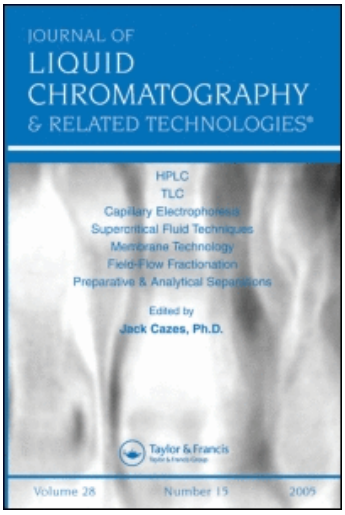


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# Diagnostic and Discriminatory Efficiency of Eight Serum Modified Nucleosides in HIV Infection and in At-Risk Subjects

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# DIAGNOSTIC AND DISCRIMINATORY EFFICIENCY OF EIGHT SERUM MODIFIED NUCLEOSIDES IN HIV INFECTION AND IN AT-RISK SUBJECTS

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## ABSTRACT

The aim of this study was to evaluate fluctuations in modified nucleoside levels in serum could be used in the diagnosis and monitoring of human immunodeficiency viral (HIV) infection. We used an HPLC technique to assay serum levels of eight modified nucleosides in three groups of patients: normal subjects, patients with HIV infection at various stages of the disease, and intravenous (i.v.) heroin users, HIV-negative. The mean levels of modified nucleosides in patients with HIV infection were 2 or 3 times higher than those of normal subjects, while they were only slightly increased in i.v. heroin users. With multivariate discriminant analysis we identified a panel of serum nucleosides that had a 92% diagnostic efficiency in the discrimination between HIV-positive subjects and i.v. heroin users, and a 96% diagnostic efficiency in distinguishing HIV-positive patients from normal subjects; this efficiency has been confirmed with a new group of incoming HIV-positive patients. Furthermore, we found that in HIV patients at stage II of the disease, classified according to the Center for Disease Control of Atlanta, those with the lowest values of the helper/suppressor (H/S) lymphocyte ratio showed the highest serum levels of modified nucleosides,

and that there was a statistically significant inverse correlation between $t^6A$ ($N^6$-threoninocarbonyl-adenosine), $m^1I$ (1-methylinosine) and $m^2_2G$ ($N^2N^2$-dimethylguanosine) levels and the H/S ratio.

## INTRODUCTION

Modified nucleosides derive from several species of cellular RNAs, in particular tRNA. They are synthesized through enzymatic modifications (methylation, acetylation, pseudouridylation etc) of the major nucleosides (e.g., adenosine, cytidine, guanosine, uridine) at post-transcriptional macromolecular level. After RNA catabolism, major nucleosides can be reutilized in cells thanks to various salvage mechanisms, or they can be further degraded to uric acid (purines) and beta-alanine (pyrimidines), while no salvage or degradation pathways exist for modified nucleosides. Therefore, this family of compounds can be considered end-products of the precursor macromolecules. Consequently, the levels of modified nucleosides in body fluids are, in principle, good indicators of the intracellular turnover rate of RNA (1-3), and more interestingly of specific tRNA molecules that have variable turnover rates (3).

In our laboratory it has been demonstrated in two experimental systems that retroviral infections are accompanied by an increased excretion rate of pseudouridine, the most abundant modified nucleoside in tRNA. In fact, inbred mice of the AKR strain, which have a very high incidence of spontaneous lymphomas, showed an increase in serum pseudouridine prior to the appearance of clinical and biological signs of the tumor (4). This increase could be related to a recombinant oncogenic retrovirus that is generated during the preneoplastic

period of AKR lymphomas and that is considered the etiologic agent of this lymphoma (4). In fact, pseudouridine levels were not increased in mice with chemically-induced lymphomas.

Further evidence that enhanced pseudouridine levels are possibly related to the above-mentioned mechanism came from a series of experiments we conducted on an experimental system consisting of chick embryo fibroblasts (CEF) infected and transformed by Rous sarcoma virus (RSV), another retrovirus. In the culture medium of these cells, pseudouridine levels were increased 60 hours after viral infection and before the appearance of any morphological sign of fibroblast transformation (5). Moreover, there was an overproduction in these cells of an isoaccepting tRNA for tryptophan, which is the primer of RSV reverse transcriptase (5).



