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ORIGINAL PAPER

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Mass spectrometric identification of modified urinary nucleosides used as potential biomedical markers by LC–ITMS coupling

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Abstract In diseases accompanied by strong metabolic disorders, like cancer and AIDS, modifying enzymes are up- or down-regulated. As a result, many different types of metabolic end-products, including abnormal amounts of modified nucleosides, are found in urine. These nucleosides are degradation products of an impaired ribonucleic acid (RNA) metabolism, which affects the nucleoside pattern in urine. In several basic experiments we elucidated the fragmentation pathways of 16 characteristic nucleosides and six corresponding nucleic bases that occur in urine using electrospray ionization ion trap MS^5 (ESI-ITMS) experiments operated in positive ionization mode. For urinary nucleoside analysis, we developed an auto-LC–MS^3 method based on prepurification via boronate gel affinity chromatography followed by reversed phase chromatography. For this purpose, an endcapped LiChroCART Superspher RP 18 column with a gradient of ammonium formate and a methanol–water mixture was used. This method gives a limit of detection of between 0.1 and 9.6 pmol for 15 standard nucleosides, depending on the basicity of the nucleoside. Overall, the detection of 36 nucleosides from urine was feasible. It was shown that this auto-LC–MS^3 method is a valuable tool for assigning nucleosides from complex biological matrices, and it may be utilized in the diagnosis of diseases associated with disorders in RNA metabolism.

Keywords Modified nucleosides · Ion trap mass spectrometry · Fragmentation pattern of nucleosides · Tumor markers · Urinary nucleosides

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Introduction

At present about 100 modified nucleosides are known, where each type of cellular RNA, like transfer RNA (tRNA), messenger RNA (mRNA), ribosomal RNA, tmRNA and small nuclear RNA, is modified to a different extent [1]. Among the different types of RNA, tRNA is generally the most heavily modified: in some higher eukrayotes up to 25% of the nucleotides show modifications. The structural modifications are subdivided into simple modifications such as base or ribose methylation, base isomerization, reduction, thiolation or deamination and hypermodifications, such as in N6-threonylcarbamoyladenosine.

All modifications are formed post-transcriptionally in RNA from the normal nucleosides adenosine, guanosine, uridine and cytidine by modifying enzymes, especially specific RNA-methyltransferases and RNA-synthetases. They play an important role in tRNA activity [2]. Modified nucleosides influence the translational efficiency and precision as well as the sensitivity to the reading context and the reading frame maintenance. In general, these modifications are believed to ensure the three-dimensional structural integrity of RNAs.

Due to the lack of specific phosphorylases for modified nucleosides, they cannot be recycled for synthesizing RNA, so they are excreted quantitatively in urine [3].

In diseases like cancer or AIDS, RNA metabolism is impaired, which is demonstrated by the altered levels of modified nucleosides in urine and serum from patients [4]. Especially in cancer diagnosis, modified nucleosides have been proposed as potential indicators for the presence of disease [5–14]. In recent studies, Dudley et al identified a rare nucleoside—5′-deoxycytidine—as a potential biomarker. Up to then, this nucleoside had only be observed in urine in conjunction with head and neck cancer [15].

Different methods have been applied for identifying and quantifying new nucleosides in urine. Many attempts have been made to quantify nucleosides using high-performance liquid chromatography (HPLC) with UV detection [16, 17] and capillary electrophoresis (CE) [18]. Recently, the coupling of HPLC, gas chromatography or capillary liquid chromatography with mass spectrometric detection via electrospray ionization ion trap mass spectrometry (ESI-ITMS) [19], ESI tandem MS [20] or thermospray ionization and fast atom bombardment (FAB) [21] has also been applied.

ESI is the most applicable ionization method for ITMS, as it may be coupled to LC. With other ionization methods, like matrix-assisted laser desorption/ionization (MALDI), electron ionization (EI), chemical ionization (CI) or FAB, this is either not possible at all or it requires interfaces, which are not very robust or lead to a lower sensitivity. Aside from this, EI is a ''hard'' ionization method that produces extensive fragmentation, and, if MALDI-PSD-MS is used for fragmentation, the decay is difficult to control. An ion trap is more applicable, as the fragmentation can be regulated precisely for special compounds by optimizing tuning parameters like amplitude and isolation width.

A triple quadrupole mass spectrometer may be coupled to LC, but fragmentation possibilities are limited to MS/MS spectra. With an ion trap, MS^n experiments are feasible, which yield the complete fragmentation pathways.

The basis for allocating known nucleosides and elucidating the structures of unknown compounds using MS^n experiments is to understand the fragmentation patterns of nucleosides. We have shown that the fragmentation patterns of nucleosides are characteristic, even for isomeric ones.

In basic studies, Nelson and McCloskey examined the fragmentation pathways of the nucleic bases adenine and uracil by collision-induced dissociation (CID). They applied specific ^{13}C and ^{15}N isotopic labeling to the nucleic bases in order to characterize fragmentational behavior [22, 23]. The predominant fragmentations included neutral loss of NH_3 and HCN in adenine and additionally CO and H_2O in the case of uracil.

