Analytical Biochemistry 539 (2017) 152–157

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Fluorometric determination of D-lactate in biological fluids

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ARTICLE INFO

Keywords:
D-lactic acid
Metabolic acidosis
Blood
Urine
Lactate dehydrogenase

ABSTRACT

Objective: D-lactic acid in the mammalian body is mainly of microbiological origin and is often located somewhere along the digestive tract. Surgical, extensive re-sectioning of the small bowel may be one of the risk factors for altered balance in the microbiological environment. Higher levels in the body may lead to D-lactate acidosis and neurotoxicity; consequently, the possibility of diagnosis of this condition is important. Several analytical procedures for D-lactate have been introduced, but it is absolutely mandatory to distinguish this metabolite from the much more abundant and naturally occurring stereoisomer L-lactate. If enzymatic analytical methods are used, it is consequently essential to eliminate the response from L-lactate and the ubiquitous enzyme L-lactate dehydrogenase (L-LDH) (and other oxido-reductases) which will interfere with the D-lactate determination heavily.

Design and methods: The present paper introduces an enzymatic-fluorometric method for determination of D-lactate in biological matrices, including blood plasma, serum and urine. Macro molecules, including enzymes, were initially precipitated by ethanol and the supernatant used for analyses. Several plasma samples were analysed with and without standard addition of both L- and D-lactate in order to validate the assay.

Results and conclusions: The procedure effectively eliminates enzyme activities that may interfere with the D-lactate quantification, resulting in the situation that L-lactate in the sample does not interfere with the determination. Intra- and inter-assay precision, accuracy and recovery of the analyte were investigated and everything suggests that this method will be acceptable for analytical as well as descriptive purposes. The analytical procedure is suitable for a semi-automated large scale set-up in the laboratory.

Introduction

Lactic acid, lactate, exists in two stereoisomer forms in biological systems: the L (+)lactate and the D (−)lactate. In eukaryotic organisms, L-lactate is the general form in the metabolic turnover. However, D-lactate may be produced during methylglyoxal metabolism [1–3] leading to minimal concentrations in tissue and blood plasma. Higher levels of D-lactate in the mammalian body originate from microorganisms, because numerous bacterial strains produce both L-and D-lactate in their metabolic turnover. Most systemic instances of elevated D-lactic levels originate from microbial activity in the digestive tract – in humans from the large intestine ("short bowel syndrome" [4,5]) – although other sections have been under suspicion after surgical procedures [6] – in cattle through the rumen ("ruminal acidosis" [7–9]). Several food items for humans are fermented by microorganisms that produce D-lactate, e.g. pickles, wine, beer, yoghurt etc. However, ingestion of food is not likely to induce systemic acidosis. Similarly, silage for ruminant nutrition may contain considerable amounts of D-lactate (e.g. [10]). However, the main fraction is metabolised or converted to the L-form in the rumen which seldom leads to D-lactic acidosis in the animal.

The conventional theory is that degradation of the D-form mainly appears to be "unspecific" and less efficient than the more abundant L-lactate. A considerable fraction of the D-lactate therefore seems to be excreted through the kidneys [11]. However, this situation has been disputed (reviewed by Ewaschuk et al. [12]). Urine may still contain considerable concentrations of D-lactate [4,13–15], just as blood samples are significant specimens for measurements in order to diagnose D-lactate acidosis or overload.

The L-form of lactate is abundant in all tissues and body fluids due to its central role in carbohydrate metabolism. Correspondingly, the enzyme L-lactate dehydrogenase (LDH, EC 1.1.1.27; mediating the oxidation of lactate to pyruvate) is almost ubiquitous in the body. It is considerably more abundant in soft tissue than in plasma but is also represented in cerebrospinal fluids and excreted in urine. Further, the urine concentration is elevated during bladder and kidney malignancies [16]. Human milk may contain high levels of LDH-activity [17], and similarly, cow's milk contains sizeable levels of L-lactate and L-lactate dehydrogenase activity as well [18,19].

Several analytical methods have been introduced to measure D-

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http://dx.doi.org/10.1016/j.ab.2017.10.026
Received 31 July 2017; Received in revised form 20 October 2017; Accepted 31 October 2017

lactate in plasma and urine and other body fluids. Ewaschuk et al. [20] have reviewed the situation. Enzymatic assays for D-lactate determination are resulting in an increase in NADH in the reaction medium. It is mandatory in this type of assays to discriminate between the NADH production from D-lactate and the NADH production developed from L-lactate and L-LDH (or due to other oxido-reductases). Consequently, L-LDH products and other non-specific enzymatic transformation need to be eliminated. Several different strategies have been followed in this respect. Cold perchloric acid precipitates proteins in the sample (and thereby enzymes). This approach was used in several studies (i.e. [13,15,21–23]). The disadvantage of this method is that the sample must be brought back to nearly neutral pH before analyses, and these processes also dilute the sample considerably. Deproteinisation by ultrafiltration was chosen by Herrara et al. [24] and Marti et al. [25], which is a rather expensive strategy, whereas Nielsen et al. [26] inactivated L-LDH (temporarily) by NaOH addition. Deproteinisation by 5% metaphosphate was considered sufficient in rumen samples [27]. Many commercial assays recommend a deproteinisation step before analyses, other assays and studies do apparently not worry about intrinsic contents of L-lactate or L-LDH.

