



# A standardised protocol for the quantification of lactate dehydrogenase activity in saliva

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## KEYWORDS

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**Summary** The literature about salivary lactate dehydrogenase (LDH) activity shows different results, conditioned by the diversity of methods used for sampling, handling and analysis. *Objetives:* The aim of this study was to design a protocol for quantifying salivary lactate dehydrogenase activity. *Methods:* Whole saliva samples were collected from 100 healthy volunteers by chewing a roll of cotton. Lactate dehydrogenase activity determinations were made with the Cobas Mira Plus II<sup>®</sup> autoanalyser using the recommendations proposed by the International Federation of Clinical Chemistry (IFCC) and the French Society of Clinical Biology (SFBC). Samples were stored at room temperature (20–22 °C), in the refrigerator (4 °C) and freezer (–20 °C). The correction capacity of the autoanalyser was evaluated by making serial determinations in samples of saliva diluted in water. *Results:* The frozen samples, but not those stored in the refrigerator or at room temperature, showed an important degradation of lactate dehydrogenase activity. When the samples were diluted in water, the lactate dehydrogenase activity varied substantially with the International Federation of Clinical Chemistry method after correction, but not when using the French Society of Clinical Biology method. *Conclusion:* For salivary lactate dehydrogenase activity quantification we suggest that samples be stored at 4 °C and analyse with the French Society of Clinical Biology method.

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## Introduction

The similarity between the profile of lactate dehydrogenase (LDH) isoenzymes in whole saliva and the oral epithelium supports the hypothesis that salivary LDH is predominantly of extraglandular origin.<sup>1,2</sup> Consequently, LDH concentration in saliva, as an expression of cellular necrosis, could be a specific indicator for oral lesions that affect the integrity of the oral mucosa.

Few studies have been published on LDH activity in saliva and the results are inconclusive,<sup>1–9</sup> probably due to the variety of methods used for handling and analysis. The aim of the present study was to design a standardised, reasonably homogeneous and reproducible method for whole saliva collection, a storage and handling system to guarantee sample stability, and a reliable analytical protocol for quantifying salivary LDH activity.

## Materials and methods

Saliva samples were collected from a study group of 100 healthy adult volunteers. Subjects were

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excluded for systemic diseases associated with an increase in serum LDH,<sup>10</sup> dental treatment in the 48 h before obtaining the saliva sample, lesions of the oral mucosa, or disturbances in salivary flow related with systemic processes or therapeutic procedures.

Salivary LDH activity was determined from whole saliva obtained after stimulating secretion by chewing a roll of cotton. It was collected non-invasively with the Salivette<sup>®</sup> kit, ref. 51.1534 (Sarstedt Ltd., Nümbrecht, Germany).<sup>11</sup> Samples were collected in the first hour of the morning, at least 8 h after eating, drinking, or toothbrushing. Samples were centrifuged immediately at  $1000 \times g$  for 10 min, then stored until analysis.

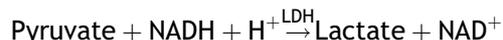
LDH determinations in whole saliva were made with the Cobas Mira Plus II<sup>®</sup> autoanalyser (Roche Diagnostics GmbH, Mannheim, Germany). LDH determinations were performed using two different methods:

- The method proposed by the International Federation of Clinical Chemistry (IFCC), standardised to 30 °C,<sup>12</sup> as recommended by the German Society of Clinical Chemistry (GSKC).<sup>13,14</sup> This method allows LDH activity to be evaluated by monitoring the NAD<sup>+</sup> reduction at 340 nm, in accordance with the following reaction:



The change in absorbance with time due to the conversion of NAD to NADH is directly proportional to LDH activity.

- The method recommended by the French Society of Clinical Biology (SFBC).<sup>15</sup> This method consists of the kinetic determination of LDH activity based on the speed of NADH oxidation standardised to 30 °C, in accordance with the following reaction:



The rate of oxidation, which is directly proportional to LDH activity, is monitored by measuring the decrease in absorbance at 340 nm.