We investigated the fragmentation patterns of 16 nucleosides occurring in urine and six corresponding nucleic bases via MS, MS^2, MS^3 and MS^4 experiments, and were able to propose the structures of the fragment

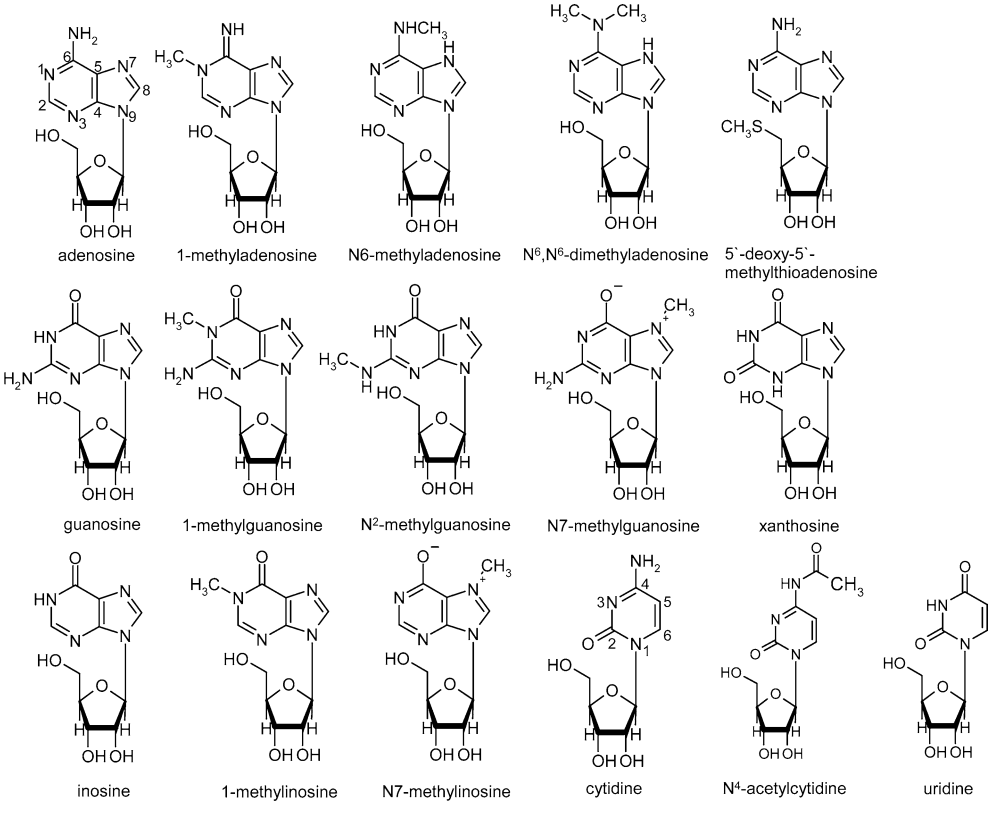

Fig. 1 Structures of the examined nucleosides

ions, analogous to the structures proposed by Nelson and McCloskey.

Dudley et al developed an LC–ITMS method for quantifying urinary nucleosides in order to compare its effectiveness when used for the study of urinary nucleoside profiles with other mass spectrometric methods [19]. They were able to quantify 17 nucleosides and identify them by their masses and MS/MS spectra by comparing them with standards. However, DHU, 3-methyluridine, guanosine and MTA could not be detected with this method and no further information about the structure of the nucleosides was obtained using only MS/MS spectra.

We developed an auto-LC–MS^3 method for separating 15 standard nucleosides, including DHU, 3-methyluridine, guanosine and MTA. We divided the LC run into 6 ms time segments with optimized tune parameters for those nucleosides eluting in this time range. These segments ensure high sensitivity and reproducibility for all nucleosides.

Besides, the MS^3 results give further information about the compounds, which is valuable for identifying nucleosides in urine by their fragmentation pattern and when looking for unknown nucleosides as tumor markers. The MS/MS spectra only show the nucleic base fragment, while the MS^3 fragmentation step gives further precious information about the structure of the nucleic base itself.

A urine sample from a breast cancer patient was examined with the newly-developed auto-LC–MS^3 method. We were able to identify 15 nucleosides by their retention times and three by comparison of the fragmentation with standard substances.

Eleven compounds were collated to known nucleosides by mass and fragmentation pattern, while seven remain unidentified.

These results show that our auto-LC–MS^3 method is a valuable tool for identifying nucleosides from complex biological matrices, and that it may be used in the diagnosis of diseases associated with disorders in RNA metabolism. It is also possible to draw conclusions about their structures based on their fragmentation patterns.

Experimental

Chemicals and materials

We used trifluoroacetic acid, ammonium formate and methanol LiChroSolv, gradient grade, purchased from Merck/VWR, Germany. Water was taken from an in-house double distillation system. The nucleoside and nucleic base standard substances were obtained from Sigma, Germany. We analyzed the nucleosides adenosine, 1-methyladenosine, N^6-methyladenosine, N^6, N^6-dimethyladenosine, 5′-deoxy-5′-methylthioadenosine (MTA), cytidine, N^4-acetylcytidine, inosine, 1-methylinosine, N^7-methylinosine, guanosine, 1-methylguanosine, N^2-methylguanosine, N^7-methylguanosine, uridine and xanthosine, and the nucleic bases adenine, cytosine, guanine, hypoxanthine, uracil and xanthine. The structures of the nucleosides are shown in Fig. 1. Affigel 601 was purchased from Biorad, Germany.

Instrumentation

The fragmentation patterns of nucleosides and the corresponding nucleic bases were compared using syringe pump infusion and MS^n experiments with an ion trap. A Bruker Esquire HCT-Ion Trap mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an ESI source was used in positive ion mode for detection. Data were acquired by Bruker EsquireControl version 5.1. For post-processing, Bruker DataAnalysis version 3.1 was used.

