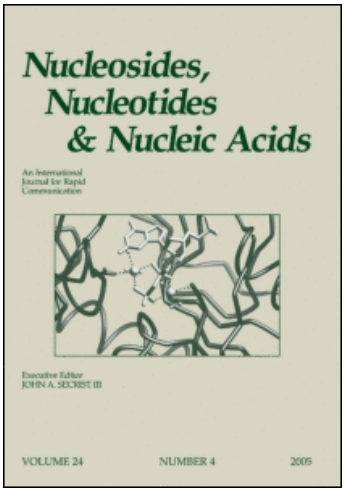

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3-Methyluridine in Normal Human Urine

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3-METHYLURIDINE IN NORMAL HUMAN URINE

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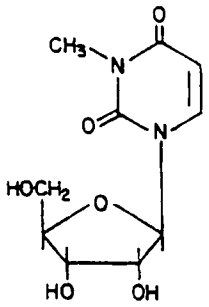
ABSTRACT

3-Methyluridine has been identified in normal human urine using gas chromatography mass spectrometry, confirming an earlier report of the presence of this modified nucleoside as a natural component of human biological fluids. Variable recoveries and the lack of quantitative data concerning normal or disease state levels of this compound make selection of 3-methyluridine a poor choice as an internal standard for the quantitation of other nucleosides in biological matrices.

INTRODUCTION

The quantitation of modified nucleosides in urine as biological markers of cancer is currently an active area of research. The suggestion by Borek[1] that modified nucleosides in urine reflect the turnover rate of tRNA and that quantitation of these compounds may provide a means for early diagnosis of tumors or permit the monitoring of a therapeutic regimen during treatment of cancer, has been investigated by a number of laboratories. The general approach to quantitation of modified nucleosides in biological fluids has been recently described[2,3] and will not be elaborated here.

The purpose of this communication is to alert researchers in this area to the presence of 3-methyluridine (**1**) in normal human urine and to caution against the use of this compound as an internal standard for the quantitation of other modified nucleosides derived from biological samples.



1, 3-methyluridine

MATERIALS AND METHODS

Materials

Reagents and containers used to prepare trimethylsilyl (TMS) derivatives were purchased from Pierce Chemical Co. Rockford, Ill.

Uridine and 3-Methyluridine were purchased from Sigma Chemical Co., St. Louis, MO.

Sample Preparation

An 80 ml urine sample from a normal subject (K.N.) was collected after a 12 hour fasting period. The sample was centrifuged to remove particulate matter and the supernatant was stored at -20 C until further treatment.

Conditions for the boronate affinity column and reversed-phase high performance liquid chromatographic (RP-HPLC) purification of the nucleoside fraction of the urine sample have been described elsewhere[4].

The dried HPLC fraction containing the modified nucleoside was dissolved in water to a concentration estimated to be 1 ug/ul. A 10 ul aliquot of the reconstituted fraction was taken to dryness at room temperature under a stream of nitrogen and traces of water removed by co-evaporation with methanol. The TMS derivative was prepared as described elsewhere[5,6].

GCMS and MS Analysis

These analyses were performed using a Varian 3400 gas chromatograph fitted with a DB-5 capillary column (30m X 0.25mm, 0.25 um film thickness) directly coupled to a Finnigan MAT90 mass spectrometer (Finnigan Corp. Palo Alto, CA). GC conditions: initial temperature, 180 C; program rate, 6° C/min to 300° C with a 5 min hold time; carrier gas, He at head pressure of 10 psig; injector was splitless at 300° C; and interface temperature was 300°C. The sample injection volume was 0.5 or 1.0 ul, depending upon the application. Mass spectrometer conditions for electron impact (70 eV EI): source temperature of 250° C; mass range scanned from m/z 70 to 1000 at a scan rate of 0.4 sec/decade and resolution of 1000. Mass spectrometer operation and data acquisition and storage utilized a DEC PDP 11/73 computer (U.S. Design, Palo Alto, CA).

Chemical ionization(CI) used ammonia as the reagent gas. The reagent gas inlet was adjusted until the ratio of the ions at m/z 18/35 was 100:1.

High resolution mass measurements were obtained on the urinary component and the reference sample using two different methods of sample introduction and mass scanning. The reference compound was analyzed using peak matching (electric sector and voltage scanning) with the material introduced into the mass spectrometer via the direct probe. The urinary component was analyzed using fast magnet scanning (2 scans per second from m/z 200 to m/z 600) with the sample introduced into the mass spectrometer from the gas chromatograph (GC). The need for a different approach to high resolution for the two materials is explained in the discussion of the results. In both experiments, the resolution was 7500 (10% valley definition).

