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DETERMINATION OF PSEUDOURIDINE IN tRNA AND IN ACID-SOLUBLE TISSUE EXTRACTS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

TOMMASO RUSSO, FRANCESCO SALVATORE and FILIBERTO CIMINO*
Istituto di Scienze Biochimiche, II Facoltà di Medicina e Chirurgia, Università degli Studi di Napoli, Via S. Pansini 5, 80131 Naples (Italy)

SUMMARY

A high-performance liquid chromatographic method to measure pseudouridine and other nucleosides in hydrolyzed unfractionated tRNA and in acid soluble tissue extracts is described.

The method is based on the following steps: (i) tRNA extraction and hydrolysis by a mixture of ribonuclease A, snake venom phosphodiesterase and bacterial alkaline phosphatase; (ii) nucleoside purification (in the case of acid soluble tissue extract) by affinity chromatography on a phenyl-boronate gel column; (iii) nucleoside separation and quantitation by high-performance liquid chromatography on an octadecylsilane column by a reversed polarity gradient elution.

The procedure allows a very accurate quantitation of pseudouridine and some other nucleosides, and its sensitivity is such that only 20 μg of tRNA are required.

The method has been utilized to compare the pseudouridine content of hydrolyzed tRNA extracted from normal and lymphomatous murine thymus, as well as the pseudouridine content in acid soluble extracts from the same tissues.

INTRODUCTION

Numerous studies have documented the occurrence of increased levels of modified nucleosides in serum and urine of cancer patients (for reviews, see refs. 1, 2). Of these compounds, pseudouridine (ψ) is the one most frequently and significantly elevated[3], and its increase is often correlated with both the stage of the disease and the response to therapy[3–5]. We have investigated the behaviour of ψ serum levels in an experimental animal system, *i.e.*, the AKR mouse strain. These mice spontaneously develop thymic lymphoma with a very high incidence (100% in female animals), and the disease shares several analogies with human acute lymphoblastic leukaemia[6]. We found[7,8] that serum ψ concentration is increased in lymphomatous mice, and, more interestingly, that it is significantly increased also in the "preleukaemic" period during which, in the absence of any clinical or histopathological evidence of the disease, a recombinant retrovirus responsible for the disease is generated[9].

Although data abound on the increased levels of modified nucleosides in cancer

patients, the biochemical basis of the phenomenon is unclear. Some results suggest that it can be related to an altered turnover rate of some tRNA subpopulations[10]. The involvement of tRNA molecules with a high ψ content as primers of reverse transcriptase for various oncogenic viruses[11], *e.g.*, $tRNA^{Pro}$ for AKR viruses and $tRNA^{Trp}$ for Rous sarcoma virus, prompted us to compare the ψ content of tRNA, extracted from normal and neoplastic AKR thymuses, with the level of free ψ in the same tissues. To investigate this correlation we devised an high-performance liquid chromatographic (HPLC) procedure for the determination of ψ in hydrolyzed tRNA and in acid-soluble tissue extracts.

MATERIALS AND METHODS

Animals and bacterial strains

AKR mice were kindly provided by C. Gurgo and S. Bridges: the colony was established from Jackson Laboratory mice. *Escherichia coli* His T^- mutant was a gift from C. B. Bruni. Bacterial cells were grown in VBC medium (0.8 m*M* $MgSO_4$, 8 m*M* citric acid, 57 m*M* K_2HPO_4, 12 m*M* $NaHNH_4PO_4$) containing 0.5% glucose, until 1 $a.u._{650\ nm}$ was reached.

