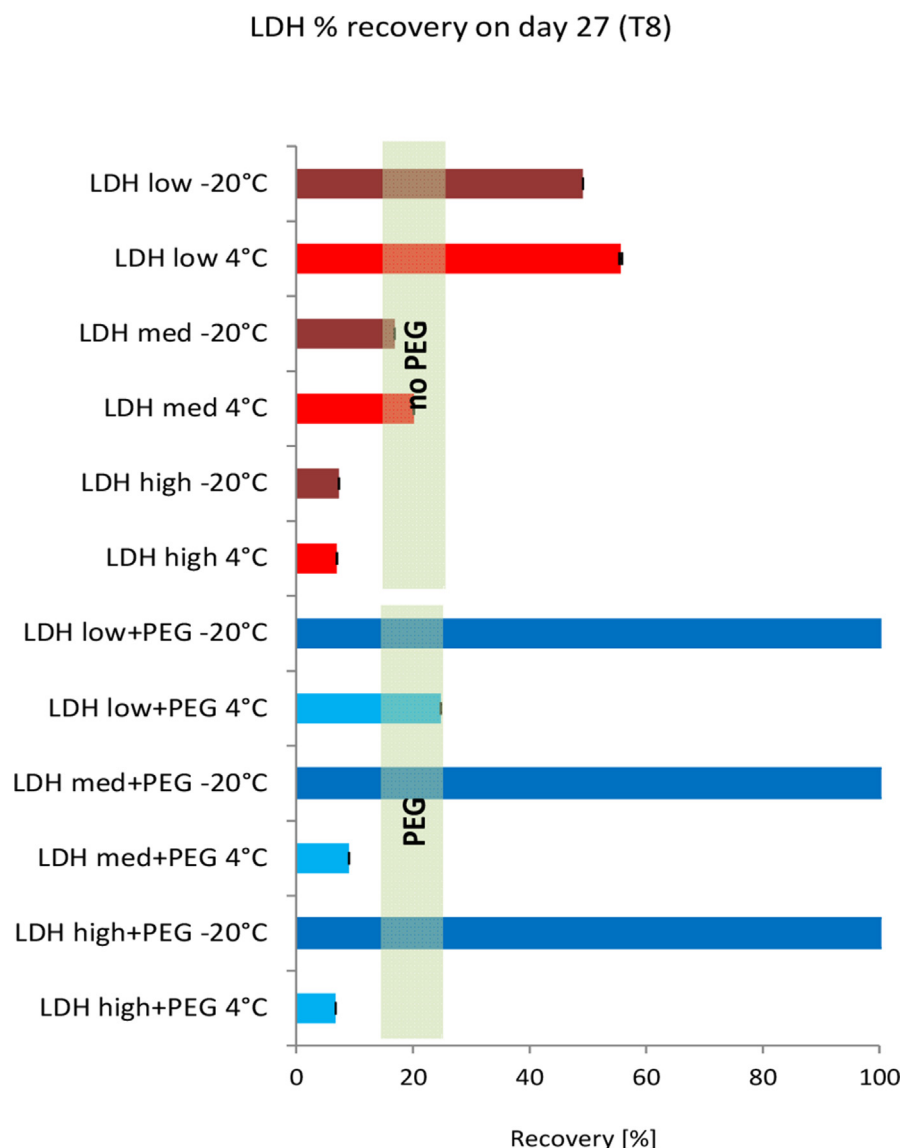


Fig. 3. Plot of measurement results on day 27 for all the 12 investigated storage conditions, showing the difference between PEG samples and no-PEG samples as well as the difference between 4 °C and −20 °C and the concentration levels low, medium and high (n = 3).

(absorbance units) are all given after mean blank subtraction of 0.058 AU.

