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Journal of Chromatography B, 803 (2004) 345–351
JOURNAL OF CHROMATOGRAPHY B
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# Liquid chromatographic method for the determination of uridine in human serum

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Received 23 October 2003; received in revised form 5 January 2004; accepted 12 January 2004

**Abstract**

To evaluate uridine levels in humans we developed a very sensitive and specific high-performance liquid chromatographic method for the determination of uridine in serum. We use techniques which are available in a standard analytical laboratory. Chromatographic analysis was carried out on a Phenomenex Aqua C18 5 μ 125A column protected by a guard cartridge system. Potassium dihydrogen phosphate buffer–acetonitrile was used as an eluent and oxypurinol as the internal standard. All sample preparation steps were done at 4 °C and the autosampler was cooled down to 4 °C. The calibration curve was linear throughout the calibration range from 0.25 to 100 μmol/l. This method was primarily established to evaluate uridine serum levels in patients with HIV infection since patients on highly active antiretroviral therapy (HAART) might develop metabolic disturbances that could lead to severe and fatal lactic acidosis due to mitochondrial toxicity. It is suggested that a limited or inadequate uridine supply is at least in part responsible for the onset of such deterioration.
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*Keyword:* Uridine

## 1. Introduction

Uridine (1-β-D-ribofuranosyluracil) is an abundant nucleoside in all cells and seems to play an important role in subcellular, cellular and tissue function and protection [1]. Although, the source of uridine in plasma is not fully understood, the concentration of uridine in tissues is tightly regulated by the enzymatic activities of uridine de novo synthesis and salvage, as well as of membrane transport. Uridine has crucial functions in regulating a large variety of biological systems.

The detection of uridine in extracellular fluids by HPLC was first described in 1981 [2]. A HPLC method suitable for the detection of uridine in HIV patients, receiving concomitant HAART and future uridine supplementation, does not exist.

Intravenous or oral supplementation of uridine has been suggested to be of potential benefit in improving the therapeutic index of cancer medication and also in abolishing the mitochondrial toxicity of anti-HIV nucleoside analogue reverse transcriptase inhibitors (NRTIs). NRTIs inhibit the synthesis of mitochondrial DNA, are associated with cytotoxicity based respiratory chain function disorders and thus possibly also affect the de novo synthesis of uridine. It has been demonstrated in vitro and in animal models that uridine may revoke such mitochondrial toxicity and this may be the case in humans as well [3–7]. Uridine is, therefore, a likely candidate for further clinical studies in HIV-patients, aimed at alleviating the mitochondrial toxicities of NRTIs. For such studies, it is important to have access to an analytical method which is able to determine the uridine concentrations in HIV-patients without interference by nucleosides used in antiretroviral drug combinations. Preliminary results from our HIV outpatients showed a clear correlation between the development of a potentially fatal lactic acidosis and low serum uridine levels.

The aim of this study is to monitor uridine levels in human serum. We present a new fast, effective and reliable method

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doi:10.1016/j.jchromb.2004.01.018

using HPLC with a special column phase. Reversed-phase high-performance liquid chromatography (RP-HPLC) is widely used in pharmaceutical analysis. For many polar compounds, however, mobile phases employing little or no organic modifier have to be used to achieve retention and separation. These highly or totally aqueous conditions can lead to the collapse of the octyl or octadecyl alkyl chains commonly used for RP-HPLC [8]. Therefore, we used a special column phase with a hydrophilic endcapping that is well suited for the analysis of polar compounds.

## 2. Experimental

### 2.1. Chemicals and reagents

Uridine (1-β-D-ribofuranosyluracil, $C_9H_{12}N_2O_6$, $M_W$: 244.2), Oxypurinol (3,7-dihydro-1H-purine-2,6-dione; $C_5H_4N_4O_2$, $M_W$: 152.11) as the internal standard (IS) and bovine serum albumin (BSA) (Albumin, bovine serum, Fraction V, minimum 96%) were obtained by Sigma–Aldrich Chemicals GmbH (Taufkirchen, Germany). Acetonitrile, potassium dihydrogen phosphate, phosphoric acid, sodium hydroxide and demineralised distilled water (gradient grade) were obtained from Merck (Darmstadt, Germany).

