aids in the removal of small amounts of DNA contamination.

The resulting purified RNA is once again quantified by measuring the absorbance at 260 nm (extinction coefficient of 1 $OD_{260}/40\ \mu g\ mL^{-1}$) and the quality assessed by the OD_{260}/OD_{280} ratio (pure RNA has a value ≥ 2.0). Following denaturing agarose gel electrophoresis, a typical cellular RNA preparation will present two prominent species representing the structural ribosomal RNAs (16S and 23S for prokaryotic cells; 18S and 28S for eukaryotic cells).

Isolation of Poly-A$^+$ Containing RNA

For many purposes, an investigator will be interested only in the messenger RNA fraction, and because the structural ribosomal RNAs represent 98% or greater of the total, measures must be taken to purify the mRNA. This is accomplished by taking advantage of the fact that most (but not all) eukaryotic mRNA molecules are distinguished by the presence of a homopolymeric adenylate sequence at the extreme 3′ end of the macromolecule (the poly A^+ tail). This stretch of 100–200 residues acts to stabilize the mRNA; however, it also serves as a convenient mechanism for purifying this particular nucleic acid species. Most of the applications are predicated on an affinity column chromatography with oligo-deoxythymidine residues of between 12 and 18 nucleotides in length (oligo-dT_{12-18}). The poly A^+ tail binds via complementary hydrogen binding to the oligo-dT and the structural RNAs fail to hybridize and bind. Conditions are changed so as to disrupt the nucleic acid interactions and the mRNA is released (desorbed) from the affinity matrix. Note that almost all mRNA species contain a poly A^+ tail and so this separation approach does not differentiate between specific mRNA species.

Summary/Future Directions

Nucleic acid extraction from biological samples was one of the enabling technologies in the development of molecular biology. It has remained largely unchanged for the past 20 years and, in its present state, continues to be a mainstay of the field. Most of the common advances have been in the automation of the process and the creation of high throughput technical platforms. The challenge for the coming years will be the further refinement of these automated applications and the creation of solid-state systems. These approaches will involve the liberation of nucleic acids from the biological samples, capture of the specific chemical form (DNA or RNA) on a solid matrix, and the subsequent analysis of the nucleic acid in that physical environment without further manipulation. Regardless of these potential technical advances, however, the essential principles will remain unchanged and the separation of nucleic acids from complex mixtures of macromolecules will be a requisite step in the characterization of genomic systems.

See also: **II/Membrane Separations:** Donnan Dialysis. **III/Nucleic Acids:** Centrifugation.

Further Reading

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidmann JG, Smith JA and Struhl K (1987) *Current Protocols in Molecular Biology*. Chichester: John Wiley and Sons.

Chirgwin JJ, Przbyla AE, MacDonald RJ and Rutter WJ (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 18: 5294–5299.

Chomczynski P and Mackey K (1995) Substitution of chloroform by bromochloropropane in the single-step method of RNA isolation. *Analytical Biochemistry* 225: 163–164.

Chomczynski P and Sacchi N (1987) Single-step method of RNA isolation by acid guanidine thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry* 162: 156–159.

Kirby KS (1957) A new method for the isolation of deoxyribonucleic acids: Evidence on the nature of bonds between deoxyribonucleic acid and protein. *Biochemistry Journal* 66: 495–504.

Sambrook J, Fritsch EF and Manitis T (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Liquid Chromatography

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Introduction

Investigations in nucleic acid biochemistry are directed toward a better understanding of how the chemical structure of nucleic acids correlated with their unique biological functions. This information

can then be used to gain a deeper insight into how cells normally regulate their metabolic activities, allows speculation on how they evolved their respective biological role(s), and potentially permits correlation of the altered structures of nucleic acids in abnormal or diseased states to biological function. An understanding of how cells behave normally and in the diseased state provides the basis for the development of rational therapeutics and improved diagnostic tools. Studies are now being undertaken in many laboratories on nucleic acid metabolites as cancer markers, and of chemical carcinogens and mutagens adducted to nucleic acids for assessment of human exposure to environmental insults. Methodological limitations have hampered the advancement and exploitation of using modified nucleosides and their signals in routine tests in clinical chemistry or as important determinant life molecules in biochemical research. The development of high resolution chromatographic methods for qualitative identification and quantitative measurement of an array of nucleosides and to obtain chemical *information on* nucleic acid components has been a challenge to analytical biochemists since the beginning of the 1960s and has evolved into a powerful tool since the mid-1980s.

Our laboratory has made extensive investigations on reversed-phase high performance liquid chromatography and ultraviolet-photodiode array detection (RP-HPLC-UV) nucleoside analysis and has developed comprehensive chromatographic methods and quantitative enzymatic RNA hydrolysis procedures. Sixty-seven known nucleosides can be identified and 31 ribonucleosides; six deoxynucleosides can be quantified directly in a single chromatographic run from an enzymatic hydrolysate of RNAs, DNAs, and in physiological fluids. In collaborative efforts with scientists across the world we have applied these methods in a number of interesting investigations. We briefly introduce RP-HPLC-UV for deoxynucleosides and ribonucleosides and place emphasis on applications in three areas:

1. RP-HPLC-UV methods for total nucleoside composition of RNAs and DNAs;
2. modified nucleosides as cancer markers and in normal metabolism; and
3. preparative isolation of unknown nucleosides in nucleic acids for structural characterization.

RP-HPLC of Nucleosides

Chromatography Information on HPLC instrumentation; chromatographic parameters for high resolution, high speed, and high sensitive separation of nucleosides; analytical and semipreparative enzymatic hydrolysis of nucleic acids; and clean-up procedures (ultrafiltration procedure and phenylboronate gel column clean-up) for ribonucleosides in physiological fluids have been presented in the literature (see 'Further Reading' section).

It is a challenge to the analytical biochemist to separate and measure such a large number of nucleosides simultaneously in a complex biological matrix. One of the major problems for nucleoside chromatography is to obtain the needed reference molecules so that the information for the essential qualitative and quantitative analytical references can be established. There are only about 20 modified ribonucleosides that can be obtained through commercial sources. We have standardized the chromatographic retention times, obtained RP-HPLC-UV spectra and determined the molar response factors for a large number of ribonucleosides. Scientists in their respective laboratories need to standardize and calibrate their analytical system for modified nucleoside analysis in a broad range of biological matrices. To overcome this limitation, we have selected three unfractionated transfer RNAs (tRNAs) – *Escherichia coli*, brewer's yeast, and calf liver – as reference sources of the nucleosides. Each of these tRNAs contain unique as well as common nucleosides and provide an array of modified nucleosides that are often encountered by researchers. Some minor differences in the modified nucleoside profile may be observed in these three tRNAs from different sources, especially for *E. coli* tRNAs. This problem can be resolved by using a reliable supplier or by standardization of a selected lot of tRNAs obtained in large quantity and of good homogeneity. **Figure 1** shows the 254 nm chromatograms from the high resolution separation of the nucleosides in the three reference tRNAs. The nucleoside peaks are identified by an assigned index number, which essentially corresponds to their respective elution order. **Table 1** gives the IUPAC names, one-letter symbol, and the index number of the nucleosides that have been determined by RP-HPLC-UV. Other ribonucleosides, which are not yet characterized by RP-HPLC-UV, are also included in this table. A total of 67 ribonucleosides have been chromatographically and spectrometrically characterized.

RP-HPLC-UV Methods for Total Nucleosides Composition of RNAs and DNAs

Nucleoside chromatography protocols for a broad array of RNAs and DNAs have been applied extensively. In general, RNA nucleoside chromatography

