Analytical Biochemistry 610 (2020) 113930

Contents lists available at ScienceDirect

Analytical Biochemistry

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

Technical note

Quantitative LC-MS/MS analysis of 5-hydroxymethyl-2′-deoxyuridine to monitor the biological activity of J-binding protein

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ARTICLE INFO

Keywords:
JBP1/2
LC-MS/MS
5-hydroxymethyl-2′-deoxyuridine
Base J

ABSTRACT

Base J replaces 1% of thymine in most kinetoplastid flagellates, and is implicated in transcription regulation. Base J is synthesized in two steps: first, a thymine base in DNA is converted to 5-hydroxymethyluracil by J-binding proteins (JBP1, JBP2); secondly, a glucosyl transferase glycosylates the 5-hydroxymethyluracil to form base J. Here, we present a highly sensitive and selective LC-MS/MS method to quantify the *in vitro* JBP1 activity on synthetic oligonucleotide substrates. The method demonstrated successful to support biochemical studies of JBPs and can be used as a template for additional JBP activity studies or for inhibitor screening in the future.

1. Introduction

Base J (β-D-glucosyl-hydroxymethyluracil) is a modified base that replaces 1% of thymine in kinetoplastid flagellates, such as *Trypanosoma*, *Leishmania* and *Crithidia* [1]. In *Leishmania*, 99% of J is located in telomeric repeats, whereas 1% is located where transcription starts or stops [2]. To form base J, a DNA incorporated thymine is first hydroxylated by J-DNA-binding proteins (JBP1 and JBP2) to form 5-hydroxymethyluracil. 5-Hydroxymethyluracil is then glucosylated by glucosyltransferase to form base J [3–5]. Subsequently, the newly formed base J residues are recognized by JBPs, leading to hydroxylation of other nearby thymine residues [6], in specific sequence patterns that support a mechanism for the sequence-specific maintenance of J upon DNA replication [7].

To investigate the biochemical activity of JBPs, a highly sensitive assay is required to monitor the conversion of the nucleobase thymine to 5-hydroxymethyluracil in DNA. Since the first application of mass spectrometry techniques in nucleic acid research, HPLC coupled with tandem mass spectrometry (LC-MS/MS) has become one of the standard methods for quantifying DNA modifications [8]. Mass spectrometry based analysis developed for base J oriented research has been described before, but this method focuses primarily on base J quantification [9]. Thus far, no LC-MS/MS assay has been described that focuses on highly sensitive and accurate quantification of dT and HOMedU to specifically and directly monitor the biochemical function of JBPs in dJ containing DNA.

Here we describe a highly sensitive LC-MS/MS method for the quantitative analysis of the deoxynucleoside 5-hydroxymethyl-2-deoxyuridine (HOMedU) incorporated in oligonucleotides. Deoxycytidine (dC) was used as an internal corrector for sample processing, and deoxythymidine (dT) was quantified to monitor the conversion of dT to HOMedU. In addition, the DNA incorporation of the nucleoside of base J (β-D-glucosyl-hydroxymethyl-deoxyuridine, dJ) was successfully monitored using qualitative LC-MS/MS analysis in a different mass spectrometer ionization mode. Using the described assay, the biological function of JPBs can successfully be investigated following straightforward processing and analytical procedures.

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https://doi.org/10.1016/j.ab.2020.113930
Received 22 March 2020; Received in revised form 25 August 2020; Accepted 27 August 2020
Available online 29 August 2020

2. Materials and methods

2.1. Chemicals and reference standards

All reference standard analytes purchased had a purity greater than 98%. dT and dC originated from Sigma-Aldrich (St. Louis, MO, USA). HOMedU was purchased from Santa Cruz Biotechnology, Inc., Dallas, TX, USA). ULC-grade acetonitrile, water, and ammonium formate and HPLC-grade methanol and isopropylalcohol were purchased from Biosolve Ltd (Valkenswaard, The Netherlands). Formic acid was from Merck (Darmstadt, Germany). Ammonium acetate, Tris (hydroxymethyl)aminomethane (Trizma® base), ethylenediaminetetraacetic acid (EDTA), alkaline phosphatase (EC 3.1.3.1), and nuclease P1 (EC 3.1.30.1) were all of molecular biological grade or higher and were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Preparation of calibration standards

