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Thermospray liquid chromatography-mass spectrometry of nucleosides and of enzymatic hydrolysates of nucleic acids

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ABSTRACT

Nucleosides dissolved in aqueous buffered solutions undergo ionization during direct introduction of the solution into a mass spectrometer using a thermospray interface. The principal ions formed represent the protonated molecule, the corresponding protonated free base, and sugar. In addition to potential utility for characterization of new nucleosides, the technique can be used to monitor nucleosides separated from enzymatic hydrolysates by liquid chromatography. The selectivity of chromatographic detection is significantly greater than with UV absorbance alone so that independent detection of components of unresolved chromatographic peaks is usually possible. Detection limits, with signal/noise >10 for most nucleosides, are approximately 0.1-1 ng per component for selected ion monitoring and 10-50 ng for full-scan mass spectra. Examples are given from the detection of modified nucleosides in enzymatic hydrolysates of 0.05 A_{260} units (2.5 ug) of rabbit liver $tRNA^{Val}$ and of unfractionated *H. volcanii* tRNA.

INTRODUCTION

There exists a great structural diversity of natural nucleosides, including more than 80 different nucleoside species in RNA and DNA (1-4). In transfer RNA more than 60 modified nucleosides are known (3), which are enzymatically synthesized by posttranscriptional processes. Under many circumstances the identification of known nucleosides, and the recognition and characterization of new ones, is a difficult task where subjectivity of assignments increases with the complexity of the mixture. This problem is particularly acute in the case of unfractionated tRNA, due to the potentially large number of components, and the possibility of encountering hypomodified or chemically degraded residues. The successful development of directly-combined high performance liquid chromatography-mass spectrometry (LC/MS) based on the thermospray interface (5-7) provides a method particularly suitable for analysis of polar molecules and is a powerful extension of the capabilities of either technique alone. We presently report on an initial study of the thermospray mass spectra of nucleosides, and of combined LC/MS of enzymatic hydrolysates of tRNA.

MATERIALS AND METHODS

Materials

Nuclease P_1 (Cat. N-8630) and alkaline phosphatase (Cat. P-5521 or P-4252) were purchased from Sigma Chemical Co. Rabbit liver $tRNA^{Val}$ was a gift from Dr. S. Nishimura, National Cancer Center Research Institute, Tokyo; *H. volcanii* tRNA was a gift from Dr. R. Gupta, Southern Illinois University.

Hydrolysis of transfer RNA

Quantities of tRNA ranging from 2-100 ug (0.04-2.0 A_{260} units) were hydrolyzed in 10-50 uL volumes of 0.01M NH_4OAc, pH 5.3, by nuclease P_1, (4 units/100 ug tRNA), for 8 hours at 37°C. Two uL 1N NH_4OH was added per 50 uL of incubation volume, with alkaline phosphatase (0.5 units/100 ug tRNA) for further incubation at 37°C for 12 hours. For smaller tRNA quantities, minimum volumes of 10 uL were maintained. Aliquots of the incubation mixture were submitted directly to LC/MS.

Liquid chromatography-mass spectrometry

Chromatography was carried out using a Beckman 332M liquid chromatograph with a Waters 440 dual wavelength (254 and 280 nm) UV absorbance monitor in series between the chromatograph and mass spectrometer. Separations of simple mixtures or single components may be obtained on a 4.6 x 75 mm 3 micron Ultrasphere ODS column (Beckman Instruments, San Jose, CA), 0.1M NH_4OAc (pH 5.6), 20% CH_3OH at 1.5 mL/min (data in Fig. 2). Relatively more complex mixtures containing components with a narrow range of polarity (e.g., nucleosides earlier eluting than adenosine) were separated on a 4.0 x 300 mm Waters uBondapak C-18 column with a flow of 1.5 mL/min of 0.1M NH_4OAc (pH 5.6) and a linear solvent program from 5 to 15% CH_3OH over 30 minutes (Fig. 5). Mixtures covering a wide polarity range were chromatographed on a 4.6 x 250 mm Supelcosil LC-18 DB with a flow of 2.0 mL/min of 0.25M NH_4OAc (pH 6.0) with a multilinear gradient to 40% CH_3CN in H_2O according to the method of Buck *et al.* (9) (Fig. 3). The entire LC effluent was passed into the mass spectrometer.

