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Original article

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Abstract – The aim of this study was to validate the measurement of glucose appearance rate using [6,6-$^2H_2$]glucose i.v. infusion in lactating dairy cows. Sample enrichments were analysed by gas chromatography/mass spectrometry. Linearity (enriched solutions) and specificity (enriched plasma) were good: for enrichments ranging between 1.6 and 6.3 mol% excess, the slopes were about 1 and the ordinates at the origin were not different from zero. For a plasma enriched at 3.74 mol% excess, repeatability and long term intralaboratory reproducibility coefficients of variation were 1.31 and 1.90%, respectively. The appearance rates were calculated by two models. The values provided by the steady-state model were not different from those provided by the non-steady-state Steele model. Both models can be used because the treatment effects were similarly discriminated regardless of the model. In our experiments analysing the nutritional effects on Ra in mid-lactating cows, the precision of the method (1.90%) was not the limiting factor to detect a significant difference in Ra compared to the statistical precision obtained with the experimental scheme (4×4 and 5×5 Latin square design). We conclude that in lactating dairy cows, the measurement of glucose fluxes with this method is relevant and minimally invasive for the animals.

dairy cows / glucose metabolism / GC-MS / [6,6-$^2H_2$]glucose / validation

Abbreviations: E: enrichment; GC-MS: gas chromatography/mass spectrometry; MPE: mol% excess; Ra: appearance rate; RSD_r: coefficient of repeatability; RSD_R: coefficient of intralaboratory reproductibility; BW: body weight.

1. INTRODUCTION

Lactating ruminants, dairy cows in particular, exhibit a high glucose requirement for milk synthesis. Glucose regulates milk volume through lactose synthesis. In addition, the glucose availability for mammary gland synthesis could be an important

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factor regulating milk composition (fatty acids and proteins) [1, 2]. On average, a cow in mid-lactation producing 25 kg·d^{-1} of milk utilises between 11 and 14 moles of glucose per day [3] and the fractional extraction from the mammary gland is about 60%. Dairy cows obtain their supply of glucose mainly from endogenous glucose production and partly from intestinal starch that escapes fermentation of carbohydrates in the rumen. Hepatic glucose production provides most of the endogenous glucose supply. The quantitative estimate of glucose supplies and requirement is an important challenge to predict and control milk yield and milk composition.

However, quantitative estimates of glucose fluxes are difficult or costly to assess in lactating dairy cows. In small lactating ruminants, two techniques are used to estimate these fluxes: total splanchnic net balance and the isotope dilution technique [4, 5]. The total splanchnic net balance is very invasive, it requires the implantation of catheters into an artery and in the portal and hepatic veins (under general anaesthesia) and measurement of blood flow from the portal vein and one liver artery [4]. In lactating dairy cows, the portal vein is very short and it remains difficult to measure portal-hepatic blood flow with an ultrasonic flow probe. To our knowledge, few experiments (9) using the total splanchnic net balance to measure glucose fluxes have been published on lactating dairy cows. By contrast, the use of isotope dilution allows the measurement of glucose appearance rates (Ra) and disappearance rates with minimal invasion of the body [4], and glucose stable isotopes, in particular [6,6-$^2H_2$] glucose, have been used extensively to measure Ra in human subjects and rodents [6]. This technique developed in the late 1970s [7] is now recognised for its safety and its precision [8]. In human subjects, [6,6-$^2H_2$]glucose is considered to be a "non-recycling tracer" and to give the best estimate of Ra [8, 6]. This aspect was not verified in ruminants; however in cattle [9], the use of the equivalent labelled radioactive tracer ([6-$^3H_2$]glucose) also leads to minimal recycling (4 to 5%). In lactating dairy cows, the intravenous (i.v.) infusion of [6,6-$^2H_2$]glucose remains costly, however the cost of the tracer has reduced progressively. This technique has begun to be used in a few studies in lactating cows [2, 10, 11] and lactating goats [5]. No validation of the analytical method has yet been performed in ruminants.

The aim of this paper was to describe the development and validation of the analysis of [6,6-$^2H_2$]glucose in plasma samples in order to obtain a repeatable measurement of Ra in dairy cows. For this purpose, an intralaboratory validation of the determination of [6,6-$^2H_2$]glucose enrichments was performed. Some of the application conditions for measuring Ra were also analysed, mainly to limit the number of sample analyses. In that connection, the possibility of reducing the number of natural abundance samples was examined. In addition, two models currently used to calculate Ra, the Steele model [12, 13] and the steady-state model [14], were compared in order to choose the model best adapted to our experimental conditions.

