Available online at www.sciencedirect.com
ScienceDirect
Journal of Pharmaceutical and Biomedical Analysis 44 (2007) 947–954
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Quantification of ^{13}C pyruvate and ^{13}C lactate in dog blood by reversed-phase liquid chromatography–electrospray ionization mass spectrometry after derivatization with 3-nitrophenylhydrazine

Steinar Uran, Kristin Eitrem Landmark *, Gro Hjellum, Tore Skotland

Research and Development, GE Healthcare, Nycoveien 2, N-0401 Oslo, Norway

Received 16 November 2006; received in revised form 28 March 2007; accepted 2 April 2007
Available online 4 April 2007

Abstract

Injection of hyperpolarized ^{13}C-labelled pyruvate (^{13}C pyruvate) is under evaluation as an agent for medical metabolic imaging by measuring formation of ^{13}C lactate using magnetic resonance spectroscopy of the ^{13}C nuclei. A quantitative method for analysis of these ^{13}C-labelled substances in dog blood was needed as part of the development of this agent and we here describe a liquid chromatography-mass spectrometry method for that purpose. Immediately after blood collection, the blood proteins were precipitated using methanol added internal standard ([U-^{13}C]pyruvate and [U-^{13}C]lactate). Prior to analysis, the compounds were derivatized using 3-nitrophenylhydrazine. Following separation on a Supelco Discovery HS C18 column, ^{13}C pyruvate and ^{13}C lactate were detected using negative electrospray ionization mass spectrometry. Calibration standards (4.5–4500 μM ^{13}C pyruvate and 9–9000 μM ^{13}C lactate) and added internal standard were used to make the calibration curves, which were fitted to a non-linear equation $y = a + bx + cx^2$ and weighted with a weighting factor of $1/y^2$. The analytical lower limit of quantification of ^{13}C pyruvate and ^{13}C lactate was 4.5 and 9 μM, respectively. The total precision of the method was below 9.2% for ^{13}C pyruvate and below 5.8% for ^{13}C lactate. The accuracy of the method showed a relative error less than 2.4% for ^{13}C pyruvate and less than 6.3% for ^{13}C lactate. The recoveries were in the range 93–115% for ^{13}C pyruvate and 70–111% for ^{13}C lactate. Both substances were stable in protein-free supernatant when stored for up to 3 weeks in a −20 °C freezer, during three freeze/thaw cycles, and when stored in an autosampler for at least 30 h.

Keywords: LC–MS; ^{13}C pyruvate; ^{13}C lactate

1. Introduction

Magnetic resonance imaging (MRI) has been a powerful tool for diagnostic medical imaging for more than 20 years. This technique produces mainly anatomical images, although also functional information may be obtained to some degree, e.g. such as motion, flow and perfusion. The use of magnetic resonance spectroscopy (MRS) has also been introduced into medical imaging and this technique can in addition provide information about metabolism; for example ^{31}P MRS can give useful information about the energy states of cells/tissues.

During the last years new possibilities has been opened for metabolic imaging of endogenous substances by using ^{13}C MRS [1,2]. This is possible following injection of hyperpolarized ^{13}C substances as hyperpolarization may increase the signal-to-noise ratio more than 10 000 times [3], thus making visualization of nuclei of low concentration possible. This new technique of using hyperpolarized ^{13}C MRS for diagnostic metabolic imaging, i.e. to obtain information about the distribution and metabolism of the injected hyperpolarized ^{13}C substance, has been reviewed recently [4,5].

One possibility for metabolic imaging using ^{13}C-labelled endogenous substances is to inject hyperpolarized ^{13}C-labelled pyruvate (^{13}C pyruvate) and measure the MRS signals for the ^{13}C nuclei during metabolism of pyruvate to lactate and other metabolites [5]. The ^{13}C MRS signals can be followed for a short period due to the rapid loss of hyperpolarization [5]. This period is too short to describe the pharmacokinetics of the injected substance and its metabolites as needed during preclinical and clinical studies for development of such an agent. Thus, it was

* Corresponding author. Tel.: +47 23 18 56 27; fax: +47 23 18 60 08.
E-mail address: Kristin.Landmark@ge.com (K.E. Landmark).

0731-7085/$ – see front matter © 2007 Published by Elsevier B.V.
doi:10.1016/j.jpba.2007.04.001

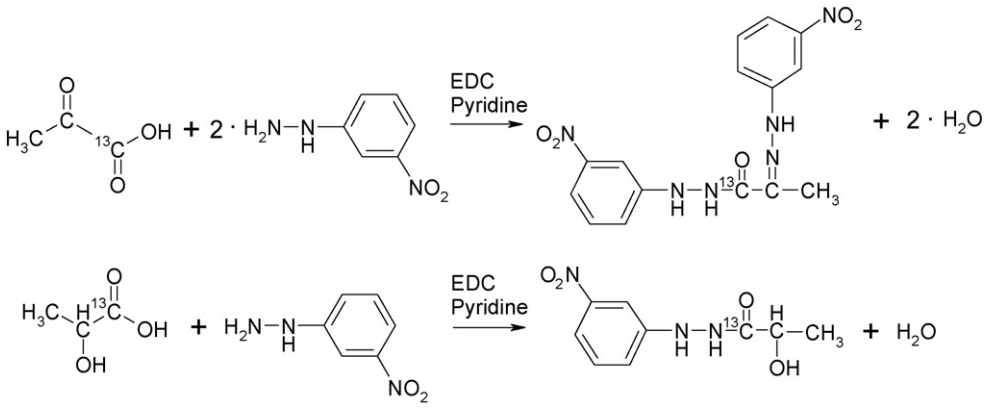

Fig. 1. Structures of pyruvate (top), lactate (bottom) and their reaction products after derivatization with 3-NPH, using EDC as a coupling reagent and catalysed by pyridine.