The results obtained in the above-mentioned experimental systems prompted us to evaluate whether fluctuations in the levels of pseudouridine and other modified nucleosides that have been estimated in human serum (6,7), and for which reference values have been recently measured by us (8), could be used in the diagnosis and monitoring of human acquired immunodeficiency syndrome (AIDS), a disease caused by HIV infection.

## MATERIALS

### Patients and Specimens

Three groups of subjects were recruited for this study. The first group consisted of 36 healthy subjects (18 men and 18 women, mean age 34.7 years)

with no evidence of chronic or acute diseases on the basis of clinical and laboratory findings.

The second group comprised 33 patients (27 men and 6 women, mean age 29.4 years) who had antibodies to HIV 1 first detected by enzyme immuno-assay (Roche, Milan, Italy) and confirmed by western blot analysis (Du Pont De Nemours Inc., Whillington, DE, USA). A history of i.v. heroin use was reported in 28 of these 33 patients (85%), and homosexual behavior in 6 of these 28, and in 2 of the non heroin users. On the basis of clinical examination and laboratory testing this second group of patients was divided into three disease-classes according to the classification of the Center for Disease Control (CDC) of Atlanta (9): 9 patients had no clinical symptoms and were classified as being at stage II of the disease (7 were i.v. heroin users, with at least 3 years of drug abuse, and 2 were homosexuals); 7 patients showed palpable lymphonodes in at least two non-inguinal areas and they were assigned to the III class (6 of these subjects had a history of i.v. heroin use of more than 7 years and 1 was homosexual); 17 patients were at stage IV of the disease as they showed the presence of one or more of the following clinical features: opportunistic infection or other infection such as oral candidosis and herpes zooster, neurologic pathologies, such as dementia and encephalopathy, wasting syndrome, neoplasias such as Kaposi sarcoma, non Hodgkin's lymphoma etc.; of these 17 patients, 15 had a history of i.v. heroin use lasting more than 5 years, and 5 were homosexuals. In a second stage of this study, serum samples from a group of 8 patients, who had antibodies

to HIV 1, but no history of heroin use, were analyzed to verify "a posteriori" the effectiveness of statistical analysis.

The third group of patients consisted of 7 i.v. heroin users who did not show HIV antibodies (mean age 28.3 years; all were male) after the tests indicated above. These subjects are considered at a high risk for HIV infection (10).

Venous blood specimens were collected from fasting subjects into vacutainer tubes (Becton-Dickinson, Rutherford, NJ, USA). Whole blood samples, using heparin as anticoagulant, were used for lymphocyte surface marker assays, while concentrations of modified nucleosides were assayed on serum specimens.



## METHODS

### Assays

Modified nucleosides were assayed by a two-step HPLC method (6,7). The nucleosides were first isolated from serum by affinity chromatography using phenylboronate gel columns, after which, the isolated nucleosides were separated and quantitated with an HP 1090M liquid chromatographic apparatus (Hewlett Packard, San Diego, CA, USA). One mL of serum was aliquoted into 1.5 mL polypropylene centrifuge tubes, and the internal standard, 3-methyluridine ($m^3U$), was added at a concentration of 0.5 nmol/mL of serum. The internal standard was employed to calculate recovery of modified nucleosides, according to the procedure reported in refs. 6 and 7. The sample was then filtered through a 25,000 - 30,000 MW cut-off membrane (YMT type, Amicon Corp., MA, USA) using the

MPS-1 centrifree system (Amicon Corp.) to eliminate the proteins. The ultrafiltrate was buffered with 250 µL of a 2.5 M ammonium acetate solution, pH 9.0. The boronate gel column was equilibrated by passing 15 mL of a 2.5 M ammonium acetate solution (pH 8.8) through the column. The sample was then loaded on the pre-equilibrated gel column. The gel was washed with 3 mL of the latter solution and after with 300 µL of a 50% methanol/water solution. The nucleosides were eluted with 5 mL of a solution of 0.02 M formic acid in 50% methanol/water, and collected in a polypropylene tube. Methanol was removed from the sample with a centrifugal evaporator and the sample was frozen and lyophilized.