2. MATERIALS AND METHODS

2.1. Cows, management

Ten lactating Holstein cows (623 ± 55 kg of body weight (BW); 26.7 ± 4.5 kg milk yield) were used for this study. They were all managed in individual tie stalls. They received restricted amounts of diets based on big bale silage according to Rigout [2, 15] to meet 100% of net energy of lactation and 110% of protein requirements [16]. Concentrate was given in eight equal portions per day every three hours and access to roughage was limited to one hour every three hours. All the cows were fitted with an indwelling catheter into the left carotid and a provisional silicone catheter was inserted into the right jugular vein for [6,6-$^2H_2$]glucose infusion. Four cows

were additionally fitted with a provisional catheter inserted into the left subcutaneous abdominal vein where it emerges from the udder [2]. Arterial samples were taken from one cow (data set 1) to perform specificity validation of the method. The other cows were arranged in two latin square designs (4×4 and 5×5, respectively) with 14 day periods [2, 15]. The treatments consisted of continuous duodenal glucose infusion or ruminal propionic acid infusion (for further information see [2, 15]).

2.2. [6,6- $^2H_2$]glucose intravenous infusion, blood sampling

A 250 mL solution of [6,6-$^2H_2$]glucose of 98.0% purity (Euriso-top, Saint Aubin, France) was prepared with sterile saline at a concentration of 59.4 ± 0.7 g·L^{-1}, and sterilised by passage through a 0.22-µm disk filter (Millipore, Saint-Quentin-en-Yvelines, France). A 50-mL volume of this solution was injected as a priming dose. The solution of [6,6-$^2H_2$]glucose was then continuously infused for 120 min at a constant rate of 1.3 ± 0.013 mL·min^{-1} with a syringe pump (Harvard apparatus, Les Ulis, France; reproducibility: 0.1%). Blood samples were collected simultaneously from arterial and venous abdominal catheters with heparinised syringes (7 mL; 12 to 30 IU·mL^{-1} of heparin lithium; Sarstedt, Nümbrecht, Germany) at 20, 15 and 10 min before injection of [6,6-$^2H_2$]glucose solution in order to measure the natural abundance level. Arterial blood was also collected during the period of i.v. infusion, at 90, 100, 110 and 120 min to measure plasma [6,6-$^2H_2$]glucose enrichments at the plateau and at 5, 10, 20, 30 45, 60 min after the infusion stopped, in order to measure the decrease in plasma enrichments. Blood samples were kept on ice and centrifuged at 2000 × *g* for 12 min at 4 °C. Plasma (3 mL) was immediately deproteinised with an equal volume of 1.2 M perchloric acid, filtered and aliquoted in 1.2 mL before being stored at –20 °C until analysis.

2.3. Determination of [6,6-$^2H_2$]glucose enrichments

Samples of deproteinised plasma were filtered on 0.22 µ filters (Millex®, Millipore Corporation, Bedford, MA) before being neutralised with 3.2 M K_2CO_3 and crystals were eliminated by two centrifugations. The supernatant (1 volume: 750 µL) was passed over an anion then a cation resin exchange column (AG 1-8: formate form and AG 50 W-X8: H+ form; 1:1 volume ratio each; Bio-Rad S.A., Ivry-sur-Seine, France) according to Kreisberg [17]. The column was rinsed 4 times with 1 volume of distilled water. The eluate was evaporated twice in a centrifugal evaporator (Savant 110 AR, Fisher Biobloc Scientific, Ilkirch, France). The eluate was evaporated firstly. The residue (200 to 250 µg of glucose) was then dissolved in 800 µL of distilled water before being re-evaporated. The second residue was derivatised with 1-butylboronic acid (30 µL) followed 12 h later by addition of 15 µL acetic acid anhydride [18]. At least one hour later, glucose enrichment was determined by gas chromatography/mass spectrometry (GC-MS) in the electron impact ionisation mode (GC 8060 chromatograph coupled to a VG Platform II, Fisons Instruments, Altrincham, England). The GC injector temperature was set at 260 °C and the transfer line was held at 280 °C. The derivative mixture (1 µL) was injected in the split (1:20) mode. A 30 m DB 5-MS, 0.25 mm inner diameter and 0.1 µm film thickness capillary column (J&W Scientific, Courtabœuf, France) was used in this study. The oven temperature program used started at 150 °C, raised by 10 °C·min^{-1} to 220 °C and then by 20 °C·min^{-1} to 300 °C at which it was kept for 3 min. At a carrier gas (He) flux of 1.2 mL·min^{-1}, the retention time of the glucose derivative was 9.0 min. The source temperature was set at 180 °C and mass spectra was conventionally recorded at 70 eV. The selected Ion Monitoring (SIM) mode was used to monitor the specific ion masses (M-57: loss of 1 t-butylgroup) for