The calibrators and reagents used were provided by the manufacturer of the commercial LDH kit (Roche Diagnostics GmbH, Mannheim, Germany). Precinorm<sup>®</sup> U and Precipath<sup>®</sup> U (Roche Diagnostics GmbH, Mannheim, Germany) were used as controls. Precinorm<sup>®</sup> U is a lyophilised control serum based on human serum with chemical additives and tissue extracts of human and animal origin added (LDH from porcine heart). In this control serum, LDH activity is within normal range or at the normal/pathological threshold. Precipath<sup>®</sup> U is a lyophilised control serum similar to Precinorm<sup>®</sup> U, with LDH activity in the pathological range. The activities of the components are lot-specific.

Variations in LDH concentration with storage temperature were assessed by mixing saliva specimens from five different patients to obtain a single sample. This sample was distributed into three Eppendorf tubes that were stored at room temperature (20–22 °C), in the refrigerator (4 °C), and in the freezer (–20 °C). This experiment was repeated 10 times with samples from 50 different patients using the two analytical methods described.

During determinations of LDH activity, the autoanalyser automatically made serial dilutions, then applied a correction factor when high LDH values were reached. The accuracy of the autoanalyser's correction capacity was evaluated by making serial determinations in samples of 100 and 200 µl of saliva from the same patient diluted in 0, 100, 200, 300 and 400 µl of water. This experiment was made with samples from 100 different patients using both analytical methods.

