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Development of an accurate and sensitive method for lactate analysis in exhaled breath condensate by LC MS/MS

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ABSTRACT

Exhaled breath condensate (EBC) is easily obtained for clinical diagnosis and prognosis for pulmonary diseases and has gained much interest in biomarker discovery research and studies. Lactate, a physiological material, is found in EBC and has been demonstrated to be a potential indicator of chronic obstructive pulmonary disease and other lung diseases. Several assays are available to detect lactate in human body fluids, and yet none is suitable for detecting lactate in EBC. Due to the very low concentration of lactate in EBC and low volume of EBC, it is very important to develop an assay to measure lactate with high sensitivity, accuracy and easy sample processing. We report here a novel LC–MS/MS based assay to measure lactate using HILIC column separation. Sample preparation was simple and straightforward through a "dilute and shoot" approach with a separation of 4 min. The limit of quantification was determined to be 0.5 µM. This assay was linear from 0.5 µM to at least 100 µM. The inter- and intra- day precision at the levels of 1 µM, 10 µM, and 100 µM were less than 3% with recovery within 5.4% of expected values. There was no ion suppression for the assay, and no carry-over was observed up to 500 µM. Furthermore, we discovered that lactate is ubiquitously present in the lab environment, which can create significant challenges for accurate detection of lactate at low concentrations. We provided practical approaches in this paper to overcome the challenges and ensure the accuracy of the assay. In summary, this article presents an accurate and sensitive method using LC–MS/MS for measuring lactate in EBC, and this method is suitable for measuring lactate concentrations for non-invasive monitoring of pulmonary functions.

1. Introduction

Exhaled Breath Condensate [1–6] (EBC) is an easily obtained material that is potentially useful in the diagnosis of various lung diseases. It is used as a source of materials to indicate physical fitness and cardio-respiratory disease [7]. As air moves through the respiratory system, it becomes humidified and exposed to metabolites, proteins and other endogenous compounds. The materials that have been shown to exist in EBC range in size from ions and dissolved gases through proteins and macromolecules [8]. These compounds can accumulate in the water vapor or aerosolized particles that constitute EBC.

Among the many materials present in EBC, lactate has been demonstrated to be a potential indicator of COPD and other lung diseases [7,9,10]. Traditionally, physiological lactate from blood or plasma is measured at low mM levels by enzymatic or Ion Selective Electrode (ISE) methods in medical laboratories [11]. Lactate has also been measured by a variety of analytical techniques including NMR, ion chromatography, capillary electrophoresis and GC/MS. However, these methods do not have the required sensitivity for measurement in EBC at µM level.

This study is to develop an accurate and sensitive assay on LC–MS/MS to measure lactate in EBC. As a small organic anion, lactate is essentially unretained in traditional reverse phase HPLC separations. Hydrophobic Interaction chromatography (HILIC) [12–16] was chosen

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Abbreviations: ACN, acetonitrile; BEH, ethylene bridged hybrid; CAS, chemical abstract service; CIL, Cambridge Isotope Laboratories, Inc; COPD, chronic obstructive pulmonary disease; CV, coefficient of variation; EBC, exhaled breath condensate; GC/MS, gas chromatography – mass spectrometry; HILIC, hydrophilic interaction chromatography; HPLC, high performance liquid chromatography; IS, (isotopically labeled) internal standard; ISE, ion selective electrode; LC–MS/MS, liquid chromatography – tandem mass spectrometry; LLOD, lower limit of detection; LLOQ, lower limit of quantification; *m/z*, mass to charge ratio; mM, millimolar; NH_4OAc, ammonium acetate; NH_4OH, ammonium hydroxide; NMR, nuclear magnetic resonance; µL, microliter; µM, micromolar; w/w, weight/weight

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for ease of use, appropriate retention of lactate and compatibility with current LC–MS/MS instrumentation. In addition to the low concentration of lactate in EBC, there are several other challenges to the use of EBC for monitoring lactate. It is difficult to obtain sufficient EBC for matrix-matched calibration curves for daily use. In addition, most EBC has endogenous lactate. We therefore assessed an alternative matrix, water, for use in calibration. Another challenge is the difficulty in obtaining matched lactate internal standards. We chose to use carbon 13 labeled sodium lactate with all three carbons labeled. The biggest challenge, however, is the broad distribution of lactate in the human body, including high concentrations on the skin surface, which leads to contamination issues unless stringent cleanliness protocols are followed.

Overall, we were interested in developing an assay for lactate in the low μM range using LC–MS/MS in EBC for clinical studies related to COPD and other pulmonary conditions.

2. Materials and methods

2.1. Chemicals and reagents

Sodium DL Lactate, > = 99.0% pure (CAS 72-17-3) was obtained from Sigma Aldrich (Aldrich 71720). $^{13}C_3$ Labeled Sodium L-Lactate, > 98% isotopically labeled (Unlabeled CAS 867-56-1) was obtained from Cambridge Isotope Laboratories, Inc. (CIL item number CLM-1579-0.5) as a 20% w/w solution in water. Ammonium Acetate, HPLC grade (CAS 631-61-8) was obtained from J. T. Baker (0599). Acetonitrile (ACN), Optima HPLC grade (CAS 75-05-8, Fisher A955)) was obtained from Fisher Chemicals, as was aqueous Ammonium Hydroxide, Certified ACS Plus grade (CAS 7664-41-7, 7732-18-5, Fisher A669)). Type 1 laboratory water (CAS 7732-18-5) was obtained with an Elga Purelab Ultra system.