[(1,2:3,5)-bis butylboronate] 6-acetate glucose : 297 and 299 for the M and M+2 ions, respectively. The response of the mass spectrometer was considered to be in a linear range when M ranged between 300 000 and 2 000 000. Each molar ratio (M+2 to M) was determined in triplicate from areas of the corresponding peaks and the glucose enrichment was then calculated by taking into account the natural abundance molar ratio [14].

In order to take into account the potential drifts in the derivative and analytical process, each batch of measurements was organised as follows: one natural abundance plasma and one enriched control plasma, another natural abundance plasma and enriched plasma samples (3–5), then repetition of one natural abundance plasma and enriched plasma samples. In addition between each sample, 3 injections of acetonitrile were performed.

2.4. Intralaboratory validation study

Solutions of non-enriched glucose (98.0% purity; Bruneau Braun, Boulogne-Billancourt, France) and [6,6-$^2H_2$]glucose (98.0 mol% excess; 98.0% purity; Cambridge Isotope Laboratories, Inc., Andover, MA) were dissolved in distilled water to perform linearity validation. Their concentrations (2 mmol·L^{-1}) corresponded to glucose concentration of deproteinised plasma. In addition, two pools of arterial plasma samples were taken from one cow (data set 1) to perform specificity validation and to calculate repeatability and reproducibility. These two samples were used as control plasma at natural abundance molar ratio and as a control at 3.74 mol% excess (MPE) enrichment.

Linearity was assessed over the 0.95–8.6 MPE range: six enrichments distributed approximately every 1.5 MPE. For each of the six enrichments, five solutions were independently prepared by dilution of the [6,6-$^2H_2$]glucose solution and the non-enriched glucose solution. The acceptance of the linear regression model and the linearity of the range of enrichment were statistically tested according to Feinberg [19] and the procedure NF V03.110 [20]. In addition, the detection limit, the critical level and the quantification limit were calculated [20] using the results of the linear regression performed to assess linearity ($\bar{y} = a_0 + a_1 \times \bar{x}$ with S_{a0} is the standard error of a_0). The detection limit is defined as the lowest enrichment detectable but not quantified with a known error risk. The critical level is defined as the lowest enrichment above the detection limit which allows us to conclude with a low error risk that the sample was not enriched artificially, and the quantification limit is defined as the lowest enrichment quantifiable with a known error risk:

$$X_{LD} = \frac{a_0 + 3 \times S_{a0}}{a1} \text{ detection limit;}$$

$$X_{LQ} = \frac{a_0 + 6 \times S_{a0}}{a1} \text{ critical level;}$$

$$X_{LQ} = \frac{a_0 + 10 \times S_{a0}}{a1} \text{ quantification limit.}$$

In addition to the linearity analysis, the specificity analysis tested the influences on the linearity of the response of the matrix (plasma) and of the reagents required for purification of the samples before derivatization (i.e. deproteinisation, neutralisation and ion-exchange chromatography). Specificity was assessed by a recovery study using a simplified procedure of NF V03.110 [20] because the standard method remained too costly in lactating cows. Plasma samples at the natural abundance molar ratio (data-set 1) were used. They were enriched by adding 11 solutions of [6,6-$^2H_2$]glucose to obtain enrichments varying within the linearity range (1.6–6.3 MPE). A linear regression between measured enrichments and calculated enrichments was determined to assess the slope of recovery and the ordinate at x = 0. Statistical analyses including the null hypothesis were performed according to Feinberg [21].

Repeatability and long-term intralaboratory reproducibility were assessed in parallel using the control plasma (3.74 MPE). Four independent replicates per day were analysed by a single operator to assess repeatability. This operation was repeated eight different days during an 8-month period to assess long-term intralaboratory reproducibility. Three different operators contributed to the analysis of reproducibility. Statistical analyses were performed according to the guidelines in ISO 5725 [22] and to Feinberg [21].

