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Journal of Chromatography B, 746 (2000) 51–62

JOURNAL OF CHROMATOGRAPHY B

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Detection and characterization of modified nucleosides in serum and urine of uremic patients using capillary liquid chromatography–frit-fast atom bombardment mass spectrometry

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Abstract

To determine RNA metabolism in uremia, capillary liquid chromatography–frit-fast atom bombardment mass spectrometry was employed for the characterization of ribonucleosides in serum and urine of uremic patients, and the profiles were compared with those of healthy subjects. We have characterized 20 nucleosides in serum and 23 nucleosides in urine from both healthy subjects and uremic patients; most of them were modified nucleosides derived from tRNA breakdown products. Four metabolites derived from allopurinol were detected as exogenous nucleosides in patients receiving allopurinol; these include allopurinol-1-riboside, oxipurinol-1-riboside, oxipurinol-7-riboside and a unknown oxipurinol riboside. The endogenous and exogenous ribonucleosides were retained at higher levels in uremic serum, and may play a contributory role as toxins responsible for clinical symptoms of uremia.

Keywords: Ribonucleosides

1. Introduction

The measurement of ribonucleosides, in particular, modified nucleosides in physiological fluids such as urine and serum has become of potential interest in clinical biochemistry. Modified nucleosides occur predominantly in transfer RNA (tRNA) and to a lesser extent in ribosomal RNA (rRNA) [1,2]. Modification such as methylation of tRNA occurs at the macromolecule level through an *S*-adenosyl-L-methionine donor during tRNA maturation process, resulting in modification of either the heterocyclic base, the ribose moiety, or both. There are no mechanisms to reincorporate the modified nucleosides into tRNA; urinary excretion of modified nucleosides and their concentrations in plasma, with efficient renal function, are good indexes of the whole-body tRNA+rRNA turnover [3]. A very high turnover of tRNA in tumor tissue has been reported to result in elevated levels of urinary modified nucleosides in patients with malignancies [4,5]. Therefore, the urinary and serum levels of modified nucleosides have been proposed as useful markers to monitor the course of malignant diseases and their response to therapy [6].

Pseudouridine (Ψ) predominantly from tRNA was reportedly increased in uremia [7], and its levels in

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0378-4347/00/$ – see front matter
PII: S0378-4347(00)00114-6

uremic patients on continuous ambulatory peritoneal dialysis (CAPD) have been found to be significantly higher than those in hemodialysis (HD) patients, suggesting an increase in the turnover of the tRNA [8]. Many other nucleosides are accumulated in uremic serum, because their concentrations are still higher than those in healthy subjects, even after-HD, as shown in the high-performance liquid chromatography (HPLC) profiles (Fig. 1). These findings suggest that some of these solutes may be uremic toxins responsible for some uremic symptoms.

Quantification of modified nucleoside levels in biological fluids has usually been performed with reversed-phase HPLC with UV detection as a rapid