MS settings

For the 16 nucleosides and six nucleic bases we optimized the tuning parameters of the Esquire Ion Trap by syringe pump infusing 100 μg/ml solutions of the nucleosides and nucleic bases in 0.1% TFA to minimize the in-source fragmentation of the fragile nucleosides. The data were acquired by manual MS^n operation over the mass range 50–500 Da in standard enhanced scan mode (8,100 m/z per second).

Extraction of nucleosides from urine

Previous to the HPLC separation, the nucleosides were isolated from urine by affinity chromatography using a phenylboronic acid gel (Affigel 601, BioRad). This method was developed by Liebich et al in 1997 [17]. About 10 ml of urine was spiked with 0.5 ml of internal standard solution (0.25 mM isoguanosine) and then put on the column. The nucleosides are bound reversibly and specifically at the *cis*-diol groups contained in the ribose structure. After washing with ammonium acetate solution (0.25 mM, pH 8.6) and MeOH–H_2O (3:2), they were eluted with 0.1 M formic acid in MeOH–H_2O (3:2). The solvent was removed using a rotary evaporator and the nucleosides were dissolved again in 0.5 ml 25 mM KH_2PO_4.

Auto-LC–MS^3 method

To perform the chromatographic separation of the nucleosides, an Agilent 1100 Series HPLC system (Agilent, Waldbronn, Germany) was used consisting of a Solvent Degasser (G 1379 A), a binary capillary pump (G 1389 A), an autosampler thermostat (G 1330 A), an autosampler MicroALS (G 1389 A), a column oven (G 1316

Table 1 Chromatographic gradient

t (min)	5 mM ammonium formate buffer, pH 5.0	Methanol–water (3:2) + 0.1% formic acid
0	100	0
30	85	15
40	40	60
50	100	0
55	100	0

Table 2 Standard mixture of 16 nucleosides, including isoguanosine used as an internal standard

Retention time, LC–MS (min)	Nucleosides (common name)	Symbol	Concentration (nmol/ml)
4.9	Dihydrouridine	D	170.8
5.3	Pseudouridine	Ψ	638.8
6.6	Cytidine	C	4.1
9.6	Uridine	U	8.2
12.0	1-Methyladenosine	m^1 A	83.7
16.8	Isoguanosine (IS)		125.0
18.7	Inosine	I	16.3
19.2	5-Methyluridine	m^5 U	16.0
20.2	Guanosine	G	4.1
24.0	3-Methyluridine	m^3 U	8.3
24.6	Xanthosine	X	16.2
28.2	1-Methylinosine	m^1 I	4.1
29.6	1-Methylguanosine	m^1 G	16.0
32.0	2-Methylguanosine	m^2 G	4.4
33.2	Adenosine	A	17.1
44.9	N^6-Methyladenosine	m^6 A	16.5
48.3	5′-Deoxy-5′-methylthioadenosine	MTA	9.1

A) and a DAD (G 1315 B). The chromatographic system consisted of a Merck LiChroCART Superspher 100 RP-18 endcapped column (150×2.0 mm i.d.; Merck, Darmstadt) and a solvent gradient of 5 mM ammonium formate buffer, pH 5.0, and methanol–water (3:2, V:V) + 0.1% FA. The column was operated at 30 °C. The flow rate was set to 125 µl/min using the gradient shown in Table 1. The LC system was coupled to the HCT-Ion Trap mass spectrometer for mass detection. The capillary voltage was set to −5 kV, the dry temperature in the electrospray source was 325 °C, the nebulizer gas was set to 25 psi and the dry gas to 7.0 l/min.

To test the LC–MS^3 method, a standard mixture of 16 modified nucleosides was used, including an internal standard, as shown in Table 2. The ratio of nucleosides in this standard mixture was designed to mirror the ratio seen in real urine samples.

For these 16 nucleosides, the tuning parameters were optimized by syringe pump infusion, starting from a method using default parameters, to minimize the in-source fragmentation of the fragile nucleosides. With these parameters optimized, we could increase the intensities of these fragile nucleosides by a factor of 2.3–3. Afterwards the run was divided into six segments, with two to four nucleosides per segment for optimum reproducibility. The full scan mass range in each segment was set to 50–500 Da in positive electrospray mode and a maximum accumulation time of 15 ms was used.

To analyze the samples, a constant neutral loss chromatogram of 132 Da was created by Bruker Data-Analysis 3.1.

This new method can be used to identify new modified nucleosides and to search for previously-known nucleosides by their fragmentation pattern.

Table 3 Fragmentation pathways in MS^n experiments of nucleic bases and corresponding nucleosides

Compound	MS	MS(2)	MS(3)	MS(4)	MS(5)
Adenosine	268	136	94	67	
			109		
			119	67	
				92	65
Adenine	136	94	67		
		119	67		
			92		
Cytidine	244	112	95	68	
			90		
			69		
Cytosine	112	69			
		95	68	40.5	
Inosine	269	137	94	67	
			110	82	
			119	92	
Hypoxanthine	137	94	67		
		110	82		
		119	77		
			92	65	
Guanosine	284	152	110	82	69.2
			135	80	
				93	
				107	80
Guanine	152	110	82	69	
		135	80		
			93		
			107	80	
Uridine	245	113	70		
			96	68	40.6
Uracil	113	70			
		96	68		
Xanthosine	285	153	110	82	55.4
			136	81	
				93	65
				108	
Xanthine	153	110	82		
		136	81		
			93		
			108		