2.5. Statistical analysis for application conditions of Ra measurements

The possibility to reduce the number of natural abundance samples was analysed by testing the effect of individuals (cows) and the effect of vessel sampling (artery or venous) on the natural abundance molar ratio. The cow effect was analysed using 10 values (5 cows × 2 replicates) from Rigout [15] according to an analysis of variance (ANOVA) including the cow and residual effect. The effects of vessel sampling on the measurement of the natural abundance molar ratio was analysed using 30 values (two missing values) from a 4 × 4 Latin square design [2]. Each batch of analysis included both the arterial (carotid) samples and the abdominal venous samples of one cow during one period. The ANOVA included, the cow, period, treatment, vessel and residual effects as sources of variation.

The steady-state model and a non-steady-state model, the Steele model [13], were compared to calculate Ra using 14 data (2 missing values) from Rigout [2]. The ANOVA include the cow, period, treatment, model of calculation, the interaction model × treatment, model × cow, model × period and residual effects as the sources of variation.

The different ANOVA were all processed using the general linear model (GLM) of SAS [23].

3. RESULTS

3.1. Intralaboratory validation study

The results of the linearity response of the GC-MS instrument for solutions with six enrichments repeated five times are presented in Table I. The model of linear regression was acceptable at $P < 0.01$ because $F > F_{0.01,\ 1,\ 24}$ (linearity line in Tab. II). In addition, the whole linearity range chosen (enrichments between 0.97 and 8.53 MPE) was validated at $P < 0.01$ because $F \leq F_{0.01,\ 4,\ 24}$ (non-linearity line in Tab. II). The calculated regression of linearity was:

$$Em = (1.0022 \pm 0.0070) \times Ec + (-0.0765 \pm 0.0387) \quad \text{(MPE)}$$

where, Em is the measured enrichment and Ec is the calculated enrichment. The

Table I. Data of linearity assay.

	MPEc[1] (%)	MPEm[2] (%)					Mean (%)
	x	y_1	y_2	y_3	y_4	y_5	
Enrichment level							
1	0.97	0.95	0.89	0.88	0.92	0.94	0.91
2	2.43	2.34	2.51	2.42	2.28	2.48	2.40
3	4.24	4.06	4.13	4.08	4.08	4.11	4.09
4	5.73	5.70	5.48	5.64	5.66	5.63	5.62
5	7.16	7.19	6.98	7.06	7.06	7.37	7.13
6	8.53	8.57	8.49	8.30	8.60	8.53	8.50

[1] Calculated mol% excess.
[2] Measured mol% excess.

Table II. Analysis of variance results for the linearity assay.

Sources of variation	df[1]	Sum of Squares	Mean Square	F values	$F_{0.01}$[2] in tables	Conclusions
Linearity	1	205.5581	205.5581	22257	7.832	$F > F_{0.01,1,24}$
Non linearity	4	0.0621	0.01553	1.681	4.218	$F \leq F_{0.01,4,24}$
Error	24	0.2217	0.00924			
Total	29	25.8418				

[1] Degree of freedom.
[2] Fisher table values at $P < 0.01$.

correlation coefficient was 0.9997. The detection limit was 0.04 MPE, the critical level was 0.16 MPE and the quantification limit was 0.31 MPE.

The matrix (plasma) and the preparation of samples before derivatization had no significant influence on the result (Fig. 1). The regression equation obtained for specificity was very close to the regression equation obtained for linearity:

$$Em = (1.005 \pm 0.027) \times Ec + (-0.049 \pm 0.112). \quad \text{(MPE)}$$

The specificity was statistically acceptable at $P < 0.01$, the slope (1.0005) was not significantly different from 1 and the ordinate at x = 0 (–0.049) was not significantly different from zero.

For the control plasma (3.74 MPE), the precision method was good as demonstrated by the following statistical data for repeatability at $P < 0.05$: $r_{95\%} = 0.14$ and $RSD_r = 1.31\%$ and long-term intralaboratory reproducibility: $R_{95\%} = 0.202$ and $RSD_R = 1.90\%$.