The sample was dissolved in 200 µL of water and 180 µL of the resulting solution were injected into an HPLC apparatus for the separation and quantitative steps. The nucleosides were eluted using the following buffers: (a) 0.01 M ammonium phosphate solution containing 2.5% methanol, pH 5.1; (b) 0.01 M ammonium phosphate solution containing 20% methanol, pH 5.3; and (c) 0.01 M ammonium phosphate solution containing 35% acetonitrile. The first two buffers are used in a gradient at the beginning of the chromatographic run for the elution of nucleosides from the column; buffer C is used in the final part of the run to elute other compounds that may be present in the column, thus preparing it for the following run. The column used was a Supelco LC 18s 150 x 4.6 mm (Supelco, Bellafonte, PA, USA). The chromatographic conditions (gradient steps, temperature, etc.) are described in detail in refs. 6 and 7.

The isolated nucleosides were analysed with the computerized photodiode array detector of the HPLC apparatus; and identified by comparing the retention

times and absorption spectra of compounds appearing at various chromatographic peaks with those of pure reference standard of known modified nucleosides. Quantitation was performed by comparing peak absorbances at given wavelengths (254 and 280 nm) with that of known amounts of pure nucleosides. The purity of the chromatographic peaks was evaluated by comparing the absorbance ratio at two wavelengths (254 and 280 nm) of nucleosides isolated from serum with pure standards.

CD4 (helper) and CD8 (suppressor) T-lymphocytes were counted with a Coulter Epics flow cytometer (Coulter Co, Hialeah, FL, USA) (11) and commercially available monoclonal antibodies (Coulter Co) (12). The helper/suppressor ratio was also calculated for all the specimens.



Statistics

The mean and standard deviation of each nucleoside tested were calculated in the three groups of subjects. The means of values obtained for each nucleoside were compared among the three groups of subjects by the one-way analysis of variance (ANOVA), followed by the Scheffè multiple comparison test (13). The diagnostic characteristics of each nucleoside were calculated according to Galen and Gambino (14). Moreover we subjected the results for each modified nucleoside assay to the multivariate discriminant analysis to assess whether their combined use allows a better diagnostic classification of the three groups of subjects studied (13). In this statistical model, modified nucleosides are independent variables, used in a linear combination, to calculate a discriminant

function by which the values corresponding to each new patient can be assigned with a certain probability to the groups compared, in our case healthy subjects, i.v. heroin users, and HIV-infected patients. Thus, the information contained in multiple independent variables, e. g., blood serum concentrations of various modified nucleosides singularly considered, is compounded in a single index.

Subsequently, the discriminant function has been used to classify a new incoming group of HIV-positive patients whose sera were analyzed in the second stage of this study to validate data obtained with the first group of patients.

Finally, linear regression analysis followed by analysis of variance of regression residues (13) was used to evaluate the relationship between the serum concentration of modified nucleosides and the helper/suppressor lymphocyte ratio.



## RESULTS

Table 1 shows the means and standard deviations of serum concentrations of modified nucleosides in healthy subjects, in the i.v. heroin user group and in patients with HIV infection. The concentration of the 8 modified nucleosides assayed were greatly elevated in HIV-positive patients as compared to the healthy subjects ($p<0.001$ by ANOVA followed by the Scheffè multiple comparison test). The mean concentration of $ac^4C$ and $m^6A$ showed the highest increments and reached mean levels almost 3 times higher than those of healthy subjects. The levels of modified nucleosides were increased also in i.v. heroin users, but to a lesser extent than in patients with HIV infection. In fact, only the mean levels of pseudouridine and $m^6A$ in i.v. heroin users were statistically different with respect

## TABLE 1

Serum Levels of Modified Nucleosides in Normal Subjects, in Intravenous Heroin Users and in HIV+ Subjects.