The present paper introduces a fluorometric determination of D-lactate where deproteinisation of the sample is performed by ethanol, and the sample content of D-lactate is concentrated by simple evaporation. The reaction equilibrium is facilitated by the fluophore itself, preventing a coupled enzymatic reaction which is practiced by other methods to avoid feedback inhibition by the product. The simple pretreatment of the sample, the ethanol precipitation, is effective in several specimens, irrespective of the fact that they initially appear opaque. The product of the pretreatment in all cases ends up as a clear sample liberated from macro molecular substances. To date, we have successfully analysed blood plasma, serum, urine, tissue homogenates, milk, rumen juice and several commercial beverages.

Materials and methods

Sample preparation

0.5 mL sample (plasma, urine, rumen juice, milk) is mixed with 1.0 mL 99% EtOH, turned a few times and centrifuged at 3500 × g for 10 min. The supernatant is used for analysis. Two times 60 μL are pipetted to black microplate and placed in an incubator at 65 °C until dried up (1 h).

Reagents

All reagents used were of analytical grade. D-lactate dehydrogenase was Sigma L2011; D-lactate and L-lactate were Sigma 71716 and 71718, respectively, both ≥99.0% purity. Resazurin (fluophore) was Sigma R-2127. NAD^+ was from Sorachim SA, Switzerland, MW 663.4. Diaphorase was DAD-301, Toyobo Enzymes.

Procedure

a) 80 μL of Tris-buffer (100 mmoL/L Tris, 20 mmoL/L KCl, pH 8.9) is added to each well on the plates with the dried-up samples. A lid is placed on the plate and the plate is left at room temp for 60 min.
b) Standards and one replicate of the samples are supplied with Tris-buffer with D-LDH and NAD^+, 80 μL (Tris 95 mmoL/L, KCl 19 mmoL/L, NAD^+ 7.3 mmoL/L, D-LDH 4.17 U/mL, Triton X-100 0.05%, pH 8.9)
c) The other half of the samples (second replicate) is supplied like b) but without enzyme
d) 30 μL fluophore and diaphorase enzyme is added to all wells (0.75 mmoL/L resazurin, 11 U diaphorase/mL, 0.05% Triton X-100, 1.5% Tween 80).

Operations like pipetting are performed by Biomek 2000 (Beckman Coulter) robotic systems. The plate is incubated at 37 °C for 50 min and read in a plate fluorimeter (FluoSTAR/Galaxy; exitation/emission 544/590 nm).

The reaction proceeds as follows:

1 .Reaction:

$$\text{D-lactate} + NAD^+ \xrightarrow{\text{D- LDH}} \text{pyruvate} + NADH + H^+$$

1. Reaction is mediated by D-lactate dehydrogenase (D-LDH; EC 1.1.1.28)
2. Reaction:

$$\text{resazurin} + NADH + H^+ \xrightarrow{\text{diaphorase}} \text{resorufin} + NAD^+$$

2. Reaction is mediated by diaphorase (EC 1.6.99.1).

In samples (including standards and control samples) receiving the enzyme D-LDH (step b), D-lactate is oxidised to pyruvate, and NAD^+ is simultaneously reduced to NADH + H^+. Further, NADH + H^+ reduces the non-fluoroscent rezasurin to the fluorescent compound resorufin, and the oxidised NAD^+ is consequently regenerated. The basic principle is described by Guilbault and Kramer [28].

In samples not receiving enzyme c), no reaction occurs. These wells are functioning as background level for the individual samples, and the measurement of these wells are subtracted from the replicates with enzyme.

Standards and control material

Samples and control material were evaluated against a standard curve. D-lactate (Sigma 71718, MW 112.06) was stored in an exsiccator. An aqueous stock solution of D-lactate was prepared from lactate, 10 mmoL/L, and KCl 200 mmoL/L. The solution was kept frozen between analyses. Standards of 0, 50, 100, 150, 250, 500, 750 and 1000 μmoL/L were manufactured. Standards were run in duplicate on every plate.

A pooled plasma sample was divided into four portions and spiked with D-lactate to 0, 100, 400 and 800 μmoL/L D-lactate. The control material was divided in 1.0 mL portions and frozen until use.

Standards and control material were treated like samples (precipitated with EtOH etc.).

Validation of the assay

Accuracy, linearity, intra- and inter-assay precision

A pooled plasma sample was spiked with 0, 40, 60, 80, 100, 150, 200, 250, 300, 400, 500, 600, 700, 800, 900 or 1000 μmoL/L D-lactate. These samples were analysed in duplicate on the same plate. This was repeated three times (days, independently). Intra-assay precision (plate) and inter-assay precision (between plates *and days*) were also estimated for this material.

Control plasma spiked with 0, 100, 400 or 800 μmoL/L D-lactate, one of each, was placed on any analysed plate, and the outcome was used to estimate (additional) inter-assay precision (n = 19).

Cross reactivity for L-lactate

A spiking procedure similar to the above mentioned (0-1000 μmoL/L) was also performed with L-lactate (one day only).