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GLUCOSE UPTAKE RATE UNCERTAINTY IN A FED BATCH BIOREACTOR

G.Sassi[(1,2)]*, and M.Bernocco*[(1)]

1. Dipartimento di Scienza dei Materiali e Ingegneria Chimica - Politecnico di Torino Corso Duca degli Abruzzi 24 10129 Torino, ITALY, guido.sassi@polito.it
2. INRIM (Istituto Nazionale di Ricerca Metrologica) Strada delle Cacce 91 - 10135 Torino, ITALY

Abstract: Glucose uptake rate is a typical quantity for bioreactor non invasive monitoring, which is candidate as metabolic assay for tissue engineering. Volumetric glucose uptake rate enables to compare bioreactor performances. Fed Batch reactors are typical in tissue engineering applications. This work aims to the identification of the metrological bottlenecks of an uptake rate measurement in a bioreactor for human cells; it offers a first metrological characterization of the quantities for a typical bioreactor protocol. Glucose uptake rate and volumetric one are measurable at 6% and 3% uncertainty respectively. The enhancement of bioreactor management protocols and design, and volume measurement may lead under 1% uncertainty.

Keywords: Uncertainty, Uptake Rate, Bioreactor, Glucose, hMSC.

1. INTRODUCTION

Glucose uptake rate is a typical parameter for bioreactor monitoring in many applications [1]. It accounts for all the phenomena involved in the chain that leads the glucose from the liquid growth medium to the cell and in cell metabolism of glucose. The most of living cells are able to consume glucose which often is the favorite substrate able to overpass cell membrane by active transport [2]. The metabolic rate of glucose uptake depends on the physiologic state of the cell and thus on the phenotype [2,3,4,5].

In tissue engineering, glucose uptake rate measurement may be used as non invasive way to monitor cell activity and retrieve information from the bioreactor [4]. Glucose uptake rate (*GUR*) is an extensive quantity that accounts for cell activity in a bioreactor, and accounts for the cell number if the transport limitations and phenotypes are stable enough. The volumetric glucose uptake rate (GUR_V) is the intensive quantity used to compare bioreactors performances. It accounts for the cell density if the transport limitations and phenotype are stable enough. *GUR* can be candidate as metabolic assay for cell counting in 2D and 3D systems.

Fed batch reactors are generally used in biology and in particular in tissue engineering. The liquid growth medium is replaced partially or completely every time interval, around 3 days are generally considered as time interval because of the duplication characteristic time of the cells.

The aim of this work has been the identification of the metrological bottlenecks of an uptake rate measurement in a fed batch bioreactor for human Mesenchymal Stem Cells (hMSCs). The goal of this paper is to focus on the procedures for the uncertainty evaluation of the glucose uptake rate. Glucose Uptake Rate, although widely used in bioreactor monitoring and design, have never been metrologically characterized and it has started to be used recently in tissue engineering [1, 4, 5, 6].

Typical management protocols of bioreactor for tissue development at a typical bioreactor size have been considered for the measurement and uncertainty calculation. To measure glucose concentration the most accurate method for blood has been considered [7,8]. The budget of uncertainty has here been used as a tool for the analysis of critical contributions to uncertainty.

2. MATERIAL AND METHOD

Mathematical modelling. The average specific glucose consumption rate (Volumetric glucose uptake rate, GUR_V) in a fed batch bioreactor may be calculated by the glucose mass balance of the liquid phase (growth medium), assuming its perfect mixing, on a time interval. It results:

$$GUR_V = \frac{C_1 - C_0}{t_1 - t_0} \tag{1}$$

Where: C_1 and C_0 [g l^{-1}] are the concentration of glucose in the growth medium at the final time t_1 [d] and initial time t_0 [d] respectively. GUR_V is the volumetric Glucose Uptake Rate [g l^{-1}d^{-1}]; sometime in literature volumetric *GUR* is found as specific consumption [4,5,6]. The absolute consumption rate *GUR* can be calculated from the GUR_V assuming the perfect mixing of the growth medium:

$$GUR = GUR_V V_m = \frac{C_1 - C_0}{t_1 - t_0} V_m \tag{2}$$

Where: V_m [ml] is the volume of the growth medium in the bioreactor. The concentration of glucose in the growth medium was measured by D-Glucose kit (Roche, Germany). Taking into account the dilution in the cuvette, the glucose concentration in the growth medium may be calculated as reported by the Roche method procedure:

$$C = \frac{V\ M_W}{\varepsilon\ d\ v\ 1000}\left[\left(A_f - A_i\right) - \left(A_{fb} - A_{ib}\right)\right] \tag{3}$$