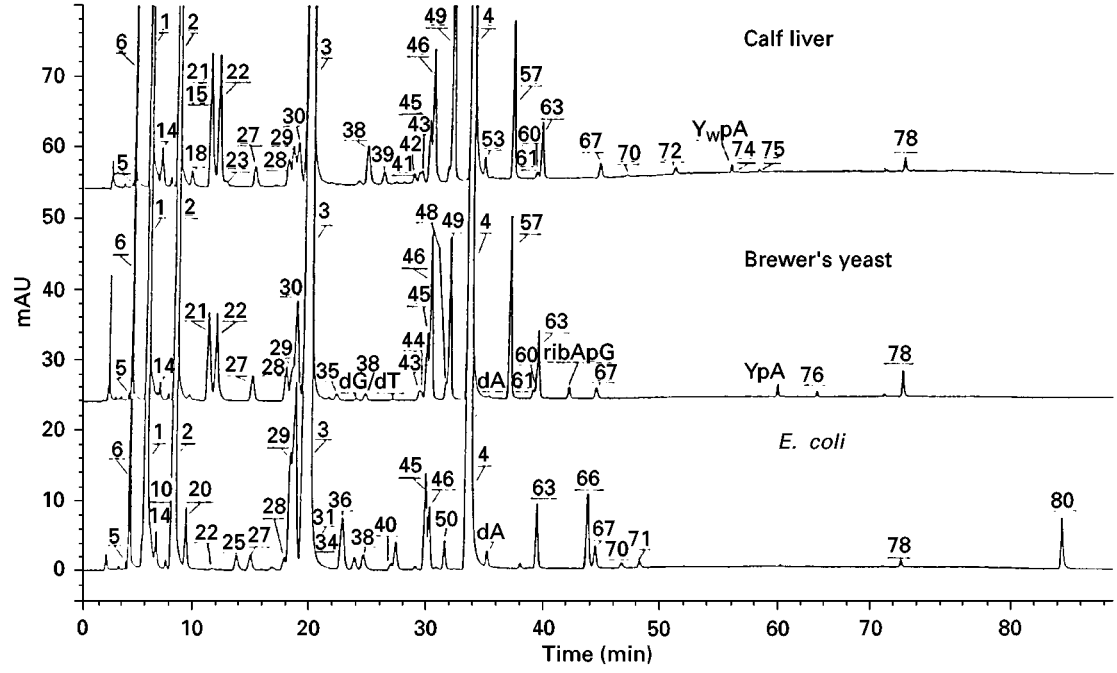

Figure 1 HPLC chromatography of reference nucleosides from unfractionated calf liver, brewer's yeast, and *E. coli* tRNAs.

requires emphasis on resolution and flexibility, and for DNA the emphasis is on accuracy and speed. There is also an ever-increasing need in biochemical analysis for high sensitivity. Recent progress in instrumentation and column technology has increased LC-UV sensitivity more than 10-fold so that low picograms of nucleosides can be quantitated routinely.

Unfractionated tRNA constitutes one of the most complicated mixtures of biopolymers known and high resolution is required for this analysis (Figure 1).

Isoacceptor tRNAs are usually available only in very small amounts (less than a few micrograms); however, an advantage with single-species tRNAs is that they are less complicated in composition. For analysis of single-species tRNAs, an intermediate resolution and higher sensitivity protocol (high speed) are generally used. An accurate identification and quantitation of the total nucleoside composition are very important in providing supplementary and confirmatory information in support of tRNA sequence studies. **Figure 2** shows the separation of nucleosides in $tRNA^{LEU}$ from bovine serum. **Table 2** gives the comparison of quantitative results from five isoacceptor tRNAs obtained by RP-HPLC-UV and compared to sequence analysis. The lower m^7G value is indicative of the instability of m^7G at alkaline pH during enzymatic hydrolysis. It is of interest that differences of one residue number for uridine in $tRNA^{Phe}$ and uridine and guanosine values in $tRNA^{Val}$ were observed from the two analytical methods. **Table 3** gives the mol% values of all the nucleosides in four bovine isoaccepting tRNAs. These four tRNAs were isolated in Dr Gerard Keith's group at the Institute for Molecular and Cellular Biology (IBMC) in Strasbourg, France. Their sequences were not yet determined at the time of HPLC analysis. These mammalian tRNAs have considerably more modifications than the tRNAs from *E. coli* (Table 2) and two unknown modified nucleosides were observed in $tRNA^{Leu}$.

Ribosomal RNA Nucleoside Analysis

Ribosomal RNA (rRNA) is a high molecular weight RNA. In *E. coli* the 70S rRNA has a molecular weight of 2.75×10^6 amu and the small subunits, 16S rRNA and 23S rRNA, have 1542 and 4718 residues, respectively. Only 10 methylated nucleosides have been reported in the 16S and 23S rRNAs. To accomplish the chromatography of rRNA for composition analysis it is necessary to separate and measure one modified nucleoside residue in *ca.* 5000 nucleotides. This demands a high column capacity so that a large amount of sample (100 μg or more) can be injected without loss of resolution. The high resolution and high speed chromatographic protocols described for tRNA nucleoside composition analysis all have adequate capacity to meet this requirement for rRNA

Table 1 Nomenclature of ribonucleosides and index numbers

IUPAC name	*One-letter symbol*	*Index no.*
Adenosines		
Adenosine	A	4
2′-*O*-Methyladenosine	Am	61
1-Methyladenosine	m^1A	21
1-Methyl-2′-*O*-methyladenosine	m^1Am	
2-Methyladenosine	m^2A	66
2-Thioadenosine	s^2A	
2-Methylthioadenosine	ms^2A	
3-Methyladenosine	m^3A	
1,3-Dimethyladenosine	m^1m^3A	
5′-Methylthioadenosine	$ms^{5'}A$	
1,N^6-Dimethyladenosine	m^1m^6A	
N^6-(*N*-Formyl-α-aminoacyl)adenosine	f^6A	
N^6-Methyladenosine	m^6A	67
N^6-Methyl-2-methylthioadenosine	ms^2m^6A	
N^6,N^6-Dimethyladenosine	m^6_2A	74
N^6-Methyl-2′-*O*-methyladenosine	m^6Am	71
2-Hydroxyadenosine	O^2A (isoG)	
N^6-Carbamoyladenosine	nc^6A	
N^6-Threoninocarbonyladenosine	tc^6A (t^6A)	63
N^6-Methyl-N^6-threoninocarbonyladenosine	mtc^6A (mt^6A)	70
N^6-Threoninocarbonyl-2-methylthioadenosine	ms^2tc^6A	72
N^6-Glycinocarbonyladenosine	gc^6A (g^6A)	50
N^6-Methyl-N^6-glycinocarbonyladenosine	mgc^6A (mg^6A)	
N^6-(Δ2-Isopentenyl)adenosine	i^6A	78
N^6-(Δ2-Isopentenyl)-2-methylthioadenosine	ms^2i^6A	80
N^6-(*cis*-4-Hydroxyisopentenyl)adenosine	cis oi^6A	
N^6-(4-Hydroxyisopentenyl)-2-methylthioadenosine	ms^2oi^6A	79
9-(2′-*O*-Ribosyl-β-D-ribofuranosyl)adenine	rA	
Inosines		
Inosine	I	29
1-Methylinosine	m^1I	43
2-Methylinosine	m^2I	
7-Methylinosine	m^7I	16
9-β-D-Ribofuranosylpurine (nebularine)	neb	
7-β-D-Ribofuranosylhypoxanthine		
Cytidines		
Cytidine	C	1
2′-*O*-Methylcytidine	C_m	27
2-Lysinocytidine (lysidine)	k^2C	
2-Thiocytidine	s^2C	20
3-Methylcytidine	m^3C	18
N^4-Methylcytidine	m^4C	22
N^4-Methyl-2′-*O*-methylcytidine	m^4Cm	
N^4-Hydroxymethylcytidine	om^4C	
N^4-Methyl-2-thio-2′-*O*-methylcytidine	m^4s^2Cm	
N^4-Acetylcytidine	ac^4C	48
5-Methylcytidine	m^5C	23
5-Methyl-2′-*O*-methylcytidine	m^5C_m	
5-Hydroxymethylcytidine	om^5C	12
Guanosines		
Guanosine	G	3
2′-*O*-Methylguanosine	G_m	45
1-Methylguanosine	m^1G	46
N^2-Methylguanosine	m^2G	49
3-Methylguanosine	m^3G	
7-Methylguanosine	m^7G	28
N^2,N^2-Dimethylguanosine	m^2_2G	57
N^2,N^2-Dimethyl-2′-*O*-methylguanosine	m^2_2Gm	