| | NORMAL SUBJECTS (n=36) | | INTRAVENOUS HEROIN USERS (n=7) | | HIV INFECTION (n=33) | |
|---|---|---|---|---|---|---|
| | M. | S.D. | M. | S.D. | M. | S.D. |
| Pseu (nmol/mL) | 2.61 | (0.31) | 3.95 | (1.17)[d] | 5.01 | (1.84)[a] |
| PCNR (pmol/mL) | 57 | (13) | 81 | (28) | 91 | (40)[a] |
| $m^1I$ (pmol/mL) | 74 | (21) | 72 | (20) | 110 | (47)[ab] |
| $m^1G$ (pmol/mL) | 22 | (4) | 29 | (7) | 39 | (16)[a] |
| $ac^4C$ (pmol/mL) | 128 | (52) | 177 | (93) | 340 | (235)[ab] |
| $m^2{}_2G$ (pmol/mL) | 21 | (2) | 26 | (10) | 36 | (13)[ab] |
| $t^6A$ (pmol/mL) | 40 | (7) | 58 | (24) | 86 | (35)[ac] |
| $m^6A$ (pmol/mL) | 12 | (2.5) | 31 | (9)[d] | 34 | (16)[a] |

[a]: significantly different with respect to the values of normal subjects ($p<0.001$ level by ANOVA and the Scheffè test for multiple comparison analysis); [b]: significantly different with respect to the values of i.v. heroin users ($p<0.05$ level by ANOVA and the Scheffè test for multiple comparison analysis); [c]: significantly different with respect to the values of i.v. heroin user ($p<0.01$ level by ANOVA and the Scheffè test for multiple comparison analysis); [d]: significantly different with respect to the values of normal subjects ($p<0.05$ level by ANOVA and the Scheffè test for multiple comparison analysis).

Pseu, pseudouridine; PCNR, 4-pyridone-3carboxamide-$N^1$-ribofuranoside; $m^1I$, 1-methylinosine; $m^1G$, 1-methylguanosine; $ac^4C$, $N^4$-acetylcytidine; $m^2{}_2G$, $N^2N^2$-dimethylguanosine; $t^6A$, $N^6$-threoninocarbonyladenosine; $m^6A$, $N^6$-methyladenosine, and ANOVA, analysis of variance.

to values obtained in healthy subjects ($p<0.05$ by ANOVA followed by the Scheffè multiple comparison test). In an attempt to identify diagnostic parameters, we transcribed the data in a scatterplot as shown in Figure 1. In HIV-positive patients the diagnostic sensitivity of each modified nucleoside was calculated using as cut-off value the upper reference limit of normal subjects (97.5% diagnostic specificity) according to previously published data (8). In this case, diagnostic