The liquid chromatograph-mass spectrometer used in this work has evolved from an earlier prototype system (5), and was constructed from components which include a Finnigan MAT hyperbolic rod quadrupole mass analyzer, an Extranuclear quadrupole power supply, and Varian NRC vacuum pumps. Except for minor details the design is similar to thermospray LC/MS systems described previously (6,7). The mass spectrometer was controlled by a Teknivent Corp. 29k data system.

The heat required to partially vaporize the LC effluent is supplied by passing an electrical current through the final portion of the metal capillary connection from the liquid chromatograph to the mass spectrometer, and is

controlled so as to maintain the temperature near the exit end of the heated portion at a constant, pre-selected value, usually in the range 240-280°C. This results in vapor temperatures within the ion sampling region of the ion source in the range of 275-300°C. With this control system the fraction of the chromatographic effluent which is vaporized can be maintained constant even though the flow rate may change. Normally the best performance is obtained when ca. 95-98% of the liquid is vaporized within the capillary.

A supersonic jet of vapor issues from the vaporizer capillary carrying unvaporized solvent and sample in the form of charged microdroplets or particles into the heated ion source. Additional heat is transferred as these droplets and particles travel through the ion source causing vaporization. If sufficient aqueous buffer is present in the effluent, ionization may be produced by ion evaporation from charged liquid droplets of sample and buffer constituents as described previously (8).

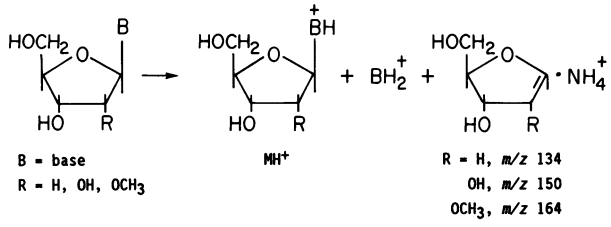
RESULTS AND DISCUSSION

High Performance Liquid Chromatography.

The C-18 reversed phase systems described by Buck *et al.* (9) and Gehrke and co-workers (10) were both found to be satisfactory from the standpoint of separation of modified nucleosides from RNA. For LC/MS the system of Buck *et al.* which employs a linear mobile phase gradient, was found to be more satisfactory because of greater ease in control of the effluent spray temperature compared with a step-gradient (10). In principle, any volatile buffer may be used with the thermospray interface (5), but $CH_3CO_2NH_4$ was found preferable to HCO_2NH_4 because of 3-10 fold greater efficiency in production of nucleoside ions.

Thermospray mass spectra of nucleosides.

Thermospray mass spectra exhibit three principal ion species: MH^+ and BH_2^+, equivalent in mass to the protonated nucleoside and the corresponding protonated free base (5,6,11), and in variable abundance a sugar-derived ion complexed with NH_4^+ from the buffer, as schematically represented below. An



B = base
R = H, OH, OCH_3

R = H, *m/z* 134
OH, *m/z* 150
OCH_3, *m/z* 164

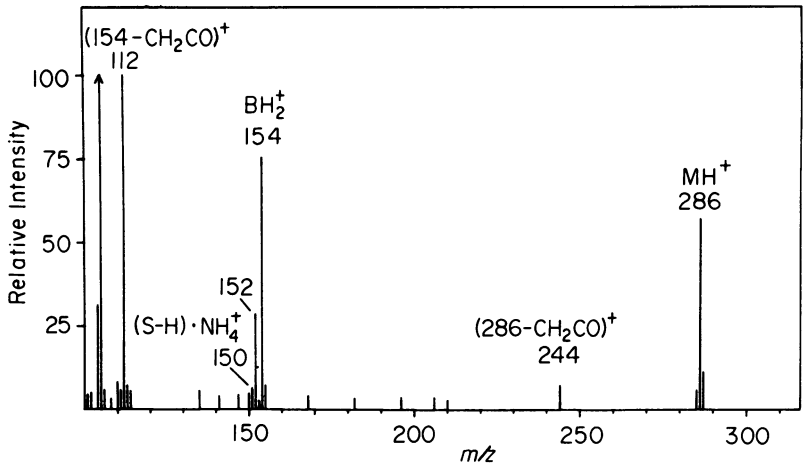


Figure 1 Thermospray mass spectrum of N^4-acetylcytidine directly acquired from an HPLC eluate of nucleosides from 0.6 A_{260} unit of unfractionated *H. volcanii* tRNA (see also Fig. 3).