Table 1 *Continued*

IUPAC name	*One-letter symbol*	*Index no.*
N^2,N^2-7′-*O*-Methyltrimethylguanosine	$m_2^2m^7G$	
Queuosine	Q	40
β-D-Mannosylqueuosine	manQ	41
β-D-Galactosylqueuosine	galQ	42
Xanthosines		
Xanthosine	X	32
1-Methylxanthosine	m^1X	
7-Methylxanthosine	m^7X	
Uridines		
Uridine	U	2
2-Thiouridine	s^2U	33
2-Thio-2′-*O*-methyluridine	s^2Um	
2-Selenouridine	Se^2U	
3-(3-Amino-3-carboxypropyl)uridine	acp^3U (nbt^3U)	32
3-Methyluridine	m^3U	37
4-Thiouridine	s^4U	36
2,4-Dithiouridine	s^2s^4U	
4-Thiouridine disulfide	$(s^4U)^2$	
5-(β-D-Ribofuranosyl)uracil (pseudouridine)	Ψ	6
5-(2′-*O*-Methyl-β-D-ribofuranosyl)uracil (2′O-methylpseudouridine)	Ψ_m	39
5-(β-D-Ribofuranosyl)-N^1-methyluracil (1-methylpseuridine)	$m^1\Psi$	17
5-(2′-*O*-Methyl-β-D-ribofuranosyl)-N^1-methyluracil (1-methyl-2′-*O*-methylpseudouridine)	$m^1\Psi_m$	
5,6-Dihydrouridine	hU (D)	5
5-Methyl-5, 6-dihydrouridine	m^5hU (m^5D)	
5-methyluridine	m^5U (T)	30
5-Methyl-2′-*O*-methyluridine	m^5U_m (T_m)	53
5-Methyl-2-thiouridine	m^5s^2U (s^2T)	52
5-Hydroxyuridine	h^5U	11
5-Carboxyhydroxymethyluridine	chm^5U	
5-Carboxymethyluridine	cm^5U	7
5-Carboxymethyl-2-thiouridine	cm^5s^2U	
5-Methoxyuridine	mo^5U	34
5-Methoxy-2-thiouridine	mo^5s^2U	55
5-Aminomethyluridine	nm^5U	
5-Aminomethyl-2-thiouridine	nm^5s^2U	
5-Methylaminomethyluridine	mnm^5U	9
5-Methylaminomethyl-2′-*O*-methyluridine	mnm^5Um	
5-Methylaminomethyl-2-thiouridine	mnm^5s^2U	25
5-Methylaminomethyl-2-selenouridine	mnm^5Se^2U	
5-Carboxymethylaminomethyluridine	$cmnm^5U$	8
5-Carboxymethylaminomethyl-2′-*O*-methyuridine	$cmnm^5Um$	
5-Carboxymethylaminomethyl-2-thiouridine	$cmnm^5s^2U$	24
5-Carbamoylmethyluridine	ncm^5U	14
5-Carbamoylmethyl-2′-*O*-methyluridine	ncm^5Um	
5-Carbamoylmethyl-2-thiouridine	ncm^5s^2U	
5-Methoxycarbonylmethyluridine	mcm^5U	44
5-Methoxycarbonylmethyl-2-thiouridine	mcm^5s^2U	60
5-Methylcarboxymethoxyuridine	$mcmo^5U$	54
5-Methylcarboxymethoxy-2-thiouridine	$mcmo^5s^2U$	68
6-Carboxyuridine (oridine)	c^6U (O)	
Hydroxywybutosine	YOH, OyW	75
Wybutosine	Yt, yW	76
Wyosine	Y, W	77

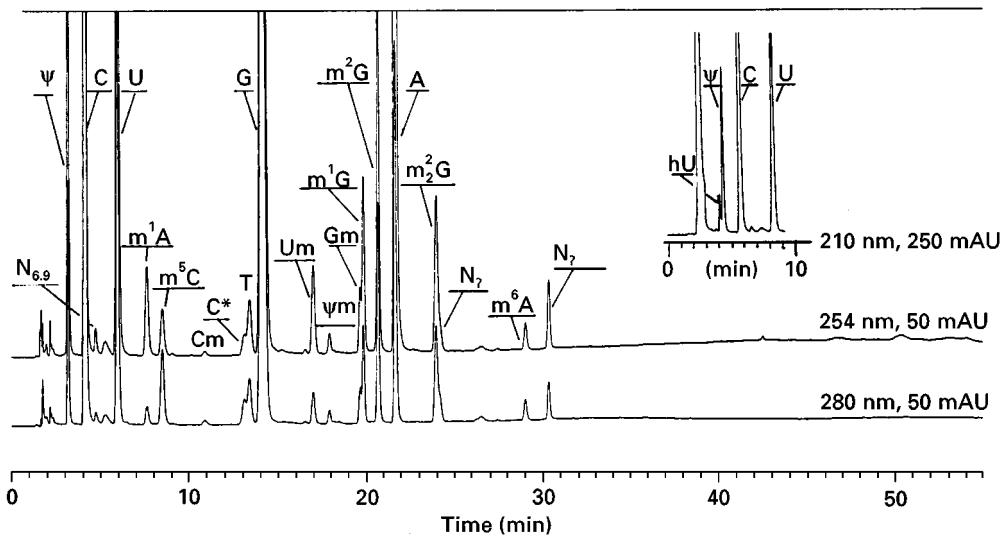

Figure 2 HPLC of nucleosides in $tRNA^{Leu}$ from bovine serum.

analysis. Chromatograms of these respective hydrolysates are presented in **Figure 3** and **Figure 4** and the quantitative results are presented in **Table 4**. Some deoxyribonucleosides were found in the enzymatic hydrolysates of the tRNA samples. However, their presence does not interfere with the measurement of any known modified ribonucleoside. This separation demonstrates the high selectivity of the

Table 2 RP-HPLC-UV for total composition of isoaccepting tRNAs

	Residue per 76 residues									
	Yeast $tRNA^{Phe}$		*E. coli $tRNA^{Phe}$*		*E. coli $tRNA^{Glu}$*		*E. coli $tRNA^{Met}$*		*E. coli $tRNA^{Val}$*	
	HPLC	*Lit.*	*HPLC*	*Lit.*	*HPLC*	*Lit.*	*HPLC*	*Lit.*	*HPLC*	*Lit.*
Major nucleoside										
C	15.8	15	20.6	21	27.1	27	25.1	25	23.2	23
U	12.1	12	8.8	8	9.0	9	8.3	8	10.1	9
G	18.3	18	22.8	23	21.9	22	23.6	24	22.1	23
A	16.0	17	14.0	14	12.9	13	13.6	14	13.8	14
Modified nucleoside										
hU	2.16	2	2.39	2			1.08	1	1.05	1
Ψ	2.05	2	2.80	3	2.02	2	1.00	1	1.18	1
m^1A	0.91	1								
mnm^5s^2U					1.24	1				
m^5C	1.98	2								
Cm	0.80	1					0.89	1		
m^7G	0.76	1	0.69	1			0.71	1	0.58	1
m^5U	1.01	1	1.01	1	1.00	1	1.00	1	1.00	1
s^4U			0.92	1			0.75	1	0.74	1
Gm	1.00	1								
m^2G	0.99	1								
m^2A					1[a]	1				
m^6A									1[a]	1
ms^2i^6A			1.01	1						
o^5U									1[b]	1

[a] Not quantitated, assumed to be one residue.
[b] Not identified, assumed to be one residue if present.

Table 3 RPLC-UV analysis of nucleoside composition in bovine isoaccepting tRNAs

Nucleosides	*Mol% of nucleosides*			
	Pro-tRNA	*Lys-tRNA*	*Thr-tRNA*	*Leu-tRNA*
Major nucleosides				
C	24.1	25.0	23.4	23.6
U	12.8	10.9	14.1	14.8
G	27.6	27.8	24.7	22.9
A	14.9	15.9	16.1	17.6
Modified nucleosides				
hU	3.08	3.97	4.21	nc
Ψ	5.92	6.35	2.46	4.98
mcm^5U	nd	nd	nd	2.84
m^3C	nd	1.36	1.05	nd
m^1A	1.07	0.94	0.94	1.12
m^5C	3.48	0.32	3.31	1.19
Cm	0.35	0.05	0.90	nd
m^7G	0.39	0.05	0.01	nd
I	0.73	0.01	0.95	1.30
$m^5U(T)$	nd	1.21	0.04	1.20
Um	0.99	0.11	0.04	1.19
Gm	0.38	0.01	1.10	0.43
m^1G	2.65	1.30	1.17	1.12
ac^4C	nd	nd	nd	0.82
m^2G	1.13	1.97	2.33	2.35
m^2_2G	0.08	0.15	1.11	1.19
t^6A	nd	2.00	2.17	nd
m^6A	0.42	0.42	0.44	0.10
Unknown 1				nc
Unknown 2				nc
Total	100.5	99.72	100.5	99.33

nd, not detected.
nc, not calculated. ncm^5U was calculated using factor for Urd. Unknown 1 is probably an unknown nucleoside. Could be a modified Ado. Unknown 2 is probably an unknown nucleoside. Could be a modified Cyd.

RP-HPLC so that the respective deoxy- and ribonucleoside are differentiated. RP-HPLC showed qualitative and quantitative differences of modification in both 16S rRNA and 23S rRNA as compared to the literature values. In 16S rRNA we found one additional residue of m^5C, and m^2G. Two nucleosides, Gm and m^4Cm, were not found. From 23S rRNA, four additional residues of Ψ, two of m^4C, one of m^5C, two of m^2G, and one of m^2A, were found by RPLC. A number of other modifications as shown in **Table 4** are in good agreement with the literature values.

Messenger RNA Nucleoside Analysis

Messenger RNAs from viral and eukaryotic cells contain a unique structure known as 'caps' that consist of an inverted 7-methylguanosine (m^7 Guo) linked to the penultimate nucleoside through a 5′–5′ triphosphate bridge. These mRNAs usually have a very low amount of internal nucleoside modification ($< 1/1000$). A highly selective RP-HPLC-UV separation using a micro anion exchange column was developed for isolation of the cap structures to enhance the resolution and sensitivity of the separation and measurement.

