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# Quantitative measurement of dihydrouridine in RNA using isotope dilution liquid chromatography–mass spectrometry (LC/MS)

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## ABSTRACT

**A method has been developed for the microscale determination of 5,6-dihydrouridine, the most common post-transcriptional modification in bacterial and eukaryotic tRNA. The method is based on stable isotope dilution liquid chromatography–mass spectrometry (LC/MS) using [1,3-$^{15}N_2$]dihydrouridine and [1,3-$^{15}N_2$]uridine as internal standards. RNA samples were enzymatically digested to nucleosides before addition of the internal standards and subsequently analyzed by LC/MS with selected ion monitoring of protonated molecular ions of the labeled and unlabeled nucleosides. Sample quantities of ~1 pmol tRNA and 5 pmol 23S rRNA were analyzed for mole% dihydrouridine. Dihydrouridine content of *Escherichia coli* tRNA$^{Ser}_{VGA}$ and tRNA$^{Thr}_{GGU}$ as controls were measured as 2.03 and 2.84 residues/tRNA molecule, representing accuracies of 98 and 95%. Overall precision values for the analyses of *E.coli* tRNA$^{Ser}_{VGA}$ and *E.coli* tRNA$^{Thr}_{GGU}$, unfractionated tRNA from *E.coli* and 23S rRNA from *E.coli* were within the range 0.43–2.4%. The mole% dihydrouridine in unfractionated tRNA and 23S rRNA from *E.coli* were determined as 1.79 and 0.0396%, corresponding to 1.4 and 1.1 residues/RNA molecule respectively.**

## INTRODUCTION

Modified nucleosides occur in DNA (1), but are particularly characteristic of tRNA, rRNA and eukaryotic mRNA (2). More than 79 different nucleosides are presently known in tRNA, the most highly modified of the RNAs from all sources (2). The modified nucleosides show considerable structural variety, from simple methylation of either the base or the O-2′ hydroxyl of ribose to much more complex types of modification in the base.

5,6-Dihydrouridine (D) is a post-transcriptionally modified nucleoside first reported as a naturally occurring constituent of RNA by Madison and Holley in 1965, in tRNA$^{Ala}$ from yeast (3). It functions to promote conformational flexibility (leading references in 4) and is the single most common form of post-transcriptional modification in tRNA from bacteria and eukaryotes (5,6), where it is found at conserved positions of the D loop in numbers up to 5 residues/tRNA. It occurs less commonly at position 47 of the variable loop of tRNA and recently has been identified in the peptidyl transferase loop of 23S rRNA from *Escherichia coli* (7). Dihydrouridine is characteristically absent from the RNA of most archaea (archaebacteria) and is present only in trace amounts in the few archaea in which it is found (8).

The method of choice for accurate quantitation of nucleosides in RNA hydrolysates has traditionally been the measurement of HPLC chromatographic peak heights or areas using UV detection and comparison with data from weighed amounts of authentic nucleoside standards (9,10). Because D, unlike all other natural nucleosides, possesses no significant chromophore, HPLC analysis with UV detection is not practical due to poor sensitivity. Its previous quantitation in RNA has been achieved by several methods. Gehrke and Kuo (11) quantitated dihydrouridine in six isoaccepting tRNAs from yeast and *E.coli* by monitoring absorbance at 210 nm, requiring 5 µg tRNA. Cerutti *et al.* (12) treated tRNA with sodium borotritiide, followed by characterization and quantitation of the labeled reduced trialcohol products. Magrath and Shaw (13) converted D of RNA to β-alanine by alkaline treatment, followed by quantitation of β-alanine with an amino acid analyzer. Molinaro *et al.* (14) estimated D in RNA by measuring the time-dependent loss of $A_{235}$ in 0.1 N KOH. Jacobsen and Hedgcoth (15) utilized a colorimetric assay for dihydropyrimidine after conversion of D in RNA to its open ring form (*N*-ribosyl-3-ureidopropionic acid) and also TLC analysis of radioactively labeled RNA digests for the quantitation of dihydrouridine. Randerath *et al.* (16) developed a tritium derivative method, which was limited by nucleoside recovery losses, to semi-quantify modified nucleosides in RNA. Johnson and Horowitz (17) utilized the latter method for estimating D content in tRNA and 23S rRNA from *E.coli.*