necessary to develop methods to quantify ^{13}C pyruvate and ^{13}C lactate in blood.

In the present article we describe method development and validation of a liquid chromatography-mass spectrometry (LC–MS) method that can be used to quantify ^{13}C pyruvate and ^{13}C lactate in dog blood. To be able to separate between the natural abundant ^{12}C pyruvate and lactate and the ^{13}C-labelled compounds following injection of ^{13}C pyruvate, MS detection has to be used. Due to the polarity and low molecular weight of pyruvate and lactate, separation and detection are not straight forward. Even if some papers describe separation and detection of pyruvate and lactate without previous derivatization [6,7], our experience was that both better chromatographic separation and better detection limits could be obtained by derivatization of the compounds prior to the LC–MS analysis. In order to analyse both pyruvate and lactate in one single run, we decided to derivatize with 3-nitrophenylhydrazine (3-NPH) using a carbodiimide coupling reagent. This reaction (2-NPH or 3-NPH) is widely used for derivatization of carboxylic acids, aldehydes and ketones [8,9]. Using this reaction, one molecule of pyruvate will react with two molecules of 3-NPH, of which one will react with the carboxylic acid functional group and one with the ketone functional group, giving a derivative with a molecular mass of 358. Lactate, on the other hand, has carboxylic acid as the single functional group, thus giving a derivative with only one molecule 3-NPH and a molecular mass of 225. The structures are shown in Fig. 1.

2. Experimental

2.1. Materials

Formic acid, acetic acid (glacial) and pyridine were analytical grade from Merck, Germany. Acetonitrile and methanol were HPLC grade from Merck, Germany. Ethanol was 96% from Arcus, Norway. 3-Nitrophenylhydrazine hydrochloride (3-NPH, ≥98% purity) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, ≥98% purity) were from Sigma–Aldrich, Germany. Sodium [1-^{13}C]pyruvate (powder, molecular weight 111.04), sodium [U-^{13}C]pyruvate (powder, molecular weight 113.02), sodium [1-^{13}C]lactate (as aqueous solution, molecular weight 113.05) and sodium [U-^{13}C]lactate (as aqueous solution, molecular weight 115.04) were from Sigma–Aldrich, Germany. The ^{13}C-labelled compounds are for simplicity in the following referred to as ^{13}C pyruvate, $^{13}C_3$ pyruvate, ^{13}C lactate and $^{13}C_3$ lactate, respectively. According to the manufacturer, the standard compounds contained approximately 99 at.% ^{13}C and the purity was approximately 99%. Water was purified by reversed osmosis, ion exchanged and filtered through a 0.45 μm filter on a Milli-Q Reagent Water System (Millipore, France).

2.2. Standards and control samples

The calibration standards were prepared daily by diluting standard stock solutions of ^{13}C pyruvate and ^{13}C lactate in water. The concentration range of the standards was approximately 4.5–4500 μM ^{13}C pyruvate and 9–9000 μM ^{13}C lactate. The quality control samples used to evaluate the precision and accuracy of the method were made by diluting the standard stock solutions of ^{13}C pyruvate and ^{13}C lactate in water to concentrations of 13.5, 224 and 3364 μM ^{13}C pyruvate and 26.5, 442 and 6634 μM ^{13}C lactate. The concentrations in the low quality control sample correspond to approximately three times the lowest calibration standard and the concentrations in the high quality control sample to approximately 75% of the highest calibration standard.

The quality control samples used to evaluate the stability of ^{13}C pyruvate and ^{13}C lactate in protein-free supernatant (made as described in Section 2.3) were made by diluting the standard stock solutions of ^{13}C pyruvate and ^{13}C lactate in protein-free supernatant to concentrations of 0, 13.5, 225 and 3379 μM ^{13}C pyruvate and 0, 26.5, 442 and 6634 μM ^{13}C lactate. Note that the zero-concentration samples are neither added ^{13}C pyruvate nor ^{13}C lactate, and in practice are "blank" samples containing only the endogenous levels of pyruvate and lactate. Usually three concentration levels in the range of the expected sample concentrations are used in control samples, but due to the endogenous levels of pyruvate and lactate in dog blood, we decided also to include non-spiked samples.