Deoxynucleoside in DNAs

The determination of the molar composition of the major and modified deoxynucleosides in high molecular weight DNAs requires a high degree of accuracy and sensitivity. Modified nucleosides in the DNA such as 5-methyldeoxycytidine (m^5dc) 6-methyldeoxyadenosine (m^6dA) and 4-methyldeoxycytidine (m^4dC) are normally present at 0.1 to 2 mol% level. Several separation systems were developed and used in our laboratory. The method that we use is dependent on the sample matrices (i.e. presence of RNA, deoxyinosine (dl), inosine (I), nucleobases, and other UV peaks) and amount of DNA sample available. An optimum amount of DNA is 10 μg. The best chromatographic system for the separation of deoxynucleosides is a two-buffer, single ramp gradient using a 150×4.6 mm Supelcosil® LC-18S column. With this column a complete separation can be achieved in less than 15 min. Dual wavelength quantitation and high quality data reduction software are essential for the analysis. The deoxynucleoside reference compounds obtained from commercial sources do not have the required purity to obtain the accuracy pair ratio, i.e. $(dC + m^5dC)/dG = 1.000$ and $dT/(dA + m^6dA) = 1.000$, from high molecular weight DNAs. Quantitation of the nucleoside composition of a large number of isolated DNA oligomer fragments and synthesized oligomers requires a high sensitivity. In this case, a 5 cm or 3 cm regular bore (3.9–4.6 mm) with 3 or 5 μm particle size columns used in an isocratic separation mode provides the separation in less than 10 min with a five-fold increase in sensitivity. Refer to the published HPLC protocols for quantitation of major and modified nucleosides in DNA. Precision and linearity of the method are presented in **Tables 5** and **6**. The high resolution separation of ribo- and deoxynucleosides is presented in **Figure 5**.

Modified Nucleosides as Cancer Markers and in Normal Metabolism

Borek stated that the hope of finding some unique metabolic products or unique components of malignant cells circulating in body fluids which can be

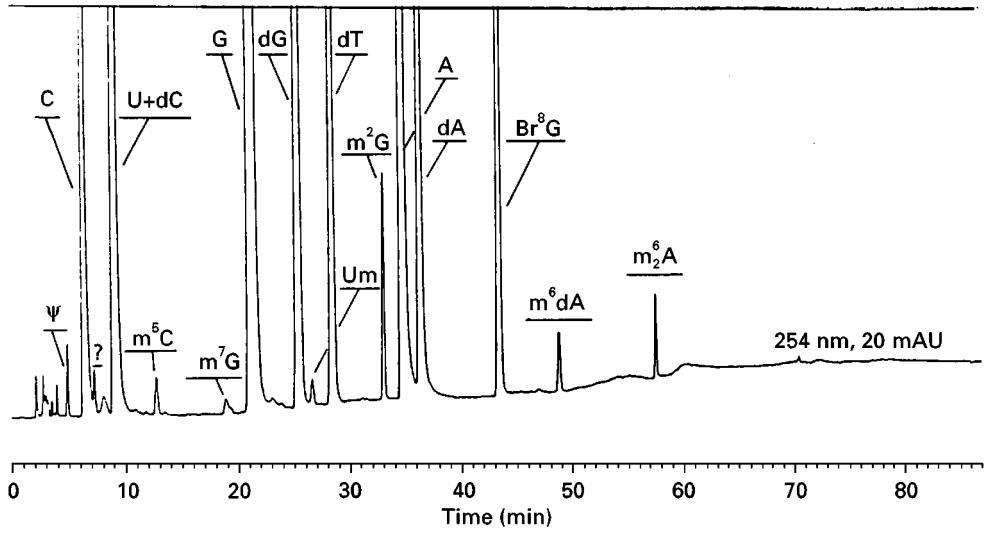

Figure 3 HPLC of nucleosides in *E. coli* 16S rRNA.

measured is as old as modern biochemistry. The term 'tumour marker' has been coined by Dr Morton K. Schwartz of the Sloan Kettering Institute for such a product. Before we consider whether we have fulfilled such a hope, we ought to define what a tumour marker should be. The requirements for an effective tumour marker are manyfold; it ought to be specified for malignancy – it should provide a minimum of false-positives and false-negatives; it ought to indicate the extensiveness of the malignancy and it should preferably diminish or hopefully disappear after effective therapy.

At an international conference held in Vienna (1982) under the auspices of the Society for Early Detection of Cancer, someone calculated that there were close to 90 reported different tumour markers. It can be stated, however, that unfortunately none of these putative tumour markers meet even partly the qualifications we have set above.

A problem is that most of the tumour markers in use today are proteins. Proteins are the peripheral end products of the molecular mechanisms of every cell. A mammalian cell is endowed with the capability of producing perhaps 10 000 or more different proteins. Unless we chance upon a protein which is either causal of a malignancy or which is a universally aberrant concomitant of malignant tissues, looking for protein products which will qualify as tumour

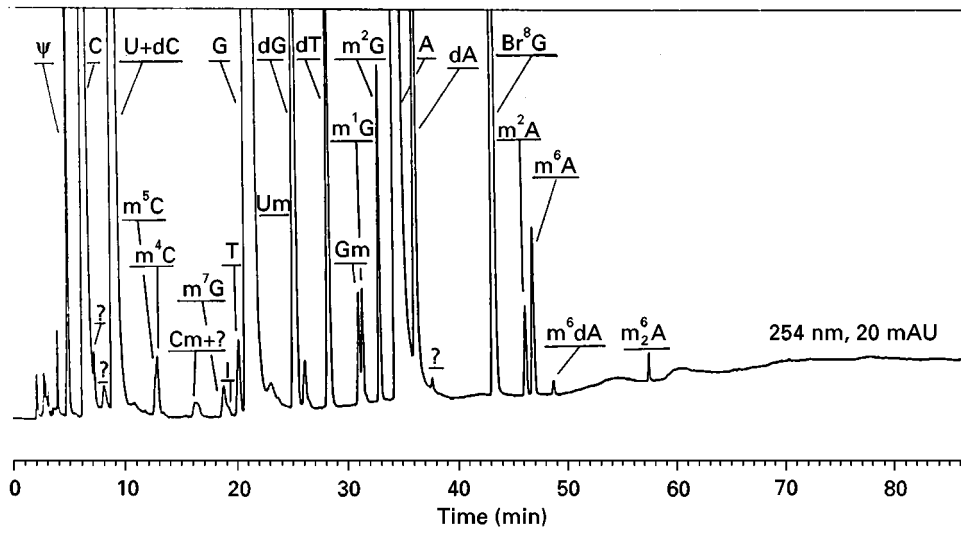

Figure 4 HPLC of nucleosides in *E. coli* 23S rRNA.

Table 4 RP-HPLC-UV quantitation nucleosides in *E. coli* 16S and 23S rRNA

Nucleosides	*Mol% in 16S rRNA*		*Mol% in 23S rRNA*	
	HPLC	*Lit.*[a]	*HPLC*	*Lit.*[b]
C	23.0	22.8	22.2	
U	20.9	20.4	20.4	
G	30.4	31.6	30.7	
A	25.1	25.2	26.0	
Total	99.4	100.0	99.3	
Residues/mol in				
Ψ	1.3	0.0	7.8	3.0
m^5C	2.0	1.0		
m^4C			1.9	0.0
m^7G	0.5	1.0	0.7	1.0
Cm + ?			0.9	1.0
T			1.7	1.0
m^3U	0.8	1.0	0.9	1.0
Gm	0.0	1.0	0.9	1.0
m^4Cm	0.0	1.0		
m^1G			0.8	1.0
m^2G	2.9	2.0	2.3	0.0
m^2A			0.9	0.0
m^6A			2.1	2.0
m^6_2A	1.6	2.0	0.2	0.0

[a] Literature values from Noller HF (1984) Structure of ribosomal RNA. In Richardson CC, Boyer PD and Meista A (eds) *Annual Review of Biochemistry* 53: 119–162.
[b] Literature values obtained from Gutell RR, Weiser B, Woese CR and Noller HF (1985) Comparative anatomy of 16-S-like ribosomal RNA. *Progress in Nucleic Acid Research and Molecular Biology* 32: 155–216.
[c] m^7G is partially lost during hydrolysis.
[d] The 210 nm signal was examined; no hU was observed.

markers in terms of the requirements listed above is hopeless.