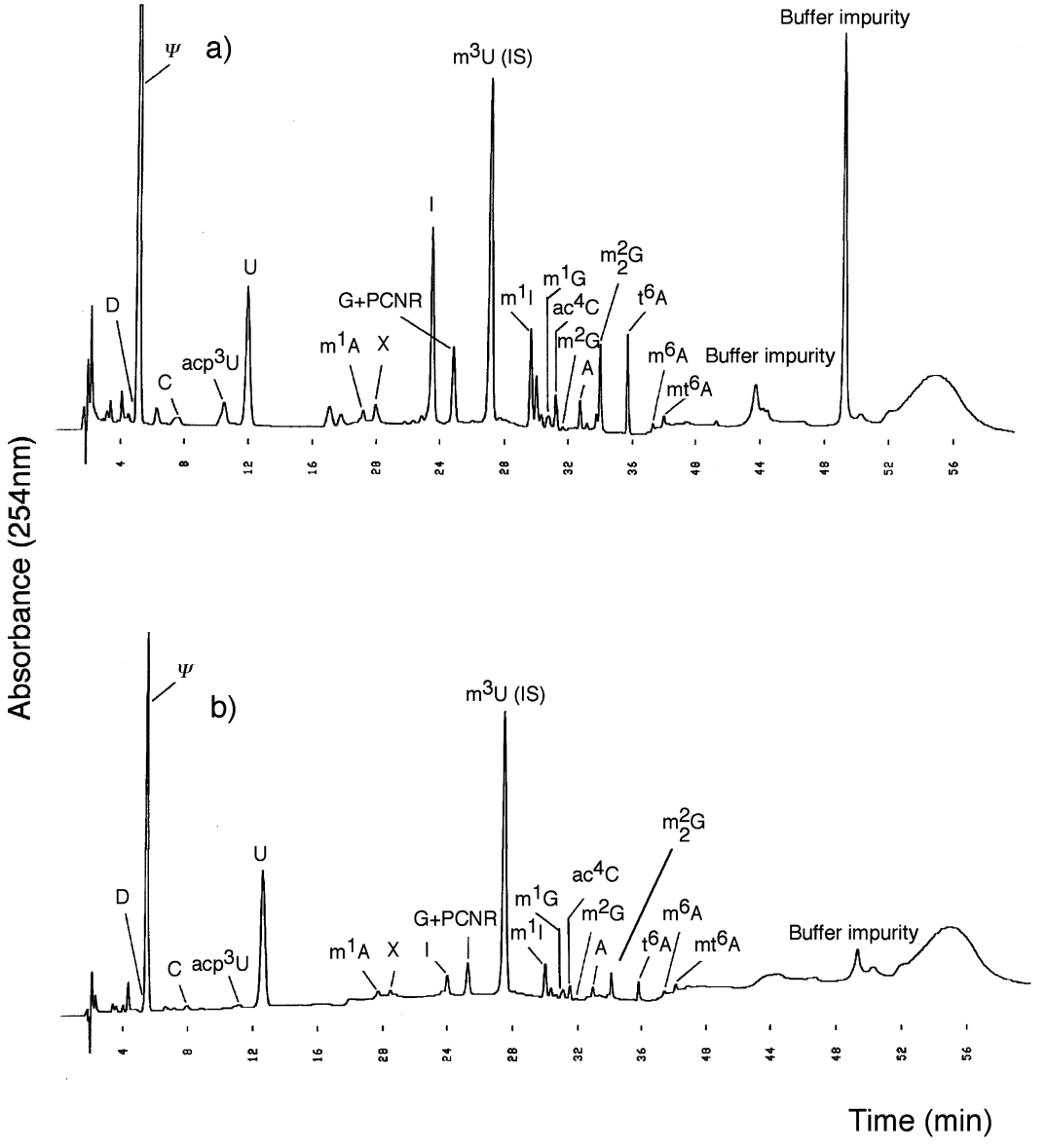

Fig. 1. HPLC separation of ribonucleosides in serum of a uremic patient (a) before hemodialysis (pre-HD) and (b) after hemodialysis (after-HD). Identification of each ribonucleoside was confirmed by frit-FAB LC–MS analysis. For the abbreviations of nucleosides, see Section 4.

and sensitive method [9], because most nucleosides have strong UV absorbance around 260 nm. However, characterization of the nucleosides by this method is based primarily on their chromatographic mobilities comparing with reference compounds, so that analytical difficulties arise when unknown or unexpected identities are encountered. Even with HPLC coupled with photodiode array UV detection, the validity of the characterization is difficult to justify especially when the peak in HPLC eluate does not represent a single component.

HPLC coupled with mass spectrometry (LC–MS), allowing on-line mass analysis of complex biological matrix, has an advantage of providing both molecular mass and structural information of individual components, which are not obtainable using UV detection. The use of mass spectrometry as a detection provides greatly enhanced specificity compared with UV detection, and permits recognition of chromatographically unresolved components in most case [10]. Nevertheless, little attempt has been made to explore the applicability of LC–MS to the analysis of nucleosides in biological fluids [11]. In this paper, we describe the application of frit-fast atom bombardment (FAB) capillary LC–MS to detect and characterize serum and urinary ribonucleosides in normal subjects and uremic patients.

2. Experimental

2.1. Chemicals

The nucleoside standards used were of analytical grade from several sources (Sigma, St. Louis, MO, USA; Kohjin, Tokyo, Japan; Nakalai Tesque, Kyoto, Japan; Wako, Osaka, Japan). For the abbreviations of nucleosides, see Section 4. t^6A was synthesized according to the method of Chheda and Hong [14], and m_2^2G was a gift from Dr. A. Yamazaki (Central Research Labs., Ajinomoto, Japan).

2.2. Urine and serum samples

Urine samples were obtained as 24-h urine from 19 undialyzed uremic patients (13 men, six women), 17 of whom receiving allopirinol, and from 11 healthy subjects (four men, seven women). Serum samples were obtained from 13 undialyzed uremic patients (seven men, six women), 11 of whom receiving allopirinol. Serum samples were also obtained from 10 healthy subjects (five men, five women). Aliquots of urine and serum were frozen immediately and maintained at −50°C.

2.3. Boronate affinity gel column

Affi-Gel 601 (Bio-Rad Labs., Richmond, CA, USA), a boronate affinity gel, was used for the prefractionation of ribonucleosides from different biological fluids, urine and deproteinized serum, according to the method described by Kuo et al. [12]. The boronate gel (1 g) was allowed to swell for 5 min in water (ca. 25 ml), and then alternately washed 10 times with methanol and water. The gel was poured into a Miniature Champagne column (5.5× 0.5 cm I.D.) with a reservoir (4 ml) (Supelco, Bellefonte, PA, USA). The inner wall of the borosilicated glass column had been treated with a silianizing reagent, dimethyldichlorosilane, to prevent gel-adhesion [13], and the column tip had been plugged with glass wool prior to gel packing. The column was equilibrated with 15 ml of 0.25 *M* ammonium acetate (pH 8.8) prior to sample loading.

2.4. Extraction of ribonucleosides from biological samples

Urine (250 μl) was pipetted into a microtest-tube (1.5 ml). Then, 100 μl of 2.5 *M* ammonium acetate (pH 9.0) containing 10 nmol of br^5U as an internal standard was added, and the mixture was vortex-mixed for several seconds. The urine sample was transferred to the preequilibrated boronated gel column with a Pasteur pipet. The sample tube was rinsed with 500 μl of 0.25 *M* ammonium acetate (pH 8.8), and the rinsed solution was transferred to the gel column. The column was washed with 3 ml of 0.25 *M* ammonium acetate (pH 8.8) and 0.3 ml of 50% MeOH in water, and eluted with 5 ml of 0.04 *M* HCOOH in 50% (v/v) MeOH in water. The collected eluate was lyophilized. The residue was dissolved in 5 μl of 0.1 *M* ammonium acetate (pH 6.0), and 3 μl of this solution was subjected to LC–MS.

Ribonucleosides were isolated from serum using the boronated gel column. After adding 20 nmol of br^{5}U as an internal standard into 1 ml of serum, the sample was filtrated through a Centricut mini V-20 (Kurabou, Osaka, Japan) by centrifugation at 2000 *g* for 3–4 h to remove proteins with molecular masses greater than 20 000. After addition of 250 μl of 2.5 *M* ammonium acetate (pH 9.0) to the ultrafiltrate, the sample was applied to the boronated gel column. Further steps were the same as for the isolation of urinary nucleosides. The residue was dissolved in 5 μl of 0.1 *M* ammonium acetate (pH 6.0), and 2-μl aliquots were injected into the LC–MS system.