Although the assays described above are generally reliable for the detection of dihydrouridine, they all have notable limitations with regard to accurate quantitative measurements. These include harsh reaction conditions with potential for base loss, lack of sensitivity, selectivity and accuracy of identification, sample loss and speed of analysis. In the case of reversed phase HPLC-based methods, D is the earliest eluting nucleoside, resulting in potential loss of selectivity due to minor UV absorbing impurities that elute just after the void volume. To overcome these limitations we have developed a rapid, sensitive and accurate assay based on stable

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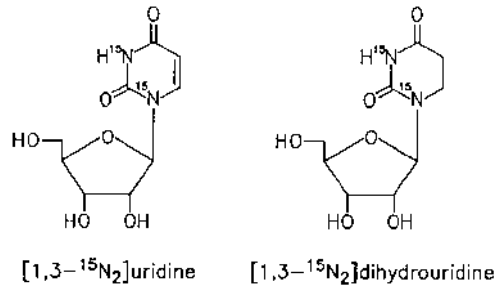


**Figure 1.** Structures of the internal standards [1,3-$^{15}N_2$]uridine and [1,3-$^{15}N_2$]dihydrouridine.

isotope dilution liquid chromatography–mass spectrometry (LC/MS) with selected ion monitoring for the direct chemical measurement of dihydrouridine in enzymatic hydrolysates of RNA.

## MATERIALS AND METHODS

### Labeled nucleosides

[1,3-$^{15}N_2$]Uridine was purchased from Cambridge Isotope Laboratories (Woburn, MA). [1,3-$^{15}N_2$]Dihydrouridine was synthesized in 95% yield by the hydrogenation of [$^{15}N_2$]uridine under atmospheric pressure using 5% rhodium on alumina catalyst in aqueous media (18) as follows. [$^{15}N_2$]Uridine (10 mg) was dissolved in 1 ml water, 7 mg 5% rhodium alumina was added and the mixture was hydrogenated. After 4 h, the catalyst was removed and the filtrate was purified by reversed phase HPLC using 25 mM $NH_4HCO_3$ containing 1% acetonitrile, pH 6.5 (flow rate 1 ml/min, $t_R$ 4.3 min). The identity of [$^{15}N_2$]dihydrouridine was verified by LC-MS: $t_R$ 3.2 min (19), $[M + H]^+$ 249. Structures of the labeled nucleosides are shown in Figure 1.

Internal standard solutions were prepared by drying each nucleoside over $P_2O_5$ for 24 h and dissolving in water to a concentration of ~1 µg/µl. The solutions of isotopically labeled nucleosides both contained 0–0.2% unlabeled and mono-[$^{15}N$]-labeled nucleoside, as measured by mass spectrometry. Exact concentrations of each solution were determined by UV absorbance at 261 nm for [$^{15}N_2$]uridine [ε = 10 100; (20)] and at 254 nm for [$^{15}N_2$]dihydrouridine. Dihydrouridine has a very low molar absorptivity at 254 nm (10). The concentration of the labeled solution was therefore determined by constructing a standard curve ($A_{254}$ versus [D]) using precisely weighed amounts of unlabeled D as standards. More dilute solutions were prepared from these stock solutions for isotope dilution experiments.

### RNA

Isoaccepting $tRNA^{Ser}_{VGA}$ and $tRNA^{Thr}_{GGU}$ from *E.coli* were purchased from Subriden Inc. (Rolling Bay, WA). Unfractionated tRNA from *E.coli* was purchased from Boehringer Mannheim Inc. (Indianapolis, IN). 23S rRNA from *E.coli* was isolated and purified as described (7).

### Enzymatic digestion of RNA

RNA was completely hydrolyzed to nucleosides using nuclease $P_1$, snake venom phosphodiesterase and bacterial alkaline phosphatase as previously reported (21).

### Directly combined LC/MS

Analysis of nucleosides in RNA digests was carried out with a mass spectrometer consisting of a non-commercial quadrupole mass analyzer, with a thermospray HPLC interface (Vestec Corp., Houston, TX), controlled by a Vector/One data system (Teknivent Corp., St Louis, MO). HPLC separations were made using a Supelcosil LC-18S column (4.6 × 250 mm) and a 3 cm Brownlee Spheri-5 $C_{18}$ precolumn, thermostatted at 31°C. The HPLC gradient elution system of Buck *et al.* (9), with 0.25 M ammonium acetate, pH 6.0, and acetonitrile, was used with minor modifications in the gradient profile (19). Mass spectra for the region *m/z* 244–250 were acquired every 0.36 s during the 10 min chromatographic elution of D and U. The instrument, procedures and interpretation of data for qualitative LC/MS analysis of nucleosides in RNA hydrolysates have been described in detail (19).