2.2. Calibration standards and linearity analysis

A stock solution of 10 mM sodium lactate was prepared gravimetrically from Sodium DL-lactate powder by weighing 11.206 mg of the purchased Sodium DL-lactate powder into a 10 mL volumetric flask and diluting to the mark with water. "Clean" or "artificial" EBC is not available commercially and EBC from any donor contains low levels of lactate. Since EBC is reported to consist of > 99% water, we chose to use water as a surrogate for EBC as a calibration solvent [17]. The IS working solution was prepared by first preparing an IS stock solution from the purchased 20% w/w solution by diluting 10 μL to 10 mL with water. The IS working solution was then prepared by diluting 100 μL of the IS stock solution to 10 mL to create the final 2 mM IS solution.

Calibrators were prepared by serial dilution from the 10 mM solution, as was the IS stock solution at 2 mM. Ten calibration solutions were prepared at concentrations of 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50 and 100 μM and the final working IS solution at 200 μM.

2.3. Chromatographic conditions

A Hydrophilic Interaction Chromatography (HILIC) method was developed using a Waters XBridge BEH Amide column (2.1 × 150 mm, 3.5 μm, 130 Å) with a matching Waters BEH Amide VanGuard Cartridge guard column (2.1 × 5 mm, 3.5 μm, 130 Å).

Solvent A was a 50:50 (v/v) mixture of acetonitrile (ACN):water containing 10 mM ammonium acetate (NH_4OAc) and was pH adjusted to pH 9 with aqueous ammonium hydroxide (NH_4OH). Solvent B was a mixture of 95:5 (v/v) ACN:water containing10 mM NH_4OAc and was also pH adjusted to pH 9 with NH_4OH. Flow was maintained at 0.5 mL/minute and the ratio of solvents A:B was maintained at 55:45 for an isocratic separation.

Separation time was 4 min, and the effluent from the liquid chromatograph was diverted to waste from the end of data acquisition until 0.5 min after the subsequent injection. 10 μL injections were used. The sample compartment of the autosampler was maintained at 15 °C, and the column was maintained at 40 °C.

Table 1
Ion Source Parameters.

Temperature	350 °C
Ionization Mode	Negative
Ionization Voltage	−4500
Resolution	Unit
Dwell Time	50 ms
Collision Energy	−15
Collision Gas	Nitrogen

2.4. Mass spectrometric analysis

Experiments were performed on a SCIEX® 6500 Q-trap mass spectrometer, operated in negative ion mode, with nitrogen as the collision gas. The source was operated at 350 °C and −4500 V, the collision energy was set to −15 V and the dwell time was 50 msec for each transition. The optimized source parameters are summarized in Table 1.

2.5. Sample collection and sample preparation

Exhaled breath condensate (EBC) was obtained from male and female volunteer donors with informed written consent as approved by the University of Rochester Institutional Review Board and Office for Human Subjects Protection. Human volunteer breath was collected using an EcoScreen® device (Erich Jaeger GmbH, Hoechberg, Germany). EBC was collected for 15 min, separated into 250-μl aliquots, frozen and stored at −80 °C.

For use, the EBC samples were thawed at room temperature, vortexed and an aliquot removed, following which the samples were refrozen. 10 μL of sample were diluted 10-fold by the addition first of 10 μL IS working solution followed by addition of 80 μL of a 55:45 mixture of the two HPLC solvents, closely matching the isocratic chromatographic conditions. The resulting mixture was again vortexed and subsequently analyzed. 10 μL injections were employed. Samples were run as a batch with calibrators bracketing the samples.

2.6. Assay validation procedures

The assay was validated for its linearity, limit of quantification, precision, accuracy, interference, ion suppression and carry-over.

2.6.1. Calibration curve and LLOQ

A number of factors forced us to use a surrogate calibration. There is no commercial source of an EBC analog, and obtaining sufficient EBC from donors for routine use would be problematic. EBC from donors also contains low, inconsistent levels of lactate. The latter was also the reason to use labeled Sodium DL-Lactate for the calibration curve in EBC, while using a non-labeled form in water. Linearity of the detection of lactate was tested in both water and EBC by preparing calibration curves. In water, standards were made by dilution of a gravimetrically prepared solution of Sodium DL-Lactate. To test that the EBC did not affect the calibration, standards of labeled sodium lactate were prepared in pooled EBC by dilution of the purchased IS solution and used to generate a calibration curve. This experiment was designed to minimize the impact of any potential contamination while evaluating the linearity of the assay.

Quantification of lactate in EBC was performed using a labeled IS as described previously. Initial surveys indicated that a calibration range of ~1 μM to ~100 μM would be adequate for the analysis of EBC samples. Ten calibration solutions (0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50 and 100 μM) were used to cover the concentration range found in most