From the linearity with PEG (Table 3) all seven points fulfilled the specifications in a split linearity (the linear range is divided in a higher and lower division, which must intersect at one point). The linear range expanded again from 0.020 U/mL to 0.409 U/mL, with 0.113 U/mL (KLP4, corresponding to 0.938 AU) as common point. The determination coefficient of both high/low was with 0.9963 and 0.9981 higher than the acceptance value of ≥ 0.99 . The deviations (RSD) ranging from 13.04% to −13.32% were within the acceptance limits of $\leq 15\%$. 1,568 AU was set as ULOQ, the LLOQ reached 0.168 AU. The system suitability test (SST) was carried out in six-fold determination (also n = 3) with a concentration of 0.077 $\mu\text{g/mL}$ and resulted in a mean absorbance of 1.315 ± 0.08 AU. The deviation was with 5.94% within the acceptance limit of $\leq 15\%$. The corresponding calibration curves and plot of residues are shown in Fig. 4.

4. Discussion

In this study an LDH stability trial under different conditions was conducted and the LDH release test system was validated under bio-analytical aspects. In addition, a literatur review about nomenclature and

specific properties of LDH isotypes was carried out.

After the clarification of LDH nomenclature in the literature, the research about specific features of LDH isotypes was considerably facilitated. Based on the studies of Lewis and Uitto [29, 30], LDH-5 is the main isoform in skin, followed by LDH-4. From the supplier of the recombinant LDH enzyme used in this study, the confirmation was obtained that also in this product, LDH-5 was the predominant isoform. This information is on one hand based on literature as the enzyme is derived from rabbit muscle, and LDH-5 is generally predominant in muscular tissue [28, 29]. On the other hand, an internal assay has also been conducted by the supplier but no more information was given due to confidentiality reasons. As the claim of LDH-5 being predominant in muscle tissue could be supported during the first part of this study, the obtained information was considered as reliable.

Conflicting recommendations exist regarding stability of total LDH and especially isotypes LDH-4 and LDH-5. According to Collins et al. and de la Peña et al. [20, 22], LDH displays cryo-sensitivity and loses part of its activity after freezing, especially if subjected to repeated freeze-thaw cycles [20]. De la Peña identified 4 °C as preferable storage temperature for salivary LDH [22]. The predominant isoforms in saliva are LDH-4 and LDH-5, identical to cutaneous LDH isoforms [22, 30, 31]. In another

Table 36-point and 7-point linearity with/without PEG including deviations and r^2 ($n = 3$).

Linearity samples	Theoretical concentration [$\mu\text{g/mL}$]	Absorbance [AU] w/o blank subtraction mean $\pm$ SD	Absorbance [AU] with blank subtraction	Calculated LDH concentration [$\mu\text{g/mL}$]	Calculated LDH concentration RSD [%]
KPL1 no PEG	0.149	1.033 ± 0.04	0.974	0.147	-1.08
KPL2 no PEG	0.074	0.560 ± 0.01	0.502	0.078	4.14
KLP3 no PEG	0.041	0.324 ± 0.01	0.265	0.043	4.16
KLP4 no PEG	0.018	0.140 ± 0.005	0.082	0.016	-14.92
KLP5 no PEG	0.012	0.110 ± 0.002	0.051	0.011	-7.48
KLP6 no PEG	0.007	0.087 ± 0.001	0.029	0.008	8.19
r^2	0.9983				
KPL1 PEG high	0.148	1.619 ± 0.03	1.568	0.148	-0.24
KPL2 PEG high	0.114	1.391 ± 0.04	1.340	0.110	-3.40
KLP3 PEG high	0.074	1.231 ± 0.03	1.180	0.084	13.04
KLP4 PEG high	0.041	0.938 ± 0.04	0.887	0.035	-13.32
r^2	0.9963				
KLP4 PEG low	0.041	0.938 ± 0.04	0.887	0.041	-0.57
KLP5 PEG low	0.018	0.480 ± 0.01	0.429	0.019	2.42
KLP6 PEG low	0.012	0.348 ± 0.02	0.297	0.013	5.00
KLP7 PEG low	0.007	0.218 ± 0.01	0.168	0.006	-11.29
r^2	0.9981				