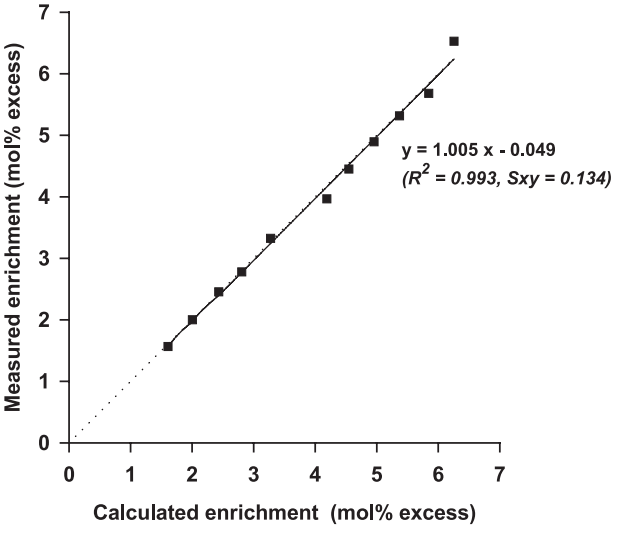

Figure 1. The specificity of the analytical method to test the matrix (plasma) effect and the influences of the purification process on the linearity of the response. Plasma samples at natural abundance were enriched by the addition of [6,6-$^2H_2$]glucose over the range of 1.6–6.3 mol% excess. The linear regression between the calculated enrichments and measured enrichments was determined to assess the slope of recovery and the ordinate at x = 0 according to Feinberg [19].

3.2. Validation of application conditions of Ra measurement

3.2.1. Measurement of natural abundance molar ratio

To analyse the cow effect on the natural abundance molar ratio, a variable MR_i was calculated using NAi, the natural abundance molar ratio for a cow i, determined in two replicates in a batch i (i = 1 to 5), Ci, the natural abundance molar ratio of the control plasma (data set 1), and $\hat{C}$ the corresponding mean for the five batches:

$$MR_i = NA_i + (\hat{C} - C_i)$$

The ANOVA did not shown any significant difference among cows (P = 0.55, SE = 0.001) in the measurement of the natural abundance molar ratio (0.0265). Additionally, in the data of Rigout [2], the determined natural abundance molar ratio was not significantly different (P = 0.50) between the samples taken from the carotid artery (molar ratio: 0.02535 ± 0.00017) and the samples taken from the subcutaneous abdominal vein (molar Ratio: 0.02519 ± 0.00017).

3.2.2. Comparison of two models for calculating Ra

Two current models are used to calculate Ra, the steady-state model that requires plateau conditions for plasma glucose concentrations and enrichments and a non-steady-state model, the Steele model. Before the experiment [2], we did not know if our procedure respected the plateau conditions required for the steady-state model. For this reason, Ra was first calculated using the Steele model equation [24]:

$$E(t) = \frac{f \times Einf - p \times V \times c(t) \times \frac{dE(t)}{dt}}{Ra + f}$$

given $Ra(Steele)_{12}$ =

$$\frac{f \times Einf - p \times V \times \left(\frac{C_1 + C_2}{2}\right) \times \left(\frac{E_2 - E_1}{t_2 - t_1}\right)}{\left(\frac{E_1 + E_2}{2}\right)} - f$$

($g \cdot min^{-1}$)

where f is the [6,6-$^2H_2$]glucose infusion rate ($g \cdot min^{-1}$), Einf is the isotopic enrichment of the infusate (98 MPE), E(t), E_1 and E_2 are enrichments (in MPE) and C(t), C_1 and C_2 are glucose concentrations measured at each sampling time (t, t_1, t_2…). The estimation of the total glucose pool (V) was required. It was calculated using the samples taken after the infusion of [6,6-$^2H_2$] glucose stopped as described by Rose [10]. This volume was about 110 ± 5 $mL \cdot kg^{-1}$ BW. In the Steele equation, it was corrected by a p fraction (P = 0.65) according to Brockman [12] leading to mean Ra about 2.07 ± 0.038 $g \cdot min^{-1}$.

However during our experiment [2], concentrations and enrichments of plasma glucose reached a plateau before measurements (between 90 and 120 min after the beginning of [6,6-$^2H_2$]glucose infusion). The individual coefficients of variation ranged from 0.65 to 3.72% for plasma glucose concentrations and from 1.39 to 4.86% for enrichments. The conditions of application of the steady-state model were respected because the CV of plasma glucose concentrations and enrichments were both smaller than 5%. In that respect, Ra was determined by the equation:

$$Ep = \frac{f \times Einf}{Ra + f}$$

given

$$Ra = f \times \left(\frac{Einf}{Ep} - 1\right) \ (g \cdot min^{-1})$$

where Ep is the plasma enrichment.