### 2.2. Chromatographic equipment and conditions

Chromatographic analyses were performed using a Beckman–Coulter Gold HPLC system (Krefeld, Germany) equipped with a solvent delivery module (126 solvent module), a diode array UV-Vis detector (model 168 NM), an autosampler (model 508) and a column oven (model jetstream 2 plus).

The chromatographic separation was achieved with a Phenomenex Aqua C18 5 μ 125A (250 mm × 2 mm) column (Phenomenex, Aschaffenburg, Germany) protected by a guard cartridge system (LC 18 security guard column (Part No ICJO4282), Phenomenex, Aschaffenburg, Germany).

The isocratic elution was performed with potassium dihydrogen phosphate buffer (0.67 M, adjusted to pH 4.0 with phosphoric acid–acetonitrile (98:2, (v/v)) filtered through a 0.45 μm cellulose nitrate membrane filter (Schleicher & Schuell, Dassel, Germany). Flow rate was 0.2 ml/min and the column temperature was controlled at 30 °C. The PDA detector was set at 260 nm. The injection volume for all samples was 100 μl and temperature of the autosampler module was set at 4 °C. Chromatograms were recorded using Beckman–Coulter 32 Karat Software.

### 2.3. Stock solutions

An amount of 24.42 mg uridine was transferred to a 100 ml volumetric flask and dissolved in 0.67 M potassium dihydrogen phosphate to yield a final concentration of 1000 μmol/l. This solution was stored at −20 °C and was stable for at least 3 months. Working solutions (concentration range of 0.25–100 μmol/l) were prepared by diluting the stock solution in 0.67 M potassium dihydrogen phosphate.

### 2.4. Internal standard (IS) preparation

Oxypurinol (10 mg) was dissolved in distilled water containing 0.1 N NaOH to achieve a final concentration of 0.65 mmol/l. This solution was stable at 4 °C for at least five months.

### 2.5. Calibration standards

Five gram BSA powder were dissolved in 100 ml distilled water to achieve a solution with a final BSA concentration of 5%. The standard curve consisted of BSA-samples spiked with uridine in eight different concentrations (in the range of 0.25–100 μmol/l). The standard curve samples, prepared on the day of use, were generated in duplicate for each analytical run.

### 2.6. Sample collection

All patient samples were collected at our outpatient care unit, division of hepatology and infectious diseases, University of Wuerzburg, Germany, after informed consent. For sample collection, standard Sarstedt® Monovettes were used (Sarstedt, Nümbrecht, Germany). Blood samples were drawn, centrifuged and serum was stored at −20 °C until further analysis. Detection of haptoglobin and lactate dehydrogenase in serum was used to rule out any significant haemolysis as erythrocytes represent uridine reservoirs.

### 2.7. Sample preparation procedure

Aliquots (0.5 ml) of BSA-standards and serum samples were pipetted into 1.5 ml snap-cap micro-centrifuge vials. The IS oxypurinol (30 μl) was added and the solutions were briefly vortex mixed. For protein precipitation, 0.7 ml of ice cold acetonitrile was added and vortex mixing was followed by incubation for 1 h at 4 °C. Samples were then centrifuged for 5 min at 21,382 g at 4 °C (Eppendorf centrifuge model 5403). The supernatant was transferred to a 5 ml glass tube and evaporated to dryness at 40 °C under a stream of nitrogen. The dried residues were reconstituted in 0.5 ml of 0.67 M potassium dihydrogen phosphate buffer (pH 4.0) and drawn into auto-sampler vials. Injection volumes of samples and standards (100 μl) were performed with a precooled (4 °C) auto-sampler.