Where: C is the concentration in the sample [g l^{-1}], ε the extinction factor 6.3 [l $mmol^{-1}$ cm^{-1}], V the final volume in the cuvette [ml], v the sample volume [ml], d the light path [cm], M_W the molecular weight [g mol^{-1}], A_i initial absorbance at 3-8 min before enzyme addiction and A_f final absorbance at 15-25 min after enzyme addiction. Subscript b refers to blank, i.e., bidistilled water instead sample, blank correction accounts for kit solution instability. ε is verified by calibration with a standard solution of glucose (0.5 g l^{-1}). Combining equations 1-3, the measurand equations resulted to be for simultaneous t_1 and t_0 measurements:

$$GUR_V = \frac{M_W}{\varepsilon\, d\, 1000} \frac{V_1/v_1 \left(A_{f,1} - A_{i,1}\right)_1 - V_0/v_0 \left(A_{f,0} - A_{i,0}\right)}{t_1 - t_0}$$

(4)

$$GUR = \frac{V_m M_W}{\varepsilon\, d\, 1000} \frac{V_1/v_1 \left(A_{f,1} - A_{i,1}\right)_1 - V_0/v_0 \left(A_{f,0} - A_{i,0}\right)}{t_1 - t_0}$$

Experimental. hMSCs were purchased from Lonza (Basel, Switzerland). They are bone marrow derived-hMSCs from donor. hMSCs were expanded and maintained in a complete non differentiating growth medium (aMEM, Listarfish) supplemented with 10% fetal bovine serum (FBS, Listarfish), 1% L-glutamine, 1% antibiotics (kanamycin). 10 bioreactors, as a part of one 24 well plate, were filled by 1 ml growth medium (Lonza, Wokingham, UK) on one carbonate scaffolds CTT1-1 (Biocoral® France). 9 bioreactors were inoculated by seeding $1x10^4$ to $8x10^5$ hMSCs and allowed to adhere for 24h. The tenth bioreactor without cells was taken as blank solution. Bioreactors were cultured in an incubator at 37°C with 5% CO_2 for 64 days with growth medium replacement every 2–3 days. Glucose concentration was measured on each replaced growth medium.

Concentration measure. The concentration of glucose in the growth medium was measured by D-Glucose kit (D-glucose UV-method, Roche, Germany) via spectrometry according to protocol provided by Roche. D-Glucose was evaluated by enzymatic reaction: Hexokinase (HK) catalyzes the phosphorylation of glucose by ATP to form glucose-6-phosphate and ADP. Following the reaction, a second enzyme, glucose-6-phosphate dehydrogenase (G6PDH) is used to catalyze oxidation of glucose-6-phosphate by $NADP^+$ to form NADPH. The NADPH formed is stoichiometrically proportional to the glucose available in the sample. The NADPH initial and final concentration was measured by Lambert Beer equation from the initial and final absorbance at 340 nm. Absorbance has been calculated by HP8452 diode array Spectrophotometer software from beam intensity at 340 nm on the sample solution and on air (used as blank solution). Cuvette 10 mm path length 4.5 ml volume (Kartel 1937) has been used. The total volume in the cuvette V is the sum of four independent measures of volume, i.e., the bidistillated water V_w (1.9 ml by Gilson P1000 micropipette), the kit suspension V_s (1.0 ml by Gilson P1000 micropipette), the kit enzyme solution V_e (0.02 ml by Gilson P20 micropipette), and the sample solution v (0.1 ml by Gilson P200 micropipette). Each single contribution has been considered. The volume of each solution was dosed by micropipettes and weighted on 0.1 mg Gibertini E42S balance. The sample solutions were diluted appropriately to reach a glucose concentration lowers than 1 g l^{-1}.

Evaluation of uncertainty. The influence quantities which compose the *GUR* are expressed in equations (4). For each quantity measurement the uncertainty has been evaluated. The uncertainty was calculated following the ISO Guide to the Expression of Uncertainty in Measurement GUM as type A or B [9]. t_0 is the replacement time at which growth medium start to be in contact with the biological system. t_1 is the replacement time when the growth medium is removed from the biological system. The blank growth medium at final time t_1 was taken as the growth medium at initial time t_0 to correct for scaffold to growth medium interactions.