Similarly, the quality control samples used to evaluate the recovery of ^{13}C pyruvate and ^{13}C lactate from whole dog blood were made by adding standard solutions of ^{13}C pyruvate and ^{13}C lactate to dog blood to concentrations of 13.8, 230 and 3501 μM ^{13}C pyruvate and 27.0, 452 and 6875 μM ^{13}C lactate. The internal standards were prepared daily by diluting standard stock solutions of $^{13}C_3$ pyruvate and $^{13}C_3$ lactate in water to concentrations equivalent to 226 μM $^{13}C_3$ pyruvate and 442 μM $^{13}C_3$ lactate in the standards and samples.

2.3. Blood sample preparation

At least 300 μl dog blood was collected in tubes containing heparin as anticoagulant. As soon as practically possible after collection (usually within 30 s), 200 μl of the blood samples were transferred to pre-chilled centrifuge tubes stored on ice containing 900 μl methanol and 20 μl internal standard, and mixed on a whirlmixer for 10 s. The exact blood weight was determined by weighing the tubes. Centrifugation of blood samples was started within 15 min after sample collection, and the tubes were centrifuged at approximately 15 000 × *g* for 15 min at 2–8 °C. The protein-free supernatant (approximately 800 μl) was transferred to cryotubes and stored below −15 °C until analysis.

2.4. Preparing standards and samples for LC–MS analysis

Each reaction vial was added 25 μl calibration standards or quality control samples, 20 μl internal standard and 155 μl methanol. The blank samples contained 45 μl water and 155 μl methanol. From the deproteinized blood samples 200 μl protein-free supernatant were added to the reaction vials. To each reaction vial 200 μl 3-NPH (0.14 M in 50% (v/v) ethanol in water) and 400 μl EDC (0.05 M in 1.5% (v/v) pyridine in ethanol) were added, the content was mixed and heated for 20 min at 60 °C, then cooled on ice. Prior to analysis, 200 μl derivatized sample and 200 μl of 8% (v/v) acetic acid in water were added to HPLC vials and mixed.

2.5. LC–MS conditions

The chromatographic system consisted of an HP 1100 series HPLC system (Agilent Technologies, USA). The derivatized compounds were separated on a Supelco Discovery HS C18, 2.1 mm × 75 mm (3 μm) column (Sigma–Aldrich, USA) with a Phenomenex C18 (ODS), 2 mm × 4 mm (3 μm) guard column (Phenomenex, USA). Mobile phase A comprised 0.1% (v/v) formic acid in water and mobile phase B was 0.1% (v/v) formic acid in acetonitrile. Separation was obtained by using a gradient starting at 20% mobile phase B, increasing to 80% B in 8 min, held at 80% B for 4 min and then back to 20% B in 1 min. The total chromatographic run time was 25 min. The flow rate was 0.2 ml/min and the column temperature was 30 °C. The injection volume was 5 μl. The HPLC system was coupled on-line to an LCQ^{TM} ion-trap mass spectrometer (ThermoElectron, USA) equipped with an electrospray (ES) ionization source. The HPLC effluent entered the MS through a steel ES ionization needle set at 3.25 kV and a heated capillary set to 220 °C. The sheath gas flow was approximately 1.2 l/min. The ion source and ion optic parameters were optimised to minimise adduct formation but maintain adequate sensitivity.

2.6. Sample analysis and validation parameters

The samples were analysed by negative-ion HPLC-ES-MS in the full scan mode. Scan range was *m/z* 220–270 for the time period of 0–7.5 min and *m/z* 350–760 for the time period of 7.5–15 min. For quantification of pyruvate, the $(M-H)^{-1}$ ion for ^{13}C pyruvate (*m/z* 358.1), $^{13}C_3$ pyruvate (*m/z* 360.1) and ^{12}C pyruvate (*m/z* 357.1) were used. For quantification of lactate, the $(M-H)^{-1}$ ion for ^{13}C lactate (*m/z* 225.1), $^{13}C_3$ lactate (*m/z* 227.1) and ^{12}C lactate (*m/z* 224.1) were used. Each sequence of analysis included single injections of the calibration standards, quality control samples, blood samples and non-derivatized injection blanks.

The calibration standards were analysed in six analytical series on different days. Each sequence contained a blank sample (i.e. without added internal standard), a zero sample (i.e. with added internal standard) and seven non-zero samples. The calibration curve for ^{13}C pyruvate was made from these calibration standards by plotting the peak area ratio of ^{13}C pyruvate (*m/z* 358.1) to $^{13}C_3$ pyruvate (*m/z* 360.1), against the theoretical concentration of ^{13}C pyruvate. Similarly, the calibration curve for ^{13}C lactate was made from the same calibration standards by plotting the peak area ratio of ^{13}C lactate (*m/z* 225.1) to $^{13}C_3$ lactate (*m/z* 227.1), against the theoretical concentration of ^{13}C lactate.