A promising marker is the tRNAs of tumour tissue. The finding of aberrant tRNA-methylating enzymes in tumour tissue prompted the study of the tRNAs themselves. Surprisingly, only a few of the tRNAs in the malignant tumour were found to be different in structure from those in the normal tissue counterpart. Dr Guy Dirheimer of Strasbourg isolated 18 different tumour-specific tRNAs and found modification on them different from those in normal counterparts. On the other hand, we have determined with the aid of Japanese colleagues that the primary sequence is the same. Perhaps to enable it to perform its many functions, tRNA is endowed with an extraordinarily complex structure. Its primary sequence consists of about 80 of the four major bases found in other RNAs: adenine, cytosine, guanine and uracil. In addition to these major bases, tRNA contains a large variety of modified bases that are unique to it. The modified bases increase in number with the complexity of the organism. Thus, for example, *E. coli* tRNA may contain only two or three, yeast tRNA may contain five or six, and mammalian tRNA may contain modified bases constituting as much as 20% of the total. It has also been shown that the tRNA methyltransferases are abnormally hyperactive in every malignant tissue. Borek found that the level of excretion of the nucleosides in urine when followed before, during, and after therapy in a malignancy that responds well to chemotherapy, that within five days of commencement of therapy in six patients with Burkitt's lymphoma, that the excretion levels in urine returned to normal and remained normal as long as the subjects were in remission.

There have been reports since the early 1950s that cancer patients excrete elevated levels of methylated purines and pyrimidines as well as other modified nucleosides. Ample evidence had indicated that increased tRNA methylase activity in neoplastic cells was a common and consistent finding, and increased excretion of modified bases in urine from cancer patients and tumour-bearing animals had also been reported. Methylation of the bases in tRNA had been

Table 5 Precision of RP-HPLC-UV for DNA total composition analysis

	Mol% of deoxynucleoside[a]				
	dC	*m^5dC*	*dG*	*dT*	*dA*
Average	20.94	1.52	22.42	27.59	27.65
SD	0.0080	0.022	0.022	0.035	0.043
%, RSD ($N = 16$)	0.038	1.43	0.096	0.13	0.15

[a] Average values from four hydrolysates; four analyses were made from each hydrolysate over a period of 2 days. Sample: DNA calf thymus DNA. SD: population standard deviation; %, RSD: relative standard deviation as a percentage.

Table 6 Linearity of RP-HPLC-UV from analysis of calf thymus DNA

μg of DNA injected	*Mol% of deoxynucleoside*[a]				
	dC	*m^5dC*	*dG*	*dT*	*dA*
8.5	20.85	1.47	22.38	27.59	27.68
5.0	20.93	1.51	22.41	27.70	27.44
2.5	20.86	1.55	22.58	27.64	27.41
1.25	20.81	1.52	22.54	27.89	27.38
0.50	20.78	1.67	22.45	27.65	27.46
Average	20.85	1.54	22.47	27.69	27.48
SD	0.051	0.068	0.076	0.10	0.10
%RSD	0.24	4.4	0.34	0.38	0.38

[a] See notes to Table 5.

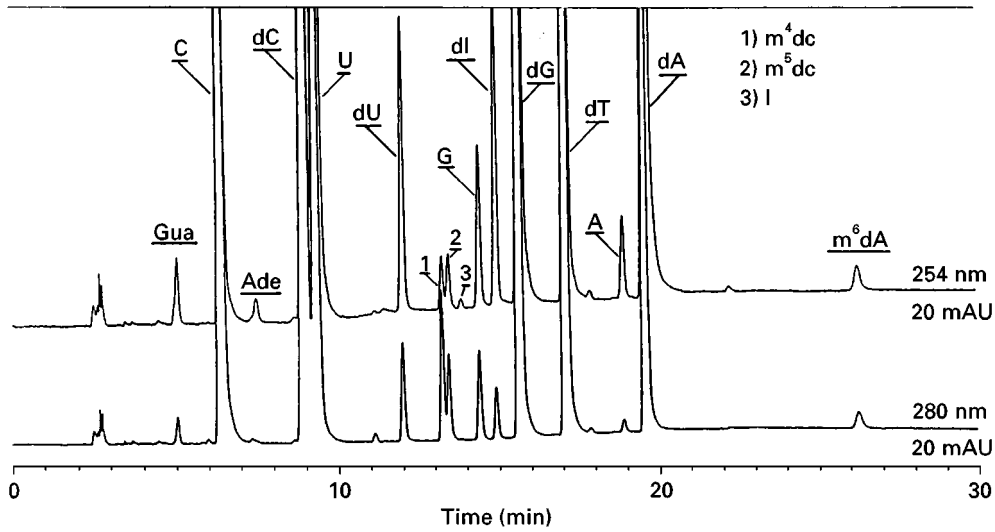

Figure 5 High resolution chromatography of ribonucleosides and deoxynucleosides.

found to occur after the macromolecule is formed, and of particular interest was that these methylated compounds were not reincorporated into the tRNA molecule but thought to be excreted intact. It has been suggested that the high turnover of a subpopulation of tRNA is the major reason for increased excretion of modified nucleosides by cancer patients. The measurement of modified ribonucleosides in body fluids as biological markers of cancer resulted largely from the studies of tRNAs by the late Dr Ernest Borek. In 1974, Dr Gordon Zubrod, then director of the National Cancer Institute (NCI), appreciated the possible value of this concept. A contract was awarded by the NCI to our laboratory at the University of Missouri to develop high resolution quantitative chromatographic methods of modified nucleosides for use in tumour marker studies. Under the leadership of Professor Charles W. Gehrke gas chromatographic and RP-HPLC-UV methods were developed for measuring modified nucleosides in urine. Later the method was further improved for measuring serum nucleosides. The RP-HPLC-UV method is far better than the gas chromatographic method for highly water-soluble nucleosides, and hence it was used in analysis for a majority of the clinical studies. **Figure 6** and **Figure 7** show the separations for ribonucleosides in human urine and serum from a patient with lung cancer and acute myelogenous leukaemia, respectively. **Figure 8** presents the recovery of nucleosides from spiked serum and normal serum.

Our research on tRNA catabolites in urine and serum has centred on analysis of the modified nucleosides following isolation of the nucleosides by boronate gel affinity chromatography. Advances in the isolation, identification and measurement of modified nucleosides have been striking, and are now providing greater insights into the value of modified nucleosides as potential tumour markers in following the course of cancer and treatment. Numerous research groups in the USA, Europe, and Japan have studied modified nucleosides and their potential relationships to cancer. A comprehensive review was presented by Zumwalt *et al*. Trewyn and Grever have provided an excellent review of urinary nucleosides in patients with leukaemia. They reviewed the available literature and discussed laboratory analyses, including methods, reference values, and multivariate analyses; clinical studies covering nonmalignant disease and infection, acute leukaemia (childhood and adult) and chronic leukaemias. They concluded that measurement of urinary nucleoside excretion offers a potential tool for monitoring disease activity in patients with acute lymphoblastic leukaemia, chronic myelo-cytic leukaemia, and perhaps chronic lymphocytic leukaemia. They also point out that additional work is necessary in following serial determinations of nucleosides at frequent intervals in patients with different types of leukaemia to assess the true value of these compounds as an accurate monitor of disease activity within the individual patient. We initiated investigations to study the correlation of the levels of serum-modified ribonucleosides with clinical status of the patient. Longitudinal serum samples were collected from leukaemia, lymphoma, and lung cancer patients. Four modified nucleosides Ψ, m_2^2G, t^6A, and m^1I, were selected to study the relationship of their levels in serum versus the course