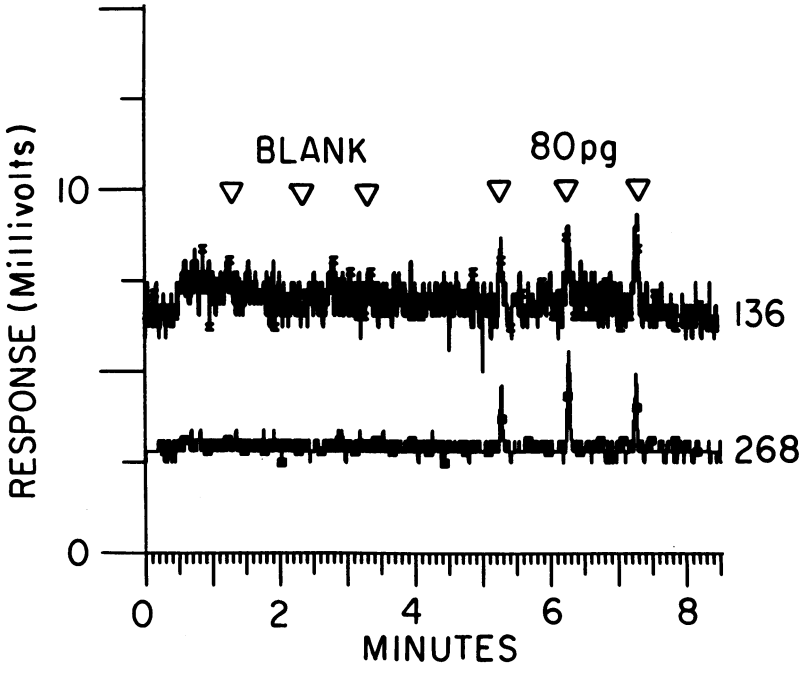


Figure 2 Mass spectrometer response in selected ion monitoring mode to triplicate injections of blanks and 80 picograms of adenosine, at one-minute intervals. Mass 136, BH_2^+ ion; mass 268, MH^+ ion.

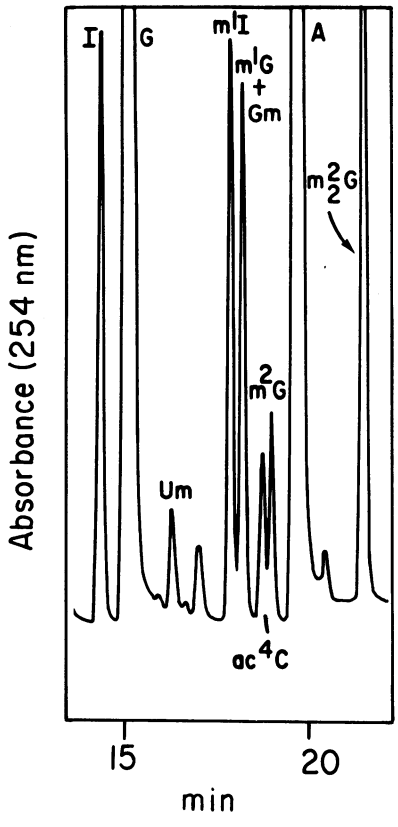


Figure 3 Portion of reversed phase chromatographic separation of nucleosides from 0.6 A_{260} units of unfractionated *H. volcanii* tRNA, with UV detection at 254 nm.

example is given in Fig. 1, which shows the mass spectrum of N^4-acetylcytidine, recorded from LC/MS of a digest of unfractionated *H. volcanii* tRNA, in which it occurs at position 34 in 5 of the 41 reported sequences (3,12). Side chain substituents in the base were found in some cases to give rise to fragment ions, such as loss of ketene from the N-acetyl moiety in both the MH^+ and BH_2^+ ions from N^4-acetylcytidine (Fig. 1). Similarly, the most abundant fragment ions in the spectra of Q nucleoside and t^6A (data not shown) are due to the protonated dihydroxycyclopenteneamino ion (*m/z* 116) and protonated threonine (*m/z* 120), respectively. Most thermospray mass spectra of ribonucleosides exhibit a peak at *m/z* 150 which is assigned as (sugar-H)·NH_4^+. This ion occurs at *m/z* 164 in spectra of 2'-O-methylribonucleosides and is hence useful in distinguishing base *vs.* sugar methylation.