2.5. LC–MS

LC–MS equipment and operating procedures used for these experiments have been reported previously [15], and are briefly described below. The gradient LC system consisted of two Shimadzu (Kyoto, Japan) LC-9A pumps, a Rheodyne (Cotati, CA, USA) Model 7125 loop injector and a Shimadzu SPD-6AV UV detector equipped with a laboratory-made micro flow cell (cell volume 0.1 μl). A flow-rate of 100 μl/min was split ca. 16:1 using a T-joint fitted to a microbore separation column (Develosil ODS-5, 250×0.3 mm I.D., 5 μm particles; Nomura, Seto, Japan) and a restriction column (Develosil ODS-5, 150×2.0 mm I.D., 5 μm particles). This column splitting provides a suitable flow-rate (4–6 μl/min) for the frit-FAB interface owing to the proper back pressure differential of the restriction column, and allows the whole sample to enter the LC–MS interface [15,16]. Gradient elution was accomplished using a mixture of 0.1 *M* ammonium acetate buffer (pH 6.0) as solvent A and 40% methanol as solvent B. Both solvents contained 0.8% (v/v) of glycerol as a FAB matrix. The gradient profile used is described in Ref. [15].

A Jeol (Tokyo, Japan) JMS-HX110 mass spectrometer equipped with an LC–frit-FAB-MS interface, with a modified SPD-6AV UV detector [15] operated at 254 nm, was used to obtain the data. The mass spectrometer was operated at an accelerating voltage of 8 kV. The FAB gun (Jeol) was operated at 6 kV with xenon. The ion source temperature was kept at 40–50°C. The mass spectra were obtained by linear scanning from 20 to 1000 u with a scan speed of 4 s per cycle, and the background was subtracted with a Jeol JMA-DA5000 data system.

3. Results and discussion

A phenylboronate affinity gel has a strong affinity for adjacent *cis* hydroxy groups (*cis*-diol) so that it effectively isolates such low-molecular-mass compounds as nucleosides, nucleotides, catecholamines and sugars from physiological fluids such as serum and urine. We attempted to examine the applicability of LC–MS to the structural characterization of ribonucleosides in serum and urine both from uremic patients and healthy subjects, according to the method of Kuo et al., who used an affinity gel column to isolate ribonucleosides from serum and urine [12].

3.1. General mass spectral features

General mass spectral features of nucleosides under frit-FAB conditions have been previously investigated [15], and are basically the same as those with standard FAB-MS. Typical examples are represented by mass spectra of uridine and peudouridine (Fig. 2). Base peaks in the spectra are usually the protonated molecules $[MH]^+$ accompanied by molecular adduct ions such as $[M.NH_4]^+$, $[MH+G]^+$, and $[M.NH_4+G]^+$ (G=glycerol). These adducts are formed with NH_4^+ and glycerol in a mobile phase. The major fragment ion observed was a protonated base ion, $[BH_2]^+$, resulting from cleavage of the glycosidic bond. $[MH]^+$ and $[BH_2]^+$ ions are usually prominent in the spectra, and the mass differences between $[MH]^+$ and $[BH_2]^+$ are 132 mass units, which represent a loss of ribose moiety, leading to the readily recognition of the analyte to be a ribonucleoside. However, C-nucleoside pseudouridine (Ψ) cannot produce $[BH_2]^+$ in the mass spectrum, where another diagnostic ion $[B+44]^+$ was observed instead of $[BH_2]^+$. The ion, $[BH_2]^+$, was very small in intensity or undetectable in the mass spectra of some modified nucleosides (D, acp^{3}U) [15]. The general mass spectral features, including fragmentations, are similar to those observed with thermospray [17] and electrospray LC–MS [18].

