
An improved method for the separation and quantitation of the modified nucleosides of transfer RNA.

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ABSTRACT

A method is described which allows a very efficient determination of the modified nucleosides of tRNA. The technique involves enzymatic degradation of the tRNA to nucleosides at pH 7.6 and their separation by two-dimensional thin-layer chromatography on cellulose-coated aluminium foils. Based on the analysis of two mammalian tRNAs it is shown that the technique is suitable for the determination of chemically unstable nucleosides as well as the ribose-methylated compounds. At least 36 of the 45 known modified nucleosides can be separated and quantitatively determined by the method described. This procedure is especially suitable for the estimation of the nucleoside composition of unlabeled tRNAs as well as for studying the post-transcriptional modifications of tRNA.

INTRODUCTION

Changes in the distribution of isoacceptor tRNAs have been noticed in many organisms. Such differences have been observed in various organs, during differentiation, in malignant cells and after treatment with hormones³. One of the possible reasons for the alterations of tRNA profiles could be due to differences in the post-transcriptional modification of tRNA. We decided therefore to find a simple and convenient method for the determination of the total nucleoside composition of isoacceptor tRNAs.

The major difficulties with the determination of the rare nucleosides² are the great variety of the compounds (about 45 different odd nucleosides) which one has to separate and their partial chemical instability at acidic as well as alkaline pH⁴⁻⁷.

In recent years several authors described methods for analysis of the base composition of RNA⁴⁻¹⁵. Due to the complexity of the problem, all of the known methods have their limitations. In some instances, complete separation of the modified nucleosides was not achieved⁶⁻¹⁵, and in others extreme conditions during the hydrolysis of the tRNA or chromatography led to partial destruction of some unstable compounds⁴⁻¹⁵. The elegant method of Randerath et al. remains restricted to the determination of the base-modified nucleosides and also causes destruction of certain bases⁵. Thus, it seemed obvious to develop a simple technique which allows a satisfactory analysis of all modified nucleosides.

In the present investigation we tried to overcome the above difficulties by enzymatic digestion of the tRNA to nucleosides at nearly neutral pH. In addition, by testing a variety of neutral and slightly acidic solvents, we found a combination of solvents which led to an optimal separation of the nucleosides by two-dimensional thin-layer chromatography (TLC). It will be shown that at least 40 nucleosides known to occur in tRNA can be separated from each other by the present method.

MATERIALS

The source of the synthetic nucleosides are indicated in Table I. ac⁴C was synthesized according to Watanabe et al.¹⁶. The ribosemethylated nucleosides, cm⁵U, and s²cm⁵U were isolated from a dinucleotide fraction of alkaline treated rat liver tRNA¹⁷. Other odd nucleosides were isolated from a variety of sources, m¹I from yeast tRNA^{Ala} 18, Q from E. coli tRNA^{Asp} 19, o₂yW from calf liver tRNA^{Phe} 20, ms²i⁶A and acp³U from E. coli tRNA^{Phe} 21, yW from yeast tRNA^{Phe} 6, 22, m²A from E. coli tRNA^{Glu} 23.

The organic solvents were obtained from Merck (Darmstadt, Germany) and Fluka (Buchs, SG, Switzerland). TLC-foils were purchased from Merck (Prod.No.5563/OO01).