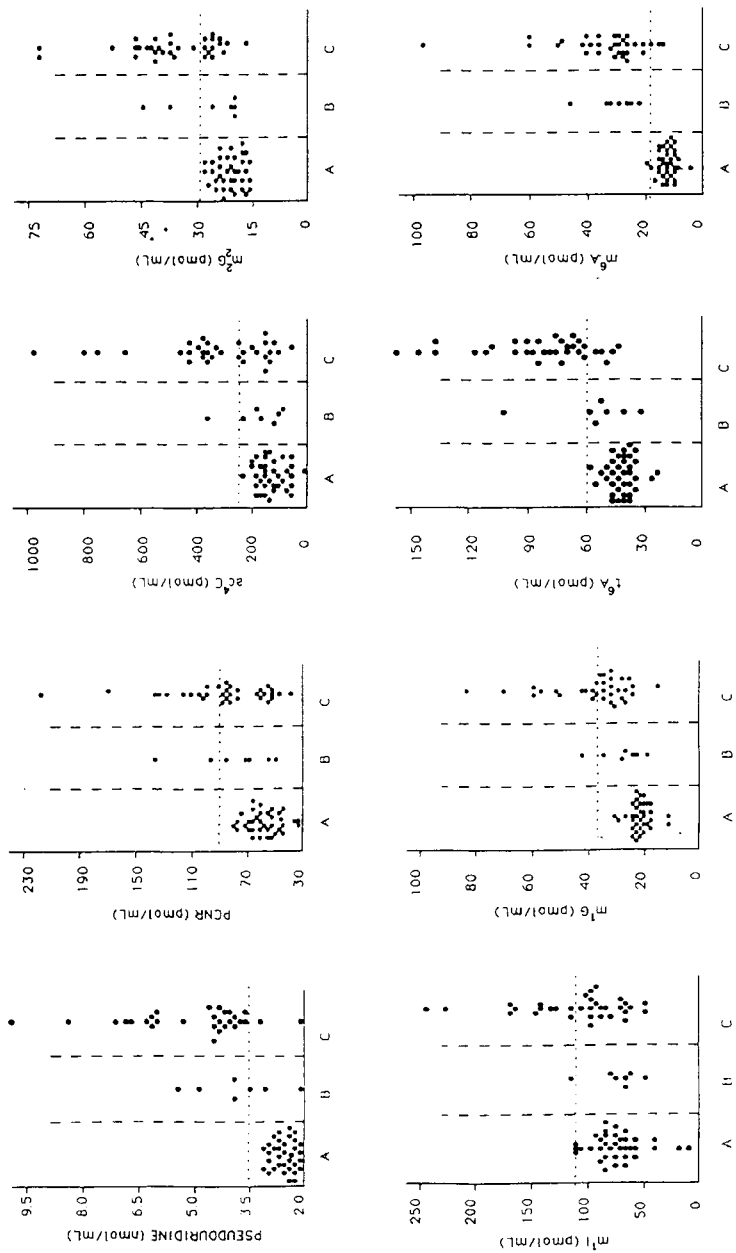


FIGURE 1: Levels of some modified nucleosides in healthy subjects (panel A), intravenous heroin users (panel B), and HIV-positive patients (panel C). Dashed horizontal lines are cut-off levels calculated according to data of normal subjects as reported previously (8).

sensitivity reached around 90% for some nucleosides (pseudouridine, $t^6A$ and $m^6A$) and ranged from 30% to 75% for the others. Diagnostic sensitivity was not satisfactory when comparing the values in the heroin users used as controls with the levels in the HIV-positive patients (Figure 1, panels B and C).

Therefore, as specified under Statistics, the serum levels of the various modified nucleosides were examined with multivariate discriminant analysis to calculate a discrimination equation. The patient score (P.S.) is calculated as follows:

$$P.S.= A_o+A_1X_1+.....A_nX_n$$

where $X_{1...n}$ are the values of the independent variables (in our case, serum modified nucleoside levels of the patients), $A_{1...n}$ are numerical coefficients resulting from discriminant analysis that maximize differences of patient scores among the various groups of patients compared, and $A_0$ is also a numerical constant.

Tables 2 and 3 show the discrimination equations applied to the comparison between healthy subjects versus HIV-positive patients, and between the latter versus i.v. heroin users, respectively. Each table also shows the percentages of cases correctly classified on the basis of calculated patient scores, and the cut-off values used for the classification. Discrimination among the groups compared is clearly very satisfactory even in the case of i. v. drug users versus HIV-positive patients. Furthermore, the discriminant equation was then applied to the 8 heroin-free HIV-positive patients who served as control for the statistical analysis to validate the results obtained: 7 of them were correctly classified (87.5

## TABLE 2

Multivariate Discriminant Analysis of Modified Nucleoside levels to Distinguish Between Healthy Subjects and HIV+ Patients (see text for details).

**Discriminant equation*:**

Patient score (PS): 11.60 + Ln [Pseu] x 0.83 - Ln [PCNR] x 0.49 - Ln [$m^1I$] x 0.33 + Ln [$m^1G$] x 0.42 + Ln [$ac^4C$] x 0.47 + Ln [$m^2_2G$] x 0.27 - Ln [$t^6A$] x 0.16 + Ln [$m^6A$] x 2.99

*the values of each nucleoside in the Patient Score formula are expressed as natural logarithm of serum concentration (see Table 1)

**Calculated cut-off** = 0.0; therefore: PS < 0 = Normal subjects, PS > 0 = HIV+

**Correctly classified cases:** Normals subjects: 100%; HIV+ patients: 91%

**Overall discrimination efficiency:** 96%



% of overall discrimination efficiency) as being HIV infected, thus confirming the efficiency of the discriminant functions calculated on the first subset of patients.