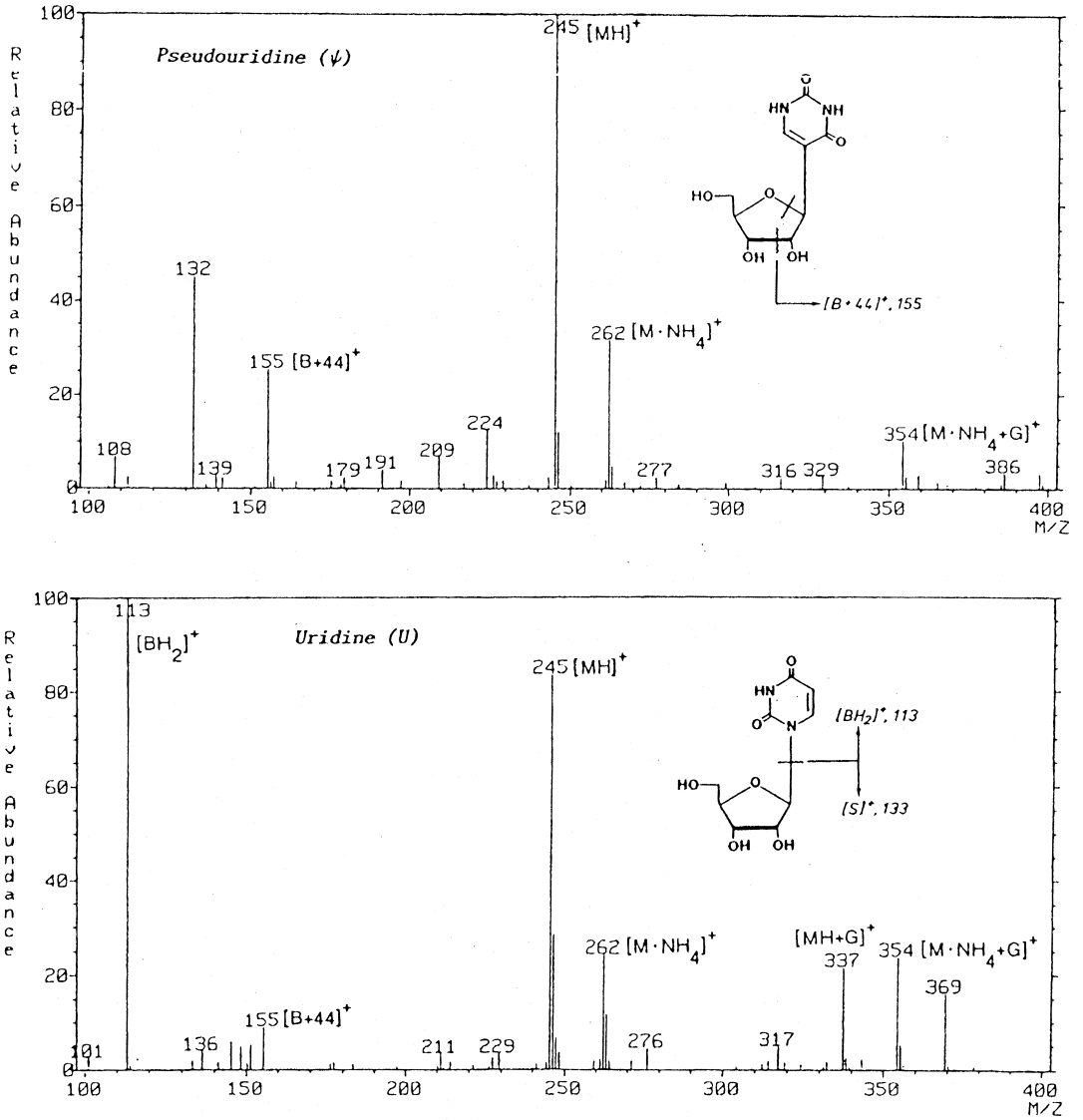

Fig. 2. Frit-FAB LC–MS mass spectra of pseudouridine (top) and uridine (bottom) in uremic serum.

3.2. Serum ribonucleosides in uremic patients and healthy subjects

In uremic patients a number of nucleosides are accumulated in serum because of reduced renal clearance. We have characterized 20 ribonucleosides in serum samples: these include four major ribonucleosides (A, C, G, U), three their metabolites (X, I, PCNR), and 13 modified ribonucleosides exclusively derived from the catabolism of tRNA. Several nucleosides could not be detected in healthy subjects (data not shown) because of their lower serum levels than those in uremic patients. The detection sensitivity for unequivocal assignment of each nucleoside can be estimated about 80 ng. A typical LC–MS profile of serum ribonuclesides in a uremic patient is shown in Fig. 3. Ribonucleosides were identified by comparing their $[MH]^+$ and $[BH_2]^+$ ions, and their retention times with those of authentic standards if the ribonucleosides of interest are available. For