Results and discussion

Syringe pump infusion experiments

Comparing nucleoside and nucleic base decay

The most common fragments from the nucleic bases adenine, cytosine, guanine, uracil and xanthine in our syringe pump infusion experiments resulted from the

Table 4 Fragmentation pathways of the 16 examined nucleosides

Nucleoside	MS	MS^2	MS^3	MS^4
Adenosine	268 (MH^+)	136 (BH^+)	119 (BH^+ $-NH_3$)	92 (119–HCN)
			109 (BH^+ $-HCN$)	
			94 (BH^+ $-NH_2CN$)	67 (94–HCN)
1-Methyladenosine	282 (MH^+)	150 (BH^+)	133 (BH^+ $-NH_3$)	106 (133–HCN)
				92 (133–CH_3CN)
			123 (BH^+ $-HCN$)	
			109 (BH^+ $-CH_3CN$)	
			94 (BH^+ $-CH_3NHCN$)	
N^6-Methyladenosine	282 (MH^+)	150 (BH^+)	133 (BH^+ $-NH_3$)	106 (133–HCN)
				92 (133–CH_2NCH)
				79 (133–2HCN)
			123 (BH^+ $-HCN$)	108
				96 (123–HCN)
				82 (123–CH_2NCH)
			108 (BH^+ $-NH_2CN$)	
			94 (BH^+ $-CH_3NHCN$)	69
				79
N^6,N^6-Dimethyladenosine	296 (MH^+)	164 (BH^+)	121 (BH^+ $-CH_3NCH_3$)	94 (121 –HCN)
				108
			149 (BH^+ $-CH_3^+$)	
			135 (BH^+ $-CH_2NH$)	
			94 (BH^+ $-CH_3NCH_2CHNH$)	
			71	
5′-Deoxy-5′-methylthioadenosine	298 (MH^+)	136 (BH^+)	119 (BH^+ $-NH_3$)	92 (119–HCN)
			109 (BH^+ $-HCN$)	82 (109–HCN)
			94 (BH^+ $-NH_2CN$)	67 (94–HCN)
Cytidine	244 (MH^+)	112 (BH^+)	95 (BH^+ $-NH_3$)	68 (95–HCN)
N^4-Acetylcytidine	286 (MH^+)	154 (BH^+)	112 (BH^+ $-CH_2CO$)	95 (112–NH_3)
				69 (112–HNCO)
Inosine	269 (MH^+)	137 (BH^+)	119 (BH^+ $-H_2O$)	92 (119–HCN)
			110 (BH^+ $-HCN$)	82 (110–CO)
			94 (BH^+ $-HCNO$)	67 (94–HCN)
1-Methylinosine	283 (MH^+)	151 (BH^+)	128	110
				98
			133 (BH^+ $-H_2O$)	79 (133–2×HCN)
				96
				106 (133–HCN)
			110 (151–CH_2NCH)	82 (110–CO)
			94 (151 –CO –CH_2NH)	
7-Methylinosine	283 (MH^+)	151 (BH^+)	133 (BH^+ $-H_2O$)	106 (133–HCN)
				79 (133–2HCN)
			124 (BH^+ $-HCN$)	
			108 (BH^+ $-HNCO$)	
			96 (BH^+ $-HCN–CO$)	
Guanosine	284 (MH^+)	152 (BH^+)	135 (BH^+ $-NH_3$)	107 (135–CO)
				93 (135–NH_2CN)
				80 (135–CO–HCN)
			110 (BH^+ -NH_2CN)	
1-Methylguanosine	298 (MH^+)	166 (BH^+)	149 (BH^+ $-NH_3$)	94 (149–NH_2CO)
			109 (BH^+ $-CH_3NCO$)	
2-Methylguanosine	298 (MH^+)	166 (BH^+)	149 (BH^+ $-NH_3$)	121 (149–CO)
			110 (BH^+ $-CH_3NHCN$)	
7-Methylguanosine	298 (MH^+)	166 (BH^+)	149 (BH^+ $-NH_3$)	121 (149–CO)
				107 (149–NH_2CN)
				94 (149–HCN–CO)
				80 (149–NH_2CN–HCN)
			124 (BH^+ $-NH_2CN$)	
N^2,N^2-Dimethyguanosine	312 (MH^+)	180 (BH^+)	162 (BH^+ $-H_2O$)	
			153 (BH^+ $-HCN$)	
			137 (BH^+ $-H_3CNCH_2$)	
			122	
			110 (137–HCN)	
Uridine	245 (MH^+)	113 (BH^+)	96 (BH^+ $-NH_3$)	68 (96–CO)
			70 (BH^+ $-HNCO$)	
Xanthosine	285 (MH^+)	153 (BH^+)	136 (BH^+ $-NH_3$)	108 (136–CO)
				81 (136–HCN–CO)
				93 (136–HNCO)
			110 (BH^+–HNCO)	82 (110–CO)