### 2.8. Specificity and selectivity

In order to evaluate levels of compounds with potential for interference with the analytical method the following compounds were determined: abacavir, adefovir, allopurinol, amprenavir, ceftriaxon, didanosine, efavirenz, flu-

conazole, folinic acid, ganciclovir, indinavir, itraconazole, lamivudine, lopinavir, methadone, methotrexate, nelfinavir, M8-metabolite of nelfinavir, nevirapine, oxazepam, oxypurinol, pyrazinamide, pyrimethamine, ranitidine, rifampicin, ritonavir, saquinavir, lopinavir, stavudine, sulfamethoxazole, sulfadoxin, trimethoprim, uric acid, zalcitabine, and zidovudine. Serum samples of patients who were taking the mentioned drugs at an established dose were evaluated for interference with IS and uridine. Other endogenous nucleosides (adenosine, cytidine, guanosine, and thymidine) were analyzed by dissolving each substance in the same manner as the calibration samples.

### 2.9. Precision and accuracy, linearity and recovery

Intra-day accuracy and precision of the method were determined by measuring nine replicate serum samples at two different concentrations of uridine (5 and 50 μmol/l).

Accuracy was calculated as the relative error of the nominal concentration. Precision was expressed in terms of relative standard deviation and obtained by analysis of variance (ANOVA) for each test concentration using the analytical run as the grouping variable.

Inter-day accuracy and precision was calculated out of three samples using two solutions with concentrations of uridine at 5 and 50 μmol/l. Analyses were performed on eight different days.

The linearity of the response for the assay was established over the concentration range of 0.25–100 μmol/l of uridine. Linearity and assay reproducibility were determined by measuring the standard concentrations in five assay runs on separate days. The linearity of calibration curves was tested with the *F*-test for lack of fit, using a weight factor of (1/concentration$^2$).

The absolute recovery was calculated by comparing peak areas obtained from freshly prepared samples with those found by direct injection of aqueous standard solutions of the same concentration.

### 2.10. Stability

Blank serum samples were spiked with an aliquot of diluted uridine stock solution to achieve concentrations of 5.0 and 50 μmol/l. Three samples of each concentration were stored at 4 °C for 24 h, at room temperature for 24 h and for 30 days at −20 °C. An additional set of three samples underwent four freeze–thaw-cycles. After the storage period, the samples were analysed and compared to freshly prepared control samples.

### 2.11. Limit of detection

The limit of detection (LOD) was defined by the lowest detectable concentration yielding a signal-to-noise ratio of three, indicating a significant difference of spiked and blank samples in serum samples of three individuals as determined by the two-tailed, paired Student's *t*-test.

### 2.12. Limit of quantification

The limit of quantification was determined by repeated analysis of spiked serum samples ($n = 3$). Precision and accuracy of the determination at the limit of quantification were calculated as described above in Section 2.9. Following international recommendations a precision (C.V. (%)) $\leq$ 20% and an accuracy (R.E. (%)) $\leq \pm 20\%$ are acceptable at the limit of quantification.

### 2.13. Calculation and data analysis

All statistical calculations were performed with the Statistical Product and Service Solutions (SPSS) for Windows, version 11.5.dt. (SPSS, Chicago, IL, USA).

## 3. Results and discussion

### 3.1. Chromatography and detection

Evaluation of uridine serum levels in HIV positive humans treated with NRTI drugs may become eminent important regarding detection and management of mitochondrial toxicity or lactic acidosis. A first description of the detection of the nucleoside uridine in extracellular fluids by HPLC was in 1981 [2]. For drugs such as the antimetabolite 5-fluorouracil, 2-deoxyuridine and other substances with chemical similarity to uridine, several detection methods have been evaluated i.e. [9], but a HPLC method suitable for the detection range expected in humans (supplemented with uridine and) receiving concomitant highly active antiretroviral therapy (HAART) does not exist. In a recently published paper rat plasma uridine levels were detected by HPLC–ESI–MS, whereas cytidine, another endogenous nucleoside, was used as IS. So far, this MS method was not evaluated in humans [10]. In contrast the described method relies on standard HPLC conditions without MS and uses oxypurinol as a non-endogenous IS. An equal baseline and very good sensitivity of our assay has been achieved. Peak shape, separation from contaminating serum compounds or metabolites as well as the separation from other endogenous substances was satisfactory.