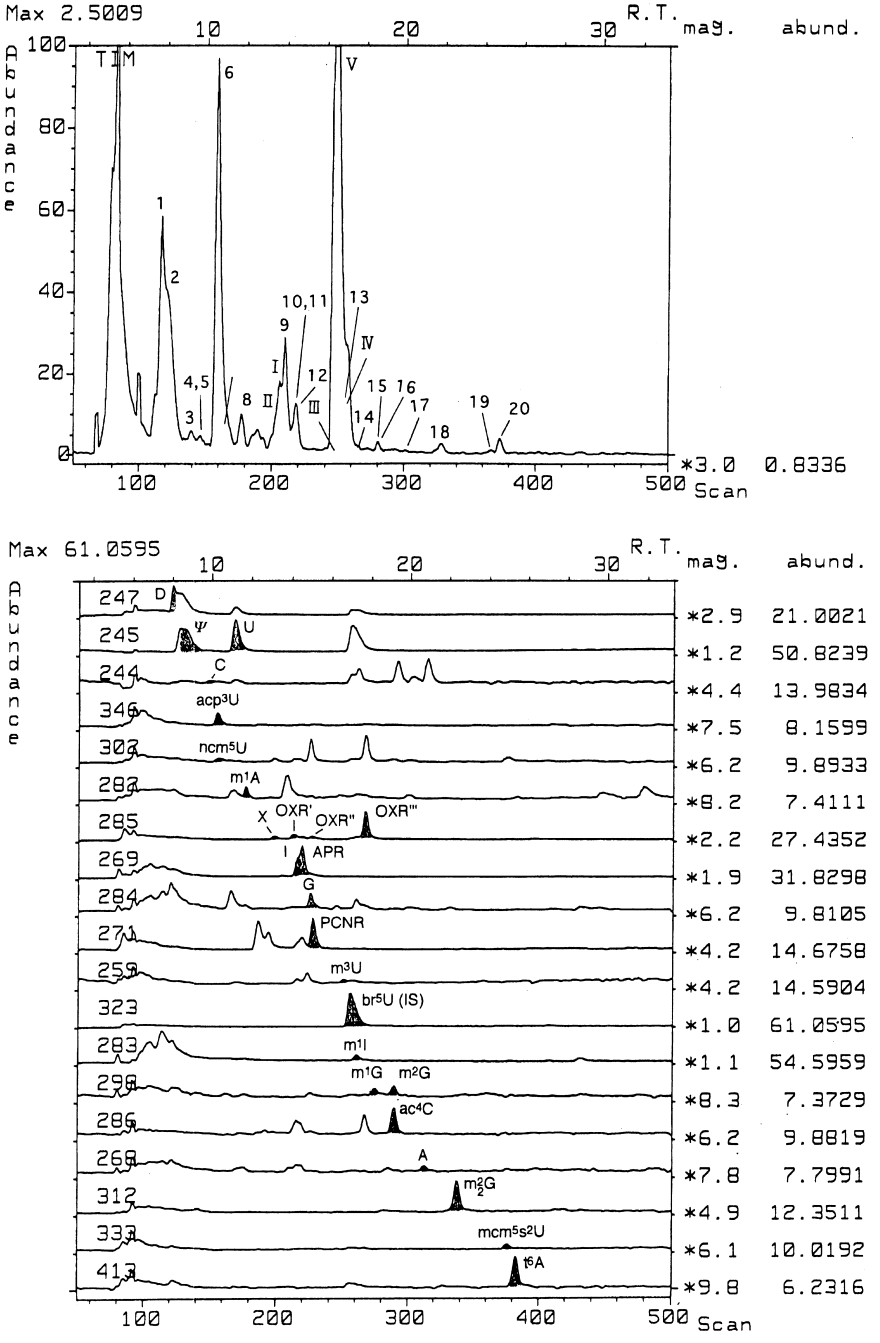

Fig. 3. Chromatographic separation of nucleosides by frit-FAB LC–MS analysis of uremic serum. LC–UV trace (top) and mass chromatograms of $[MH]^+$ ions for the ribonucleosides (bottom). The peaks were identified as follows: 1, D; 2, Ψ; 3, C; 4, acp^3U; 5, ncm^5U; 6, U; 7, m^1A; 8, X; 9, I; 9, I; 10, G; 11, PCNR; 12, m^3U; 13, m^1I; 14, m^1G; 15, m^2G; 16, ac^4C; 17, A; 18, m_2^2G; 19, mcm^5s^2U; 20, t^6A; I, OXR′; II, APR; III, OXR″; IV, OXR‴; V, br^5U.

example, a modified nucleoside, 1-methylinosine (m^1I), which nearly coeluted with 5-bromouridine (br^5U) as an internal standard, was characterized by the chromatographically identical alignment of the $[BH_2]^+$ (*m*/*z* 151) and $[MH]^+$ (*m*/*z* 283), which are distinguished from $[MH]^+$ (*m*/*z* 323) and $[M.NH_4]^+$ (*m*/*z* 340) derived from br^5U (Fig. 4). The mass spectrum obtained at 26.5 min was identical to that of authentic m^1I except for interference from coeluting br^5U (data not shown).

Some ribonucleosides showed additional fragmentation which helped to verify the chemical structure. A loss of bromine from $[MH]^+$ and $[BH_2]^+$ was observed in the mass spectrum of br^5U giving peaks corresponding to *m*/*z* 245 and 113, respectively, due to FAB-induced decomposition in the gas phase [19]. Similarly, the highly modified nucleoside t^6A showed a loss of threonyl side chain moiety from $[MH]^+$ and $[BH_2]^+$ to form protonated adenosine (*m*/*z* 268) and adenine (*m*/*z* 136), respectively, suggesting t^6A to be an adenosine derivative.

3.3. Urine ribonucleosides in uremic patients and healthy subjects

Screening of nucleosides in urinary samples was accomplished by mass responses for the $[MH]^+$ and $[BH_2]^+$ as described above for the serum sample. A number of ribonucleosides were characterized in the urine sample. Furthermore, modified nucleosides, m^5C, mt^6A and mcm^5s^2U, were detected in the urine sample. Although urinary concentration of ribonucleosides was somewhat altered in uremic patients, the same species of ribonucleosides were observed in the healthy subject, except for presence of several unknown peaks. In separate experiments to quantify several representative nucleosides in normal subjects and uremic patients by HPLC, urinary excretion of inosine and some other nucleosides was decreased in the uremic patients. Interestingly, the serum level of inosine was also decreased in uremic patients, suggesting that the production of inosine is reduced in uremia [20].