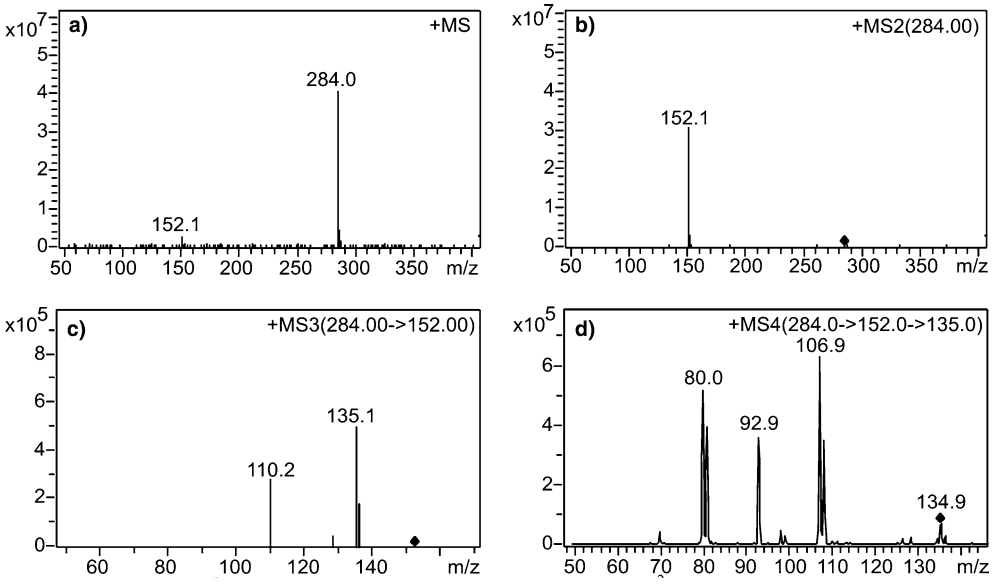

Fig. 2a–d Ion trap MS^n spectra of guanosine: (**a**) MS spectrum; (**b**) MS^2 spectrum of m/z 284; (**c**) MS^3 spectrum of m/z 152; (**d**) MS^4 spectrum of m/z 135

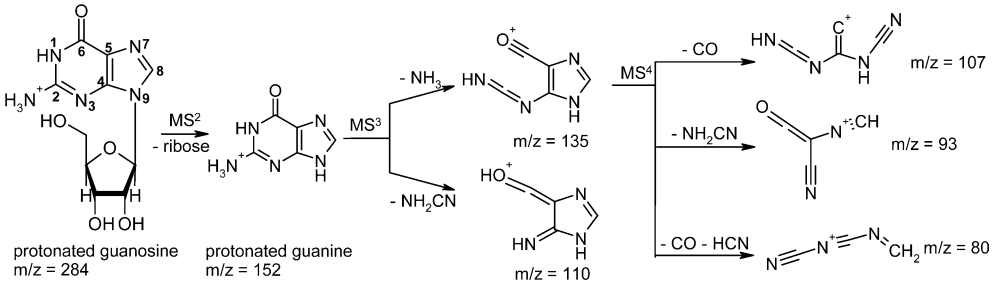

Fig. 3 Proposed fragmentation pathway of protonated guanosine

loss of ammonia, followed by the elimination of HCN or CO. In the case of hypoxanthine, water was eliminated, as was the case for uracil although only to a very small extent.

Nelson and McCloskey examined the CID of the nucleic bases adenine and uracil [22, 23]. They also described the loss of ammonia in both uracil and adenine, the loss of CO and H_2O in the case of uracil, and the loss of HCN in adenine. We were able to show that the fragmentation of these nucleic bases with ESI-ITMS agrees with the results of Nelson and McCloskey, despite the different activation energy of the collision cell.

The initial dissociation in the fragmentation pathways of all nucleosides is the decay into the protonated nucleic base and the neutral ribose moiety, caused by the weak N–C bond.

Our investigations show that the fragmentations of the nucleic bases matches perfectly with the fragmentations of the corresponding nucleosides. As seen in Table 3, the MS^2 spectra of the nucleic bases agree with the MS^3 spectra of the associated bases; further MS^n experiments show the same analogy.

MS^n experiments on nucleosides

All of the results from our basic MS^n experiments on nucleosides are summarized in Table 4.

In the MS^3 spectra of the 16 measured nucleosides, the peaks with the highest intensity were usually those resulting from the loss of ammonia. Another common reaction was the expulsion of HCN. The most common elimination in MS^4 experiments was also HCN. Another prevalent fragmentation for nucleosides with keto groups after loss of ammonia was the expulsion of CO.

As Nelson and McCloskey describe for adenine [22], the initial loss of ammonia from adenosine and 5′-deoxy-5′-methythioadenosine, which have identical nucleic bases, also derives from N^6 and N^1. In N^6-methyladenosine, N^6 is blocked by a methyl group, so the only possible expulsion of ammonia is derived from N^1, while in 1-methyladenosine, ammonia must be eliminated from N^6, as N^1 is blocked.

Another possibility for the ring-opening fragmentation step is the loss of HCN, which also takes place in all of the aforementioned nucleosides.

Guanosine, another purine-based nucleoside, possesses an amino group at the C-2 position, and thus it is likely to lose ammonia from either N^2 or N^1. Further fragmentation of the resulting fragment with m/z 135 produces a fragment with m/z 107, which indicates loss of CO (C-6 and O^6). This leads to the assumption that the preferred position for loss of ammonia must be N^1; otherwise the elimination of CO is impossible. The three

