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Modified high-performance liquid chromatography assay for the measurement of 2′-deoxyuridine in human plasma and its application to pharmacodynamic studies of antimetabolite drugs

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Abstract

A new method is presented for the HPLC determination of plasma 2′-deoxyuridine (dUrd). Briefly, 1 ml of human plasma is deproteinised with perchloric acid followed by purification by solid-phase extraction using a non-polar high-capacity polymeric sorbent. The dUrd is separated on a C_{18} reversed-phase column using a mobile-phase of 0.05% v/v trifluoroacetic acid in water, with a retention time of 8.5 min at a flow-rate of 1.25 ml min^{-1}. Quantitation is by UV detection at 261 nm using a photodiode array detector. The limit of quantitation is 6 n*M* with a linear response over the measured range 6–400 n*M*. Both intra- and inter-day RSD and bias are typically less than 13%. Chromatograms and pharmacodynamic data from a Phase 1 Clinical Trial of a new antifolate drug, ZD9331 are included to illustrate the utility of the method. They show the increase in circulating dUrd as a result of drug inhibition of the target enzyme thymidylate synthase. The method has the significant advantages of ease and simplicity over earlier methods and may be applied to the analysis of other nucleoside species. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Thymidylate synthase (TS) catalyses the reductive methylation of 2′-deoxyuridine-5′-monophosphate (dUMP) to 2′-deoxythymidine-5′-monophosphate (TMP) using 5,10-methylene tetrahydrofolate as the cofactor and methyl donor for this reaction. A number of antimetabolite drugs used for the treatment of cancer inhibit this reaction. Examples of these are 5-fluorouracil, whose active metabolite 5-fluoro-2′-deoxyuridine-5′-monophosphate (FdUMP) binds to the pyrimidine binding site of TS, while other antifolate drugs such as raltitrexed (Tomudex™) specifically bind to the folate binding site [1]. Methotrexate acts primarily through inhibition of the enzyme dihydrofolate reductase, causing depletion of the folate pool and thus producing indirect inhibition of TS [1]. New folate-based TS inhibitors continue to be evaluated in clinical studies. Rational clinical development of new agents should incorporate, where possible pharmacodynamic endpoints. Previous studies have described the measurement of plasma 2′-deoxyuridine as a surrogate endpoint of TS inhibition for the antifolate TS inhibitors, CB3717, raltitrexed and nolatrexed in preclinical models and in clinical studies [2–5]. Inhibition of TS

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produces a rapid increase in the intracellular deoxyuridine monophosphate (dUMP) pool and depletion of the intracellular thymidine nucleotide pool [6,7]. This depletion in turn causes the release of feedback inhibition by thymidine triphosphate on the enzyme deoxycytidylate deaminase (which catalyses the conversion of deoxycytidine to dUMP). Dephosphorylation of dUMP to dUrd leads to efflux of this membrane permeable nucleoside which can then be detected in the plasma. This effect can be seen in both non-tumour- and tumour-bearing mice supporting the belief that the vast majority of dUrd is released from normal proliferating tissues such as intestinal epithelium and bone marrow rather than the tumour [2]. Nevertheless measurement of this surrogate marker of TS inhibition can guide clinical studies giving both information as to the length of time that the enzyme is inhibited and the effect of escalating doses.

The very low levels of dUrd in human plasma have an associated multistep preparation and HPLC analysis that is not commensurate with high throughput analysis. Described below is a highly sensitive method employing a simple extraction procedure and a one-step HPLC analysis with UV detection. Examples of the application of this method are taken from a recent Phase I clinical study with the antifolate TS inhibitor ZD9331.