PEG = polyethylene glycol, KLP = common name for calibration standards, AU = absorbance units, RSD = residual standard deviation, r^2 = correlation coefficient.

study with salivary LDH [21] LDH was least stable at 4 °C, with room temperature obtaining a better recovery and the best results with storage at -20 °C. Services described activity loss of LDH-4 and LDH-5 with storage at 4 °C and -20 °C but reported longer preservation in frozen samples compared to refrigerated ones or those stored at room temperature [33]. Shain et al. did not find any instability/loss of activity of LDH-4 and LDH-5 after six weeks of storage, neither at 4 °C nor frozen at -20 °C [23]. Rohaya et al. investigated LDH stability at room temperature, 4 °C and -20 °C in presence of three different protectors [25]. The addition of PEG prevented degradation for two weeks at all three temperatures with a recovery of $\geq 98\%$, while glycerol seemed to be suitable only for frozen samples and ethylenediaminetetraacetic acid (EDTA) only for room temperature. Due to those contrasting results, an own stability study was conducted. As from Rohaya's study, PEG provided better results than glycerol and EDTA [25], PEG was chosen as protective agent as described. In this study, a low recovery for samples without PEG at 4 °C and -20 °C was obtained, with only slight variation between those two conditions, but differences between the concentrations. Storage at 4 °C in presence of PEG delayed the stability decrease for up to 2 days, being most visible in the high concentrated sample and partially supporting the finding of Rohaya et al. [25]. This PEG-derived preservation decreased, and from day 6 the recovery dropped down to the levels of storage

without PEG. Storage of LDH at -20 °C in the presence of PEG displayed the highest recovery for the complete four week period. The results indicate, that the individual effects of concentration, temperature and cryoprotector are negligible while the combination of high concentration, low temperature and cryoprotection had a significant and synergistic effect on the results. The best stability of LDH is therefore given at -20 °C in presence of PEG with a higher concentration while for the other conditions a marked instability was observed. These findings are in accordance with some researchers [21] but contrasting to others [20, 22, 23]. Some of these inconsistencies may stem from variation in sample handling, preparation and measurement due to different study protocols or enzyme composition, as already discussed by De la Peña [22]. Accordingly, as various recombinant LDH enzymes are available which differ in activity and isotype composition, their properties regarding stability and preferable storage temperature may differ as well, partially explaining the controversial results. Variation in laboratory procedures such as thawing of the enzyme before usage (thawing time, using heat or passive thawing at RT), incubation time (strongly depends on enzyme activity, usual variation from 5 min to 30 min), measurement wavelength, amount of cryoprotector, and equipment related restrictions may be another contributing factor.

The modified LDH assay used in this study was subjected to a partial

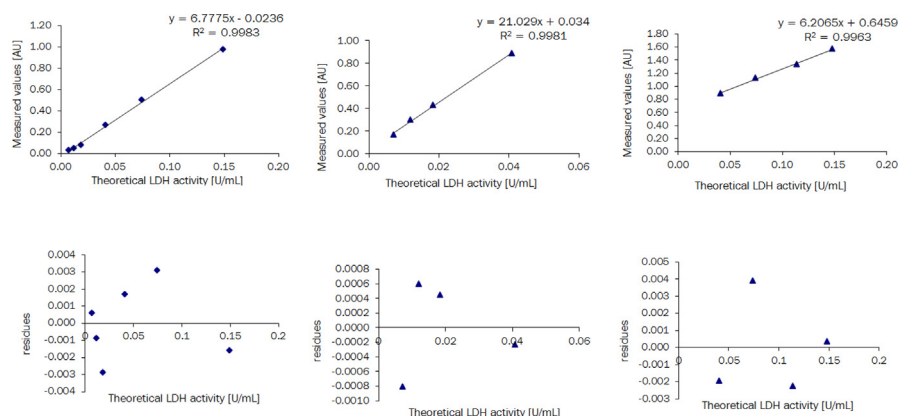


Fig. 4. Calibration curves and plot of residues. Left: 6 point calibration without PEG; middle: 7 point calibration with PEG, low part of the split linearity; right: 7 point calibration with PEG, high part of the split linearity ($n = 3$).