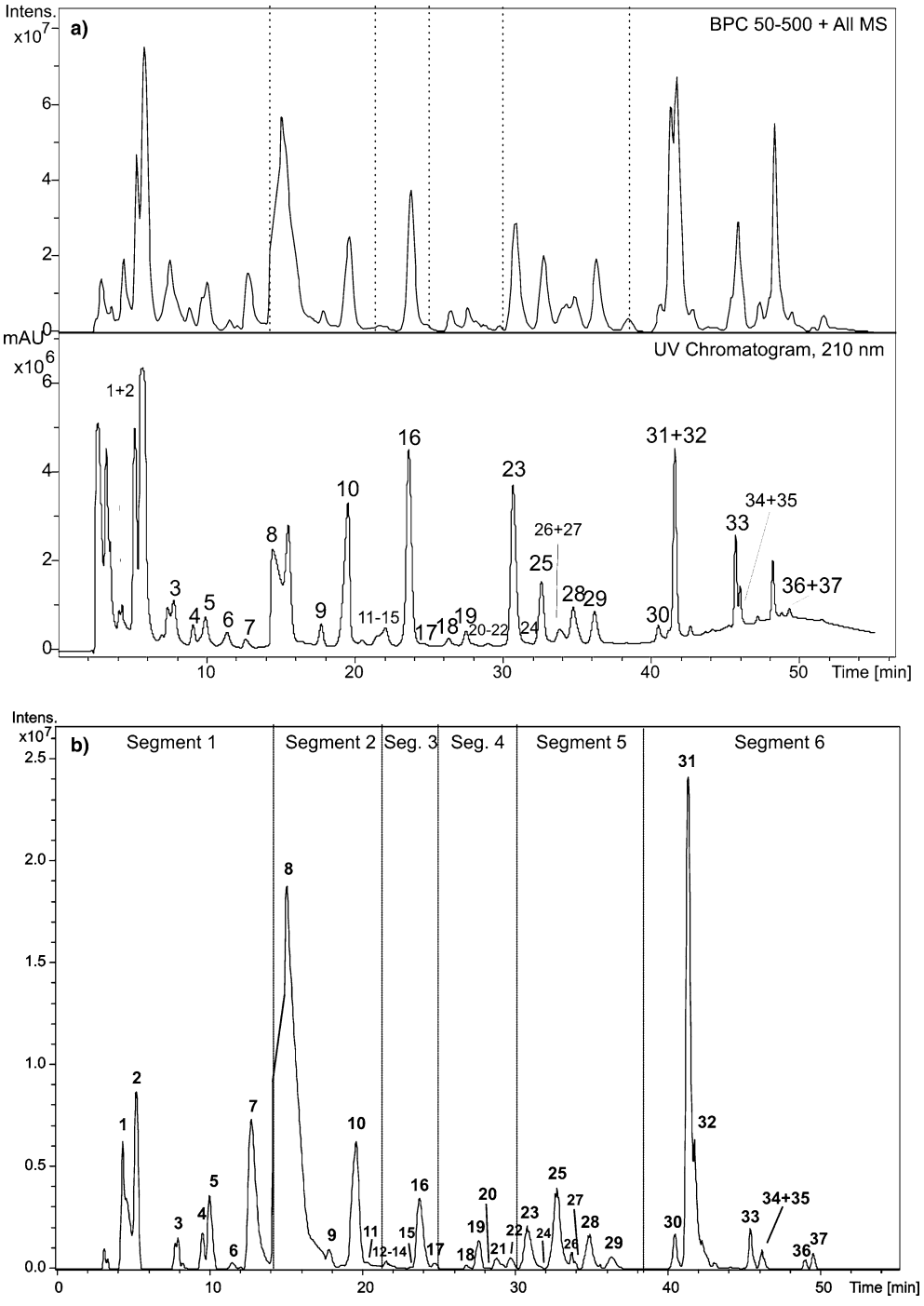

Fig. 4a–b **a** Base peak chromatogram and UV chromatogram of urine sample. **b** Constant neutral loss chromatogram (132 u) of urine sample

isomeric methylguanosines that were examined show similar fragmentation ions in MS^2 and MS^3. Like guanosine, they lose ammonia in the MS^3 experiments, producing a fragment with m/z 149. As N^1 is blocked by the methyl group in 1-methylguanosine, the loss of ammonia presumably happens at N^2. The other MS^2 fragment with m/z 109 is presumably caused by loss of CH_3NCO (C-1, N^1, C-6 and O^6). The fragment with m/z 94 in the MS^3 spectrum of the fragment with m/z 149 presumably originates from the loss of HCN (C^1 and N^1) and CO (C-6 and O^6).

2-Methylguanosine shows different fragmentations in MS^3 and MS^4 spectra, resulting from the different positions of the methyl group (N^2). This configuration leads to the presumption that ammonia is released from N^1 and CH_3NHCN (C_{methyl}, N^2, C-2, N^3) in the next fragmentation cycle, producing a fragment with m/z 110.

The fragment with m/z 124, occurring in the MS^3 spectrum of 7-methylguanosine, presumably results from loss of NH_2CN (N^2, C-2, N^3). A loss of 42 often indicates loss of NH_2CN in nucleosides, which also occurs with guanosine and adenosine.

As described, the three methylguanosines differ in their MS^3 spectra, which allows us to differentiate between them.

Figure 2 shows MS^n spectra of guanosine as an example. We were able to suggest the fragmentation pathways and structures of all of the fragments shown in Fig. 3.

Inosine and its derivatives, unlike all of the other nucleosides featuring a keto group, are characterized by the loss of H_2O (O^6), but not of ammonia, in the MS^3 spectra of the nucleic base. The major difference from the other nucleosides is the unblocked C-2 position. Substituents like amino groups or keto groups in the C-2 position probably destabilize the N^1–C-2 bond, while this bond is more stable in inosine and so elimination of H_2O is preferred.

1-Methylinosine and 7-methylinosine, like the methylated guanosines, differ in their MS^3 and MS^4 spectra, resulting from the different positions of the methyl groups.