DISCUSSION

The presence of 3-methyluridine in normal human urine has been known for some time[7] and has been identified in our laboratory using capillary gas chromatography-mass spectrometry (GCMS). The low resolution mass spectrum of the urine sample component is compared to a reference sample of 3-methyluridine in Figure 1. Structural assignments are based on high resolution mass measurements, described below and shown in Table 1, and are consistent with structure/fragmentation studies reported earlier[5]. Ion designations are those used in Reference 5.

Major points of interest[5] in the low resolution mass spectra of the TMS derivatives of 3-methyluridine reference sample and urinary component include a) the presence of molecular and M-15 ions at m/z 474 and 459, respectively. These ions establish the molecular weight of the compound, which was verified by chemical ionization (CI) (MH+ at m/z 475; 100% RI). Subtraction of 216 daltons, 72 mass units for each of the 3 TMS groups added, from the molecular ion gives a free molecular weight of the nucleoside of 258 daltons, which corresponds to the addition of a methyl group to uridine; b) the presence of an unmodified pentafuranose sugar is indicated by the ions at m/z 349, 348, 259, 243, 217, 169 and 103. The S and S-H ions reveal that the added methyl group is not in the ribose portion of the nucleoside, leading to the conclusion that the aglycone must be a methylated uracil; c) ions representing the aglycone and portions of the sugar all of which are shifted by 14 mass units relative to the spectrum of uridine-TMS_3[5]. Ions of interest in this regard are designated B+H (m/z 126, 1.2%), B+58 (m/z 183, 7.7%), B+74 (m/z 199, 4.3%), B+102 (m/z 227, 5.9%), B+188 (m/z 313, 6.1%) and B+204 (m/z 329, 4.0%); and d) ion intensities and mass values identical within experimental error for the urinary component and the reference sample. Consideration of ion