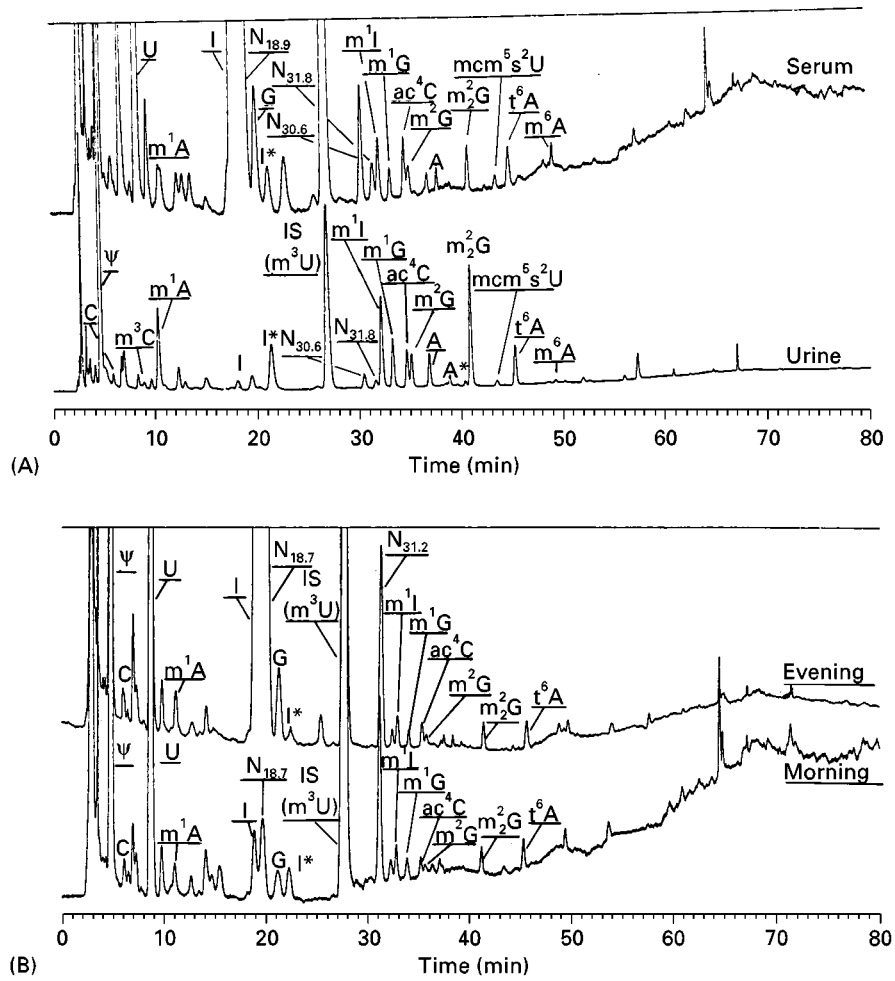

Figure 6 RP-HPLC-UV separations of nucleosides in (A) human urine and (B) serum from a lung cancer patient and a chromatogram for a morning and evening sample.

of disease. Serum pseudouridine levels showed a direct relationship to total RNA turnover. N^2,N^2-Dimethylguanosine and N^6-threoinocarbonyladenosine, which are only found in tRNA, showed that their concentrations in serum reflects the state of tRNA catabolism. 1-Methylinosine is a very interesting modified nucleoside. The concentration of serum m^1I in the normal population is quite high (65 ± 21 nmol mL^{-1}) and is one of the commonly elevated nucleosides found in cancer patients.

The origin of serum 1-methylinosine is not completely clear at this time. It can be accounted for partially from direct tRNA turnover and deamination of m^1A by adenosine deaminase in serum. We also studied longitudinal collected normal serum samples and found that the four target nucleosides levels in serum are constant during one day (7.30 a.m., 12.00 noon and 5.00 p.m.), and over 14 days. For cancer patients we plotted the ratio of each nucleoside to the average concentration found from 94 normal subjects as a percentage. The results of the longitudinal studies from one selected leukaemia patient, one lymphoma, and one with lung cancer are presented here. The modified nucleoside level from the respective patients over time are presented in **Figure 9**, **Figure 10** and **Figure 11**. The patient description and correlation of clinical status and modified nucleoside levels are as follows.

Patient MP-K (Acute Myelogenous Leukaemia): Patient Description (Figure 9)

A 31-year-old white male smoker was admitted on 12 June 1991 with fever. Work-up showed severe leucocytoses, with increased white cell count up to 244 000. The patient went into pulmonary oedema; a bone marrow test was performed and leucophoresis started. Bone marrow showed acute myelogenous leukaemia. Chemotherapy and antibiotics were given. Sample collection was started 10 days after

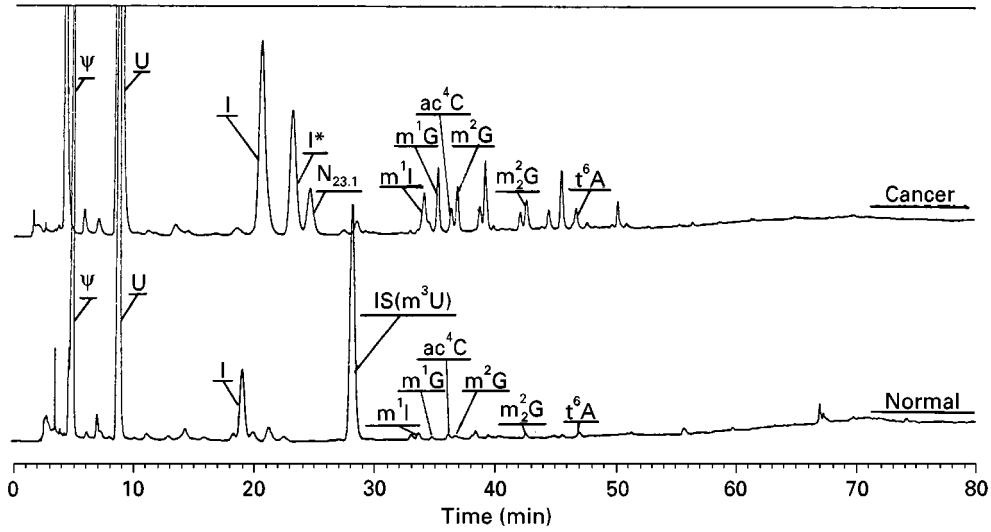

Figure 7 RP-HPLC-UV separations of nucleosides from patient with acute myelogenous leukemia versus normal.

diagnosis. The patient recovered and on 2 July 1991 a bone marrow test was again done and there was no evidence of leukaemia. However, 2 weeks later a repeated bone marrow showed relapse of disease. Chemotherapy was again given on 18 July 1991. A bone marrow 2 weeks after treatment (day 38) showed persistent disease. In the meantime the patient developed appendicitis and surgery was performed but the patient died postoperatively.

Clinical status and modified nucleoside levels (**Figure 9**) We started collecting samples on this patient right after induction chemotherapy was given. A bone marrow test done by the time sample no. 2 was drawn showed no evidence of leukaemia. However, there was already a slight increase in modified nucleoside levels. Relapse of the disease was clinically suspected and confirmed by bone marrow and there was a marked increase in all modified nucleoside levels. Reinduction chemotherapy was given and correspondingly there was a decrease in levels of modified nucleosides. A bone marrow test was performed at day 38 of the study, which showed presence of leukaemic cells at a time when modified nucleoside levels were also increasing. From this date, a good clinical correlation of the level of markers increased with clinical deterioration of the patient.

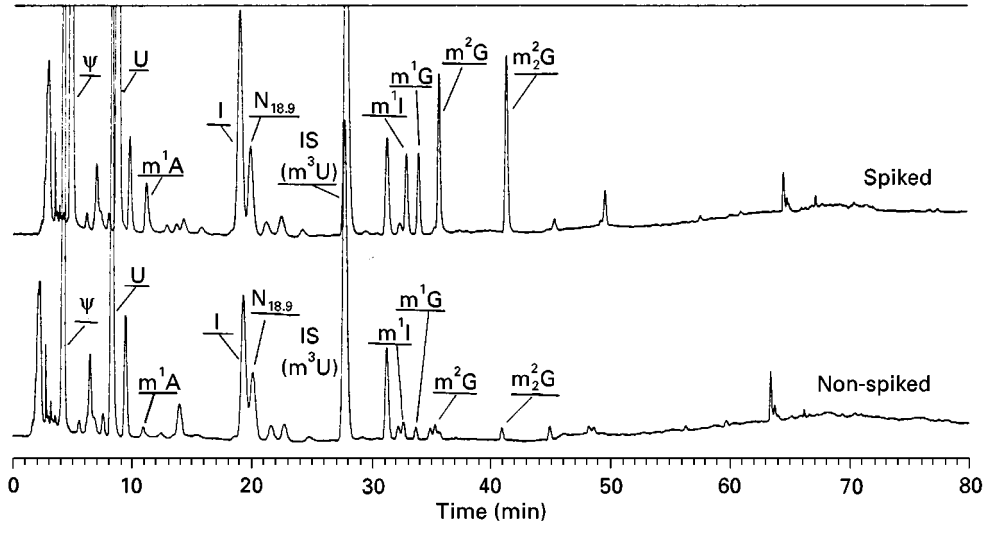

Figure 8 Recovery of nucleosides from spiked and normal serum.