Glucose Ra calculated with the steady-state model is not different than with the Steele model (2.08 vs. 2.07 $g \cdot min^{-1}$, SEM = 0.038; P = 0.9). In addition, the calculation

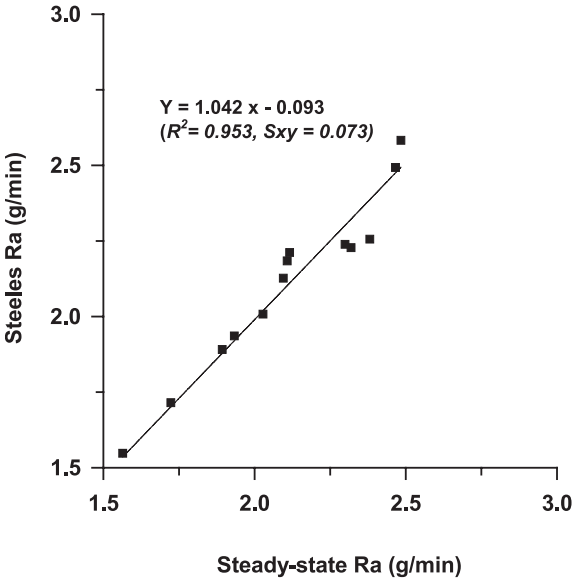

Figure 2. Linear relation obtained between the appearance rate (Ra) calculated using the Steele model and the steady-state model.

method did not interfere with the treatment measurements (duodenal infusions of glucose or water, see [2]) because the interaction between the model of calculation and treatment was not significant (P = 0.8). This also applied to the effects due to the cow or to the period of measurement (no significant interaction between the model and cow: P = 0.9 or between model and period: P = 0.9). Moreover, as shown in Figure 2, parallel responses for Ra values were obtained with the two models. The linear regression between Ra(Steele) and Ra exhibited a slope that was not significantly different from 1 ($t_{12\ ddl} = 0.63$, $P > 0.5$) and the ordinate at the origin was not different from zero:

$$\text{Ra(Steele)} = (1.04 \pm 0.067) \times \text{Ra} + (-0.09 \pm 0.140) \quad (\text{g}\cdot\text{min}^{-1})$$

4. DISCUSSION

The objective of this study was to analyse the feasibility and the validity of measuring glucose Ra using a [6,6-$^2H_2$]glucose i.v. infusion in lactating ruminants, mainly in dairy cows. Infusions of [6,6-$^2H_2$]glucose and blood sampling were easily performed with minimal discomfort for goats [5] and cows [2, 15]. In our experience, the measurement of glucose Ra with this technique was easier, less invasive and safer for the dairy cows than the measurement of total splanchnic net balance [25]. In addition, the laboratory technique for the determination of plasma enrichments was validated.

4.1. A validated analytical method allowing to reduce the number of analyses

With our method described, a standard curve for each batch of analysis is not required because both the linearity and the specificity were good. In both linearity and specificity measurements, the slopes of enrichment responses and the ordinates at the origin (x = 0) were not significantly different from 1 and 0, respectively. The absence of a need for a standard curve for each batch of analysis is interesting because standard curves with biological enriched samples (plasma) are not practical to produce [14]. In that regards, the number of analyses to be performed may be reduced in each batch if the calculations are based on enrichments.

Repeatability and intralaboratory reproducibility were assessed by an enrichment (3.74 MPE) corresponding to the mean enrichment of plasmas obtained during our experiments [2, 15]. The coefficients obtained for the present method (RSD_r = 1.31%; RSD_R = 1.90%) were within the range of variation of methods [7, 26] including a separation technique (GC-MS), which is noteworthy because the present method includes all the steps of sample extraction and preparation before GC-MS injection.

Nevertheless in the future, when beginning a series of analysis that corresponds to an experiment (100 to 200 blood samples), the first batch of analyses will include a natural abundance plasma, the control plasma and a standard curve. This first batch will allow to verify if the peaks (M) obtained with solutions and plasmas are within the linearity range of the GC-MS linearity (M between 300 000 and 2 000 000). The standard curve will be used to verify the linearity (ordinate at the origin not different from zero and slope about 1). A maintenance of the ion source could be required to obtain correct values. In addition, the control plasma (at 3.74 MPE) that corresponded to enrichments obtained in our experiments (2.5 to 4.5%) will be introduced into each batch to continue to follow long-term intralaboratory reproducibility and construct a control card. Lastly, the calculation of enrichments implies a correct measurement of natural abundance molar ratio to take into account the "basal" response. Interestingly, the basal response was not affected by the animal chosen (cow) and by the vessel used for blood sampling, suggesting that a same plasma may be used for all natural abundance ratio measurements in the future.