Several variables were found to influence the abundances of ions produced. Derivatives of cytidine were found to give 2-5 fold greater sensitivity for both MH^+ and BH_2^+ ions than from other nucleosides. 7-Methylguanosine (m^7G)

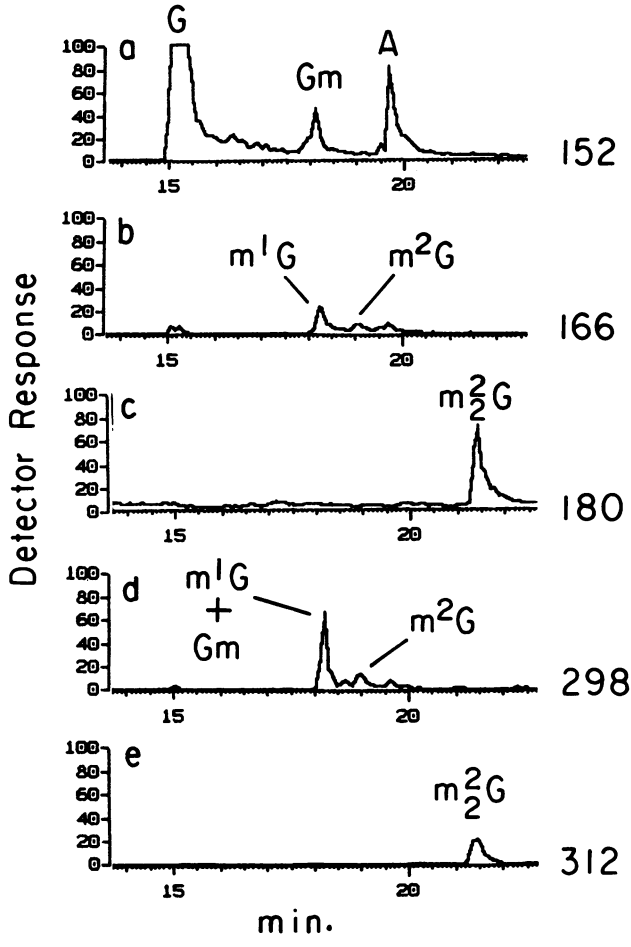


Figure 4 Selected ion recording from data acquired during the separation shown in Fig. 3. Mass values monitored are shown to the right of each panel. (a), (b), (c), BH_2^+ ions; (d), (e), MH^+ ions.

produced a very low MH^+ ion but an abundant BH_2^+ (*m/z* 166), at the appropriate HPLC elution position for m^7G. Guanosine derivatives were generally found to exhibit broader thermospray chromatographic profiles compared with peak profiles (from UV detection) of the material entering the mass spectrometer. This was judged to be due to thermal formation of the free base in the thermospray stream.

Stability of operation and sensitivity of detection were found to depend strongly upon maintenance of constant temperature in the spray, with the opti-