Calibration curves were constructed for the quantification of dT, HOMedU and dC. The calibration curve consisted of 8 calibration standards in water in the following concentration range: 0.1–100 ng/mL for dT and HOMedU, and 10–1000 ng/mL for dC. The calibration curve ranges were chosen based on the sequence of the processed oligonucleotides (section 2.3) and the amount of DNA that was processed (~1–2 μg DNA). Linear regression with a weighting factor of $1/x^2$ was applied, where x equals the concentration of the analyte. The calibration curves were considered acceptable if 75% of all non-zero calibration standards were within ±15% of the nominal concentration, or ±20% for the lower limit of quantification. Calibration standards were prepared fresh for each experiment.

2.3. Selection of dT, HOMedU and dJ containing oligodeoxyribonucleotides

To investigate the selectivity and sensitivity of the LC-MS/MS method for the analysis of dT and HOMedU in oligonucleotides of interest, single-stranded and double-stranded 14-mer oligonucleotides were purchased from TriLink BioTechnologies (San Diego, CA, USA) with sequence CAGCAGC***X***GCAACA containing either dT or HOMedU in the place of X. In addition, a base J oligonucleotide (AGC***J***GCAACAA) was used for qualitative analysis of dJ in this 11-mer oligonucleotide.

For the analysis of the thymidine hydroxylase activity of JBP1, purified proteins were used with a 14-mer oligonucleotide substrate to convert dT into HOMedU under different conditions, as previously described [10].

2.4. Enzymatic digestion of oligodeoxyribonucleotides into nucleosides

Aliquots of 20 μL oligonucleotides in water (~1–2 μg DNA) were transferred to 1.5 mL reaction tubes and incubated at 95 °C for 3 min followed by rapid cooling on ice. To each reaction tube, 4 units of Nuclease P1 and 100 μL of digest buffer (containing 0.04 mM deferoxamine mesylate, 3.25 mM ammonium acetate (pH 5.0), and 0.5 mM zinc chloride) were added, after which the mixture was incubated at 65 °C for 10 min to allow for nucleotide release from the DNA structure. After this, 20 μL of Tris/HCL pH 8.5 and 4 units of alkaline phosphatase were added and vortex mixed. The sample was then incubated at 37 °C for 1 h to allow for nucleotide to nucleoside conversion. After the incubation, 20 μL of 300 mM ammonium acetate pH 5.0 was added to stop the reaction, and the samples were then evaporated under nitrogen to dryness at 40 °C. The dried samples were reconstituted in 50 μL of 5 mM ammonium acetate in water/acetonitrile (2:98, v/v) and stored at 4 °C prior to LC-MS/MS analysis.

3. Chromatography and mass spectrometry

The LC-MS/MS method was based on a previously validated method for the quantification of β-decitabine DNA incorporation [11]. For quantification, the LC-MS/MS system consisted of a QTRAP 5500 tandem mass spectrometer (Sciex, Framingham, USA) coupled to an HPLC Acquity I Class pump (Waters, Milford, MA, USA). The HPLC system was equipped with a FTN I-Class autosampler and a I-Class column oven (Waters). Data acquisition was performed using Analyst 1.6.2. Software (Sciex).

Chromatographic separation of dT, HOMedU, dC, and dJ was achieved using a Waters Nova-Pak Silica column (150 × 3.9 mm, 4 μm). Analytes were separated using gradient elution with 5 mM ammonium formate in water (mobile phase A) and 5 mM ammonium formate in water – acetonitrile (2:98, v/v) (mobile phase B). A flow rate of 1.4 mL per minute was applied and the column oven was set to 30 °C. The elution gradient was as follows: mobile phase B: 97% (0–3.2 min), 97 → 90% (3.2–7.2 min), 90 → 2% (7.2–7.3 min), 2% (7.3–11.3 min), 2 → 97% (11.3–11.4 min), 97% (11.3–14.0 min). The divert valve was set in place to direct the flow to the mass spectrometer from 2.0 to 7.5 min and to the waste for the remainder of the acquisition time to protect the MS from contaminants.