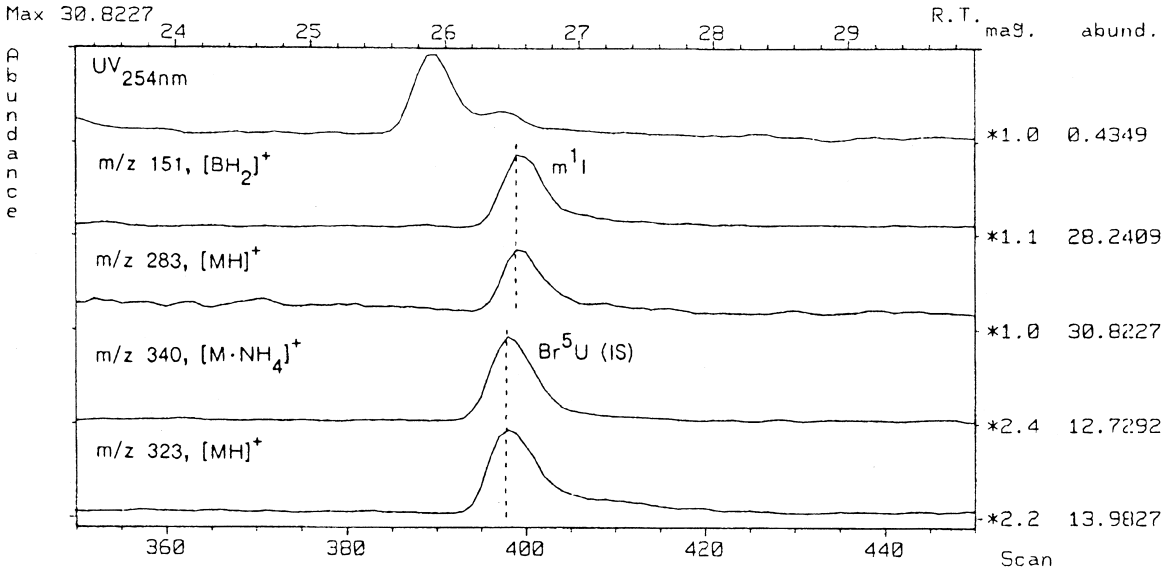

Fig. 4. 1-Methylinosine (m^1I) and 5-bromouridine (br^5U), an internal standard, in uremic serum ultrafiltate: partial HPLC chromatogram (UV 254 nm) and mass chromatograms of *m*/*z* 151 and 283, representing $[MH]^+$ and $[BH_2]^+$ ions for m^1I, and *m*/*z* 340 and 323, representing $[M.NH_4]^+$ and $[MH]^+$ ions for br^5U, respectively. In the chromatograms, the mass channel time scale lags the UV scale by approximately 32 s (eight scan), due to the transit time between UV and mass detection.

3.4. Detection of allopurinol-derived ribonucleosides

Partial LC–MS profiles of urine ribonucleosides obtained from a normal subject and a uremic patient are shown in Figs. 5 and 6, respectively. In the elution region between m^1A and m^1G, additional peaks designated as peaks 2–4 and 6 appeared only in the uremic patient, but not in the normal subject. In the normal subject (Fig. 5) peaks 1 and 5 could be assigned to xanthosine (X) and inosine (I), respectively, by comparing their $[MH]^+$ and $[BH_2]^+$ ions, and their retention times with their authentic standards. On the other hand, peak 6 observed in the uremic patient showed mass response similar to that for I, but it eluted slightly earlier than authentic I under the reversed-phase HPLC conditions (Fig. 6) [21]. Mass spectra of peaks 1 to 6 taken from Figs. 5 and 6 are shown in Fig. 7 along with their plausible structures. The mass spectrum of peak 6 was appar-

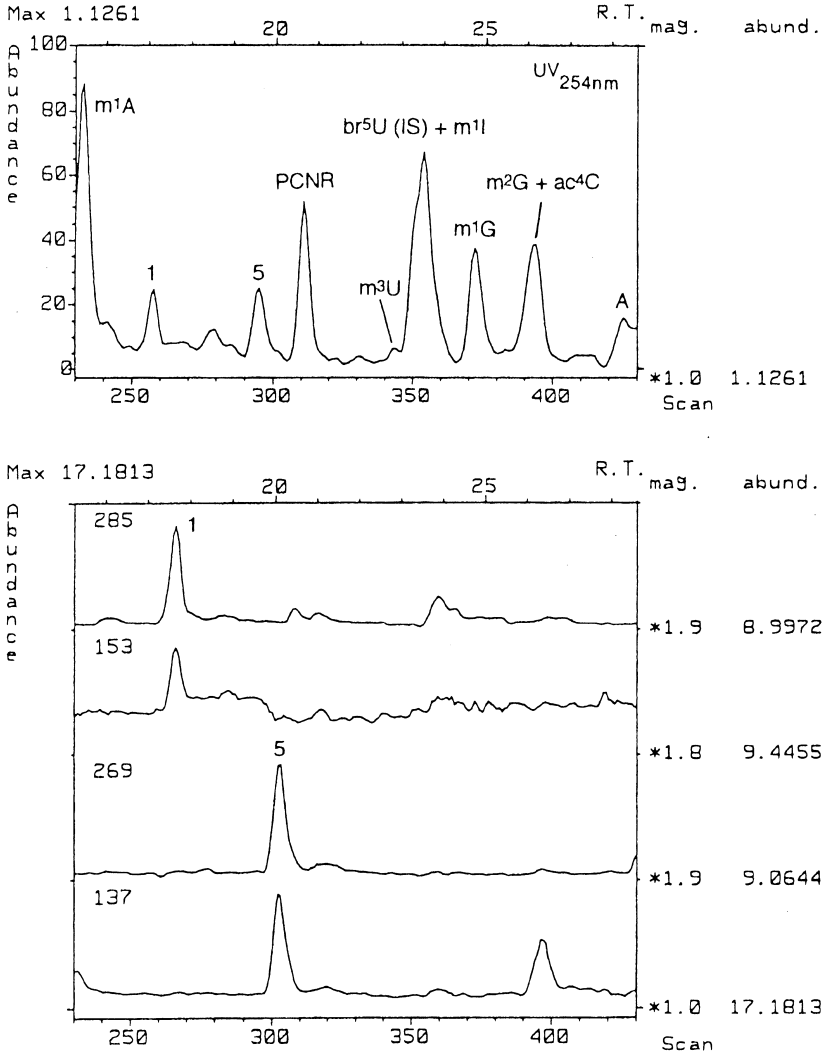

Fig. 5. Frit-FAB LC–MS of ribonucleosides in normal urine. The HPLC–UV (254 nm) chromatogram (top) and mass chromatograms of m/z 285 and 153, representing the $[MH]^+$ and $[BH_2]^+$ ions, respectively, for xanthosine (peak 1), and m/z 269 and 137, representing the $[MH]^+$ and $[BH_2]^+$ ions, respectively, for inosine (peak 5) (bottom).