Table I: Sources and Rf-values of nucleosides on TLC

Nucleoside (source)*	Rf-values (x 100) in the solvents:						
	A	B	C	D	E	F	G
A (B)	42	11	80	70	49	29	19
g ⁶ A (Sc)	6	9	50	62	13	4	22
i ⁶ A (S)	91	5	96	92	90	89	87
m ¹ A (S)	34	66	77	62	39	20	5
m ⁶ A (S)	56	13	83	84	65	52	35
mt ⁶ A (Ch)	13	27	55	87	58	12	43
t ⁶ A (Sc)	8	30	56	80	29	7	23
I (B)	17	39	48	64	33	23	8
C (B)	26	61	67	64	43	23	8
ac ⁴ C	40	45	67	79	55	53	27
m ³ C (C)	44	80	72	69	50	34	9
m ⁵ C (S)	38	51	73	69	48	28	9
G (B)	13	30	48	57	29	18	9
m ¹ G (S)	21	23	53	66	40	32	14
m ² G (S)	27	20	58	67	41	29	14
m ₂ ² G (S)	36	17	73	69	46	37	16
m ₇ ² G (S)	40	61	76	46	34	10	4
U (B)	21	58	48	71	50	42	25
hU (C)	17	67	46	70	39	34	14
T (C)	38	50	56	76	51	53	29
Ψ (C)	12	62	38	59	29	20	7

*B = Boehringer GmbH, C = Calbiochem, S = Sigma Chem.Co.,
Ch = Dr. G.B. Chheda, Roswell Park Memorial Institute,
Buffalo, New York, U.S.A., Sc = Dr. M.P. Schweizer,
ICN Nucleic Acid Research Institute, Irvine, Calif.

METHODS

Determination of Rf-values of synthetic nucleosides. 1-10 µg of each standard constituent were applied 2 cm from the bottom edge of TLC-plates (Avicel 1440, Schleicher und Schuell). The plates were transferred to tanks (Desaga, Heidelberg) which were lined by a filter paper and which were presaturated by shaking with 100 ml of one of the following solvents just before chromatography:

A 1-butanol - isobutyric acid - conc. ammonium hydroxide - water (75 : 37.5 : 2.5 : 25, by vol.)²⁴

B saturated ammonium sulfate - 0.1 M sodium acetate pH 6 - isopropanol (79 : 19 : 2, by vol.)²⁵

- C isobutyric acid - 0.5 M ammonium hydroxide - 0.1 M EDTA
(100 : 60 : 1.6, by vol.)²⁶
- D tert. butanol - 0.08 M formic acid - isoamyl alcohol
(50 : 50 : 2, by vol.)²⁷
- E isopropanol - 5% ammonium acetate pH 3.5 (60 : 25, by vol.)²⁸
- F acetonitrile - ethyl acetate - 1-butanol - isopropanol -
0.04 M ammonium formate pH 7.6 (7 : 2 : 1 : 1 : 2.7, by vol.)
- G tert. amylalcohol - methyl ethyl ketone - acetonitrile -
ethyl acetate - water - conc. formic acid (4 : 2 : 1.5 :
2 : 1.5 : 0.18, by vol.)⁵

The chromatograms were developed at 20-21°C until the solvent front migrated 15 cm from the origin. The plates were dried and the nucleosides localized under UV-light. Dihydro-uridine was detected on reaction with p-dimethylamino-benzaldehyde²⁹.

Enzymatic hydrolysis of tRNA. Prior to hydrolysis, the tRNA was desalted as described previously⁷. About 2-3 A₂₆₀ units tRNA were hydrolysed overnight at 37°C in a siliconized test tube in 0.2 ml 0.02 M ammonium formate pH 7.6, 5 · 10⁻⁴ M MgCl₂, containing 25 µg pancreatic RNase (Boehringer GmbH, Mannheim), 30 µg snake venom phosphodiesterase (Worthington, N.J.) and 25 µg alkaline phosphatase (Worthington, Code BAPF). Subsequently, the digest was treated for 2 h at 37°C with 5 µg ribonuclease T₁. The original alkaline phosphatase preparation was dialysed four times against 250 vol. of 10⁻³ M MgCl₂ and stored at -30°C in small portions.

Application of the hydrolysate to TLC-foils. The hydrolysis of the tRNA was carried out in a relatively large volume in order to avoid losses of poorly soluble compounds. We developed a simple procedure to apply large samples conveniently onto the chromatograms as described below.