The precision of the method, i.e., intra-day, inter-day and total precision, was evaluated by analysing quality control samples in triplicate in six analytical series. Precision data was determined by ANOVA single-factor data analysis. The results of total mean concentration ($mean_t$), the pooled within-run standard deviation ($S.D._{w(p)}$), the between-run standard deviation ($S.D._b$) and total standard deviation ($S.D._t$) was calculated. Accuracy was determined by comparing the concentrations of ^{13}C pyruvate and ^{13}C lactate in the quality control samples to the nominal concentrations and expressed as the relative error (R.E.) of the measurements. Recoveries of ^{13}C pyruvate and ^{13}C lactate were determined using dog blood samples added three concentration levels of the analytes. Quality control samples at three concentrations, analysed in triplicate, were also used to validate long-term stability of pyruvate and lactate in water (23 days storage in a −20 °C freezer, i.e. below −15 °C).

Quality control samples, one at endogenous level and at three additional concentrations, analysed in triplicate, were used to validate long-term stability of ^{13}C pyruvate and ^{13}C lactate in protein-free supernatant (21 days storage below −15 °C), stability of derivatized ^{13}C pyruvate and ^{13}C lactate in protein-free supernatant for 7 days storage at 2–8 °C, stability of ^{13}C pyruvate and ^{13}C lactate in protein-free supernatant after three freeze/thaw cycles (approximately −20 and 25 °C) and the stability of processed ^{13}C pyruvate and ^{13}C lactate samples during approximately 35 h in the autosampler.

To correct for the ^{13}C contribution from natural abundant ^{12}C pyruvate and ^{12}C lactate in the blood samples, the amount of ^{12}C pyruvate and ^{12}C lactate in the samples were determined using the calibration curves for the ^{13}C-labelled compounds. The ^{13}C concentration of pyruvate and lactate in the samples was then corrected with the theoretical ^{13}C isotope contribution, i.e. 18.7% and 11.1% for pyruvate and lactate, respectively. This correction is necessary because the MS detects ions with different isotope compositions at different masses. The rather high isotope effects for pyruvate and lactate in the present method is due to that it is the derivatized form of pyruvate and lactate that are detected (see Fig. 1).

2.7. Data handling

Xcalibur chromatographic software, Version 1.3, was used for data sampling, integration, preparation of calibration curves and concentration determinations. GraphPad Prism, Version 4.00, was used for preparation of calibration curves and statistical calculations. Microsoft Excel 2002 SP-2 was used for statistical calculations.

3. Results and discussion

3.1. Method development

Because both pyruvate and lactate are known to be very unstable in plasma and whole blood, it is not recommended to store such samples without any previous sample preparation, and precipitation of blood proteins using perchloric acid is commonly used [10,11]. We had some concerns about using a strong acid because of the following derivatization and use of LC–MS. Thus, we tested acetonitrile and methanol as organic precipitation agents and obtained similar results with methanol as that obtained with perchloric acid (data not shown). The immediate precipitation with methanol added internal standard made it possible to store the samples deproteinized and ready for derivatization. Initially we added an extra clean-up step by extracting the protein-free supernatant using ethyl acetate. This extra step was time-consuming, and since for our purpose it added little value to the method, we found it unnecessary. Nevertheless, extracting the samples prior to injection will make the samples cleaner, and consequently, give better protection of the analytical column and the MS. In addition, extraction of the samples may allow up-concentration of the samples, and then improve the detection limits. Early in the method development we experienced some problem with the formation of isomeric peaks of pyruvate, i.e. possibly *syn*/*anti* isomers as previously reported [12]. However, immediate cooling of the reaction mixture followed by dilution with an equal volume of 8% (v/v) acetic acid prior to the LC–MS analysis, minimised this problem. The type and concentration of the acid was chosen based on initial experiments (data not shown). Another issue to be aware of is ion suppression. At the highest standard concentrations we could observe a decrease in the absolute peak areas, but the use of the internal standards compensated for this decrease, i.e. the peak area ratios between the standard and internal standard were not affected.

3.2. Separation and specificity of the method

Fig. 2 shows typical chromatograms of a blank dog blood sample and a dog blood sample added ^{13}C pyruvate, ^{13}C lactate and the internal standards. The peaks representing the endogenous levels of ^{12}C pyruvate and ^{12}C lactate, and their respective ^{13}C isotope contribution are clearly seen in the blank dog blood sample, which also shows that there are no endogenous peaks interfering with the internal standards $^{13}C_3$ pyruvate and $^{13}C_3$ lactate. The chromatogram of dog blood sample added ^{13}C pyruvate, ^{13}C lactate and the internal standards, show increased ^{13}C peak areas as well as well-defined peaks for the internal standards.