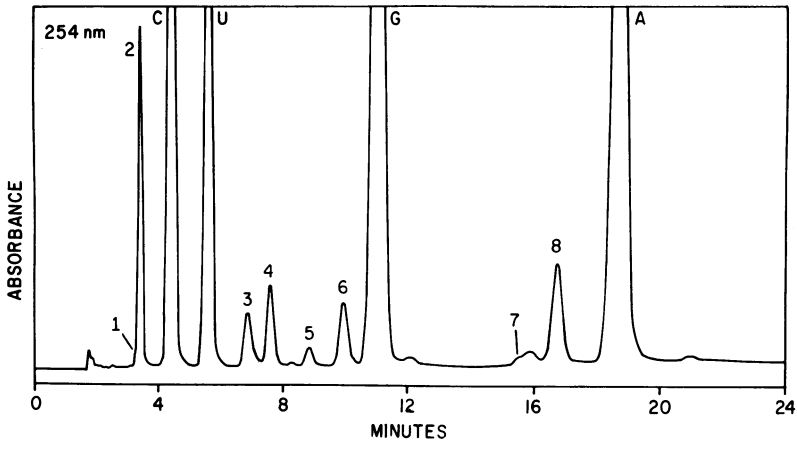


Figure 5 Reversed phase chromatographic separation of nucleosides from 0.05 A_{260} units (2.5 ug) of rabbit liver $tRNA^{Val}$, with UV detection at 254 nm.

mum temperature for more polar nucleosides such as guanosine (275°C) being 10-30° lower than for less polar nucleosides (e.g., adenosine 290°C). No effort has been made in these initial studies to quantitatively relate ion abundances to nucleoside concentrations, though response for adenosine was found to be linear over more than two orders of magnitude. Under favorable conditions for most nucleosides, detection limits in the range 0.1-10 ng using selected ion monitoring, or 10-50 ng for scanned spectra were obtained. As an example, the response from replicate injections of 80 pg of adenosine is shown in Fig. 2. In practice, practical sensitivity limits pertaining to analysis of complex mixtures, such as hydrolysates of unfractionated tRNA, were more often found to be established more from chemical interferences than by instrumental limits.

Thermospray LC/MS is potentially useful for the examination of enzymatic digests of RNA or DNA, for the identification of known modified nucleosides, or as an important initial step in the characterization of new structurally unknown constituents. Mass spectrometric data can be effectively used in conjunction with chromatographic retention (k') values and UV multiwavelength absorbance ratios (from a UV detector placed in series between the chromatograph and mass spectrometer), acquired in a single experiment.

Substantial selectivity is offered by the use of masses of the nucleoside and corresponding base as a detection parameters for HPLC. As a consequence, components in general need not be chromatographically resolved for detection, a characteristic of particular value for the examination of hydrolysates of

Table 1. Chromatographic and Mass Spectrometric Data from Modified Nucleosides Detected from Rabbit Liver $tRNA^{Val}$

Peak Fig. 5	k'	A_{254}/A_{280}	Assignment	*m/z* (rel. int.) MH^+	*m/z* (rel. int.) BH_2^+
1	0.77	--	dihydrouridine	247(100)	115(-)
2	0.88	2.5	pseudouridine	245(100)	113(-)
3	2.89	6.3	1-methyladenosine	282(6)	150(100)
4	3.26	0.66	5-methylcytidine	258(2)	126(100)
5	4.03	1.0	2'-methylcytidine	258(6)	112(100)
6	4.60	2.4	7-methylguanosine	298(-)	166(81)
			inosine	269(-)	137(100)
7	7.86	2.5	1-methylguanosine	298(12)	166(100)
8	8.49	1.8	N^2-methylguanosine	298(1)	166(100)

unfractionated tRNA. An example is given by a section of the chromatogram resulting from a digest of unfractionated *H. volcanii* tRNA, with UV detection (Fig. 3) and mass spectrometric detection (Fig. 4) carried out in the same experiment. The isomers m^1G and Gm co-elute at approximately 18 minutes (Fig. 3) but can be independently resolved (Fig. 4) because the base ions BH_2^+ (from 1-methylguanine and guanine, respectively) differ in mass by 14 daltons. Hence, in the reconstructed ion current profiles shown in Fig. 4, the protonated guanine moiety (*m/z* 152) represents the Gm component (Fig. 4a) and protonated 1-methylguanine (*m/z* 166) represents the m^1G component (Fig. 4b), with the apexes of the two profiles (Fig. 4a vs 4b) differing by about 12 seconds. The *m/z* 180 channel responds to the base-dimethylated component m_2^2G (Fig. 4c) but not to the other constituents seen in Fig. 3. From the molecular ion (MH^+) channels, *m/z* 298 shows response to monomethylated guanosines (Fig. 4d) and *m/z* 312 to dimethylated guanosines (Fig. 4e). In some cases responses from minor ions of very abundant components such as the major unmodified nucleosides will appear at the appropriate (and predictable) elution time. The effect is seen for adenosine in the *m/z* 152 channel (Fig. 4a) at 19.5 minutes.