At low serum concentrations, uridine showed partial loss of substance over 24 h if samples were stored at room temperature. These findings were not described by former investigations on uridine and we were able to avoid such complications by cooling samples to 4 °C or handling them under frozen conditions. Therefore, the autosampler was permanently kept at 4 °C.

It has been described previously, that a collapse of the octyl or octadecyl alkyl chains commonly used for RP-HPLC [8], can occur under highly or total aqueous con-

ditions. For retention of small polar molecules by HPLC the use of a highly aqueous layer is recommended. To perform optimal peak shape, the eluent of our method contains a small amount of organic modifier. The chosen composition of the eluent in combination with the hydrophilic endcapping of the column phase was well suited for good separation and quantification of uridine in human serum samples. So, a loss of retention and selectivity of the analytical column was prevented for more than 400 injections.

For the preparation of the standard curves a 5% BSA solution was used, a concentration similar to the physiological serum albumin fraction in man. Human serum albumin (HSA) was not applied since there is no advantage when compared to BSA but HSA was much less cost effective.

### 3.2. Specificity and selectivity

Serum samples from both healthy individuals and HIV infected patients were devoid of interference near the retention times of uridine and the internal standard. This was applicable only for persons not taking allopurinol for elevated uric acid levels or gout. Patients taking allopurinol could not be evaluated due to the endogenous metabolism from allopurinol to oxypurinol by the enzyme xanthinoxidase and interference with the IS. Mean uridine serum levels (±standard deviation of the mean) in healthy controls were $6.45 \pm 2.34$ μmol/l and did not differ significantly from patients with HIV at different stages of the disease ($6.23 \pm 1.98$ μmol/l).

The analysis of serum samples containing different antiretroviral drugs as well as frequently used medication in the group of HIV infected individuals, showed no interference neither with the preparation procedure nor with the analytical method or any concomitant used drugs. A clear distinction of the chromatographic peaks to a certain NRTI was not intended in this chromatographic setting but serum of the tested individuals on different NRTI based regimens was devoid of interference with uridine or the IS.

### 3.3. Limit of quantification and detection

The limit of detection of uridine in serum was determined at 0.05 μmol/l. The lower limit of quantification was reached at a concentration of 0.25 μmol/l (Fig. 1). Fig. 2 shows a chromatogram with a blank BSA sample. Endogenous levels ranged from 3 to 10 μmol/l in healthy volunteers. As in individuals substituted with different amounts of uridine preparations, first repeat measurements determined uridine serum ranges up to 100 μmol/l [3], the selected range for the quantification of uridine in serum was adapted to this level. Whether a further extension in range is helpful or not remains open.

### 3.4. Stability

The stability of uridine under various conditions is shown in Table 1. Uridine is stable for at least 24 h if handled at 4 °C or kept frozen.

### 3.5. Accuracy, precision, linearity and recovery of the assay

Intra-day accuracy was 2.07% (−2.16%) and precision was 4.41% (2.06%) for uridine at a concentration of 5 (50) μmol/l.

Inter-day accuracy and precision measurements are shown in Table 2. Precision (C.V. (%)) was below 4% and accuracy (R.E. (%)) below 3%.