On the overall group of HIV patients we then segregated the results in the drug-free HIV-positive patients from those in the drug-using HIV-positive patients, as shown in Table 4. From the table it emerges that there is no significant difference, by Student's t test, in the levels of modified nucleosides between the heroin-users and the heroin-free subjects. We have also calculated for both groups, the differences with respect to control subjects (see Table 1) showing that there

**TABLE 3**

Multivariate Discriminant Analysis of Modified Nucleoside levels to Distinguish Between Intravenous Heroin Users and HIV+ Patients (see text for details).

**Discriminant equation*:**

Patient score (PS): -0.96 + Ln [Pseu] x 1.12 - Ln [PCNR] x 0.68 + Ln [$m^1I$] x 0.51 - Ln [$m^1G$] x 0.8 + Ln [$ac^4C$] x 0.62 + Ln [$m^2_2G$] x 0.86 + Ln [$t^6A$] x 1.75 - Ln [$m^6A$] x 1.17

*the values of each nucleoside in the Patient Score formula are expressed as natural logarithm of serum concentration (see Table 1)

**Calculated cut-off** = -0.37; therefore: PS < -0.37 = i.v. heroin users, PS > -0.37 = HIV+

**Correctly classified cases**: I.v. heroin users: 86%; HIV+ patients: 94%

**Overall discrimination efficiency:** 92%



is for all modified nucleosides examined a significant difference ranging from $p<0.01$ to $p<0.001$.

Figure 2 shows the increases of modified nucleosides mean levels of the overall group of HIV-positive patients and of the HIV-negative intravenous heroin users, expressed as percentage of increase with respect to the mean of control values. There is clearly a great increase of modified nucleosides serum levels in the HIV-positive patients with respect to those both in the normal subjects and in

## TABLE 4

Serum Levels of Modified Nucleosides in HIV+ Subjects Segregated into Heroin Users and Heroin Free Subjects.

| | HIV+ PATIENTS | | | | |
|---|---|---|---|---|---|
| | HEROIN USERS (n=27) | | HEROIN FREE (n=14) | | Student's t test |
| | M. | S.D. | M. | S.D. | n.s. |
| Pseu (nmol/mL) | 5.09 | (1.85) | 3.96 | (1.22) | n.s. |
| PCNR (pmol/mL) | 88 | (41) | 67 | (27) | n.s. |
| $m^1I$ (pmol/mL) | 105 | (44) | 123 | (45) | n.s. |
| $m^1G$ (pmol/mL) | 37 | (13) | 39 | (17) | n.s. |
| $ac^4C$ (pmol/mL) | 329 | (229) | 283 | (157) | n.s. |
| $m^2_2G$ (pmol/mL) | 34 | (11) | 33 | (14) | n.s. |
| $t^6A$ (pmol/mL) | 82 | (31) | 67 | (32) | n.s. |
| $m^6A$ (pmol/mL) | 35 | (18) | 26 | (11) | n.s. |

Pseu, pseudouridine; PCNR, 4-pyridone-3carboxamide-$N^1$-ribofuranoside; $m^1I$, 1-methylinosine; $m^1G$, 1-methylguanosine; $ac^4C$, $N^4$-acetylcytidine; $m^2_2G$, $N^2N^2$-dimethylguanosine; $t^6A$, $N^6$-threoninocarbonyladenosine; $m^6A$, $N^6$-methyladenosine, and ANOVA, analysis of variance.