A 0.2 ml pipette was fitted with a thread by means of a thin wire loop inserted into the tip. The pipette was fixed with two clamps and placed horizontally on the chromatogram. The thread served as a wick to transfer the sample onto the

origin of the chromatogram. A hair-dryer, regulated by a variable transformer, was used to blow air constantly in order to obtain a spot with a diameter of about 15 mm.

Two-dimensional separation of tRNA-hydrolysates on cellulose-coated aluminium-foils. The digests were placed on 20x50 cm TLC-foils (Merck, Prod.No.5563/0001) and separated in the first dimension by descending chromatography for 24 h at 20-21°C in solvent A. The separation was carried out in a paper-chromatography tank "Shandon", England, Panglas TLC chromatank) containing a 20x50 cm filter-paper which was wetted with the solvent just before the start of the chromatography. Under these conditions the solvent front moves about 30 cm from the origin.

After development in solvent A, the TLC-foil was dried by evaporation with a ventilator for 3 h at 20°C. In the second dimension the TLC was developed for about 4 h by ascending chromatography in solvent B as described above. Solvent B was used only once.

Localisation of the nucleosides. The localisation of the nucleosides was usually carried out under the UV-light at 254 nm.

Identification of barely visible compounds such as ac⁴C, s²cm⁵U, t⁶A, etc. was often facilitated by inspection of their characteristic fluorescence or phosphorescence at 77°K^{7,30}.

Quantitative estimation of the nucleosides. Each spot was carefully scraped from the TLC and the nucleosides were eluted in 0.5 ml H₂O. Spectra were taken at pH 6, 1, and 13 in a Beckman spectrophotometer (Model 25). The extinction-coefficients used were the values from Hall¹¹. The methylated guanosines were calculated with the corrected values from Randerath et al.⁵ For the determination of dihydrouridine, small squares in the expected position were eluted and the decrease of UV-absorption at 230 nm in 0.5 M NaOH was measured.

RESULTS AND DISCUSSION

Enzymatic hydrolysis of tRNA. Among the methods available for

total enzymatic hydrolysis of tRNA. a combined treatment with pancreatic ribonuclease, snake venom phosphodiesterase and alkaline phosphatase is the most suitable one. (The use of ribonuclease T_2 was avoided since it fails to cleave the phosphodiester bonds at the 3'-side of certain modified nucleosides³¹). The snake venom phosphodiesterase and the alkaline phosphatase have their pH optima at slightly alkaline pH, and hydrolysis of RNA with these enzymes between pH 8 and 9 often leads to destruction of some labile nucleosides^{5,7,11}. We have found that snake venom phosphodiesterase and alkaline phosphatase can be successfully used in combination with pancreatic ribonuclease at pH 7.6. Under these conditions no degradation of labile nucleosides occurs. We have observed that one of the enzymes used contains traces of a guanyl-ribonuclease and this results in production of small amounts of guanosine 2'-3'-cyclic phosphate. To eliminate the cyclic nucleotide we subsequently treat the hydrolysate with ribonuclease T_1 . It is to be noted that ribonuclease T_1 cannot be included in the initial hydrolysis since this results in incomplete digestion, for example due to the formation of oligonucleotides terminating in cyclic phosphates^{31,32} which are resistant to attack by phosphomonoesterase.

It is known that the snake venom phosphodiesterase cleaves rather poorly at the phosphodiester bonds at the 5'-side of pseudouridine, of N-6-aminoacylated nucleosides, and of ribose-methylated nucleosides^{31,33,34}. Therefore, we subjected the oligonucleotides C-U-mt⁶A-A- Ψ -C-C-A-U-Um-Gp, m₂²G-A- Ψ p and the alkaline stable dinucleotides of rat liver tRNA to our hydrolysis conditions. It was found that all these very resistant compounds are totally cleaved by the digestion method used. Furthermore, we obtained all resistant nucleosides in the hydrolysates of purified tRNAs in the expected amounts.

It is advisable to use a highly purified preparation of alkaline phosphatase. Less pure preparations of this enzyme (such as alkaline phosphatase from Worthington, Code BAPSF) have been observed to contain up to 50 A₂₆₀ units RNA per mg

of protein.