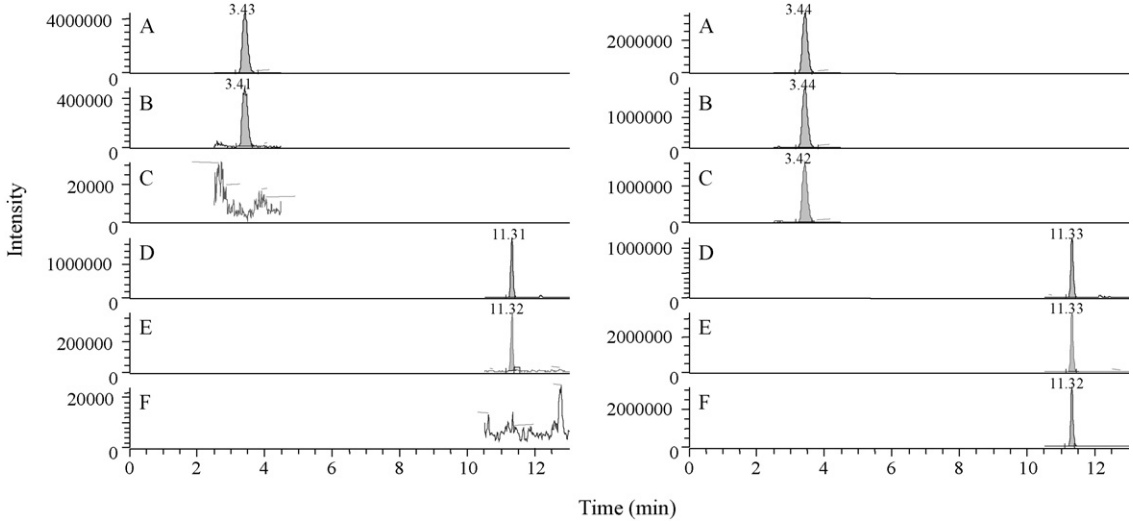

Fig. 2. Typical chromatograms of LC–MS analysis in full scan mode; blank dog blood sample to the left and dog blood sample added 224 μM ^{13}C pyruvate, 442 μM ^{13}C lactate and internal standards to the right. (A) *m/z* 224.1 (^{12}C lactate); (B) *m/z* 225.1 (^{13}C lactate); (C) *m/z* 227.1 ($^{13}C_3$ lactate as internal standard); (D) *m/z* 357.1 (^{12}C pyruvate); (E) *m/z* 358.1 (^{13}C pyruvate); (F) *m/z* 360.1 ($^{13}C_3$ pyruvate as internal standard).

Table 1
The endogenous level of ^{12}C pyruvate and ^{12}C lactate and their respective ^{13}C isotope contribution in a pool of blood from Beagle dogs

Pyruvate sample no.	Measured ^{12}C m/z 357 (μM)	Theoretical isotope effect m/z 358 (μM)	Measured m/z 358 (μM)	Apparent endogenous level of ^{13}C (μM)[a]
1	93.0	17.4	19.4	2
2	94.8	17.7	18.6	0.9
3	99.5	18.6	17.8	−0.8
Lactate sample no.	**Measured ^{12}C m/z 224 (μM)**	**Theoretical isotope effect m/z 225 (μM)**	**Measured m/z 225 (μM)**	**Apparent endogenous level of ^{13}C (μM)[b]**
1	993	110	105	−5
2	990	110	109	−1
3	959	106	107	1

[a] Measured m/z 358 − (measured $^{12}C \times 0.187$).
[b] Measured m/z 225 − (measured $^{12}C \times 0.111$).

The endogenous levels of ^{12}C pyruvate and ^{12}C lactate from a pool of blood obtained from male Beagle dogs, and their respective ^{13}C isotope contribution, were calculated using the ^{13}C calibration standards. The corrected ^{13}C concentrations for both pyruvate and lactate were then estimated and found to be below the lower limit of detection (LLOQ) of the method, and the difference between the theoretical and actual measured ^{13}C concentrations are well within the accuracy of the method (Table 1). Further, the endogenous levels of pyruvate and lactate measured are comparable to published data in both dog [13] and man [14].

A blank sample was routinely analysed after the highest calibration standard, and the peak area of ^{13}C pyruvate (m/z 358.1) and ^{13}C lactate (m/z 225.1) found in the blank sample was compared to the peak area of ^{13}C pyruvate and ^{13}C lactate in the standard. A carry-over in the range of 0.12–0.31% was then observed for ^{13}C pyruvate, but no carry-over was detected for ^{13}C lactate. The minor carry-over peak is most probably due to the high concentration found in the highest ^{13}C pyruvate calibration standard and was considered not to affect the usefulness of the method.

The retention times of ^{13}C pyruvate and ^{13}C lactate increased slightly both during a sequence of analysis and between the different sequences (data not shown). This was due to a gradual deterioration of the column used in the validation study. This column was therefore changed, and an improved precision was obtained after changing to the new column. After performing this validation study we have analysed more than 1000 samples with the same analytical column without any significant change in the properties of that column.

3.3. Quantification

To be able to determine the correct concentration of a substance from a biological matrix using LC–MS, it is important to use an internal standard. Due to ion-suppression effects, it is best if that standard is as equal to the analyte as possible. As the $^{13}C_3$ labelled variants of pyruvate and lactate are commercially available, they were the obvious choice to be used as internal standards for this method. It should, however, be kept in mind that the m/z ratio between the analyte and internal standard is only 2 units, which lead to a minor isotope effect from the standards to the internal standards. This isotope effect can easily be accounted for in the quantification software if considered necessary.