4.2. The model of Ra calculation may also reduce the number of analyses

Similar values of Ra were obtained using the non-steady-state Steele model and the steady state model when the conditions of the steady-state model were respected for plasma glucose concentrations and enrichment (both CV < 5%). The coefficient of variation of Ra measurements was not reduced by using the Steele model (8.5 and 6.5%, for the Steele and steady-state model, respectively) as already observed in sheep when comparing these methods using a radioactive tracer [12]. In addition, the Steele model requires more blood sampling than the steady state model because the volume of the miscible glucose pool should be estimated by determining tracer elimination (10 samples instead of 4 per Ra measurements). In addition, the value of the volume of the miscible glucose pool may be dependant on the method of calculation. In the present study, the volume of the calculated miscible glucose pool (110 ± 5 mL·kg^{-1} BW) was lower than that reported in sheep [12, 27] with a different method of calculation while it was only slightly lower than the volumes reported in lactating dairy cows (141 ± 3.5 mL·kg^{-1} BW and 121.5 ± 4.7 mL·kg^{-1} BW) with the same method of calculation [10, 11]. In that respect, since the treatments are equally discriminated by both models of calculation, it seems less costly to use the steady-state model, which requires less sample measurement, in future experiments with protocols similar to that of Rigout [2] if the conditions of the steady-state model are respected.

4.3. Attainable precision for detecting significant differences between Ra

The ability to detect significant differences between Ra measurements depends on the precision of the method and on the experimental design. In our experiments [2, 15], a 4×4 and 5×5 Latin square design were used to analyse nutritional effects on Ra in mid-lactating cows. The smallest significant difference in Ra that could be statistically detected at $P < 0.05$ was about 200 g·d^{-1} (i.e. 8% for an Ra of 2500 g·d^{-1}).

The precision of the method was not limiting compared to the statistical precision. The detectable difference between the

enrichment (E) due to the precision of the method was about 0.143 MPE ($2 \times [RSD_R \times 100] \times E$ with $RSD_R = 1.90\%$ for the control plasma at 3.74 MPE). The corresponding significant difference expressed in Ra (for a Ra about 2500 $g \cdot d^{-1}$) was 115 $g \cdot d^{-1}$, less than the experimental design significance detection limit (200 $g \cdot d^{-1}$).

Consequently, the [6,6-$^2H_2$]glucose i.v. infusion rate (59.4 $mg \cdot min^{-1}$) was not a limiting factor for detecting significant differences in Ra because it led to enrichments in the plasma control value (3.74 MPE) and to a significance detection limit of about 115 $g \cdot d^{-1}$. In that connection, infusions might be reduced slightly but not substantially because the RSD_R decreases with enrichment [26] according to the Horwitz model [19]. Assuming that for plasma enriched at only 2 MPE, the detectable difference between enrichments (E) will remain at about 0.143 MPE, the smallest difference detectable due to the precision of the method (170 to 200 $g \cdot d^{-1}$) would be close to the statistical detecting limit.

5. CONCLUSION

In our experience, using stable isotope techniques to measure glucose fluxes in lactating dairy cows is easy to perform and less invasive than the difficult technique of total splanchnic net balance. In this study, we validated the adaptation of the technique for analysing plasma enrichments in [6,6-$^2H_2$]glucose enrichments and the use of the steady-state model at plateau conditions in lactating dairy cows. This methodology allows a reduction in the number of analyses required to measure Ra and consequently the cost of this measurement. The precision of the method was not the limiting factor for detecting significant differences when comparing nutritional treatments in lactating dairy cows using a 4×4 or 5×5 Latin square design. However, with the current GC-MS instrument, the quantity of infused tracer to infuse cannot be considerably reduced. To greatly reduce experimental costs, by reducing the quantity of the infused tracer, other instrumentation will be required such as GC-P-IRMS for ^2H measurement. However, the limit of the new GC-P-IRMS technique is considering the M+1 isotopomer in its analyses.

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