UV absorbance: it is calculated by the software of HP8452 diode array spectrophotometer. Stability of the absorbance measurement was evaluated by monitoring absorbance on 8 hour. 20 different samples of growth medium in the range 0 to 1 g l^{-1} of glucose and 2 standard solutions at 0.5 g l^{-1} of glucose were continuously monitored before and during enzymatic reaction for a total time of 1 hour. The intervals before and after enzyme addiction have been considered for uncertainty evaluation. Measurements at t_1 and t_0 have been done simultaneously to avoid kit solution instability correction. Cuvette absorption reproducibility does not affect uncertainty because an absorption difference with the same cuvette is considered.

Molecular Weight: it has been considered to not contribute to the GUR uncertainty.

Light path: cuvette dimensions were measured by mechanical devices on 15 disposable cuvettes.

Volumes of solutions: The total volume in the cuvette V is the sum of four independent measures of volume, i.e., the bidistillate water V_w, the kit suspension V_s, the kit enzyme solution V_e, and the sample solution v. Each single contribution has been considered. All volumes were dosed by micropipettes. Two measurement procedures have been considered to calculate volumes uncertainty: micropipette uncertainty from calibration and weighing volumes on an analytical balance.

Initial and final time: their uncertainty was calculated from the information retrieved from the experimental protocol of bioreactor management.

Volume of the total amount of growth medium: its uncertainty has been calculated from the information retrieved from the experimental protocol of bioreactor management and considering the identified sources of uncertainty.

3. RESULTS AND DISCUSSION

Uncertainty of influence quantities. For each of the influence quantities expressed in equations (4) the uncertainty was evaluated.

UV Absorbance: the stability analysis of the absorbance in air, calculated by the software of the spectrophotometer, reports a variability and a drift in time giving an uncertainty lower than 0.0002 on 30 minutes, i.e., the maximum time necessary for glucose concentration measurement. A random value of absorbance in a time interval before and after enzyme addiction has been considered for initial and final absorbance respectively. A uniform distribution between the maximal and minimal values observed on each time interval has been considered for all the monitored solutions to calculate uncertainty. For 8 to 3 minutes before and 15 to 25 minutes after enzyme addiction the uncertainty of absorbance has been calculated to be lower than 0.003.

Light path: Uncertainty on a single cuvette and variability among different cuvettes has been calculated. Total uncertainty resulted to be lower than 0.02 mm in agreement with the standard for quartz cuvettes [10], but lower than data available for glass cuvettes [11].

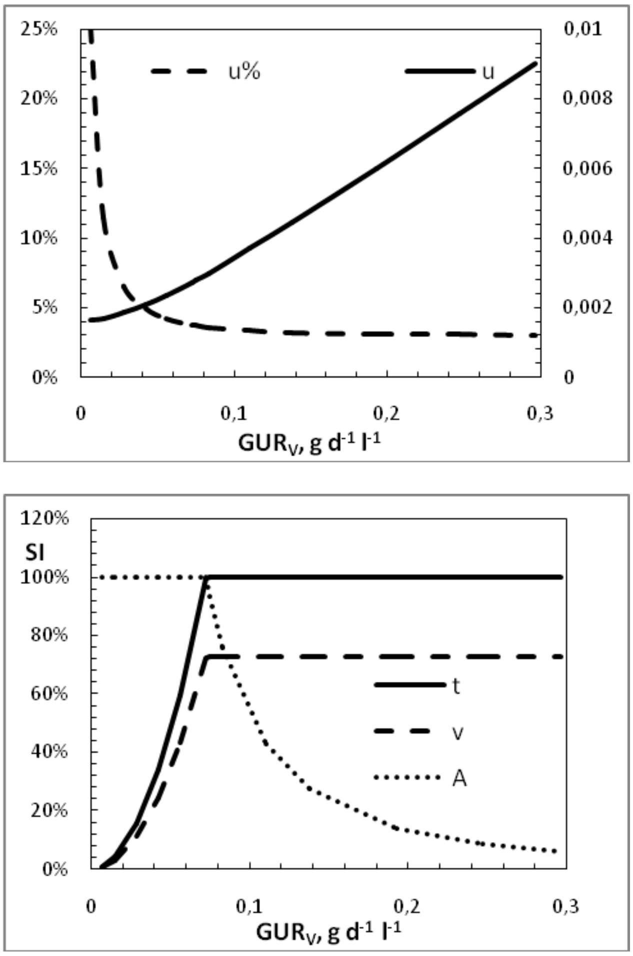


Figure 1. GURV uncertainty and Significance Index of the main contributions (time *t*, sample volume *v* and absorbance *A*)