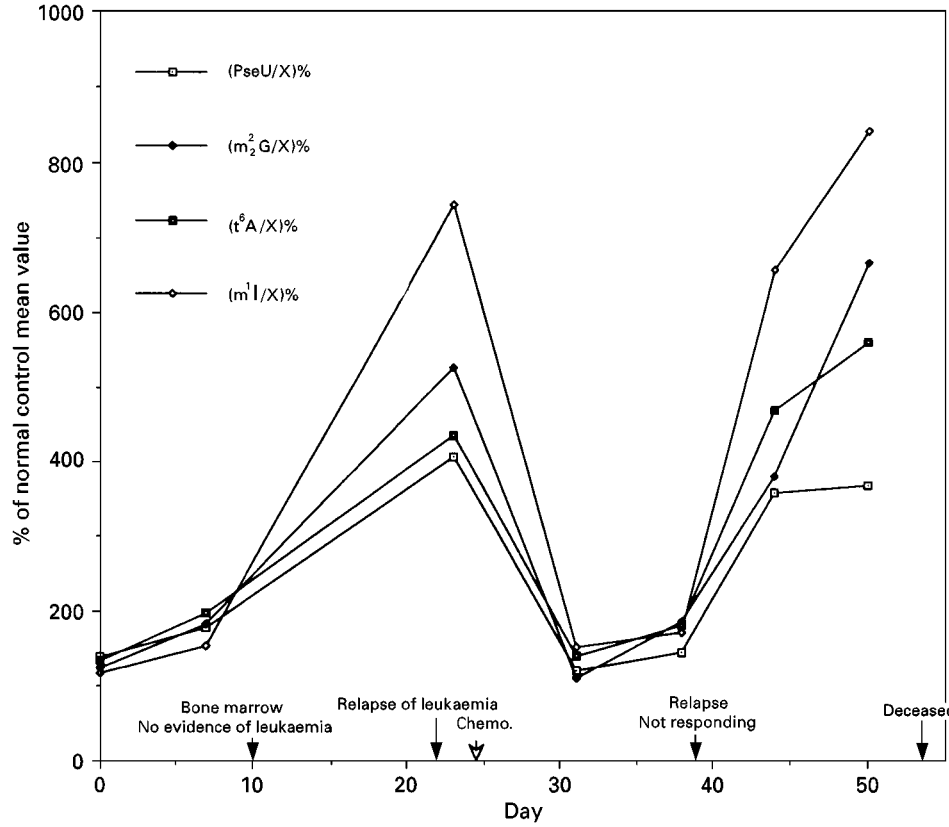

Figure 9 Serum-modified nucleoside levels as cancer biomarkers in patient with acute myelogenous leukaemia.

Patient MP-Y (Non-Hodgkin Lymphoma): Patient Description (Figure 10)

A 60-year-old white female smoker presented on 1 June 1991 with weakness in the lower extremities and magnetic resonance imaging (MRI) showed evidence of cord impression. Biopsy showed non-Hodgkin lymphoma, large cell type. Postoperatively the patient received radiation therapy. The patient was started on chemotherapy after assessment of disease. On physical examination she had axillary adenopathy and computed axial tomography (CAT) scan of the chest showed chest wall disease and a pleural effusion. CAT scan of the abdomen showed metastasis to the lumbar spine. Sample collection started on 20 September 1991 while the patient was on therapy. Evaluation of her disease showed response by physical examination (axillary lymph node decreased size), neurological examination improving and by CAT scan with decreased pleural fluid. CAT scan of the abdomen showed no evidence of disease. The patient continued the same regimen of chemotherapy. In February (day 140) the patient went into respiratory distress and increased pleural effusion was detected. However, cytology and bronchoscopy found no evidence of lymphoma. Chemotherapy was continued as soon as the patient recovered. Shortly after the patient was again admitted to hospital with fever. The patient also complained of a chest wall mass that had been growing in the last few weeks. The patient died shortly after this from cardiorespiratory arrest before any treatment was given. Autopsy showed lymphoma.

Clinical status and modified nucleoside levels (Figure 10) By the time the patient was included in the study she was already on chemotherapy and clinically responding. Modified nucleoside levels were quite steady until day 175, when there was a gradual increase and a peak around day 250 of the study. Clinically the patient was having lung problems and an infiltrate and new plural effusion by lymphoma was never documented; however, all four modified nucleoside levels increased to 250–350% of the normal control mean values. The modified nucleoside levels continued to increase and remained higher. The patient died from cardiorespiratory arrest. Lymphoma was later proved present by biopsy in the chest wall mass. There was a good clinical correlation of

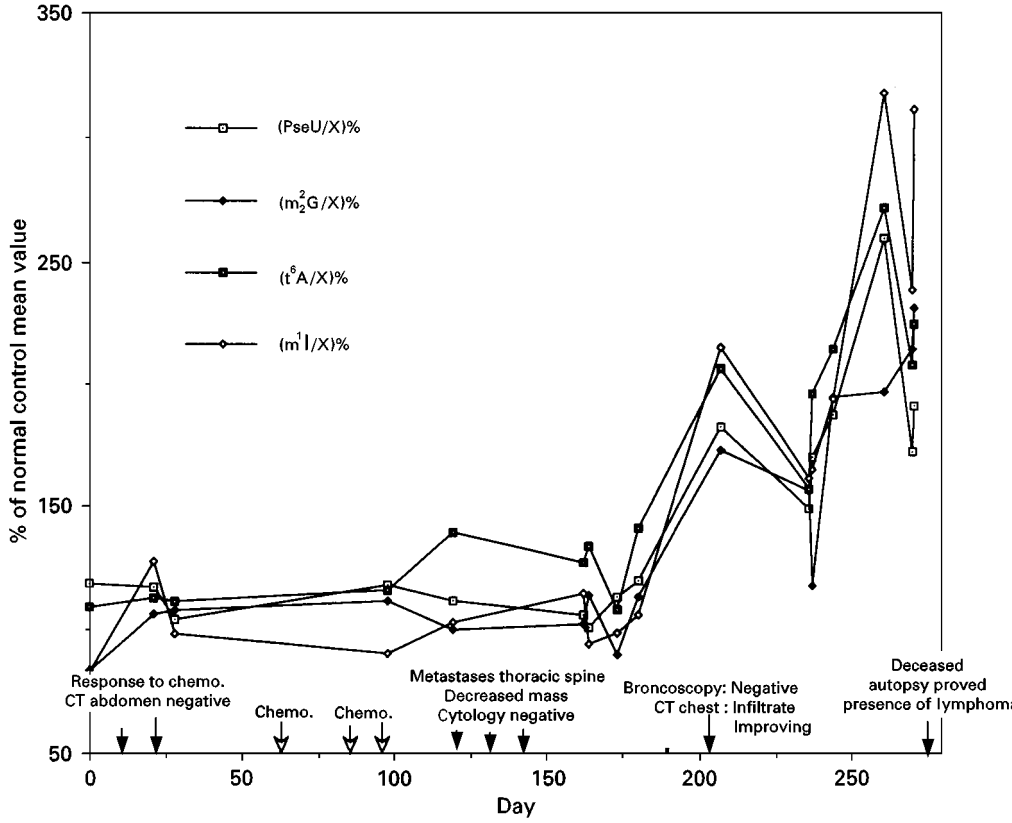

Figure 10 Serum-modified nucleoside levels as cancer biomarkers in lymphoma patient.

the four serum modified nucleosides levels and lymphoma.

Patient LRH (Adenocarcinoma of the Lung): Patient Description (Figure 11)

A 58-year-old white male smoker presented in April 1993. Biopsy and MRI showed poorly differentiated adenocarcinoma of the lung with metastases to the adrenal gland (adrenal mass) and no pleural effusion. Chemotherapy was started on 26 May 1993. This patient was not responding to chemotherapy and his clinical status was gradually deteriorating. In October 1993 (weak 23) the patient was in the end stage of the disease.

Clinical status and modified nucleoside levels (**Figure 11**) This patient was not responding to treatments. His clinical status was continually deteriorating. The progression of disease correlated with increased levels of modified nucleosides. When the disease reached the end stage, the levels of all four modified nucleosides were $> 300\%$ higher than the normal control mean values. In this case the modified nucleoside levels clearly correlated with the progress of the disease.

Preparative Isolation of Unknown Nucleosides in Nucleic Acids for Structural Characterization

Knowledge of the chemical structure of nucleoside modifications in nucleic acids is essential for increasing our understanding of their chemical structure and biological function relationships. tRNA is one of the most heterogeneous biopolymers known. It not only has a variety of functions within the cell, but also contains a much larger proportion of modified nucleosides than other nucleic acids; more than 60 modified nucleosides have been characterized in tRNA. With our newly developed RP-HPLC-UV nucleoside chromatography methodology, providing its enhanced resolution and sensitivity, many new modified nucleosides have been detected and identified. As tRNA research investigations are conducted on more complex organisms it is highly likely that additional modified nucleosides will be discovered, as we have