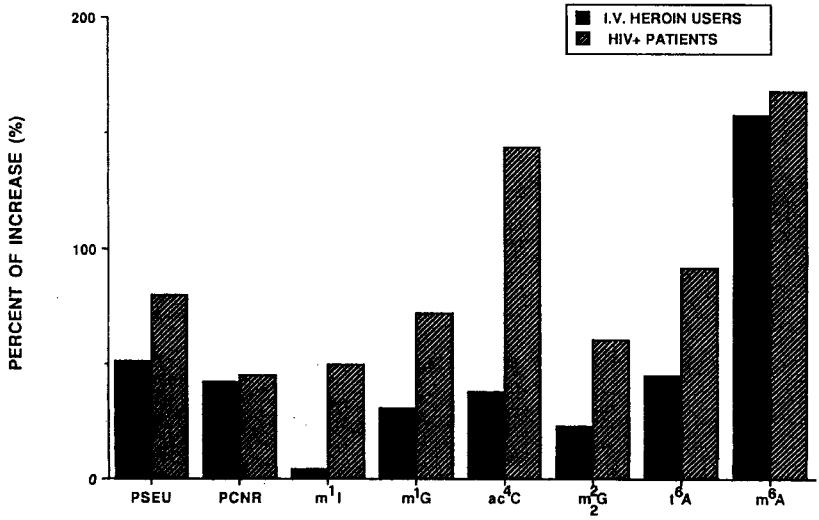


FIGURE 2: Mean levels of modified nucleosides in intravenous heroin users and in HIV-positive patients, expressed as per cent of increase with respect to the mean levels found in healthy subjects.

HIV-negative heroin users, with the exception of pseudouridine, PCNR and $m^6A$ whose increases are not much higher than those of HIV-negative heroin users.

Furthermore, by using the discriminant analysis, the efficiency of discrimination of each of the two groups of HIV-positive patients of Table 4 from both the normal subjects and the HIV-negative heroin users is in any case about 90%.

As expected, the helper vs suppressor lymphocytes ratio was decreased in HIV-infected patients and values were particularly low in patients at CDC stages III and IV, mean values being 0.13 and 0.40, respectively. In the 9 patients at stage II, the mean helper/suppressor ratio was higher (0.55) showing values comprised between 1.00 (a value usually found in healthy subjects) and 0.13. In these patients the helper/suppressor ratio showed a statistically significant inverse correlation with the serum concentration of three nucleosides, $m^1I$, $t^6A$ and $m^2_2G$ (Figure 3). This finding suggests that these three serum indexes could be used to monitor the progression of AIDS.



## DISCUSSION

Until recently, studies on correlations between pathophysiologic conditions and modified nucleoside levels in blood serum were limited to pseudouridine, which was found to be a marker of neoplasia including leukemias and lymphomas (1, 15-18). In an attempt to widen the diagnostic potential of these compounds, we used a recently devised HPLC procedure (6, 7) to evaluate eight modified nucleosides in blood serum, and to determine reference values in healthy subjects