validation to ensure sufficient accuracy and reproducibility. To be considered as valid, the correlation coefficient r^2 and deviations had to fulfill the corresponding acceptance criteria of the FDA bioanalytic guideline. Hereby, the focus was set on establishing a linear range, evaluating if a good correlation could be achieved and if the measurement system is suitable for the intended application. If these requirements were met, the investigation of further parameters was deemed unnecessary for this study. As with six, respectively seven points a full calibration curve was achieved, all the deviations for the linearity and SST were below 15 % and the r^2 was above 0.99 as specified in the guideline, it was concluded that the test system is validated for the application on *in vitro* viable skin models with which new insights about dermatological disease processes can be gained. This may improve therapeutic possibilities and the wellbeing of humans and animals.

Compared to several other methods of viability determination, the LDH release assay is non-destructive. A skin cultivation study over two weeks with regular measurements of the same skin specimen would not be possible with e.g. the MTT or WST test, for which the cell layers/tissue generally have to be damaged. The LDH release assay is also easy to use, non-expensive and fast. It is traditionally designed for cell culture systems and often used without being validated first. With the adaption of the test system to tissue usage and partial validation, a higher degree of comparability and standardization is reached, providing an advantage over other methods.

Declarations

Author contribution statement

I. Bauhammer: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

M. Sacha: Conceived and designed the experiments.

E. Haltner: Contributed reagents, materials, analysis tools or data.

Funding statement

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

References

- [1] S.F. Thomsen, Atopic dermatitis: natural history, diagnosis, and treatment, *ISRN Allergy* 2014 (2014) 354250.
- [2] R.S. Mohammedamin, et al., Increasing incidence of skin disorders in children? A comparison between 1987 and 2001, *BMC Dermatol.* 6 (1) (2006) 4.
- [3] V.A. Fadok, Treating resistant skin infections in dogs, *Today's Vet. Pract. – Dermatol. Details* 4 (3) (2014).
- [4] S.S. Oberemok, A.R. Shalita, Acne vulgaris, II: treatment, *Cutis* 70 (2) (2002) 111–114.
- [5] C.N. Spaulding, et al., Precision antimicrobial therapeutics: the path of least resistance? *npj Biofilms Microbiomes* 4 (1) (2018) 4.
- [6] L.M. Bebell, A.N. Muir, Antibiotic use and emerging resistance—how can resource-limited countries turn the tide? *Global Heart* 9 (3) (2014) 347–358.
- [7] V.L. Tsang, S.N. Bhatia, Three-dimensional tissue fabrication, *Adv. Drug Deliv. Rev.* 56 (11) (2004) 1635–1647.
- [8] J.B. Kim, R. Stein, M.J. O'Hare, Three-dimensional in vitro tissue culture models of breast cancer—a review, *Breast Canc. Res. Treat.* 85 (3) (2004) 281–291.
- [9] R. Kolar, Animal experimentation, *Sci. Eng. Ethics* 12 (1) (2006) 111–122.
- [10] M. Bekoff, *Encyclopedia of Animal Rights and Welfare*, 2 ed., ABC-CLIO/Greenwood Publishing Group, 2009, p. 685.
- [11] P. Erkekoglu, B.K. Giray, N. Basaran, 3R Principle and Alternative Toxicity Testing Methods, vol. 36, 2011, pp. 101–117.