In the case of isoaccepting tRNAs or other oligonucleotides of defined composition, the resulting chromatograms are somewhat simpler and direct correlation can readily be made between mass spectrometer response and UV detection for each component. Fig. 5 shows the HPLC separation of an enzymatic digest of 0.20 A_{260} units (10 ug) of rabbit liver $tRNA^{Val}$, which contains eight modified nucleosides (13). Mass spectrometric data recorded from full scans in the same experiment (same HPLC injection) are shown in Table 1, along with additional

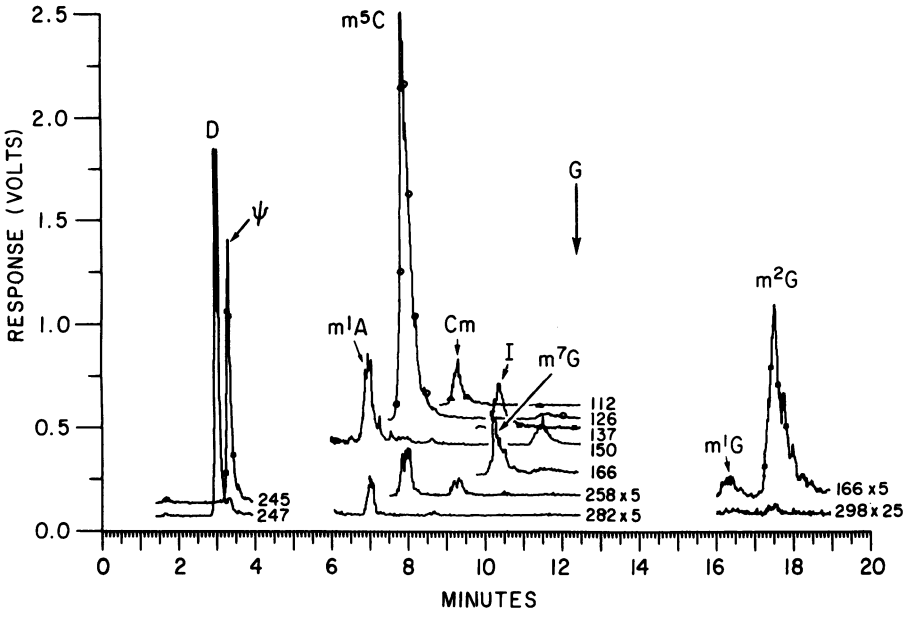


Figure 6 Selected ion recording corresponding to the chromatogram shown in Fig. 5. BH_2^+ and MH^+ ions monitored: D (247), Ψ (245), m^1A (150, 282), m^5C (126, 258), Cm (112, 258), m^7G (166), I (137), m^1G (166), m^2G (166, 298).

chromatographic data. The identities of nucleosides based on mass spectra, chromatographic retention values and UV absorbance ratios, match those expected from sequence data of Jank *et al.* (13). A complimentary approach is one in which the presence of anticipated nucleosides, or of certain structural features, can be tested by selected ion monitoring, in which the mass spectrometer is programmed to detect pre-determined mass values which are characteristic of the components in questions. Using this method, a composite chromatogram derived from a single LC/MS run of 0.05 A_{260} units of the same sample used for Fig. 5 is shown in Fig. 6. For simplicity of presentation only the relevant regions of each selected ion profile are shown; ions from the four major nucleosides are omitted but the elution position of guanosine is shown as a reference point. A minor peak at 16.3 minutes on the *m/z* 166 channel corresponds to 1-methylguanosine (BH_2^+ ion), which is not present in the established sequence and is judged to result from a minor tRNA impurity in the sample. A clear response from dihydrouridine is obtained by mass spectrometric detection (3.0 minutes, Fig. 6), even though the molar absorbtivity is very low for useful UV detection (peak 1, Fig. 5).

In summary, directly combined LC/MS is concluded to be a potentially useful technique, complementary to chromatographic methods, for studies of posttranscriptional modification patterns in tRNA, for verification of nucleoside composition established from sequence data, and for the detection of new or unexpected nucleosides in unfractionated tRNA.

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