The mass spectrometer was operated in the negative ionization mode for dT, HOMedU and dC analysis, and in the positive ionization mode for dJ analysis. The following mass-to-charge ratio (m/z) transitions were monitored: 241 → 125 for dT, 257 → 124 for HOMedU, and 226 → 110 for dC in the negative ionization mode and 421 → 125 and 421 → 143 for dJ in the positive ionization mode. For dJ, a second m/z transition was monitored for qualification purposes, as a reference standard was lacking. Proposed fragmentation patterns of monitored m/z transitions can be found in Fig. 1. Source and gas parameters were optimized for the HOMedU transition window according to Table 1.

4. Results and discussion

4.1. Method development

4.1.1. Chromatography and mass spectrometry

The aim of this research was to develop an LC-MS/MS method to quantify dT to HOMedU conversion in oligonucleotides by JBPs. As the conversion rate was expected to be low, focus during method development was on the assay selectivity and sensitivity of detecting small amounts of HOMedU in the presence of other nucleosides. During method development, the sensitivity for the detection of HOMedU appeared to better in the negative than the positive ion mode. Although the sensitivity of dT and dC appeared to be better in the positive ionization mode, the relatively higher concentrations as compared to HOMedU allowed for detection in the negative ionization mode as well. After optimizing the mass spectrometer for HOMedU analysis, lower limits of quantification of 0.1 ng/mL for HOMedU and dT were reached with a signal-to-noise ratio of >10.

4.1.2. Sample pretreatment

One potential way to quantify HOMedU formation is to release the modified nucleobase (5-hydroxymethyluracil) from the DNA structure during sample processing by single-stranded specific monofunctional uracil-DNA glycosylase 1 (SMUG1). SMUG1 is an uracil-DNA glycosylase that is able to remove uracil and 5-hydroxymethyluracil from single- and double-stranded DNA [12]. This way, the released amount of 5-hydroxymethyluracil following SMUG sample pretreatment can be quantified, allowing for interpretation of JBP activity. A drawback of using this approach is that only 5-hydroxymetyluracil will be released, making it unable to detect the nucleobases thymine and base J following the same sample pretreatment procedure. By enzymatically digesting the DNA into single nucleosides using alkaline phosphatase and nuclease P1, dT, HOMedU and dJ are all released from the DNA structure at the

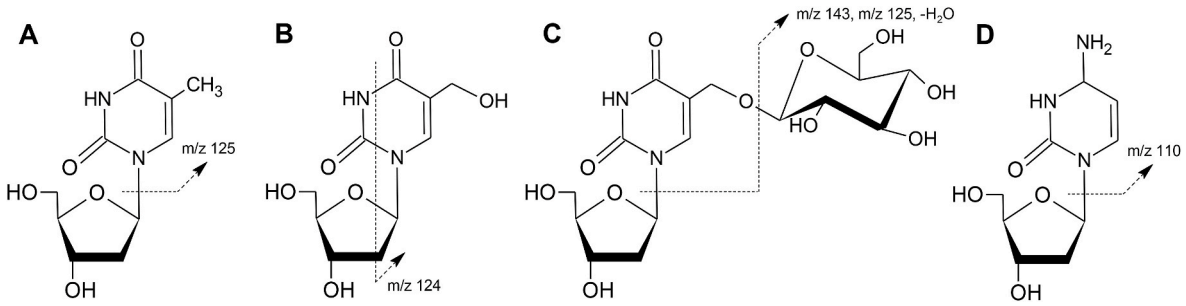

Fig. 1. Chemical structures of **(A)** deoxythymidine (dT), **(B)** 5-hydroxymethyl-2′-deoxyuridine (HOMedU), **(C)** β-D-glucosyl-hydroxymethyl-deoxyuridine (dJ), and **(D)** deoxycytidine (dC), with proposed location of fragmentation.

Table 1
General and analyte specific mass spectromic settings.