Separation of nucleoside standards in different solvents. In recent years many different chromatographic techniques were used for separation of modified nucleosides. However, none of the described systems are completely satisfactory, since either only insufficient separation was achieved or partial destruction of labile nucleosides occurred in the strongly acidic pH or the alkaline solvents used⁴⁻¹⁵. With the goal to find an optimal two-dimensional TLC-system for separation of the nucleosides, including the labile compounds m^7G , m^1A , ac^4C , m^3C , t^6A , g^6A , mt^6A , hU. we tested seven neutral or slightly acidic solvents. The Rf-values of the nucleosides in these solvents are shown in Table I. Out of the combinations of the Rf-values of the nucleosides in two solvents, only a two-dimensional TLC in the solvent systems A and B yields a complete separation of the nucleosides tested. Knowledge of the properties of the other solvent systems may be helpful for special tasks.

Separation of tRNA hydrolysates on cellulose-coated aluminium-foils. The nucleosides show relatively low mobility in solvent A. Therefore, for the separation of tRNA hydrolysates, we replaced the conventional TLC by descending chromatography on commercially available TLC-foils³⁵. Figure 1 shows the two-dimensional separation of 40 nucleosides in the solvents A and B. The figure is based on the separation of an enzymatic digest of rat tRNA₃^{Ser 36}. The chromatographic positions of modified nucleosides which do not occur in this tRNA were deduced from hydrolysates of other tRNAs, of alkaline stable dinucleotides of rat liver tRNA, or by chromatography of synthetic compounds (mcm^5U , g^6A , m^3U , m^6A , ho^5U). In instances when two components showed a similar chromatographic mobility (m^7G and m^1A , m^1G and m^2G , etc.), we verified the separation of the two compounds by chromatography of mixtures of two digested tRNAs or by cochromatography of synthetic nucleoside with the hydrolysate of a tRNA. Overlaps of two nucleosides were only found

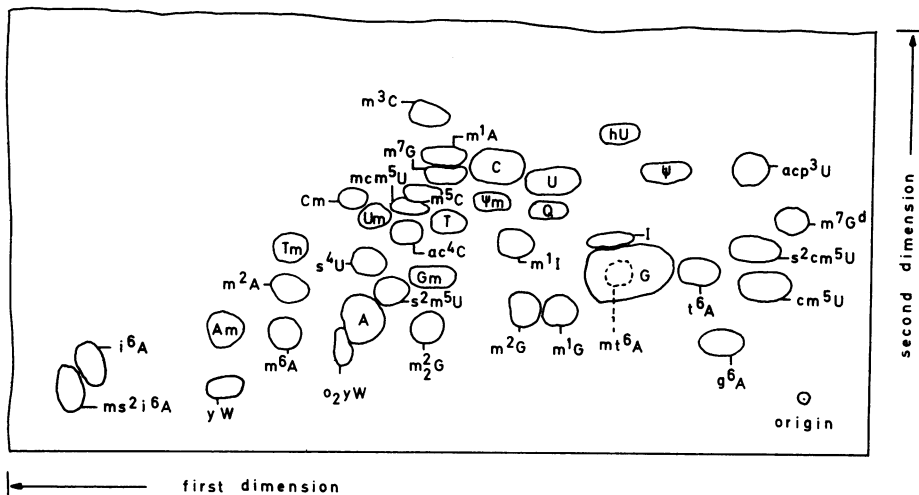


Fig. 1
Two-dimensional separation of nucleosides on cellulose coated aluminium-foils. The nucleosides were separated in the first dimension by descending chromatography in solvent A for 24 h. The front has travelled 30 cm. The separation in the second dimension was carried out by ascending chromatography (4 h) in solvent B.

in three cases.