Nelson et al also examined the decays of uracil and its derivatives by CID [23]. They discovered two basic pathways of fragmentation. We only observed one of these pathways in our ESI examinations of uridine: in the further fragmentation of the protonated base fragment with m/z 113, ammonia is eliminated (N^3), followed by loss of CO (C-4, O^4). The other pathway described by Nelson and McCloskey starts with the loss of H_2O (equally from O^2 and O^4), followed by the elimination of HNCO, predominantly from N^3, C-2 and O^2 [23]. We determined a loss of HNCO as well, but no expulsion of H_2O. A reason for this could be the use of different collision gases, helium in the ion trap and argon in the collision cell. Argon is heavier and thus more efficient at generating fragment ions [24].

Xanthosine shows similar behavior to uridine. Like uridine, xanthosine features two keto groups in equal positions, which are separated by an N-atom. As in uridine, no elimination of H_2O was observed, but loss of HNCO, presumably either from N^1, C-2 and O^2, or from N^1, C-6 and O^6. In both cases, the expulsion of CO, producing a fragment with m/z 82, is possible.

The other ring-opening fragmentation option is the loss of ammonia. The subsequent expulsion of HNCO (m/z 43, N^3, C-2, O^2) and CO (m/z 28, O^2 or O^6), followed by loss of HCN, are possible if ammonia is lost from N^1.

Cytidine is another pyrimidine-based nucleoside that loses ammonia in the first fragmentation cycle. This is assumed to happen at either N^3 or N^4, according to Nelson and McCloskey, in each case producing the same fragment with m/z 95. Further fragmentation results in a fragment with m/z 68 via loss of HCN (N^4 and C-4).

In N^4-acetylcytidine, in the first step, the acetyl group is eliminated, producing the protonated cytosine.

Auto-LC–MS^3 method

Reproducibility

To investigate the reproducibility of the method, the nucleoside standard solution was injected 11 times. The standard deviations of the retention times of the 16 nucleosides were between 0.02 and 0.36 min.

Limit of detection

To determine the detection limits for the auto-LC–MS^3 method, the nucleoside standard solution was diluted 1:10, 1:50, 1:100, 1:500, 1:1,000 and 1:5,000. This series was injected with increasing concentration. The limits of detection were determined by the concentration that gave a signal-to-noise ratio of 3, which occurred between 0.1 and 9.6 pmol.

Urine sample

A urine sample of a 63-year-old breast cancer patient suffering from a carcinoma ductale in situ (DCIS) was examined with the new auto-LC–MS^3 method. The post-processed constant neutral loss chromatogram (132 u) is shown in Fig. 4. In this CNL chromatogram, only those compounds losing 132 u in the MS^2 fragmentation step are shown, which applies to nucleosides as they decay in a neutral ribose moiety and the nucleic base fragment. Thus, it enables us to search for nucleosides specifically, but only for those containing the unmodified ribose.

Using the developed auto-LC–MS^3 method, we were able to detect 36 compounds in the urine sample producing fragments 132 Da less than the parent mass in MS^2 experiments. Additionally, the MS^3 experiments yielded information on the structures of the detected compounds, based on the results of our syringe pump infusion experiments. The nucleosides DHU, cytidine, uridine, 1-methyladenosine, 5-methyluridine, 3-methyluridine, xanthosine, 1-methylinosine, 1-methylguanosine, 2-methylguanosine, adenosine, N^6-methyadenosine and MTA were identified before in this chromatographic system and were among those contained in the standard solution. Even without considering the retention times, these nucleosides could be assigned to the correct compounds by comparing the characteristic fragmentation pattern with the results from our previous MS^n experiments. Three nucleosides that were not contained in the nucleoside standard solution could be identified using only their fragmentation patterns. These are N^4-acetylcytidine, which was first isolated from human urine and characterized by Uziel and Taylor in 1978 [25], 7-methylguanosine, another nucleoside known to be present in urine [10, 26], and N^2,N^2-dimethylguanosine,

Table 5 Compounds from urine sample separation showing constant neutral loss of 132 u

	Nucleoside	MS	MS(2)	MS(3)
1	?	255	123	80, 96, 106
2	5,6-Dihydrouridine	247	115	73, 98
3	Cytidine	244	112	69, 95
4	**?**	228	96	68, 78
5	*3-3-Amino-3-carboxypropyl-uridine*	346	214	113, 168, 197
6	Uridine	245	113	70, 96
7	***Methylcytidine***	258	126	69, 83, 95, 109
8	1-Methyladenosine	282	150	82, 94, 109, 123, 133
9	***Ribosylpyridinonecarboxamide***	271	139	78, 93, 122
10	Isoguanosine (internal standard)	284	152	135
11	?	300	168	95, 112, 150
12	5-Methyluridine	259	127	82, 110
13	Inosine	269	137	84, 94, 110, 119
14	**7-Methylguanosine**	298	166	96, 107, 124, 142, 149
15	Guanosine	284	152	110, 128, 135
16	***Ribosylpyridinonecarboxamide***	271	139	122
17	*N^1,N^6-Dimethyladenosine*	296	164	96, 108, 123, 135, 149
18	3-Methyluridine	259	127	96, 109
19	Xanthosine	285	153	110, 136
20	*Adenosinisomer?*	268	136	94, 119
21	?	293	161	91, 116, 134, 143
22	*5-Methylaminomethyl-2-selenouridine*	351	219	155, 173, 201
23	Methylinosine	283	151	94, 110, 128
24	*N6-Succinyladenosine*	384	252	136, 148, 162, 192, 206, 234
25	1-Methylguanosine	298	166	109, 128, 136, 149
26	**N^4-Acetylcytidine**	286	154	112
27	?	293	161	91, 106, 116, 134, 143
28	2-Methylguanosine	298	166	110, 149, 135, 128
29	Adenosine	268	136	94, 112, 119, 109
30	*2-Methylthio-N6-(cishydroxyisopentenyl)adenosine*	398	266	194, 165, 182, 248, 231
31	***Trimethylguanosine***	326	194	124, 136, 149, 167, 179
32	N^2,N^2-Dimethylguanosine	312	180	110, 122, 137, 153, 162
33	N6-Methyladenosine	282	150	94, 123, 133
34	*N^6-Threonylcarbamoyladenosine*	413	281	150, 162
35	?	313	181	96, 110, 124, 137, 167
36	5′-Deoxy-5′-methylthioadenosine	298	163	97, 115, 145
37	?	459	327	182, 208