Volume of solutions: the bidistillated water V_w was measured with 0.02 ml uncertainty by calibrated micropipette and 0.0005 ml by calibrated balance; the kit suspension V_s was measured with 0.02 ml uncertainty by calibrated micropipette and 0.0005 ml by calibrated balance; the kit enzyme solution V_e was measured with 0.002 ml uncertainty by calibrated micropipette and 0.0005 ml by calibrated balance; the sample solution v was measured with 0.002 ml uncertainty by calibrated micropipette and 0.0005 ml by calibrated balance.

Time: protocols for bioreactor management reports the day and the part of the day in which samples were withdrawn and bioreactor was fed. A uniform distribution on 4 hour interval was considered for uncertainty calculation. Uncertainty resulted to be 0.05 days for both initial and final time.

Volume of the total amount of growth medium: V_m is affected by the residual volumes at replacement and its variability, the uncertainty of the added growth medium volumes and the growth medium evaporation during reaction. All of them contribute to the uncertainty of V_m. For the actual protocol of bioreactor, the relative uncertainty of V_m was roughly evaluated to be 10% in 1 ml bioreactors.

Volumetric Glucose Uptake Rate (*GUR$_V$*). GUR_V was observed to range between 0 to 0.3 g $l^{-1}d^{-1}$ in agreement with literature data [4,5,6]. On the range the uncertainty vary from 0.002 to 0.01 g $l^{-1}d^{-1}$, while relative uncertainty is around 3% for GUR_V values over 0.1 g $l^{-1}d^{-1}$ and sharply increase below it. Figure 1 reports the Significance Index (*SI*) for the contribution of the sample volume, time and absorbance uncertainties. Significance index *SI* is the ratio between the term related to the quantity and the largest term in the sum of contributions defined by the GUM [9]. Absorbance uncertainty gives the main contribution when GUR_V is lower than 0.072 g $l^{-1}d^{-1}$, and it is not relevant over 0.2 g $l^{-1}d^{-1}$. Time and sample volume uncertainty gives a relevant contribution to GUR_V uncertainty over 0.02 g $l^{-1}d^{-1}$, time contribution become the main one over 0.072 g $l^{-1}d^{-1}$.

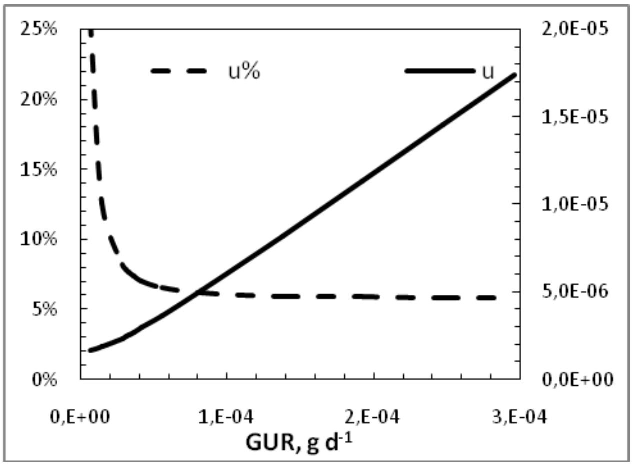
Glucose Uptake Rate (*GUR*). In the experimental tests, *GUR* was observed to range between 0-3x10^{-4}g d^{-1}. The uncertainty of the *GUR* varies in the range 0.2-1.8x10^{-5}g d^{-1}, while relative uncertainty is around 6% for *GUR* values over 1x10^{-4}g d^{-1} and sharply increases below it. Figure 1 report the Significance Index (*SI*) for the contribution of reactor growth medium volume, sample volume, time and absorbance uncertainties. Absorbance uncertainty gives the main contribution when *GUR* is lower than 3.3x10^{-5}g d^{-1}, and it is not relevant over 10^{-4}g d^{-1}. The volume of the growth medium gives a relevant contribution over 10^{-5}g d^{-1} and becomes the main contribution over than 3.3x10^{-5}g d^{-1}. Time and sample volume uncertainty gives a relevant contribution to *GUR* uncertainty over 2x10^{-5}g d^{-1}.