tRNA extraction and hydrolysis

Thymuses (1.20 g) from 2-month-old AKR mice were homogenized in 3 ml of 0.14 *M* NaCl, 0.05 *M* sodium acetate, pH 5.1, containing 0.3% sodium dodecyl sulphate (SDS) and then centrifuged at 13,000 *g* for 1 h. The supernatant was centrifuged at 100,000 *g* for 90 min, and used for tRNA extraction. Bacteria from 100-ml cultures were collected by centrifugation and disrupted by sonication in 3 ml of the above buffer. tRNA extraction was performed by mixing the cell or the tissue preparations with an equal volume of freshly distilled phenol, saturated with the same buffer, and shaking the mixture for 30 min at room temperature. After centrifugation at 6000 *g* for 30 min, the nucleic acids present in the aqueous phase were precipitated with 2.5 volumes of ice-cold ethanol in the presence of 2% potassium acetate, pH 5.1. After centrifugation and washing with 70% ethanol, the nucleic acid fraction was dried under vacuum, and then resuspended in 1 ml of 0.1 *M* Tris–HCl, 0.01 *M* $MgCl_2$, pH 7.5. tRNA was purified by ion-exchange chromatography on a 5 × 1.5 cm DE-52 (Whatman) column, by eluting with 1 *M* sodium chloride solution, in the same Tris–HCl–$MgCl_2$ buffer. Contamination and/or degradation of the prepared tRNA was tested by polyacrylamide gel electrophoresis: no more than 10% of 5S RNA was found.

Dried tRNA (2 $a.u._{260\ nm}$) was hydrolyzed according to Cimino *et al.*[12] by incubation at 37°C for 18 h with 25 μg of ribonuclease A (Sigma), 30 μg of snake venom phosphodiesterase (Sigma) and 32 μg of bacterial alkaline phosphatase (Sigma) in 0.5 m*M* $MgCl_2$, 80 m*M* ammonium formate, pH 7.8, in a final volume of 50 μl.

Nucleoside purification from acid-soluble tissue extract

Thymuses (0.70 g) from 2-month-old AKR mice were homogenized in 1.5 ml of 0.1 *M* Tris–HCl, pH 7.8, at 4°C. A 200-μl aliquot was centrifuged at 13,000 *g* for 1 h and the supernatant was utilized for protein determination, performed according

to Bradford[13], using bovine serum albumin as standard protein. The remaining homogenate (1.3 ml) was rapidly mixed with an equal volume of 15% trichloroacetic acid (TCA). After centrifugation the supernatant was buffered to pH 8 with 2.5 *M* ammonium acetate, pH 9.5. Nucleosides present in the acid-soluble extract were purified by affinity chromatography on Affi-Gel 601 (Bio-Rad Labs.) as previously described[14]. After lyophilization the samples were treated with 64 μg of bacterial alkaline phosphatase in 0.01 *M* Tris–HCl, pH 7.8, at 37°C, to convert the 5′-nucleotides copurified by the affinity chromatography into nucleosides (3′-nucleotides are not retained on the phenylboronate column).

HPLC

A 25-μl volume of diluted samples (1:20 for tRNA and 1:100 for acid-soluble extract) was chromatographed on the HPLC equipment described in detail elsewhere[14]. The nucleosides were eluted by means of a reverse-polarity gradient generated with an M660 solvent programmer (Waters Associates), which regulates the flow-rate of two pumps so that the resulting total flow-rate through the column (μBondapak C_{18}, Waters Associates) was kept constant at 1 ml/min. It was thus possible linearly to decrease the polarity of the eluent by increasing the percentage of the "organic" phase (methanol–water, 50:50) in the "aqueous" phase (0.01 *M* $NH_4H_2PO_4$, pH 5.1). Both "organic" and "aqueous" phases were prepared with water purified on a Milli-Q system (Millipore), filtered through 0.45-μm filters (Millipore) and degassed under vacuum. The column temperature was regulated by a column jacket connected with a thermostat bath. Two linear gradients were used: from 0 to 200 ml of methanol per l or from 0 to 300 ml of methanol per l, both in 30 min.

The eluted compounds were detected by measuring the absorbance at 254 or 280 nm with an absorbance units full scale of 0.02 or 0.05. The UV profiles were printed and integrated (valley-to-valley) with a Hewlett-Packard 3385 A laboratory data system, which quantitates nucleosides by the external standard method.