The calibration curves were fitted to a non-linear equation $y = a + bx + cx^2$ with a weighting factor $1/y^2$. A statistical test (F-test, which quantifies the relationship between the relative increase in sum of squares and the relative increase in degrees of freedom) to evaluate linear versus quadratic regression was performed. For ^{13}C pyruvate the values from the F-test ($P = 0.05$) ranged from 2 to 27, clearly showing that non-linear regression fits the data significantly better than linear regression. For ^{13}C

Table 2
The estimated regression parameters of the ^{13}C pyruvate and ^{13}C lactate calibration curves fitted to the equation: $y = a + bx + cx^2$ and weighted by $1/y^2$

Regression coefficient ^{13}C pyruvate	Intercept (a)	Slope (b)	Curve (c)	Correlation coefficient (r^2)
Mean ($n = 6$)	0.00258	0.00426	−0.000000179	0.997
S.D.	0.00196	0.0000822	0.0000000219	0.00158
R.S.D. (%)	na	1.9	−12.3	0.16
95% confidence	0.00206	na	na	na
Regression coefficient ^{13}C lactate	**Intercept (a)**	**Slope (b)**	**Curve (c)**	**Correlation coefficient (r^2)**
Mean ($n = 6$)	−0.000635	0.00218	−0.0000000118	0.999
S.D.	0.000725	0.0000716	0.0000000008	0.000911
R.S.D. (%)	na	3.3	na	0.09
95% confidence	0.000761	na	na	na

na = not applicable.

Table 3
Precision of the method, i.e. intra-day, inter-day and total precision for ^{13}C pyruvate and ^{13}C lactate at three different concentrations

Pyruvate control sample	Mean$_t$ (μM)	S.D.$_{w(p)}$ (μM)	S.D.$_b$ (μM)	S.D.$_t$ (μM)	R.S.D.$_{w(p)}$ (%)	R.S.D.$_b$ (%)	R.S.D.$_t$ (%)
Low (13.5 μM)	13.2	1.20	na[a]	na[a]	9.1	na[b]	9.1[b]
Medium (224 μM)	229	8.90	2.42	9.22	3.9	1.1	4.0
High (3364 μM)	3284	272	na[a]	na[a]	8.3	na[b]	8.3[b]
Lactate control sample	**Mean$_t$ (μM)**	**S.D.$_{w(p)}$ (μM)**	**S.D.$_b$ (μM)**	**S.D.$_t$ (μM)**	**R.S.D.$_{w(p)}$ (%)**	**R.S.D.$_b$ (%)**	**R.S.D.$_t$ (%)**
Low (26.5 μM)	27.0	1.53	na[a]	na[a]	5.7	na[b]	5.7[b]
Medium (442 μM)	469	11.4	11.4	16.1	2.4	2.4	3.4
High (6634 μM)	6909	171	147	226	2.5	2.1	3.3

$n = 3 \times 6$ at each concentration. na = not applicable.
[a] S.D.$_b$ considered not significant, i.e. $F < F_{crit}$ in the ANOVA single-factor analysis.
[b] R.S.D.$_t$ is set equal to R.S.D.$_{w(p)}$ in cases where S.D.$_b$ is na.

lactate the values from the *F*-test ranged from 0.05 to 21. Even if the results from the *F*-test were not conclusive, they indicate that non-linear regression fits the data better in four of six curves. Further, back-calculation of the calibration standards showed a better overall fit using non-linear regression for ^{13}C lactate as well (data not shown). Thus, non-linear regression was selected for both ^{13}C pyruvate and ^{13}C lactate analysis. Weighting was found to be essential to give optimal fit in the entire concentration range. The estimated curve parameters with standard deviations (S.D.), the correlation coefficients (r^2) and the 95% confidence interval of the *Y*-intercepts from six series of analysis are listed in Table 2 and shown to be highly reproducible. The goodness of fit of the calibration points to the calibration curve using the non-linear equations was also estimated and the largest deviation from the theoretical concentrations were 2.3% relative error (R.E.) for ^{13}C pyruvate and −3.3% R.E. for ^{13}C lactate (data not shown), indicating an acceptable fit of the calibration points to the calibration curves.

3.4. Limit of quantification

According to published recommendations [15] the LLOQ of a method can be set to a specific concentration provided that the precision and accuracy at this concentration is below 20% relative standard deviation (R.S.D.) and ±20% R.E., respectively. The lowest calibration standard at 4.48 μM ^{13}C pyruvate and 8.85 μM ^{13}C lactate was chosen as the LLOQ of the method, and three replicates of this standard were analysed in six analytical sequences. The precision was 20.8% R.S.D. and 16.6% R.S.D. for ^{13}C pyruvate and ^{13}C lactate, respectively. The accuracy was 3.2% R.E. and 8.0% R.E. for ^{13}C pyruvate and ^{13}C lactate, respectively. However, the LLOQ could be lowered by small modifications of the method, i.e. by modifying volumes used in blood sample preparation and in the derivatization process, in addition to extraction and up-concentrating of the samples as outlined in Section 3.1. We did not see any reason, however, to further improve the LLOQ of the method, as the ^{13}C peaks caused by the isotope effects of the endogenous level of pyruvate and lactate were detected with the present method.