2. Experimental

2.1. Chemicals and reagents

Pure 2′-deoxyuridine ($C_9H_{12}N_2O_5$, M.W. 228), thymidine phosphorylase (EC 2.4.2.4), and human serum albumin were obtained from Sigma UK (Poole, UK). [5-^{3}H]-2′-deoxyuridine, 23 Ci mmol^{-1} was obtained from Moravek Biochemicals (Brea, CA, USA). HPLC-grade solvents were obtained from Laserchrom Analytical (Rochester, UK). Solid-phase extraction (SPE) columns, Isolute ENV+ 100 mg sorbent×3 ml column volume were obtained from Jones Chromatography (Hengoed, UK). Radiolabelled dUrd standards were counted using Hionic Fluor scintillation fluid (Packard UK, Pangbourne, UK). All other chemicals were of HPLC grade or equivalent purity. Plasma for the estimation of sample dUrd recovery was obtained from blood from normal volunteers. Blood was collected into heparin containing Vacutainers (Becton Dickinson, Meylan, France), placed on ice and centrifuged at 4°C at 500 *g* for 10 min within 15 min of collection before separating the plasma layer and freezing. Water for all purposes was purified using an Elgastat Maxima HPLC purifier (Elga, High Wycombe, UK).

2.2. Stock solutions

A stock solution for generating the dUrd calibration curve was prepared by dissolving 12.2 mg of 2′-dUrd in 1000 ml of water followed by a 1/20 serial dilution in water. A series of four further halving dilutions were made to produce a standard series equivalent to a plasma dUrd concentration range 25–400 n*M*.

For the assessment of assay precision and accuracy known amounts of dUrd were spiked into 'simulated plasma'. This consisted of 4 g/100 ml of human serum albumin in phosphate buffered saline (PBS) containing 0.21 g l^{-1} potassium dihydrogen phosphate, 9.0 g l^{-1} sodium chloride and 0.73 g l^{-1} disodium hydrogen phosphate.

2.3. Apparatus and chromatographic conditions

The solid-phase extraction eluates were evaporated to dryness using a Jouan 10.22 centrifugal evaporator (Jouan, St. Herblain, France). Liquid scintillation counting was carried out on a Tricarb 2200CA (Packard UK). The HPLC system consisted of a Waters 2690 Separations Module (Waters UK, Watford, UK) equipped with column oven, sample cooling, mobile-phase vacuum degassing and a Waters 996 photodiode array detector. Chromatographic control, data collection and processing were carried out using Waters Millennium software. Additional statistical and linear regression analysis as part of the method validation study was carried out using GraphPad Prism 3.0 software (Graphpad SoftWare, San Diego, CA, USA). The separation system consisted of a 250×4.6 mm Supelcosil LC-18 5-μm particle size stainless-steel column fitted with a 20 mm Supelguard LC-18 guard-column (Supelco UK, Poole, UK). The mobile-phase consisted of 0.05% v/v trifluoroacetic acid in water, filtered through a

0.22-μm cellulose nitrate membrane filter (Sigma UK). The flow-rate was 1.25 $ml\ min^{-1}$ with a column temperature of 45°C. The detector was set-up to collect spectra over the range 190–300 nm at five spectra per second measuring dUrd concentrations at the λ_{max} of 261 nm. The sample injection vials were held at 4°C. The injection volume for all samples and standards was 40 μl and the plasma dUrd peak identity was confirmed with reference to a stored spectrum of pure dUrd reference standard.

2.4. Sample preparation

Frozen plasma samples were quickly thawed and duplicate 1.0 ml aliquots transferred to 2.0 ml polypropylene microcentrifuge tubes (Sigma UK) containing 50 μl ice-cold 70% v/v perchloric acid and allowed to stand on ice for 10 min. The tube contents were diluted with 500 μl water and centrifuged at 10 000 *g* for 10 min at 4°C. Perchlorate was removed by precipitation with 50 μl of 5 *M* potassium hydroxide and 250 μl of 3 *M* potassium hydrogen carbonate. After brief centrifugation (10 000 $g\times10$ min), 200 μl of 0.5 *M* disodium hydrogen phosphate pH 7.0 buffer was added to the obtained supernatant to adjust the final pH of the extracts. The samples were loaded onto the SPE columns previously wetted with one column volume of methanol, equilibrated with one column volume 0.05 *M* disodium hydrogen phosphate buffer pH 7.0 and allowed to run through by gravity. The columns were rinsed with 1 ml of the pH 7.0 buffer followed by 1 ml of water. Column-bound dUrd was eluted using one column volume methanol into 12×75 mm Pyrex glass tubes. The methanolic extracts were evaporated to dryness in a vacuum centrifuge without chamber heating. The dried residues were reconstituted in 150 μl of water and transferred to injection vials before being frozen and stored at −20°C pending analysis.