General settings			
Run duration (min)			14
Ion spray voltage (V)			5000
Ion Source Gas 1 (au)			60
Ion Source Gas 2 (au)			30
Curtain gas (au)			30
Collision gas (au)			10
Temperature (°C)			450
Dwell time (msec)			100
	deoxycytidine (dC)	5-hydroxymethyl-2′-deoxyuridine (HOMedU)	deoxythymidine (dT)
Parent mass	226	257	241
Product mass	110	124	125
Collision energy (V)	21	21	21
Collision exit potential (V)	14	14	14
Declustering potential (V)	96	96	96
Retention time (min)	5.3	1.7	1.3

same time, allowing LC-MS/MS detection of all three nucleosides using a single sample pretreatment method.

In previous work, we were able to quantify modified nucleosides after DNA digestion to nucleosides prior to LC-MS/MS analysis [11]. To test this approach for JBP1 activity analysis, we performed different experiments to investigate the assay selectivity and sensitivity for dT and HOMedU analysis. First, we processed single stranded DNA containing either dT or HOMedU in the sequence. In the processed sample containing dT, HOMedU was not detected and vice versa. We slightly adjusted the processing method for double stranded DNA analysis by adding a 3 min incubation step at 95 °C followed by rapid cooling to convert the dsDNA to ssDNA prior to further processing. The final sample pretreatment procedure used is visualized in Fig. 2.

4.1.3. dC as an internal corrector for HOMedU quantification

To correct for incomplete release of nucleosides from the oligonucleotides during DNA digestion, dC was used as an internal corrector. For each processed oligonucleotide, the sequence, and therefore the amount of dC was known, allowing to correction for incomplete DNA digestion. By expressing both dT and HOMedU as a molar ratio to the released amount of dC, both analytes can be more accurately quantified.

4.1.4. Qualitative analysis of base J

Although the main interest in research on the biochemical function of JBP activity lies in the monitoring of nuclear dT to HOMedU conversion, following the complete process of dT to HOMedU to dJ conversion might be of interest for future experiments. For this reason, the possibilities to add dJ to the developed LC-MS/MS assay were explored. For dJ, no signal was found in the negative ionization mode but was detected in the positive ionization mode, as can be seen in Fig. 3. As no dJ reference standard was available, a primer containing dJ and a negative control were used to qualify dJ in the chromatogram based on known *m/z* transitions used in a previously developed LC-MS/MS assay [9]. As can be seen in the chromatogram, dJ is chromatographically separated from dT, HOMedU and dC. This allows for LC-MS/MS analysis of dJ using the same LC system, with the MS operating in the positive ionization mode.

4.2. Method application

4.2.1. Quantification of HOmedU in oligonucleotides

The developed assay was used to quantify HOMedU concentrations in oligonucleotides, to measure the thymine hydroxylation activity of JBP1 *in vitro*. [10] In short, purified proteins with a 14-mer

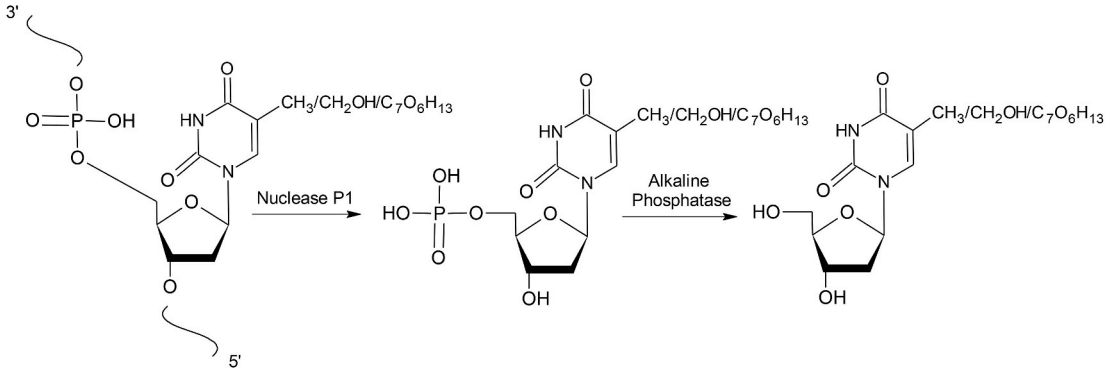

Fig. 2. Enzymatic degradation of DNA incorporated deoxythymidine (CH_3), 5-hydroxymethyl-2′-deoxyuridine (CH_2OH), or β-D-glucosyl-hydroxymethyl-deoxyuridine ($C_7O_6H_{13}$) monophosphate to their respective nucleosides during sample processing.