3.5. The precision and accuracy of the method

The precision of the method i.e., intra-day, inter-day and total precision, was calculated as described in Section 2.6. The results of total mean concentration (mean$_t$) for each quality control sample are shown together with the pooled within-run standard deviation (S.D.$_{w(p)}$), between-run standard deviation (S.D.$_b$) and total standard deviation (S.D.$_t$) in Table 3. The intra-day precision of the method, expressed as the pooled within-run R.S.D., was better than 9.2% for ^{13}C pyruvate and 5.8% for ^{13}C lactate, showing that the analysis of the quality control samples were reproducible. The inter-day precision of the method, expressed as the between-run R.S.D., was better than 1.2%

Table 4
Accuracy of the method for ^{13}C pyruvate and ^{13}C lactate at three different concentrations

Pyruvate control sample	Theoretical concentration (μM ^{13}C pyruvate)	Mean determined concentration (μM ^{13}C pyruvate)	Relative error (% of theoretical)
Low	13.5	13.2	−1.9
Medium	224	229	2.3
High	3364	3284	−2.4
Lactate control sample	**Theoretical concentration (μM ^{13}C lactate)**	**Mean determined concentration (μM ^{13}C lactate)**	**Relative error (% of theoretical)**
Low	26.5	27.0	2.0
Medium	442	469	6.2
High	6634	6909	4.1

$n = 3 \times 6$ at each concentration.

Table 5
Recovery of ^{13}C pyruvate and ^{13}C lactate added to dog blood at three concentrations

Added ^{13}C pyruvate (μM)	Measured ^{12}C *m/z* 357[a]	Measured ^{13}C *m/z* 358[a]	Corrected ^{13}C[b] (μM)	% recovery (mean ± S.D.)
13.8	171 ± 12	45 ± 2.4	12.8 ± 1.7	93 ± 13
230	182 ± 0.9	297 ± 10	263 ± 10	115 ± 4.4
3501	218 ± 18	3878 ± 250	3837 ± 246	110 ± 7.0
Added ^{13}C lactate (μM)	Measured ^{12}C *m/z* 224[a]	Measured ^{13}C *m/z* 225[a]	Corrected ^{13}C[b] (μM)	% recovery (mean ± S.D.)
27.0	1582 ± 27	194 ± 9.5	18.9 ± 6.7	70 ± 25[c]
452	1606 ± 31	681 ± 11	503 ± 8.1	111 ± 1.8
6875	1649 ± 61	7331 ± 261	7148 ± 267	104 ± 3.9

[a] Mean determined concentration ± S.D. (μM, $n = 3$).
[b] Corrected for the ^{13}C isotope effect as described in Section 2.6.
[c] The low recovery probably due to an sample replicate outlier, see details in Section 3.6.

for ^{13}C pyruvate and 2.5% for ^{13}C lactate, showing that the between-run variation did not contribute significantly to the total variation. The total precision of the method was subsequently calculated to be better than 9.2% for ^{13}C pyruvate and 5.8% for ^{13}C lactate, indicating an acceptable overall precision of the method.

The accuracy of the method was found to be between −2.4% and 2.3% R.E. for ^{13}C pyruvate and between 2.0% and 6.2% R.E. for ^{13}C lactate (Table 4), showing an acceptable accuracy of the method.

3.6. Recovery of the method

The recoveries of the method were determined to be 93%, 115% and 110% for ^{13}C pyruvate and 70%, 111% and 104% for ^{13}C lactate, for low, medium and high concentrations, respectively (Table 5). The repeatability at each concentration level of the recovery blood samples were within ±15%, except for the low ^{13}C lactate recovery sample. One of the three sample replicates had a recovery of only 44% for ^{13}C lactate and 78% for ^{13}C pyruvate which resulted in the lower recovery at this level. Excluding this sample (statistically on the limit of being an outlier, data not shown), gives recoveries of 100% and 83% for ^{13}C pyruvate and ^{13}C lactate, respectively. This shows that the "real" recoveries probably are higher than the ones given. Since the added ^{13}C lactate concentration at this level is only about 17% of the signal observed due to the isotope effects of the endogenous lactate concentration, this is not considered to affect the usefulness of the method.

3.7. Sample stability

When ^{13}C pyruvate and ^{13}C lactate were dissolved at three concentration levels in water and stored below −15 °C for 23 days, the recoveries were in the range 93.3–101.5% and 97.8–105.6% for ^{13}C pyruvate and ^{13}C lactate, respectively. When ^{13}C pyruvate and ^{13}C lactate were dissolved at four concentration levels (i.e. including the endogenous level) in protein-free supernatant and stored below −15 °C for 3 weeks, the recoveries were in the range 87.6–108.7% and 103.5–108.9% for ^{13}C pyruvate and ^{13}C lactate, respectively. Further, during three freeze/thaw cycles the recoveries were in the range 97.1–104.3% and 101.6–110.0% for ^{13}C pyruvate and ^{13}C lactate, respectively, except for the two lowest ^{13}C lactate concentrations, which showed recoveries of 79.9% and 83.4%, respectively. When derivatized samples at the four concentrations tested were stored at 2–8 °C for 7 days, the recoveries were in the range 102.1–104.9% and 98.1–110.7% for ^{13}C pyruvate and ^{13}C lactate, respectively.