### Measurement of mole% uridine in unfractionated tRNA from *E.coli*

Using weighed amounts of authentic nucleosides, standard curves (nmol nucleoside versus $A_{254}$) for pseudouridine (ψ), cytidine (C), uridine (U), 5-methyluridine ($m^5U$), guanosine (G), 7-methylguanosine ($m^7G$) and adenosine (A) were constructed based on HPLC chromatographic peak heights, using UV detection (data not shown). These calibration curves were then used in conjunction with HPLC chromatograms of *E.coli* tRNA digests to calculate the molar proportion of uridine in tRNA from *E.coli.*

### Construction of calibration curves for D and U

To verify the spectroscopically determined concentrations of the isotopically labeled internal standard solutions, they were calibrated by reverse isotope dilution using primary standards of unlabeled U and D. In addition, this procedure provides a test of the accuracy of mass spectrometrically measured isotope ratios in mixtures of the labeled and unlabeled nucleosides. For this standardization, four samples were prepared for each nucleoside by mixing known amounts of primary standard and labeled internal standard solutions to achieve four different optimum isotope ratios (*m/z* 245/247 for U, *m/z* 247/249 for D) in the mixtures. These solutions were then analyzed by LC/MS. The peak area ratios were calculated automatically by the data system of the mass spectrometer. Contributions from natural abundance heavy isotopes were taken into account through the calibration curve. Each measurement was performed in triplicate for statistical purposes. The data were then subjected to a linear least squares analysis.

### Measurement of nucleoside molar ratios

The quantitative assay consists of the addition of isotopically labeled U and D to the RNA digest (1 µg tRNA, 3 µg 23S rRNA) prior to analysis by LC/MS. Quantitation of U and D in RNA is accomplished by selected ion monitoring of their $MH^+$ ions and those of the corresponding isotopically labeled internal standards. The mass values of these ions are: U, 245; D, 247; [$^{15}N_2$]uridine,

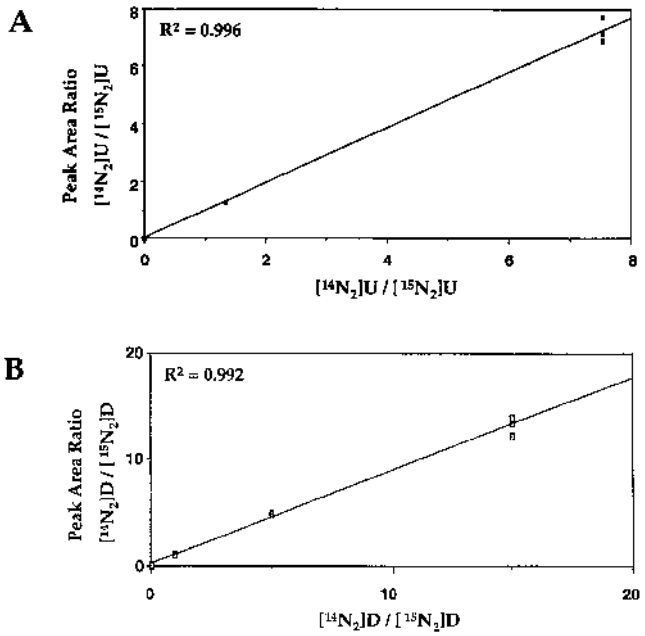


**Figure 2.** Calibration curves for LC/MS analyses: (**A**) uridine; (**B**) dihydrouridine. The plots show calculated selected ion peak area ratios versus the ratio of molar quantities of unlabeled to labeled nucleoside injected into the chromatograph.

247; [$^{15}N_2$]dihydrouridine, 249. Although dihydrouridine and [$^{15}N_2$]uridine possess the same mass, their signals are distinguished by a difference in retention time. The peak area ratios (245/247 for U, 247/249 for D) are used to derive the amounts of U and D in the RNA. Since the mole% of U in the RNA is known [as calculated from sequence data (5,22) or as measured from HPLC chromatographic peak heights], the mole% of D can be determined from the U:D ratio. This approach is similar in principle to the GC/MS method earlier developed for quantitative determination of 5-methylcytosine in DNA (23).