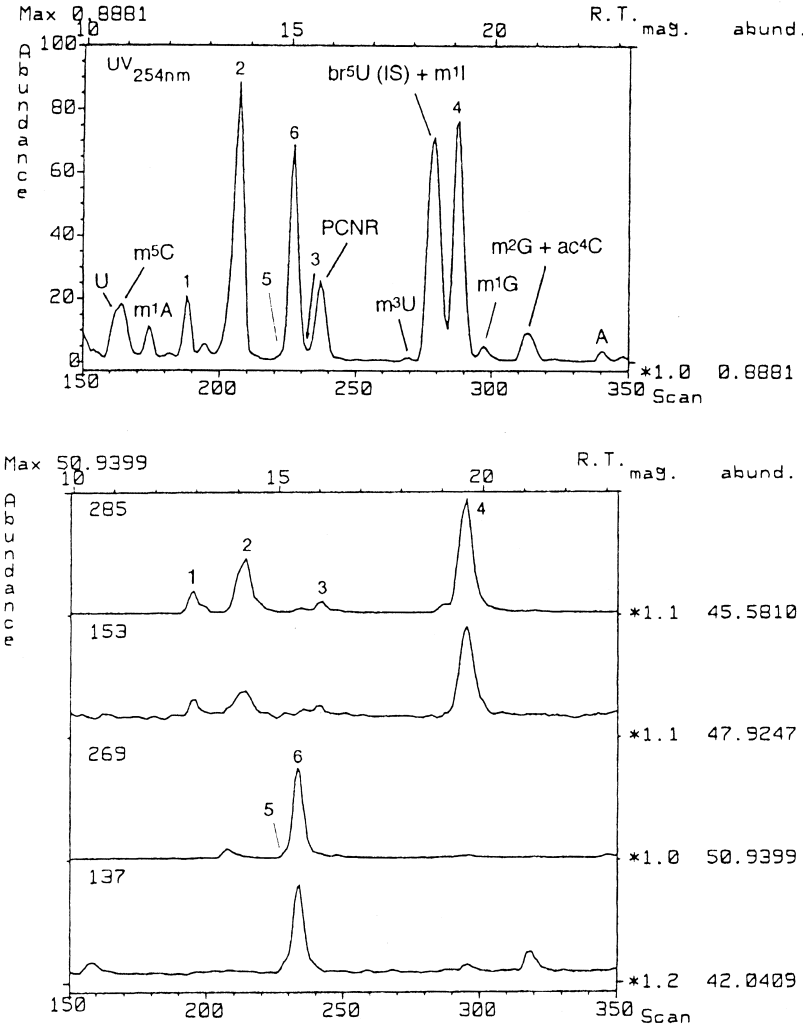

Fig. 6. Frit-FAB LC–MS of ribonucleosides in uremic urine. The HPLC–UV (254 nm) chromatogram (top) and mass chromatograms of m/z 285 and 153, representing the $[MH]^+$ and $[BH_2]^+$ ions, respectively, for xanthosine (peak 1) and its structural isomers (peaks 2–4), and m/z 269 and 137, representing the $[MH]^+$ and $[BH_2]^+$ ions, respectively, for inosine (peak 5) and its structural isomer, allopurinol-1-riboside (peak 6) (bottom).

ently different from that of peak 5 in terms of intensity ratio of $[MH]^+/[BH_2]^+$. Thus, peak 6 was assigned as a structural isomer of I. Furthermore, X and its three structural isomers designated as peaks 1 to 4 were detected in the uremic patients. Peaks 2–4 and 6 were also observed in serum of uremic patients (Fig. 3), indicating that these peaks may be attributed to metabolites of a drug, presumably allopurinol, because the uremic patients had a history of chronic allopurinol therapy.

Allopurinol [4-hyroxypyrazolo (3,4-d)pyrimidine], an analogue of hypoxanthine, is converted to its principal metabolite, oxipurinol [4,6-dihyroxypyrazolo (3,4-d)pyrimidine], by xanthine oxidase. Ribonucleosides of allopurinol and oxipurinol were known to be detected as final metabolites in urine of