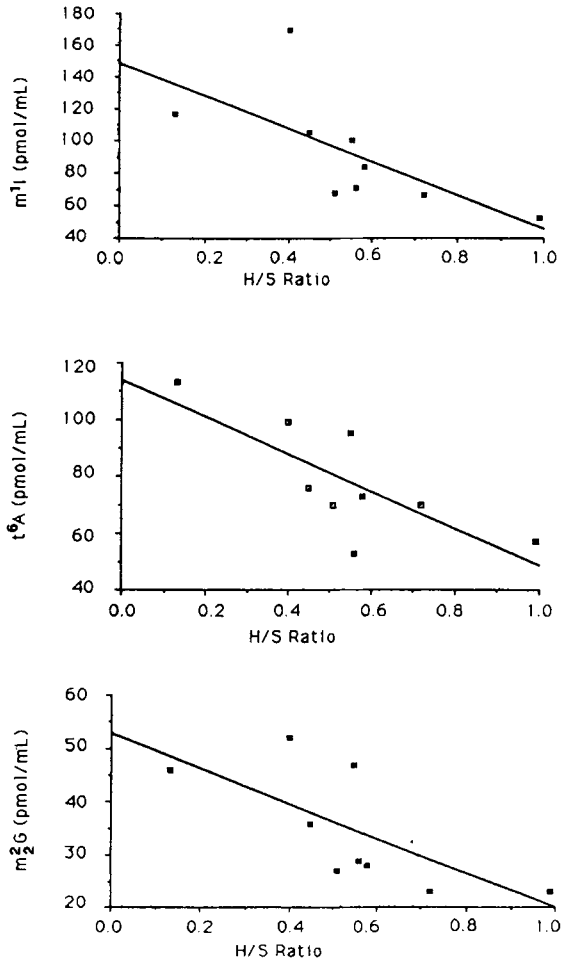


FIGURE 3: Inverse correlation between serum values either of $m^2_2G$ or $t^6A$ or $m^1I$ and the H/S lymphocite ratio in II stage HIV-positive patients.

of both the USA and Italy (8). Now we have extended our study to investigate the profile of these eight compounds in the serum of AIDS victims and at-risk subjects with respect to healthy subjects. Our results show, for the first time, that the concentrations of all the eight modified nucleosides measured are greatly enhanced in AIDS patients. In some instances ($m^6A$, $ac^4C$) levels reached three times the upper normal level. In i.v. heroin users, increases were much smaller, being significant only for pseudouridine and $m^6A$. This latter observation suggests that at least some of these modified nucleosides estimated in sera could signal the state of altered tRNA turnover that seems to accompany the early phases of HIV infection in patients that are still seronegative. Indeed, Schoch et al. (3) demonstrated that increased urinary excretion levels of $m^2{}_2G$ and $t^6A$ are specifically attributable to the turnover of total tRNA molecules. It remains to be seen whether the generalized, as well as single species, increases of tRNA turnover are effectively related to the early phases of HIV infection, as suggested by the results obtained in the two retroviral experimental systems mentioned in the Introduction (4, 5), or whether they depend on other concurrent diseases or on a direct effect of heroin. However, our preliminary results showing no difference between heroin and non heroin user subsets of HIV-positive patients seem to rule out that drug consumption plays a primary role in the increase of modified nucleosides serum levels that we observed in HIV-positive patients. Methods for the detection of viral RNA by amplification or viral culture are required to test this possibility, and to establish whether or not the altered RNA turnover is associated with RNA viral multiplication.

The possibility of estimating eight modified nucleosides in the same HPLC run (6, 7), and of quantitating them by automated integration of peak areas, widens the number of markers that can be used to signal disease states, to monitor their evolution and also to improve the differential diagnosis between confounding clinical situations. In fact, with discriminant multivariate analysis it is possible to use combinations of several nucleosides, each one bearing a different weight, to enhance the discrimination power between two physiopathological conditions. In our case, we were able to identify the HIV-infected patients with an overall efficiency of 90-95%, and to correctly classify 100% of normal subjects. The mean value of each modified nucleoside was higher, though in general not statistically significant, in heroin abusers than in normal subjects. We were able to discriminate between HIV-positive patients and the heroin group only by using a combination of modified nucleosides obtained with discriminant multivariate analysis; with this panel, discrimination reached about 90%. Therefore, the simple and fairly fast assay of eight modified nucleosides in a single HPLC run has significant discrimination power. Ongoing experiments in our laboratory seem to indicate that the use of panels of modified nucleosides, identified with discriminant multivariate analysis, could be effective also in differentiating between various types of leukemias and lymphomas. Furthermore, by monitoring some modified nucleosides ($m^6I$, $t^6A$, and $m^2{}_2G$) we were able to obtain a linear inverse correlation with the lymphocyte helper/suppressor cell ratio which is a recognized signal of the progression of HIV-positive infection. Taken together, the data

presented here also support the use of these nucleosides in monitoring the evolution of the disease status.

In conclusion, this study indicates that panels of several modified nucleosides, which can be estimated in a single HPLC run, can be useful to help in the diagnosis of HIV-positive patients, to discriminate, not only from normal, but also from at-risk subjects who are not yet seropositive, and finally to monitor the evolution of the disease at an early stage of HIV infection. The latter point is of particular relevance in the drug management of this condition.



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