## RESULTS AND DISCUSSION

The calibration curves for U and D are shown in Figure 2. Verification that the calibration solutions of labeled and unlabeled nucleosides did not contain interfering species of equal mass was achieved by MS analysis of samples containing labeled or unlabeled nucleoside in the absence of the other. The enzyme solutions used for hydrolysis were similarly tested for absence of interfering ions at the appropriate retention times.

The D calibration curve shows that the experimentally measured ratios were in good agreement with the actual ratios of unlabeled/labeled nucleoside ($r^2$ = 0.992). A similar result was found for the U calibration curve, with one exception. The experimentally measured ratio corresponding to the mixture containing labeled and unlabeled U in a ratio of 15.4:1 was 12.4, an error of ~19%. This data point was therefore removed from the curve illustrated in Figure 2A. Because data contained in the U calibration curve were more accurate and precise for ratios from 1.3:1 to 7.5:1 ($r^2$ = 0.996), care was taken in the RNA assays to approximate these values.

Carry-over in the LC/MS system during sequential analyses of samples with different isotope ratios can adversely affect the accuracy of subsequent measurements. It will also affect the precision of the overall analysis. In this study, the memory effect was evaluated by running a blank LC/MS experiment following the analysis of a sample containing labeled and unlabeled nucleosides. No memory effect was observed during the blank runs.

### Quantitative measurement of dihydrouridine in RNA

The calibrated internal standard solutions of [$^{15}N_2$]uridine and [$^{15}N_2$]dihydrouridine were used to quantitatively measure dihydrouridine in RNAs of defined D content, *E.coli* $tRNA^{Ser}_{VGA}$ and $tRNA^{Thr}_{GGU}$, as well as in unfractionated tRNA and in 23S rRNA from *E.coli*. Typical selected ion chromatograms of the protonated molecular ion species of D, [$^{15}N_2$]dihydrouridine, U and [$^{15}N_2$]uridine from *E.coli* $tRNA^{Ser}_{VGA}$ are shown in Figure 3. Three replicate measurements of D content were made for each of the four samples. Mean values and relative standard deviations were calculated for each and are tabulated in Table 1. The calculations of D content in *E.coli* $tRNA^{Ser}_{VGA}$ and *E.coli* $tRNA^{Thr}_{GGU}$ have precisions ranging from 0.43 to 2.4% and accuracies of 98 and 95% respectively. The mole% of U in unfractionated tRNA from *E.coli* was found to be 14.78% by HPLC analysis of *E.coli* tRNA digests. This value was calculated using UV detection in conjunction with absorbance standard curves of ψ, C, U, $m^5U$, G, $m^7G$ and A (data not shown). D mole%$_{calc.}$ for unfractionated tRNA from *E.coli* is 2.16%, when estimated as the average mole% of D in 43 tRNA sequences compiled by Sprinzl *et al.* (5). Previous estimates of D content in *E.coli* tRNA by Jacobson and Hedgcoth (15), Johnson and Horowitz (17) and Cerutti *et al.* (12) were 1.98, 1.94 and 2.5 mole% D respectively. The mass spectrometrically measured value of 1.79% (Table 1) suggests reasonably accurate values were obtained with the former two methods, but that a considerable error was obtained in the latter measurement (although the comparisons are subject to variations in dihydrouridine levels between *E.coli* samples). The difference between our value and the value calculated simply from sequence data indicates that in *E.coli*, isoaccepting tRNAs with lower D content represent a higher proportion of the transfer RNA population than those with higher D content.

**Table 1.** Quantitation of dihydrouridine in four RNA species

| | U (mole%)[a] | U:$D_{calc.}$ | U:$D_{found}$ | D (mole%)[a,b] |
|---|---|---|---|---|
| *E.col* $tRNA^{Ser}_{VGA}$ | 11.36 | 5.00 | 4.91 | 2.31 (0.01) |
| *E.coli* $tRNA^{Thr}_{GGU}$ | 17.11 | 4.33 | 4.56 | 3.74 (0.09) |
| *E.coli* unfractionated tRNA | 15.53 | | 8.65 | 1.79 (0.03) |
| *E.coli* 23S rRNA | 20.19 | | 510 | 0.0396 (0.001) |

[a]Mole% are given as residues/100 nucleotides.
[b]Standard deviations are given in parentheses.