RESULTS AND DISCUSSION

Fig. 1 shows a typical chromatographic profile of a standard mixture of fifteen nucleosides separated by using a linear gradient from 0 to 200 ml of methanol per l in 30 min at 36 ± 0.5°C. The described chromatographic conditions gave a good resolution of the four "major" and eleven modified nucleosides; ψ was eluted with a retention time (RT) of 5.62 ± 0.05 min in the first part of the chromatogram. Table I reports the RTs of all the nucleosides of Fig. 1 and of a further six nucleosides chromatographed under the same conditions. Although in some cases co-elution occurred, most of the compounds were well separated. However, by varying the gradient or the column temperature some unresolved compounds could be separated. For example, ribothymidine was well separated from guanosine when chromatography was performed at 25°C, and N^2,N^2-dimethylguanosine was separated from other compounds when the elution gradient was from 0 to 300 ml of methanol per l.

ψ in the hydrolyzed bulk tRNA from murine thymus and from bacteria, and in the acid-soluble extracts, was identified by comparing the RT and the absorbance

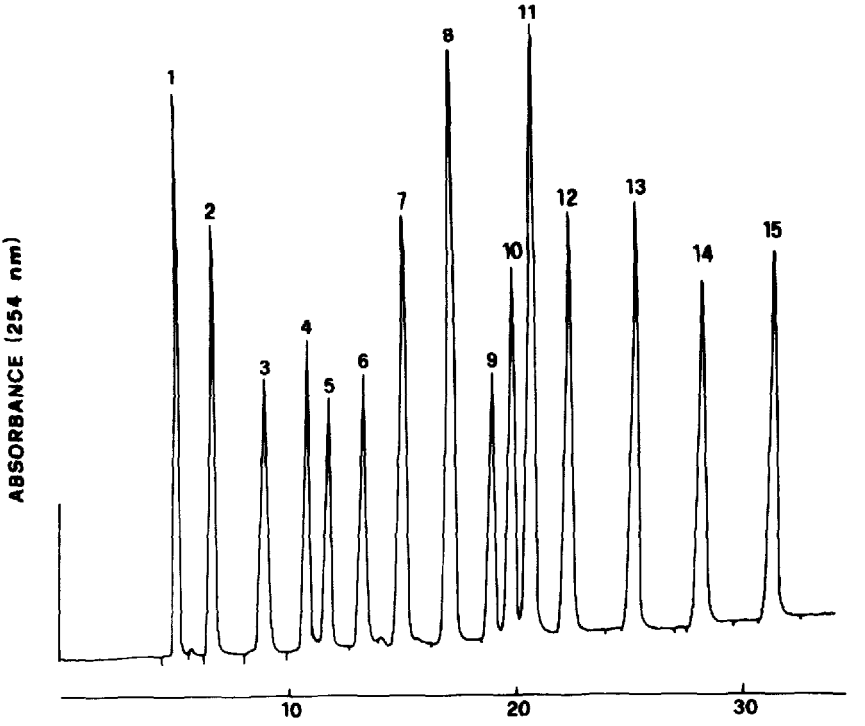

Fig. 1. HPLC profile of a standard mixture containing the following nucleosides: 1 = pseudouridine; 2 = cytidine; 3 = uridine; 4 = 1-methyladenosine; 5 = 5-methylcytidine; 6 = 7-methylguanosine; 7 = guanosine; 8 = 3-methyluridine; 9 = 1-methylinosine; 10 = 1-methylguanosine; 11 = 4-acetylcytidine; 12 = adenosine; 13 = N^2,N^2-dimethylguanosine; 14 = 2′,O-methyladenosine; 15 = 6-methyladenosine. The nucleoside amount in 25 μl injected mixture ranged between 0.250 and 0.500 nmol. The chromatographic conditions are those reported under Materials and methods. The linear gradient was from 0 to 200 ml of methanol per l; the column temperature was 36 ± 0.5°C.

ratio (A_{280}/A_{254}) of the peak (5.62 ± 0.04 min and 0.540, respectively) with those of the pure compound (5.62 ± 0.05 min and 0.536, respectively). The same procedure confirmed that ψ is not contaminated with other compounds.