Bold: nucleosides identified by retention times and fragmentation; *italics*: proposed nucleosides; ?: unknown compounds

identified in human urine by Chheda et al in 1969 [27]. N^4-Acetylcytidine and N^2,N^2-dimethyguanosine were found to be elevated in urine samples from patients suffering from colon cancer [28] and in sera from lung cancer patients [29].

The urinary levels of N^4-acetylcytidine in mice were elevated after tumor induction even before the tumor was diagnosable [30].

Other peaks were from a methylcytidine, a structural isomer of adenosine, $N^{1,6}$-dimethyladenosine, N^2,$N^{2,7}$-trimethylguanosine, N^6-threonylcarbamoyladenosine (t6A), 3-(3-amino-3-carboxypropyl)-uridine, 5-methylamino-methyl-2-selenouridine, N^6-succinyladenosine, 2-methylthio-N^6-(cishydroxyisopentenyl)-adenosine, and two isomers of PCNR, the latter not being nucleosides but metabolites of NAD or NMN [31]. These peaks were

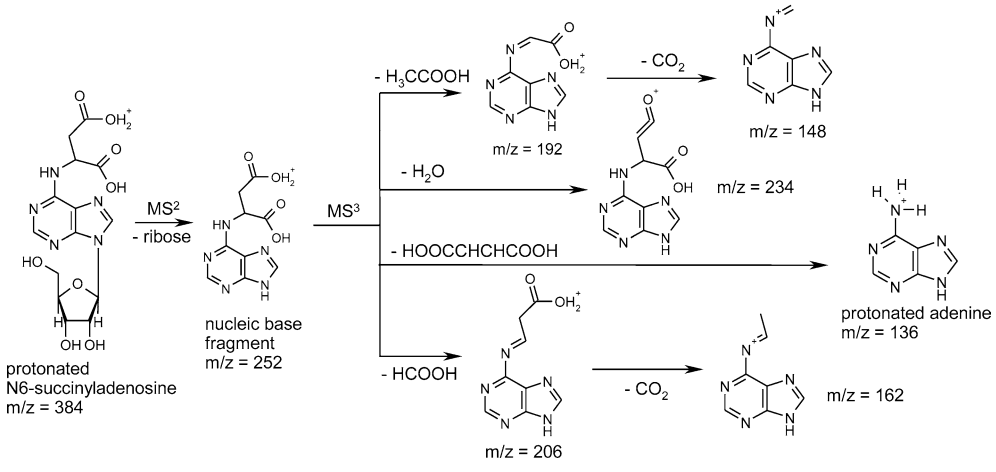

Fig. 5 Proposed fragmentation pathway for N^6-succinyladenosine

identified by considering the mass and fragmentation patterns, as summarized in Table 5. However, these results require further confirmation. All of the above-mentioned nucleosides are known to be present in urine or tRNA [1, 3, 32].

As an example, the proposed fragmentation pathway of N^6-succinyladenosine is shown in Fig. 5. Water may be eliminated from either of the carboxyl groups. Loss of formic acid and acetic acid leads to the fragments m/z 206 and m/z 192. Both of these fragments may lose CO_2, resulting in the fragments m/z 148 and m/z 162. The fragment m/z 136 is probably the protonated nucleic base adenine.

Furthermore, seven peaks occurred in the neutral loss chromatogram that could not be collated to any known nucleosides. These results are also shown in Table 5. For example, the fragmentation pattern of compound 11 (m/z 300) includes the fragments 112 and 95, which could be related to a cytidine-related nucleoside, as these are fragments which are characteristic fragments of both cytidine and N^4-acetylcytidine. The mass difference of 18 u between the base fragment and m/z 150 indicates the loss of water, which correlates with a cytidine-related nucleoside, as this contains an oxygen atom which might be eliminated.

Conclusions

Sixteen nucleosides and six corresponding nucleic bases have been examined by direct MS^n experiments via syringe infusion. The fragmentation of the nucleic bases is analogous to the further fragmentation of the nucleic base fragments of the corresponding nucleosides in MS spectra. The MS^n spectra of 16 standard nucleosides have been interpreted and the probable structures of the fragments have been found.

The observations made in these basic experiments lead us to a better understanding of the fragmentation behavior of nucleosides, which can also be used for other nucleosides with similar structures.

We developed a new sensitive auto-LC–MS^3 method, which enabled us to identify known nucleosides not only by retention time, but also by mass and fragmentation pattern. A urine sample of a breast cancer patient was examined, and based on the results of our fragmentation experiments, 36 compounds could be identified as nucleosides, including several not previously identified in urine.

Using post-processed constant neutral loss chromatograms in connection with the fragmentation, it was possible to search for unknown nucleosides and propose their structures.

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