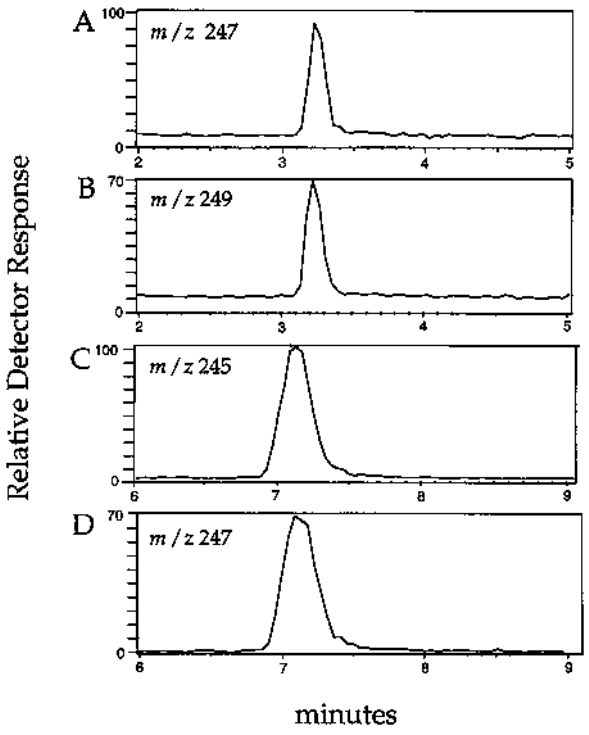


**Figure 3.** Selected ion chromatograms reconstructed from mass spectra for $MH^+$ ions from *E.coli* $tRNA^{Ser}_{VGA}$: (**A**) dihydrouridine, *m/z* 247; (**B**) [$^{15}N_2$]dihydrouridine, *m/z* 249; (**C**) uridine, *m/z* 245; (**D**) [$^{15}N_2$]uridine, *m/z* 247.

The mole% of U in 23S rRNA from *E.coli* as calculated from the gene sequence (22) is 20.19%. Dihydrouridine content of *E.coli* 23S rRNA is found to be 0.0396%, in contrast to 0.10% found by Johnson and Horowitz (17), obtained using the semi-quantitative tritium derivative method (16). However, problems with the latter method are suggested by the finding in the same study (17) of dihydrouridine in 16S rRNA, which was absent when rigorously screened by LC/MS of total rRNA digests and of fractionated RNase $T_1$ hydrolysis products (24). The mass spectrometrically determined value corresponds to one residue of dihydrouridine in 2908 nt and indicates that the D residue located at position 2449 in *E.coli* 23S rRNA (7) is the only D residue occurring in the molecule.

Directly combined chromatography-mass spectrometry with stable isotope dilution is an analytical method capable of both high accuracy and high selectivity (25). The goal of the present work was to develop a method for the microscale (pg–low ng) analysis of D in RNA. This report describes the successful quantitation, using isotope dilution LC/MS, of D in tRNA. It has also been shown that this method can be extended to the analysis of other RNA molecules, including rRNA, without variation. Furthermore, this method should be adaptable without change to electrospray and similar ionization methods.

The present method has several advantages over previously described methods for the quantitation of D in RNA. It is 5–500 times more sensitive than any method described to date, requiring <1 µg tRNA (~1 pmol D) or 3 µg rRNA (~5 pmol D) for analysis. It is more accurate than other methods involving HPLC analysis, which produce errors of 5% for modified nucleosides which absorb in the 254–280 nm region of UV (10). In the case of dihydrouridine, the accuracy obtained using UV detection at 210 nm (11) was reported as 92–95% using 5 µg purified $tRNA^{Phe}$ in which early eluting impurities were minimized. Selectivity in the region of the chromatogram where D elutes with UV-absorbing impurities following the void volume may be compromised in HPLC analyses, but is greatly increased by the high selectivity afforded by the mass spectrometer as detector. The analyses described in this report are quite rapid, taking 10 min to complete for each digest. Finally, the use of an internal standard provides a means of compensating for potential variations in nucleoside concentration due to adsorptive losses during chromatography and sample handling.

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