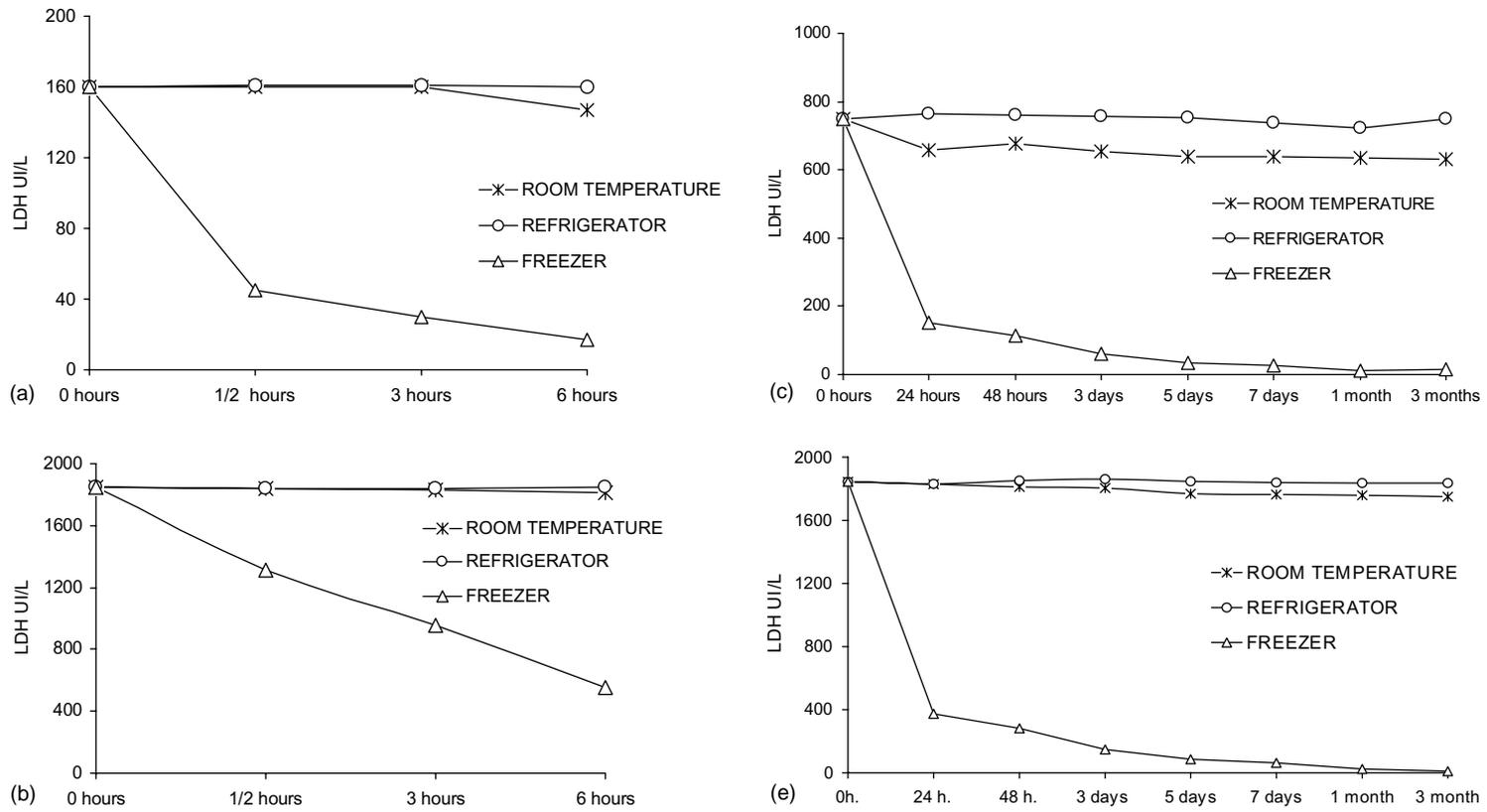
## Results

The samples that had been frozen (–20 °C) showed an important decrease in LDH activity after 30 min of storage. This loss of activity continued progressively and was not observed in samples stored in the refrigerator (4 °C) (Fig. 1a and b). At room temperature, we detected only a slight decrease in LDH concentration in the first 24 h, followed by stabilisation (Fig. 2a and b). This finding was confirmed in 50 samples from different patients using both the IFCC and SFBC methods.

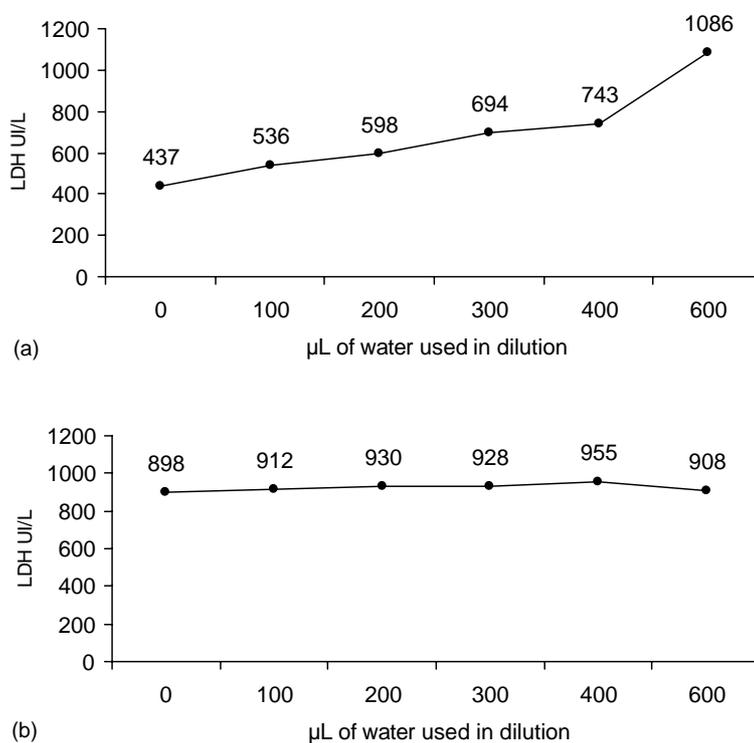
When the saliva sample was diluted in water (100–400 µl), the LDH concentration varied substantially with the IFCC analytical methods after correction (Fig. 2a). In contrast, LDH concentration did not vary significantly when using the SFBC method (Fig. 2b). In this case, similar results were obtained with 100 and 200 µl of saliva, indicating that the sample volume analysed did not influence results. These findings were reproduced with specimens obtained from 50 different individuals.