Occasionally, about 30% of the O_2yW was found in adenosine and m^5C was slightly contaminated with mcm^5U . mt^6A was found to comigrate with guanosine. However, the latter problem can be solved by cutting the chromatogram between G and U after the chromatography in the first dimension and by a threefold successive development of the part containing G and mt^6A in solvent G. In this solvent mt^6A has a relatively high mobility, whereas G, ψ and hU barely migrate. mt^6A can be recovered from the foil after this treatment and the rest of the compounds can be separated in solvent B in the usual manner.

We have obtained highly reproduceable separations in these two solvent systems. However, it is extremely important to note that the drying of the chromatograms after the first dimension has to be carried out as mentioned. A longer time of drying led to diffuse spots, whereas insufficient drying gives rise to slight contaminations between C and U. We have found that ventilation for 3 h at room-temperature is optimal for the sepa-

rations in the second dimension. Another important parameter is the size of the spot at the origin. G and m^2G are poorly soluble in neutral solvents and therefore tend to tail in the first dimension. This can be avoided if one spots the hydrolysate at the origin with a diameter of 15 mm. This, however, does not affect the separation, since the nucleosides are focussed to smaller spots during chromatography.

Quantitative analysis of the nucleoside composition of tRNA.

Table II shows the quantitative analysis of the nucleoside composition of two mammalian tRNAs with known structure. The values of the four determinations are in good agreement with

Table II: Base compositions of rat tRNA^{Ser}₃ and of calf tRNA^{Phe}

Nucleoside ²	Rat tRNA ^{Ser} ₃		Calf tRNA ^{Phe}			
	expect- ed (36)	observed Expt.1 Expt.2		expect- ed (20)	observed Expt.1 Expt.2	
A	13	12.8	13.1	16	16.0	15.8
m^1A	1	1.1	1.1	2	1.8	1.8
mt^6A	1	0.9	1.0			
C	19	19.8	19.2	16	15.6	15.8
ac^4C	1	0.8	0.9			
m^3C	2	2.1	2.2			
m^5C	1	1.1	1.1	1	1.2	1.0
Cm				1	0.9	0.9
G	25	24.6	25.4	18	18.2	18.5
m^2G				1	0.9	0.8
m_2^2G	1	0.9	1.0	1	0.9	0.8
m^7G				1	1.1	0.9
Gm	1	0.8	1.2	1	1.0	1.0
U	11	11.8	10.5	9.5	10.1	10.4
hU	3	2.8	2.8	3	2.7	2.9
Ψ	4	3.6	4.2	4	3.6	3.9
T	1	0.8	1.0	0.5	0.6	0.6
Um	1	1.0	1.0			
o_2y^W				1	0.6*	0.8

* about 30% of o_2y^W was covered by adenosine

the expected results. The fact that mt^6A , ac^4C , m^1A and m^7G are obtained as full residues shows that neither during hydrolysis nor during separation of the nucleosides any detectable decomposition of these labile compounds has occurred. This demonstrates the usefulness of this technique for analysis of purified tRNAs.

Certain odd nucleosides occur in less than 0.05 residues per molecule of total tRNA⁶. It is obvious that these compounds cannot be detected with optical methods in hydrolysates of total tRNA. We are currently using the above described method in combination with in vivo labeling of tRNA³⁷ for quantitative estimation of these odd nucleosides.

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- 2 Unusual abbreviations: The nomenclature of nucleosides follows the rules of IUPAC-IUB Commission on Biochemical Nomenclature, *J.Mol.Biol.* (1971) 55, 299-305. These abbreviations are listed in ref. 11. yW = Wybutosine, o₂yW = peroxy-Wybutosine, m^7G^d = alkaline degradation product of m^7G , t^6A (g^6A) = \underline{N} -[\underline{N} -(9- β -D-ribofuranosylpurin-6-yl)carbamoyl]threonine resp. (glycine), mt^6A = \underline{N} -[\underline{N}' -methyl- \underline{N} -(9- β -D-ribofuranosylpurin-6-yl)carbamoyl]threonine, acp^3U = 3-(3-amino-3-carboxypropyl)uridine.
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