Uncertainty reduction strategies. The opportunities for reducing uncertainty need to individuate the uncertainty sources that give a relevant contribution to the total uncertainty. Absorbance uncertainty depends on the reaction reproducibility and stability. To reduce uncertainty at low GUR_V and *GUR* values the kit solutions have to be enhanced.

At higher rates, time is a relevant value for both the quantities under study. Protocols of management of bioreactor must be studied in order to reduce time uncertainty. The target uncertainty is 10 minutes to reach a *SI* of 1%, i.e, a negligible effect with 3 days time period. A lower uncertainty could be target if the monitoring frequency must be improved. Automatic bioreactor can reach 1 minute uncertainty.

In the high rate range the contribution of sample volume is relevant, mainly for GUR_V. The weighting of the volume reduces uncertainty. GUR_V uncertainty can be reduced to 0.85% and 0.80% with 10 and 1 min time uncertainty respectively. Sample volume and absorbance gives the most relevant contributions.

The same strategy reduces *GUR* uncertainty from 6.3% to 5.5%, because the main contribution comes from the volume of the liquid growth medium. To reduce liquid growth medium volume uncertainty it is necessary to design accurately the bioreactor and its procedure of management. The limit GUR uncertainty is around 2% when V_m uncertainty is lower than 1% and *GUR* is higher than $1x10^{-4}$g d^{-1}. The main contribution comes from absorbance or from V_m if *GUR* is over $1.7x10^{-4}$g d^{-1}.



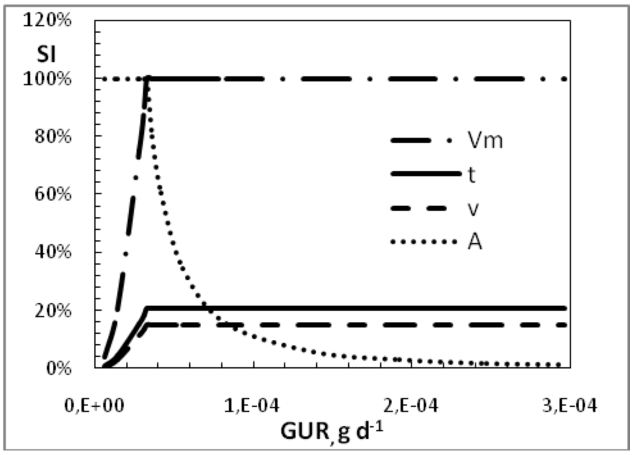


Figure 2. GUR uncertainty and Significance Index of the main contributions (bioreactor volume V_m, time *t*, sample volume *v* and absorbance *A*)

4. CONCLUSIONS

Volumetric Glucose Uptake Rate and Glucose Uptake Rate measurements have been analyzed in order to calculate uncertainty, identify bottleneck and opportunities to enhance the accuracy of the method. Volumetric Glucose Uptake Rate is an intensive quantity but the bioreactor size is not a direct influence quantity. Its actual uncertainty has been calculated around 3% over a threshold of GUR_V. GUR_V uncertainty can be reduced by reducing the uncertainty of sample volume, e.g., by weighing the volume, and of time interval, i.e., changing bioreactor management protocols. 0.8% is here considered as the minimal expected uncertainty for the method. A longer time interval could enhance the measurement, but the information would be averaged on a longer period. Glucose Uptake Rate is an extensive quantity and the volume of the growth medium in the bioreactor is the main quantity affecting uncertainty. Its actual uncertainty has been calculated around 6% over a threshold of *GUR*. To reduce *GUR* uncertainty to 3% it is necessary to modify the bioreactor management protocol and the bioreactor design to have reproducible residual volumes at the replacement and to reduce liquid evaporation. For a target uncertainty below 3%, GUR_V uncertainty must be reduced too. GUR_V and *GUR* are available measurement for tissue engineering application. The opportunities of uncertainty reduction make this measurement convenient for its use as metabolic assay for cell counting.

5. REFERENCES

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