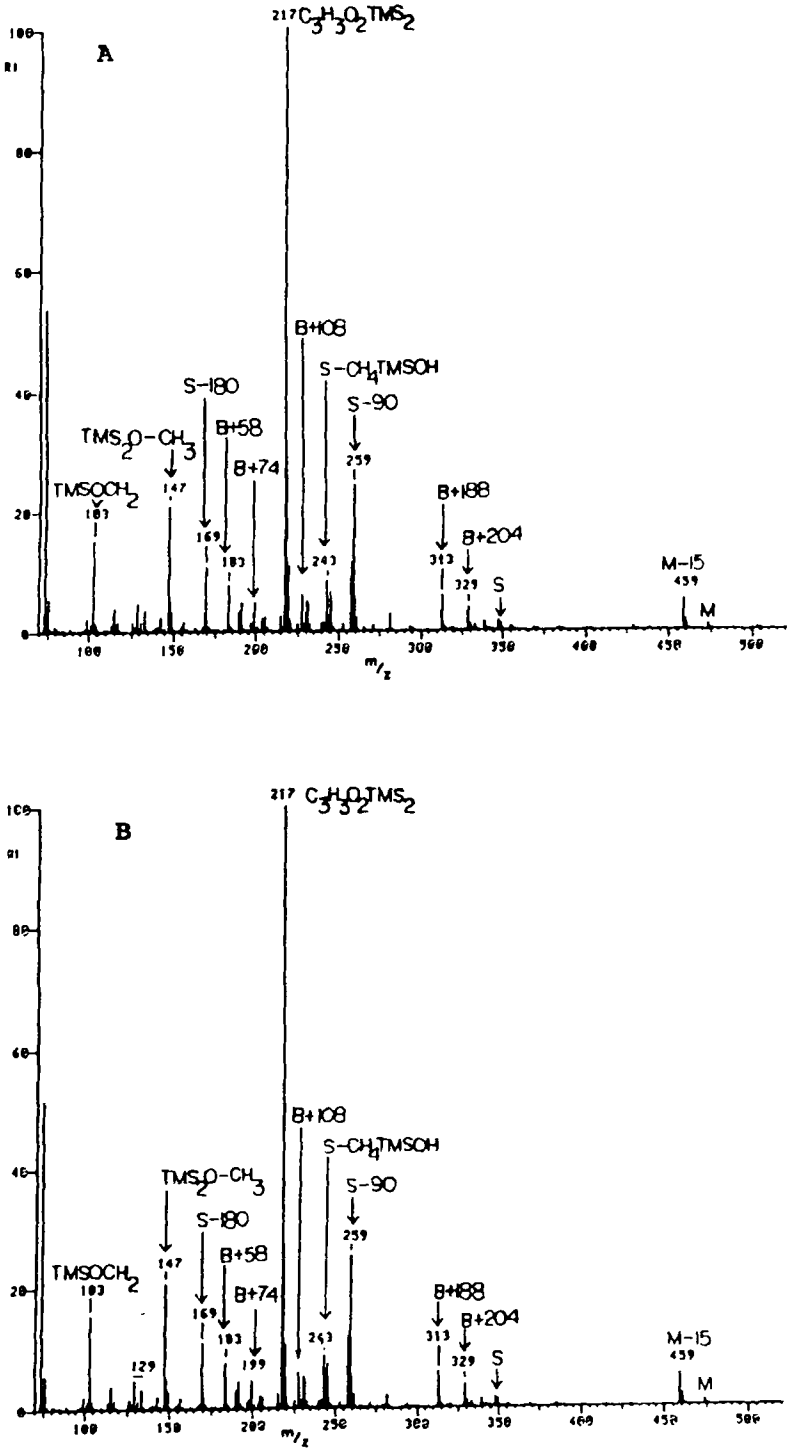


FIGURE 1. The 70 eV Low Resolution Spectrum of a) reference 3-methyluridine (TMS_3), and b) the urinary component assigned as 3-methyluridine (TMS_3).

TABLE 1.

Mass Spectral Assignments of Reference and Urine Component of 3-Methyluridine (TMS_3):

m/z	Elemental Composition	(error in millimass units, mmu)	Structural Assignment[5,8]
474.2045 474.2009	$C_{19}H_{38}N_2O_6Si_3$	(0.8mmu) (-2.8mmu)*	Molecular ion
459.1809 459.1772	$C_{18}H_{35}N_2O_6Si_3$	(0.6mmu) (-3.1mmu)*	M-15
349.1658 349.1648	$C_{14}H_{33}O_4Si_3$	(-2.9mmu) (-3.9mmu)*	S
348.1574	$C_{14}H_{32}O_4Si_3$	(-3.4mmu)*	S-H
329.1344 329.1375	$C_{13}H_{25}N_2O_4Si_2$	(-0.9mmu) (-2.2mmu)*	B+204
313.1406 313.1404	$C_{13}H_{25}N_2O_3Si_2$	(0.2mmu) (0.0mmu)*	B+188
259.1196	$C_{11}H_{23}O_3Si_2$	(1.0mmu)	S-90
243.0873	$C_{10}H_{19}O_3Si_2$	(0.0mmu)	S-CH_4-TMS-OH
227.0856	$C_9H_{15}N_2O_3Si$	(0.4mmu)	B+102
217.1079	$C_9H_{21}O_2Si_2$	(-0.1mmu)	$C_3H_3O_2TMS_2$
199.0877	$C_8H_{15}N_2O_2Si$	(-2.6mmu)	B+74
183.0583	$C_7H_{11}N_2O_2Si$	(-0.7mmu)	B+58
169.0683	$C_8H_{13}O_2Si$	(-0.2mmu)	S-180
147.0663	$C_5H_{15}OSi_2$	(0.2mmu)	[TMS-O=Si$(CH_3)_2$]+
103.0586	$C_4H_{11}OSi$	(0.7mmu)	[TMS-O-CH_2]+

(*Values for the urine component)

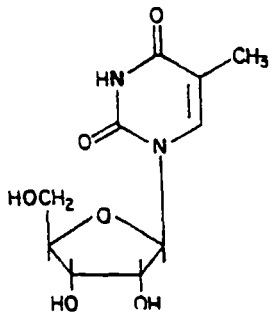
intensities is important in excluding the possibility of an alpha glycosidic linkage and/or the presence of an epimeric hydroxyl group in the sugar. Although definitive correlations have not yet been established that permit assignment of structures to epimeric sugars, e.g., ribo, arabino, lyxo and xylo nucleosides, subtle differences do exist in the mass spectra of these samples[5,8,9], and ion intensities have been used to distinguish alpha from beta glycosidic bonds[5]. Moreover, the

use of boronate affinity column, with specificity for cis-diol groups, during sample purification significantly reduces the possibility of the of presence arabino or xylo sugars. The correspondence in elution times of the urinary component and reference sample, determined using RP-HPLC[10] and capillary gas chromatography[11], is also strong evidence that the compounds have identical structures. Minor modifications to the structure of a nucleoside, e.g., the presence of an epimeric hydroxyl or a change from beta to alpha, are detectable by differences in retention times in the gas chromatogram[12].