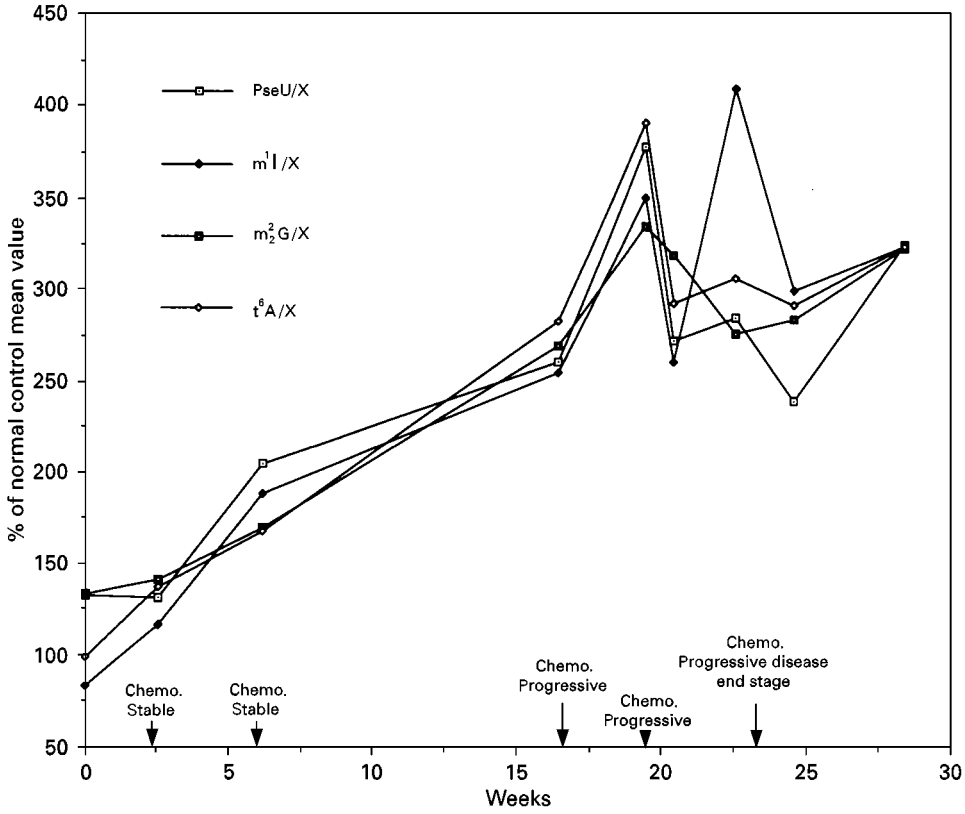

Figure 11 Serum-modified nucleoside levels as cancer biomarkers in patient with adenocarcinoma of the lung.

observed many unidentified nucleosides in urine and serum. The information on modified nucleosides in human tRNAs is limited, and further investigation should be conducted.

For purification of specific tRNAs, various types of chromatographic and electrophoretic procedures have been used. Because of the complexity of the initial mixture, the first purification step is generally not for high selectivity but for high capacity. We therefore used the countercurrent distribution (CCD) method, which is mild and serves as a first preparation step with a high capacity. This CCD method has been adapted from Holley and Merrill and by Dirheimer and Ebel. This technique permits separation of quantities of tRNAs as high as 5–6 g.

In our research we introduced standard RPLC-UV methodologies for the analysis of nucleosides and nucleoside composition of RNAs, detailed the chromatographic protocols, developed the 'nucleoside columns', and gave the essential requirements needed in the HPLC instrumentation. Three optimized systems with particular emphasis placed on resolution, speed, and sensitivity are described. In addition, three unfractionated tRNAs were selected: *E. coli*, yeast, and calf liver as sources of 'reference nucleosides' to establish the performance of the chromatography; also a quantitative enzymatic hydrolysis protocol to release exotically modified nucleosides from tRNAs was described. We have addressed the analytical characterization of nucleosides in nucleic acids, and chromatography and modification of nucleosides from the perspective of additional chromatographic methodologies for isolation of the nucleic acids, quantitative enzymatic hydrolysis, high resolution preparative HPLC, and affinity chromatography to obtain the pure single-species nucleosides for UV absorption spectroscopy and interfaced mass spectrometry identification. In addition, we described experiments on the determination of the structure–spectrum relationships, composition, and conformation using an array of advanced analytical techniques of HPLC-UV, FT-IR, nuclear magnetic resonance and mass spectrometry (MS), as well as structure–RP-HPLC retention relationships. In these studies, a consortium of scientists from different institutions have combined their expertise and present a comprehensive discussion of the isolation and analytical–structural characterization of tRNAs, oligonucleotides, and nucleosides in RNA and DNA. Two modified nucleosides, A* and G* in yeast initiator tRNA (initiator $tRNA^{Met}$) at positions 64 and 65 in the T-Ψ stem were identified as an unmodified

guanosine at position 65, and for A* as *O*-β-D-ribofuranosyl-(1″–2′)-adenosine in position 64. We elucidated that the final structure for A* at position 64 in yeast initiator $tRNA^{Met}$ was established as an *O*-ribofuranosyl-(1″–2′)-adenosine-5″-phosphate linked by a 3′5′-phosphodiester bond to G at the position 65.

An unknown U* nucleoside in position 34 isolated from yeast mitochondrial $tRNA^{Leu}$ was characterized as $cmnm^5U$ by HPLC-UV-MS.

Also, we have confirmed m^3U, an unknown modified uridine, in the 16S colicin fragment from *E. coli* rRNA, and report the structural characterization of a catabolite in canine urine as 5-hydroxymethycytidine (om^5C).

This report describes the 'research tools' we have developed and are using in analytical characterization of modified nucleosides and dinucleosides in RNAs and which will be of value to others in molecular biology investigations.

A good clinical correlation was observed in patient management using four cancer modified nucleoside biomarkers in following the course of disease and treatment.

See also: **II / Chromatography: Liquid:** Detectors: Ultraviolet and Visible Detection; Mechanisms: Reversed Phases.

Further Reading

Borek E (1971) Introductions to symposium: tRNA and rRNA modification 1. Differentiation and neoplasia. *Cancer Research* 31: 596–597.

Borek E (1972) The morass of tumor markers. *Bulletin of Molecular Biology and Medicine* 10: 103–117.

Borek E and Kerr SJ (1972) Atypical transfer RNAs and their origin in neoplastic cells. *Advances in Cancer Research* 15: 163–192.

Borek E, Baliga BS, Gehrke CW, Kuo KC and Waalkes TP (1977) High turnover rate of transfer RNA in tumor tissue. *Cancer Research* 37: 3362–3366.

Desgres J, Keith G, Kuo KC and Gehrke CW (1989) Presence of phosphorylated O-ribosyladenosine in T-Ψ stem of yeast methionine initiator tRNA. *Nucleic Acids Research* 17: 865–882.

Dirheimer G and Ebel JP (1967) Fractionnement des rRNA de Levure de biere par distribution en countre-courant. *Bulletin de la Société Chimique et Biologique* 49: 1679–1687.

Gehrke CW and Kuo KC (1989) Ribonucleoside analysis by reversed-phase high performance liquid chromatography. *Journal of Chromatography* 471: 3–36.

Gehrke CW, McClune RA, Gama-Sosa MA, Ehrlich M and Kuo KC (1984) Quantitative RP-HPLC of major and modified nucleosides in DNA. *Journal of Chromatography* 301: 199–219.

Gehrke CW and Kuo K (eds) (1990) *Chromatography and Modification of Nucleosides*. Amsterdam: Elsevier.

Holley RW and Merrill SH (1959) Counter-current distribution of an active ribonucleic acid. *Journal of the American Chemical Society* 55: 735.

Kuchino Y, Borek E, Grunberger D *et al.* (1982) Changes of post-transcriptional modification of large base in tumor-specific $tRNA^{Phe}$. *Nucleic Acids Research* 10: 6421–6432.

Kuo KC, McCune RA, Gehrke CW, Midgett R and Ehrilich M (1980) Quantitative reversed-phase high performance liquid chromatographic determination of major and modified deoxyribonucleosides in DNA. *Nucleic Acids Research* 8: 4763–4776.

Kuo KC, Smith CE, Shi Z, Agris PF and Gehrke CW (1986) Quantitative measurement of mRNA cap 0 and cap 1 structures by high-performance liquid chromatography. *Journal of Chromatography and Biomedical Applications* 378: 361–374.

Kuo KC, Esposito F, McEntire JE and Gehrke CW (1987) Nucleoside profiles by HPLC-UV in serum and urine of controls and cancer patients. In: Cimino F, Birkmayer GD, Klavins JV, Pimentel E and Salvatore F (eds) *Human Tumor Markers*. Berlin: Gruyter, pp. 519–544.

Martin RP, Sibler A, Gehrke CW *et al.* (1990) 5-Carboxymethylaminomethyluridine is found in the anticodon of yeast mitochondrial tRNAs recognizing two-codon families ending in a purine. *Biochemistry* 29(4): 956–959.

Trewyn RW and Grever MR (1986) Urinary nucleosides in leukemia: laboratory and clinical applications. *CRC Critical Reviews in Clinical Laboratory Sciences* 24: 555.

Thin-Layer (Planar) Chromatography

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Introduction

Thin-layer (TLC; or planar chromatography) is well suited to the separation of nucleic acids. One of the