Thus, ^{13}C pyruvate and ^{13}C lactate are considered adequately stable during 23 days storage below −15 °C when dissolved in water, during 3 weeks storage below −15 °C when dissolved in protein-free supernatant and during three freeze/thaw cycles when dissolved in protein-free supernatant. Further, derivatized samples of ^{13}C pyruvate and ^{13}C lactate were found to be adequately stable during 7 days storage at 2–8 °C and processed samples of ^{13}C pyruvate and ^{13}C lactate in protein-free supernatant were found to be stable during approximately 35 h storage in sample for injection at 25 °C (data not shown).

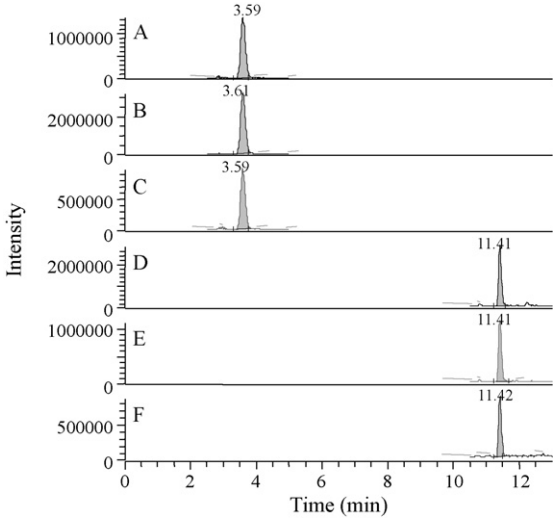

Fig. 3. Representative chromatogram of LC–MS analysis in full scan mode of a dog blood sample obtained 1 min after injection of ^{13}C pyruvate test item, and added internal standards. (A) *m/z* 224.1 (^{12}C lactate); (B) *m/z* 225.1 (^{13}C lactate); (C) *m/z* 227.1 ($^{13}C_3$ lactate as internal standard); (D) *m/z* 357.1 (^{12}C pyruvate); (E) *m/z* 358.1 (^{13}C pyruvate); (F) *m/z* 360.1 ($^{13}C_3$ pyruvate as internal standard).

3.8. Analysis of a blood sample following injection of ^{13}C pyruvate

The utility of the method is shown by analysing a blood sample obtained 1 min after injection of 5 ml test item (approximately 210 mg ^{13}C pyruvate) per kg body weight into one Beagle dog (Fig. 3). Complete kinetic data from preclinical studies will be published elsewhere.

3.9. Summary

An analytical method for determination of ^{13}C pyruvate and ^{13}C lactate in dog blood has been developed and validated and found to be suitable for analysis of these substances following injection of ^{13}C pyruvate in dogs.

References

[1] K. Golman, J.H. Ardenkjær-Larsen, J.S. Petersson, S. Månsson, I. Leunbach, Proc. Natl. Acad. Sci. 100 (2003) 10435–10439.
[2] K. Golman, R. In't Zandt, M. Thaning, Proc. Natl. Acad. Sci. 103 (2006) 11270–11275.
[3] J.H. Ardenkjær-Larsen, B. Fridlund, A. Gram, G. Hansson, L. Hansson, M.H. Lerche, R. Servin, M. Thaning, K. Golman, Proc. Natl. Acad. Sci. 100 (2003) 10158–10163.
[4] S. Månsson, E. Johansson, P. Magnusson, C.-M. Chai, G. Hansson, J.S. Petersson, F. Ståhlberg, K. Golman, Eur. Radiol. 16 (2006) 57–67.
[5] K. Golman, J.S. Petersson, Acad. Radiol. 13 (2006) 932–942.
[6] G. Minniti, R. Cerone, E. De Toni, Am. Clin. Lab. 20 (2001) 21–23.
[7] R.H. Haas, J. Breuer, M. Hammen, J. Chromatogr. 425 (1988) 47–57.
[8] R. Peters, J. Hellenbrand, Y. Mengerink, S. Van der Wal, J. Chromatogr. A 1031 (2004) 35–50.
[9] H. Miwa, Anal. Chim. Acta 465 (2002) 237–255.
[10] C.-K. Chuang, T.-J. Wang, C.-Y. Yeung, W.-S. Hsieh, D.-S. Lin, S.-C. Ho, S.-P. Lin, Clin. Biochem. 39 (2006) 74–77.
[11] F.M. Carragher, J.R. Bonham, J.M. Smith, Ann. Clin. Biochem. 40 (2003) 313–320.
[12] H. Terada, T. Hayashi, S. Kawai, T. Ohno, J. Chromatogr. 130 (1977) 281–286.
[13] L.S. Jacobson, R.G. Lobetti, Am. J. Vet. Res. 66 (2005) 244–250.
[14] K.J. Foster, K.G. Alberti, L. Hinks, B. Lloyd, A. Postle, P. Smythe, D.C. Turnell, R. Walton, Clin. Chem. 24 (1978) 1568–1572.
[15] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittman, S. Spector, Pharm. Res. 9 (1992) 588–592.