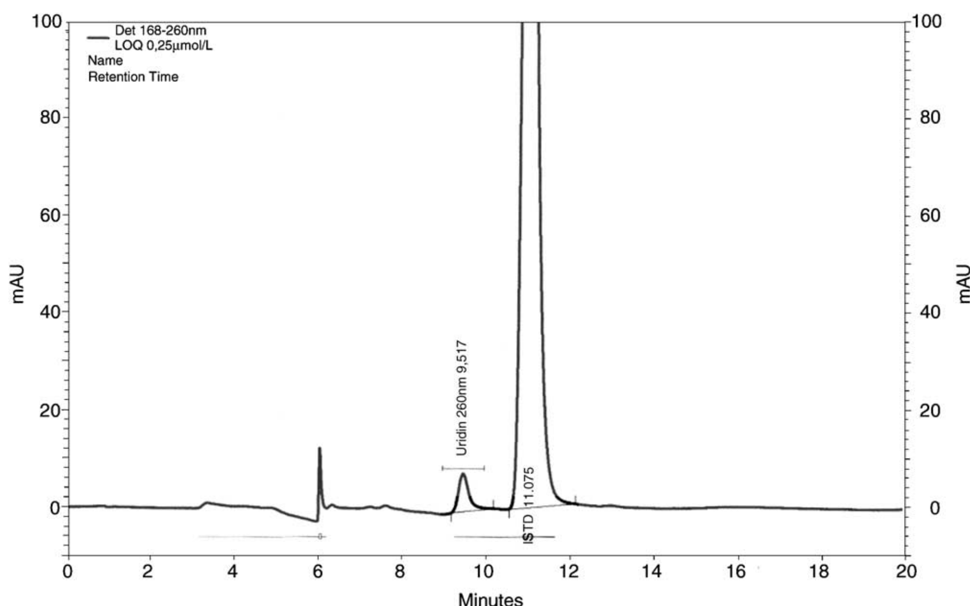


Fig. 1. Uridine at LOQ of 0.25 μmol/l (RT: 9.517 min).

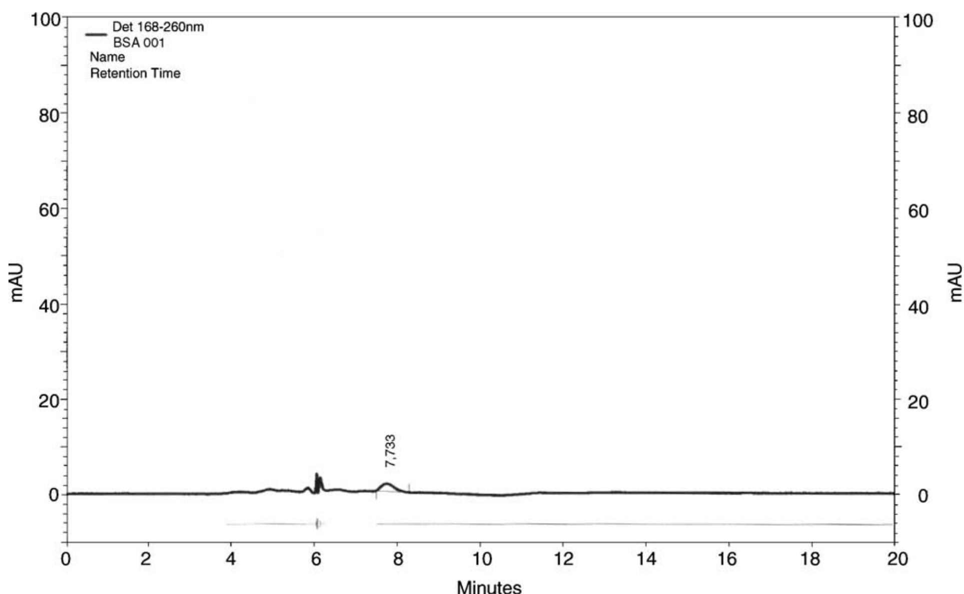


Fig. 2. Blank 5% BSA sample (without spiked uridine).

Using the ratios of the observed peak areas for uridine and the internal standard in eight spiked samples analysed in duplicate, the standard curves showed a correlation coefficient of 0.999849 (range: 0.25–100 μmol/l) as determined by least-square analysis. All calibration curves were proven to be linear in the range of 0.25–100 μmol/l. Recovery for uridine concentrations of 0.25, 1, 5, 50 and 100 μmol/l was at 95 ± 5.3% for the tested samples.

Table 1
Stability of uridine serum samples at different concentrations: accuracy (R.E. (%)) and precision (C.V. (%)) over 24 h at room temperature, over 24 h at 4 °C (nine samples for each concentration), after 30 days at −20 °C and after four freeze–thaw cycles (each for $n = 3$ samples) were evaluated