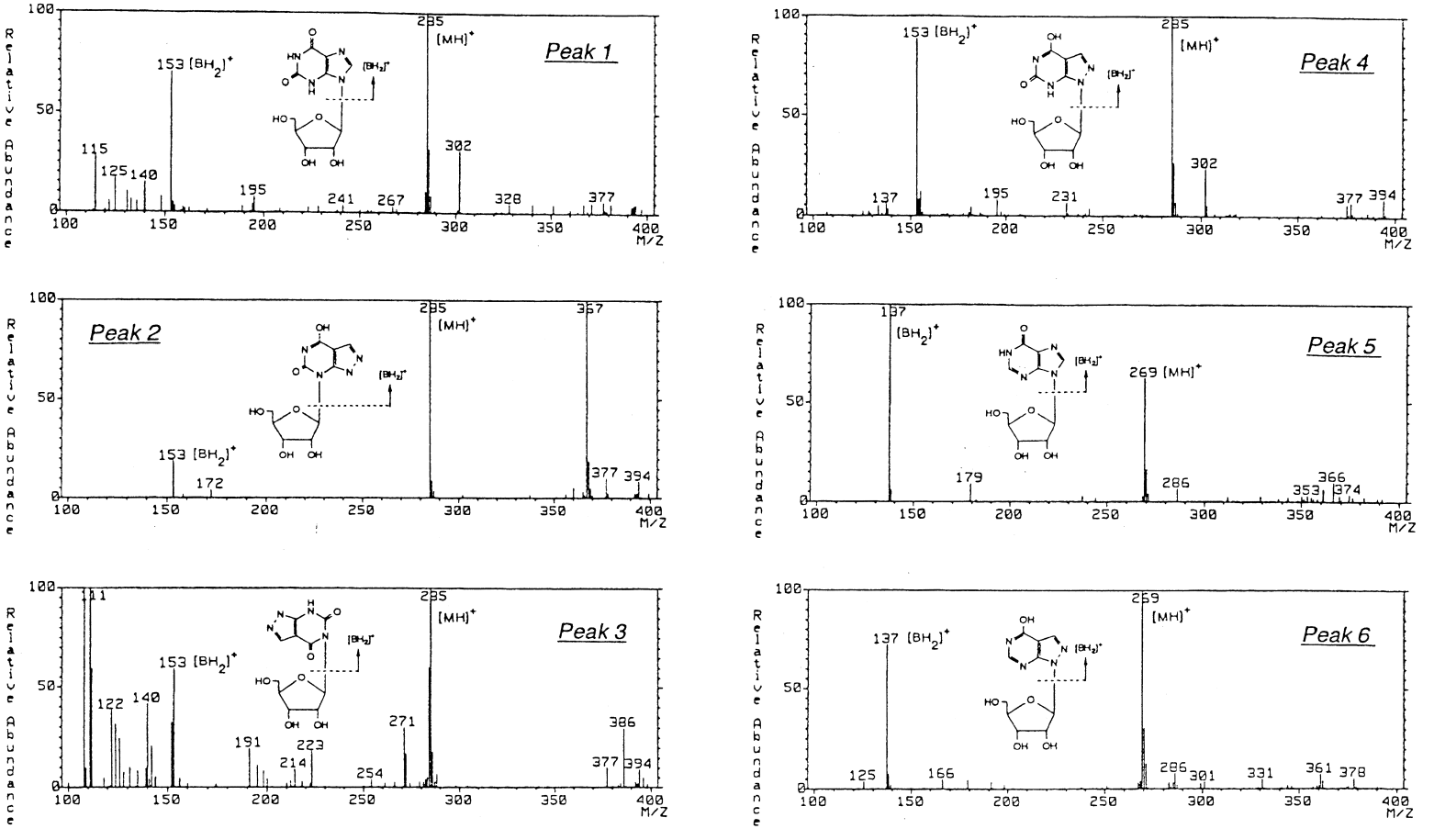

Fig. 7. Frit-FAB LC–MS spectra of peaks 1–6, corresponding to xanthosine (X), oxipurinol-7-riboside (7-OXR), unknown oxipurinol riboside (5-OXR), oxipurinol-1-riboside (1-OXR), inosine (I) and allopurinol-1-riboside (APR), respectively.

patients receiving this drug; these include allopurinol-1-riboside, oxipurinol-1- and -7-ribosides [21–24]. Nucleotides of allopurinol and oxipurinol have been implicated in the inhibition of orotidylic-decarboxylase (ODC, EC 4.1.1.23), causing the allopurinol-induced orotidinuria and orotic aciduria [24]. Although we do not have authentic standards of these ribonucleosides, chromatographic elution on reversed-phase HPLC have been well documented, where elution order of these ribonucleosides was allopurinol-1-riboside, oxipurinol-1-ribosides and oxipurinol-7-ribosides [23]. Therefore, peak 6 was tentatively assigned to allopurinol-1-riboside, and two major oxipurinol related components, peaks 2 and 4, may correspond to oxipurinol-1-riboside and oxipurinol-7-riboside, respectively (Fig. 6).

It is interesting that peak 3, an additional unexpected oxipurinol riboside, was present at a lower concentration, because its occurrence has not so far been known in any biological sources obtained from patients receiving allopurinol. The structure in terms of the site of attachment of the sugar moiety to the base and anomeric configuration of ribose cannot be determined by the mass spectrum in the present experiments. We suggest that peak 3 may be 5-substitution of oxipurinol with β-configuration of ribonucleoside. Allopurinol and oxipurinol have shown to be converted by purine nucleoside phosphorylase to the 1-ribosyl derivatives, and oxipurinol also converted by orotate phosphoribosyltransferase (OPRT, EC 2.4.2.10) to the 7-ribosyl derivative. Such anabolic reactions of oxipurinol by OPRT may lead to formation of 5-ribosyl derivative, if oxipurinol, a pyrimidine analogue (pyrazolopyrimidines), is recognized as a substrate by OPRT enzyme.

In conclusion, frit-FAB LC–MS has proved to be efficiently applicable to the analysis of ribonucleosides in serum and urine for the first time. By using the LC–MS, 20 RNA derived nucleosides and four allopurinol-derived nucleosides could be characterized in urine and serum samples of uremic patients. These RNA end products were accumulated in the serum of uremic patients at abnormally higher levels. Their retention in the blood and possibly in tissue compartments may potentially exert cumulative toxic actions, working singly or synergistically which would be of significance in the pathogenesis of some uremic symptoms [25].

4. Abbreviations

D	5,6-Dihydrouridine
Ψ	Pseudouridine
C	Cytidine
acp^3U	3-(3-Amino-3-carboxypropyl)uridine
U	Uridine
m^1A	1-Methyladenosine
X	Xanthosine
OXR′	Oxipurinol-7-riboside
I	Inosine
APR	Allopurinol-1-riboside
OXR″	Oxipurinol-5-riboside
G	Guanosine
PCNR	4-Pyridone-3-carboxamide-N^1-ribofuranoside
m^1I	1-Methylinosine
OXR‴	Oxipurinol-1-riboside
m^1G	1-Methylguanosine
ac^4C	N^4-Acetylcytidine
m^2G	N^2-Methylguanosine
A	Adenosine
m_2^2G	N^2,N^2-Dimethylguanosine
mcm^5s^2U	5-Methoxycarbonylmethyl-2-thiouridine
t^6A	N^6-Threonylcarbamoyladenosine
m^6A	N^6-Methyladenosine
mt^6A	N^6-Methyl-N^6-threonylcarbamoyladenosine
ms^2t^6A	N^6-Threonylcarbamoyl-2-methylthioadenosine

Acknowledgements

The authors are grateful to Ms. Mariko Usami and Ms. Hisako Sugiyama for their skillful technical assistance, and to Dr. A. Yamazaki for his generosity in providing N^2,N^2-dimethylguanosine standard.

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