High resolution mass measurements were also performed and established the elemental composition of the ions as assigned using the low resolution and linked scan data (see Table 1). It should be noted that the difference in accuracy of mass assignment between the reference sample and urinary component is a result of the methods used in obtaining the mass values. Measurements on the reference sample were made by slow scanning (10 seconds/decade) with the sample being evaporated from the solids probe. This method was used with the reference material because the sample was pure and quantity of material was not a limiting factor. On the other hand, the urinary component was present in a mixture, and mass chromatograms, using the low resolution data, revealed the presence of an artifact having an ion at m/z 474 which was not sample related. These circumstances, therefore, required that the analysis be performed using a faster scan rate (0.5 seconds/decade) over a limited mass range (m/z 200 to m/z 600) on the eluant from the capillary gas chromatographic column, conditions which provide less accurate mass measurements. In spite of this difficulty, elemental composition of the structurally important ions in the urinary component were established. Determination of the fragmentation pathways for the M+. and MH+ ions, coincidence in retention times and high

resolution data confirms the structural assignment of the urinary component.

Although the above mentioned data established the structural features of the sugar and the general characteristics of the aglycone, consideration was given to other known uridine analogs that would have the same molecular weight as the 3-methyluridine identified in the urine sample. Analogs requiring special attention include 5-methyluridine(thymine riboside)(2), a minor component of tRNA[13], 6-methyluridine(3), and 1-methylpseudouridine(4), a compound recently identified in the tRNA of archaebacteria[14]. The possibilities 2 and 3 can, however, be excluded from consideration since both would form a per-TMS derivative containing at least one additional TMS group. Reference spectra of 2 and 3[15] are significantly different from 1, eliminating these compounds from further consideration. Compound 4, being a C-nucleoside, would be expected to show a considerably different mass spectrum[5,8,15], with few of the sugar ions being observed. Comparison of the spectrum of urinary 3-methyluridine $(TMS)_3$ with the published spectrum of 4[14] does, indeed, show significant differences, which eliminates 4 as being the urinary component. However, in contrast to the expected formation of a TMS_4 derivative of 4, the major species formed is the TMS_3 derivative, which produces ions having the same nominal



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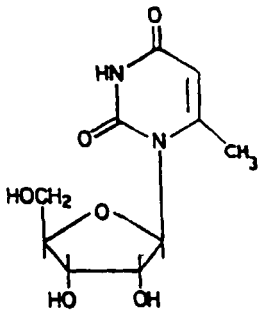

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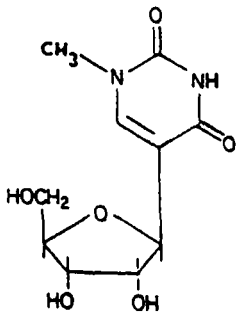

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and exact mass as ions in the spectrum of urinary 3-methyluridine, although intensity values are considerably different.

With the confirmed presence of 3-methyluridine in the urine of a normal patient, the use of this material as an internal standard for the quantitation of other nucleosides in biological samples may lead to erroneous results. Although the results of the present study are not quantitative, earlier work[7] suggests that the levels of 3-methyluridine in normal human urine is in the 0.8 mg/24 hour range. Also of significance is that recoveries of 3-methyluridine are variable and dependent on the method used for purification of the nucleoside fraction. Of particular importance is the degradation of 3-methyluridine to 3-methyluracil in the presence of boronate[7]. Since the most commonly used procedures for the isolation of the nucleoside fraction from biological samples does, in fact, utilize boronate affinity columns, quantitative results are very likely to be in error if 3-methyluridine is used as the internal standard. Finally, the value of 0.8 mg/24 hour urine sample is an estimate. No studies have been reported that provide more accurate values, and the levels of 3-methyluridine could vary considerably between subjects or may be altered in the presence of disease. Thus, when 3-methyluridine is used as an internal standard for the quantitation of nucleosides in biological samples, the results may be in error from two sources, occurrence of the sample in variable amounts as a natural component in the biological sample and degradation of the standard during purification or separation on the boronate column.

Based on the above results and discussion, the selection of an internal standard for the quantitation of nucleosides from biological samples should be restricted to synthetic analogs that do not incorporate naturally-occurring purine or pyrimidine rings.

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10. The RP-HPLC retention time was 22.57 min. which corresponds to the retention time for the standard.

11. Gas chromatographic retention times were 21.38 min. for the urinary component and 21.40 for the reference sample of 3-methyluridine.

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