| | Deployed uridine concentrations (μmol/l) | |
|---|---|---|
| | 5 | 50 |
| (A) Over 24 h at room temperature | | |
| Mean | 1.23 | 46.34 |
| STD | 0.07 | 0.32 |
| C.V. (%) | 5.90 | 0.70 |
| R.E. (%) | 75.47 | 7.33 |
| (B) 24 h at 4 °C | | |
| Mean | 4.90 | 51.08 |
| STD | 0.22 | 1.05 |
| C.V. (%) | 4.41 | 2.06 |
| R.E. (%) | 2.07 | −2.16 |
| (C) 30 days at −20 °C | | |
| Mean | 5.04 | 52.44 |
| STD | 0.06 | 0.32 |
| C.V. (%) | 1.24 | 0.60 |
| R.E. (%) | −0.80 | −4.89 |
| (D) Four freeze–thaw-cycles | | |
| Mean | 4.82 | 50.46 |
| STD | 0.03 | 0.17 |
| C.V. (%) | 0.72 | 0.34 |
| R.E. (%) | 3.6 | −0.92 |

### 3.6. Analysis of patient samples

Blood samples were taken from healthy volunteers and from patients with different stages of HIV infection. Different regimens of a HAART including NRTIs, non-nucleoside

Table 2
Inter-day accuracy (R.E. (%)) and precision (C.V. (%)) for the analysis of uridine in three serum samples daily over 8 different days (cycles 1–8, $n = 3$ samples each)

| Cycles | | Deployed uridine concentrations (μmol/l) | |
|---|---|---|---|
| | | 5 | 50 |
| | | Detected uridine concentrations | |
| 1 | Mean | 5.18 | 51.34 |
| | STD | 0.475 | 1.294 |
| 2 | Mean | 5.05 | 50.95 |
| | STD | 0.2 | 0.064 |
| 3 | Mean | 5.01 | 50.52 |
| | STD | 0.396 | 1.065 |
| 4 | Mean | 5.07 | 51.66 |
| | STD | 0.267 | 0.096 |
| 5 | Mean | 4.7 | 51.65 |
| | STD | 0.10 | 0.945 |
| 6 | Mean | 5.015 | 52.22 |
| | STD | 0.015 | 1.143 |
| 7 | Mean | 4.93 | 52.34 |
| | STD | 0.023 | 0.886 |
| 8 | Mean | 4.85 | 51.23 |
| | STD | 0.031 | 0.170 |
| 1–8 | Mean | 4.98 | 51.49 |
| | STD | 0.187 | 0.511 |
| | C.V. (%) | 3.76 | 0.99 |
| | R.E. (%) | 0.48 | −2.98 |

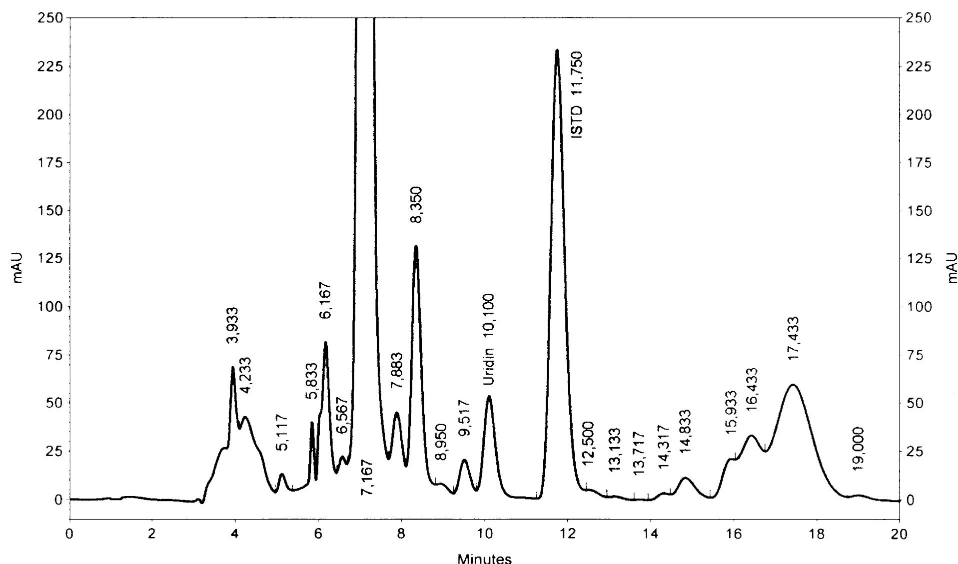


Fig. 3. Patient with HIV under antiretroviral combination therapy and an uridine level of 4.79 μmol/l (RT: 10.100 min). Antiretroviral therapy consisted of ddI and d4T at the time of evaluation.