- [12] A. Akhtar, The flaws and human harms of animal experimentation, *Camb. Q. Healthc. Ethics* 24 (04) (2015) 404–419. The Humane Society Institute for Science and Policy Animal Studies Repository.
- [13] M.B. Bracken, Why animal studies are often poor predictors of human reactions to exposure, *J. R. Soc. Med.* 102 (3) (2009) 120–122.
- [14] A.O. Barel, M. Paye, H.I. Maibach, *Handbook of Cosmetic Science and Technology*, 4 ed., CRC Press, Boca Raton, 2014, p. 725.
- [15] J. Birchall, et al., Cutaneous DNA delivery and gene expression in ex vivo human skin explants via wet-etch micro-fabricated micro-needles, *J. Drug Target.* 13 (7) (2005) 415–421.
- [16] J. Kao, F.K. Patterson, J. Hall, Skin penetration and metabolism of topically applied chemicals in six mammalian species, including man: an in vitro study with benzo[a]pyrene and testosterone, *Toxicol. Appl. Pharmacol.* 81 (3, Part 1) (1985) 502–516.
- [17] R.P. Chilcott, et al., Transepidermal water loss does not correlate with skin barrier function in vitro, *J. Investig. Dermatol.* 118 (5) (2002) 871–875.
- [18] M.J. Stoddart, WST-8 analysis of cell viability during osteogenesis of human mesenchymal stem cells, *Methods Mol. Biol.* 740 (2011) 21–25.
- [19] S.K. Bopp, T. Lettieri, Comparison of four different colorimetric and fluorometric cytotoxicity assays in a zebrafish liver cell line, *BMC Pharmacol.* 8 (2008) 8.
- [20] S.S. Collins, J.T. Keeton, S.B. Smith, Lactate dehydrogenase enzyme activity in raw, cured, and heated porcine muscle, *J. Agric. Food Chem.* 39 (7) (1991) 1294–1297.
- [21] E. Jacobs, et al., Stability of lactate dehydrogenase at different storage temperatures, *Clin. Biochem.* 19 (3) (1986) 183–188.
- [22] V.A. de la Peña, et al., A standardised protocol for the quantification of lactate dehydrogenase activity in saliva, *Arch. Oral Biol.* 49 (1) (2004) 23–27.
- [23] S.A. Shain, et al., Creatine kinase and lactate dehydrogenase: stability of isoenzymes and their activity in stored human plasma and prostatic tissue extracts and effect of sample dilution, *Clin. Chem.* 29 (5) (1983) 832–835.
- [24] H. Bisswanger, Enzyme assays, *Perspect. in Sci.* 1 (1) (2014) 41–55.
- [25] M.A.W. Rohaya, et al., Stability of human salivary lactate dehydrogenase in the presence of ethylenediaminetetraacetic acid, glycerol and polyethylene glycol at various temperatures: preliminary study, *J. Biol. Sci.* 10 (6) (2010) 520–525.
- [26] Roche, in: *Cytotoxicity Detection Kit Plus [LDH] Manual*, Roche, 2016.
- [27] FDA, *Guidance for Industry, Bioanalytical Method Validation*, 2001.
- [28] A.F. Krieg, L.J. Rosenblum, J.B. Henry, Lactate dehydrogenase isoenzymes a comparison of pyruvate-to-lactate and lactate-to-pyruvate assays, *Clin. Chem.* 13 (3) (1967) 196–203.
- [29] J.B. Bartolone, et al., *Cosmetic Compositions Containing Lactate Dehydrogenase Inhibitors*, Chesebrough-Pond's Inc, USA, 1998, p. 18.
- [30] C. Lewis Jr., M. Schmitt, F.B. Hershey, Heterogeneity of lactic dehydrogenase of human skin, *J. Investig. Dermatol.* 48 (3) (1967) 221–225.
- [31] J. Uitto, et al., Lactate dehydrogenase in pseudoxanthoma elasticum. Total activity and isoenzyme distribution in the skin, *J. Investig. Dermatol.* 59 (2) (1972) 192–195.
- [32] J. Uitto, H. Turto, S. Lindy, Assay of lactate dehydrogenase in human skin: total activity and isoenzyme composition, *Clin. Chim. Acta* 36 (1) (1972) 43–50.
- [33] B.L. Services, Lactate dehydrogenase (LD), Plasma or Serum, 2018.