## Discussion

It is not known if the increase in serum LDH detected in relation to certain pathological conditions<sup>10</sup> entails an increase in salivary LDH and/or changes in its isoenzyme distribution. In the present study, the predominant isoenzyme profile was not specified, so we excluded patients with systemic diseases that raise serum LDH. The high LDH concentration in red blood cells, especially isoenzymes LDH-1 and LDH-2,<sup>10</sup> forced us to reject saliva samples obtained



**Figure 1** Variations in LDH activity with storage temperature, using the IFCC (a and b) and SFBC (c and d) analytical methods (saliva specimens from five different patients were mixed to obtain a single sample).



**Figure 2** Effect of sample dilution (100 µL of saliva) on salivary LDH activity using the IFCC (a) and SFBC (b) analytical methods (each point is the mean of 50 cases).

within 48 h of dental treatment, due to the possibility of blood contamination. Since LDH is a marker of cell death and tissue degradation, patients with lesions of the oral mucosa were excluded. In order to minimise the effect of circadian disturbances and exogenous stimuli on salivary flow, all samples were obtained in the first hour of the morning after waking up. The same rationale was used to exclude patients with systemic processes or patients undergoing therapeutic procedures that could decrease saliva production.

The Salivette<sup>®</sup> kit is a simple saliva collection system that was introduced in 1986<sup>11</sup> in an attempt to provide a standard method for studying saliva samples. At present, three different types of Salivette<sup>®</sup> kits are commercialised, which differ in the characteristics of the cotton roll used: Model 51.1534-001 has a cotton roll with citric acid, Model 51.1534-002, a cotton roll coated by neutral polyester, and Model 51.1534, used in the present study, is uncoated and does not contain salivary stimulants. The Salivette<sup>®</sup> method is cheap and allows samples to be obtained painlessly and hygienically without the intervention of specialised personnel. It does not change salivary protein concentration.<sup>16</sup>

Most published papers offer little or no information on the storage temperature of specimens.<sup>1,3-9</sup> Only Nagler et al. mentioned that, after centrifuging, the saliva samples for LDH activity quantification

would be kept at 4 °C until analysis.<sup>2</sup> In this study, we observed progressive enzyme degradation for up to 24 h in samples that had been frozen (−20 °C), with almost 90% loss of activity regardless of the analytical method used. This finding could be attributed to the lability at −20 °C of isoenzymes LDH-4 and LDH-5,<sup>18,19</sup> which are predominant in saliva.<sup>1,2,17</sup>

The analytical method used in most studies of LDH activity in saliva was the proposed by GSKC.<sup>1,2,6-9</sup> Among other characteristics, there is less inhibition of LDH, principally LDH-1, by pyruvate. Nevertheless, high pyruvate concentrations are necessary to detect LDH-5. This technique is optimised for the quantification of LDH-1,<sup>18,19</sup> but LDH-1 is the least abundant fraction in saliva. We found that when this technique was applied to diluted saliva samples to evaluate the correction capacity of the autoanalyser, LDH activity increased gradually, doubling in value at dilutions of more than 400 µL of water. The dilution of samples in water reduces the ionic strength of the saliva and its buffer capacity, increasing pH to the optimal reaction value for this analytical method.<sup>12</sup> This explains why higher LDH values are obtained. In addition, depending on the initial pH of the saliva, LDH values vary with dilution in water. The reaction takes place at different pH values, thus increasing the bias in the results, simultaneously affecting other

measurement magnitudes (final concentration, sensitivity, absorbance, etc.).

The SFBC analytical method uses lower reagent concentrations, so there is less possibility of contamination. There is a greater change in absorbance per time unit, so kinetic measures are more precise and the test is faster. In addition, once the work reagents are prepared they are more stable.<sup>10</sup> With this analytical method we did not appreciate variations in LDH concentration when diluting the saliva sample, since the optimal pH (7.4) of the reaction<sup>15</sup> is lower than with the IFCC technique.

To summarise, we suggest that salivary LDH activity must be quantified in samples collected using the Salivette<sup>®</sup> that have been stored at 4 °C until processing and analysed with the SFBC method.

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