reverse transcriptase inhibitors (NNRTI) and protease inhibitors (PI) were applied in our selected patients, according to international recommendations. As stated above, median uridine levels were not different between the controls and patients with HIV. Three investigated HIV patients developed lactic acidosis during follow up. Interestingly, their uridine serum levels were all in the lower range (3–5 μmol/l). The trigger causing mitochondrial toxicity has still to be discussed. A combination of low uridine levels and a HAART containing deoxy-NRTIs, a subgroup of NRTIs, might be associated with a higher risk for metabolic disturbances. Therefore, monitoring uridine serum levels in patients receiving HAART could be a helpful tool for risk stratification and patient guidance. This method is suggested to become a standard procedure for monitoring HIV patients with HAART. Fig. 3 shows spontaneous serum uridine levels of a patient with HIV within the lower range; Fig. 4 shows another HIV patient with a higher value. Both patients were taking deoxy-NRTIs.

As uridine shows a rapid clearance [11], high supplementation doses (10–12 g per day) may be needed to provide and sustain effective plasma levels in patients [12,13].

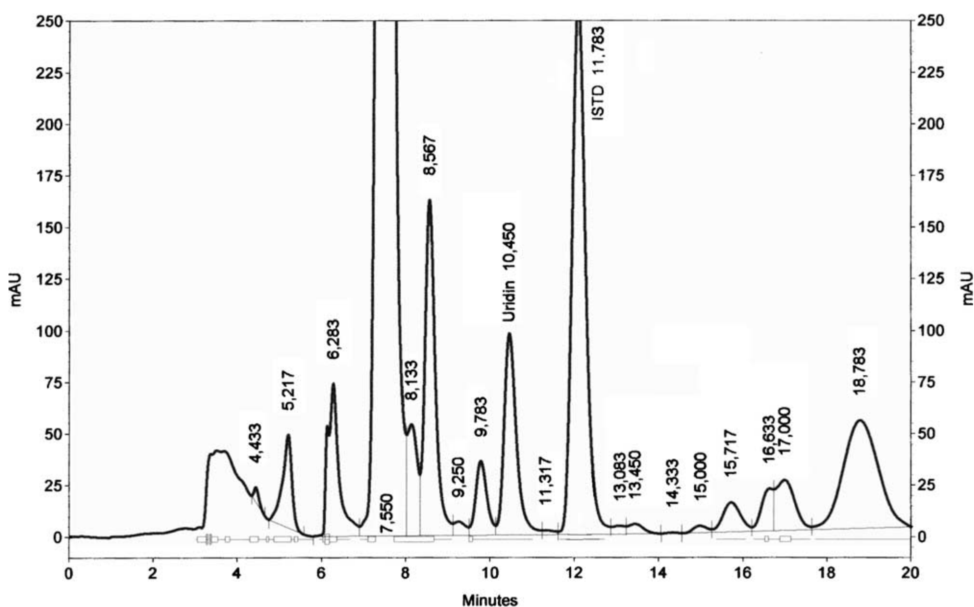


Fig. 4. Patient with HIV under antiretroviral combination therapy and an uridine level of 9.02 μmol/l (RT: 10.450 min). Antiretroviral therapy consisted of ddI, abacavir, lopinavir and ritonavir at the time of evaluation.

## 4. Conclusion

We describe a validated assay for the evaluation of human serum on uridine that was proven reliable, fast and convenient associated with a high sensitivity for the detection of uridine. The assay is easy, cost effective and can be performed at a standard hospital setting. Application of this test is focused on HIV patients treated with a (deoxy-)NRTI regime, since they have a potential risk of drug associated mitochondrial toxicity up to a lactic acidosis. The described assay was performed to monitor uridine serum levels, to provide new data on possible reversal effects and primary protection for potential devastating metabolic disturbances caused by reduced serum uridine.

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