Fig. 2 shows the chromatographic profile of the UV-absorbing compounds present in the hydrolyzed tRNA, extracted from normal murine thymus. The fifteen peaks identified included ψ, all the "major" nucleosides and six modified nucleosides, all of which are present in most eukaryotic tRNAs. Among the unidentified peaks, X_2 could be contaminated with 4-thiouridine and 3-methyluridine. Deoxyribonucleosides were found in low amounts; their concentrations (about 3 nmol of each deoxynucleoside per 2 $a.u._{260\ nm}$ of tRNA) indicate that the contaminating DNA in the tRNA preparation is about 5% of the total nucleic acids.

The accuracy of the method was evaluated by quantitating the ψ content of a given amount of commercially available tRNA isoaccepting species (Sigma Chemicals), *i.e.*, $tRNA^{Val}$ and $tRNA^{Tyr}$ from *E. coli*, whose sequences are known. We found 0.92 ± 0.05 mol of ψ per mol of $tRNA^{Val}$ and 1.95 ± 0.09 per mol of $tRNA^{Tyr}$, compared to the expected 1 or 2 mol, respectively. Furthermore, the comparison between the ψ content of hydrolyzed tRNA from *E. coli* wild type and from *E. coli* His T^- mutant showed that tRNA from the mutant has a ψ content of about 20%

TABLE I

HPLC RETENTION TIMES OF "MAJOR" AND MODIFIED NUCLEOSIDES

n.s. = Not separated; p.s. = poorly separated; 6-isopentenyladenosine is still retained on the column after 60 min. HPLC conditions as in Fig. 1. Retention times are averages from at least three different experiments in which the nucleosides (0.250–0.500 nmol per 25 μl) were analyzed individually. The compounds with very similar retention times were analyzed also simultaneously.

Nucleoside	*Retention time (min)*		*Nucleoside*	*Retention time (min)*	
Pseudouridine	5.62		Deoxyguanosine	19.12	
Cytidine	7.30		Thymidine	20.07	p.s.
Uridine	9.70				
Deoxycytidine	10.95		1-Methylinosine	20.36	
1-Methyladenosine	11.75		1-Methylguanosine	21.30	
			4-Acetylcytidine	22.19	n.s.
5-Methylcytidine	12.70				
7-Methylguanosine	14.35		N^2-Methylguanosine	22.24	
5-Methyluridine	16.18	n.s.	Adenosine	24.01	
			Deoxyadenosine	26.82	p.s.
Guanosine	16.28				
4-Thiouridine	17.50	p.s.	N^2,N^2-Dimethylguanosine	27.19	
			2'-O-Methyladenosine	30.36	
3-Methyluridine	18.38		6-Methyladenosine	33.80	