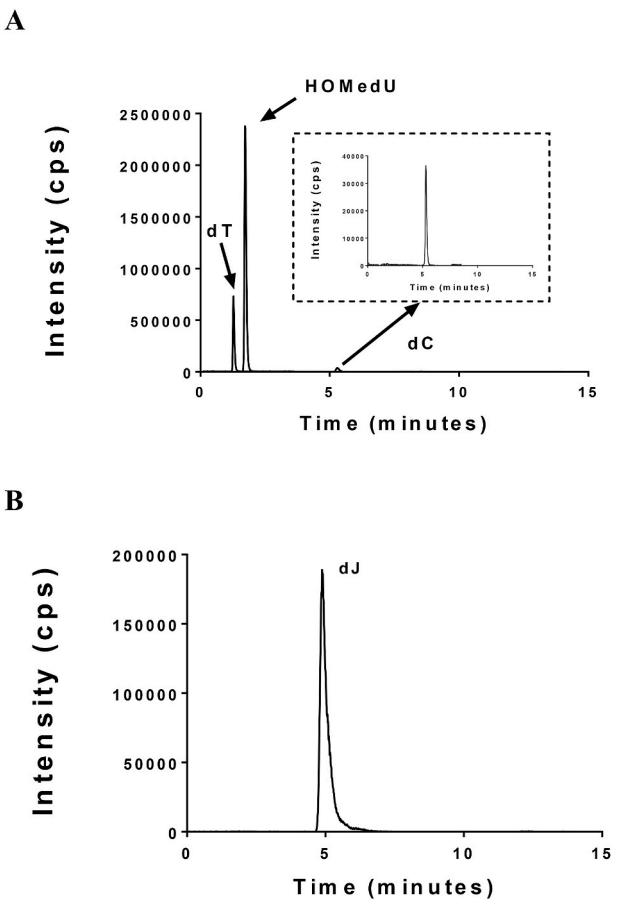

Fig. 3. Representative ion chromatograms of **(A)** deoxythymidine (dT, *m/z* 241 → 125), 5-hydroxymethyl-2′-deoxyuridine (HOMedU, *m/z* 257 → 124) and deoxycytidine (dC, *m/z* 226 → 110) in the negative ionization mode and **(B)** β-D-glucosyl-hydroxymethyl-deoxyuridine (dJ, *m/z* 421 → 125) in the positive ionization mode.

oligonucleotide were used to convert dT to HOMedU. Using the developed assay, we were able to detect and chromatographically separate dT and HOMedU following DNA degradation to nucleosides. A representative chromatogram of a processed oligonucleotide sample (~1 μg DNA) containing dT, HOMedU, dJ and dC can be found in Fig. 3.

5. Conclusion

An LC-MS/MS method was developed to quantify the amount of dT and HOMedU directly in oligonucleotides using dC as an internal corrector. Using this method, the enzymatic function of JBP1 could be monitored *in vitro* by quantification of the conversion of dT to HOMedU. The presence of dJ can be monitored by qualitative analysis using the same LC-MS/MS method operated in the positive ionization mode. The developed method is currently used for the support of biochemical functioning studies of JBP1; the assay is compatible with high-throughput approaches for screening for JBP1 inhibitors.

Declarations of interest

None.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CRediT authorship contribution statement

Jeroen Roosendaal: Conceptualization, Methodology, Validation, Investigation, Resources, Project administration, Writing - original draft. **Tatjana Heidebrecht:** Investigation, Resources, Writing - review & editing. **Hilde Rosing:** Supervision, Writing - review & editing. **Anastassis Perrakis:** Conceptualization, Writing - review & editing. **Jos H. Beijnen:** Conceptualization, Supervision, Writing - review & editing.

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