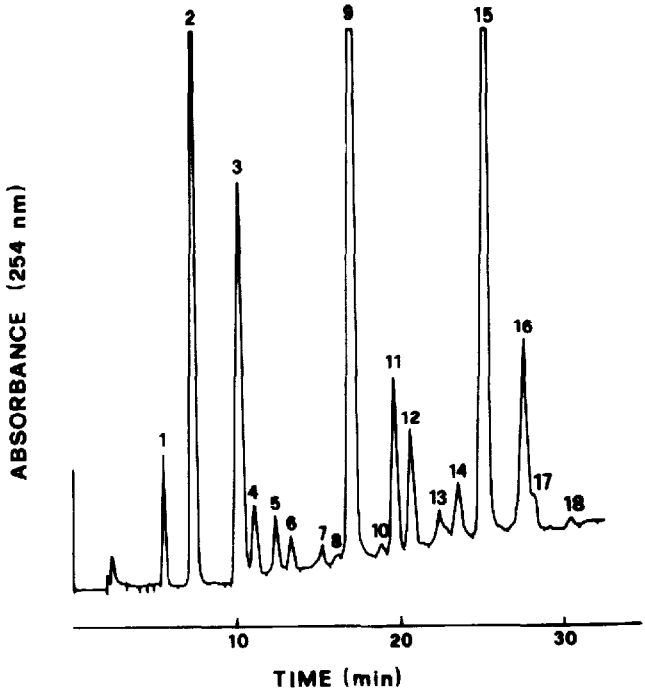

Fig. 2. HPLC profile of nucleosides present in hydrolyzed tRNA. The identified nucleosides are: 1 = pseudouridine; 2 = cytidine; 3 = uridine; 4 = deoxycytidine; 5 = 1-methyladenosine; 6 = 5-methylcytidine; 7 = 7-methylguanosine; 9 = guanosine; 11 = deoxyguanosine; 12 = thymidine; 13 = 1-methylguanosine; 14 = N^2-methylguanosine; 15 = adenosine; 16 = deoxyadenosine; 17 = N^2,N^2-dimethylguanosine. Peaks 8, 10 and 18 have not been identified. The chromatographic conditions are as in Fig. 1.

TABLE II

PSEUDOURIDINE CONTENT IN tRNA AND IN ACID-SOLUBLE EXTRACT FROM NORMAL AND NEOPLASTIC AKR THYMUSES

	Pseudouridine (mol per 100 mol of "major" nucleosides)	
	Normal thymus	*Lymphomatous thymus*
tRNA	3.79	3.98
Acid-soluble extract*	0.30	0.59

* The concentrations of ψ and "major" nucleosides in these samples include the aliquots of the respective 5'-nucleotides enzymatically dephosphorylated (see Materials and methods). The preparation of the samples and the ψ determination were performed as described under Materials and methods.

lower than that from the wild type. This appears to be in good agreement with what is known about the properties of His T^- mutant, namely the absence of the tRNA-pseudouridine synthase responsible for the biosynthesis of the ψ residue in the anticodon region[15].

The method also displays a good sensitivity: 25 pmol is the minimum detection limit for ψ[14]; it is thus suitable for quantitating the ψ content of very small samples of tRNA or acid-soluble tissue extract, such as those prepared from 250–500 mg of tissue. This is particularly useful when small specimens of tissue have to be handled, as in the case of mice.

The results of ψ determination in unfractionated tRNA and in acid-soluble extracts from murine thymus are reported in Table II. The ψ content of tRNA from normal thymus is very similar to that from lymphomatous tissue, whereas the concentration in acid-soluble extracts is higher in lymphomatous tissue. Given that tRNA-pseudouridine synthase activity in lymphomatous thymus has been found to be twice as higher as in normal tissue[16], whereas the ψ content of tRNA is the same in both types of tissue (see Table I), one can speculate that the serum ψ increase in neoplastic mice is due to a higher tRNA turnover in lymphomatous thymus.

Several methods for the determination of nucleosides in hydrolyzed tRNA have recently been described. The chromatographic–radiometric method of Randerath *et al.*[17] is very sensitive for the determination of many "major" and modified nucleosides. However, it is unsuitable for ψ determination, because ψ undergoes a chemical modification during the trialcohol labelling. Two other methods are based on the HPLC separation of nucleosides. The first[18] is characterized by a two-step isocratic elution of nucleosides, and the second[19] is based on a multistep gradient elution; both methods are performed on a reversed-phase column. In addition to giving a precise quantitation of ψ and the "major" nucleosides, comparable to that obtained with our procedure, both methods can be used to quantitate accurately many other modified nucleosides. However, for ψ determination, the single-step gradient elution used in our method has the advantage of being faster and requiring less complex HPLC equipment than the two procedures cited above[18,19]. Hence, our method is particularly suitable when numerous RNA samples have to be screened for ψ content.

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