2.5. Plasma dUrd recovery standards

The plasma dUrd values obtained were corrected for losses on processing. This was estimated by spiking triplicate 1.0 ml aliquots of normal human plasma with 50 μl 1/1000 diluted [5-^{3}H]-2′-dUrd (activity=1 $\mu Ci\ ml^{-1}$, final activity in plasma=0.05 $\mu Ci\ ml^{-1}$) and processing these along with sample plasmas. Stocks of labelled dUrd were assayed by HPLC every 3 months and the value for dUrd activity was corrected for degradative losses. The activities of the spiked standards were counted and compared to triplicate 50-μl aliquots of the labelled dUrd spike to obtain the necessary correction factor.

2.6. Data analysis

The peak area of dUrd was used as the assay parameter and the calculated values obtained subsequently corrected for extraction losses as described in Section 2.5 above. Standard calibration curves were obtained from single injections of 40 μl of standard dUrd at concentrations corresponding to 25, 50, 100, 200 and 400 n*M*. The curve was generated by using a $1/x$ weighted least-squares curve fit to the obtained data points. The quality of fit was evaluated by comparison of calculated to nominal values (bias). The criterion for accepting any curve was that all data points should have a bias of less than ±20%. The linearity of the calibration was confirmed using the coefficient of determination (r^2).

Plasma dUrd results were recorded as the mean of duplicate assays and the pharmacodynamic changes in plasma dUrd concentration expressed as the percentage change relative to drug pretreatment plasma dUrd levels.

2.7. Pharmacodynamic studies

dUrd concentrations were measured in plasma samples obtained from cancer patients enrolled in an AstraZeneca conducted Phase 1 clinical trial (ZD9331 Trial 0004). The subjects were treated with a 30-min infusion of ZD9331 at a dose of 130 $mg\ m^{-2}$ on days 1 and 8 [8]. Five-milliliter blood samples for plasma dUrd measurement were collected at various times into heparin-containing Vacutainers, immediately placed on ice and centrifuged at 4°C no later than 10 min after collection for 10 min at approximately 500 *g*. The plasma layer was removed, frozen and stored at –20°C pending analysis. After thawing the plasma was split into two replicate samples for analysis. To monitor batch-to-batch performance of the assay, a pair of quality control standards was included with each batch of

clinical samples. These standards consisted of a large stock of individually frozen 1.2-ml pooled-plasma samples from normal volunteers. An analytical HPLC run consisted of calibration standards followed by analytical samples and quality control standards. The run time allowed was 12 min with a delay of 24 min between injections. After every four plasma samples, retained material was washed off the column by running a mobile-phase gradient up to 50% acetonitrile over 60 min followed by a 40 min re-equilibration with the original mobile phase.

3. Results

3.1. Validation

3.1.1. Identity

The identity of the plasma peak corresponding in retention time to that of the dUrd reference standard was established by three means. (1) The UV spectrum of the putative plasma dUrd peak was matched to that of the dUrd reference standard. (2) The co-elution of the plasma dUrd peak with that of the radiolabelled dUrd was seen. (3) The disappearance of the dUrd peak occurred through specific enzymatic elimination. This was accomplished by adding thymidine phosphorylase (10 units added in 500 μl PBS ml^{-1} plasma, incubated for 30 min at 25°C) thereby converting dUrd to uracil and 2-deoxyribose-1-phosphate.

3.1.2. Precision and accuracy

The precision and accuracy of the method were evaluated by firstly determining the linearity and reproducibility of the standard curve. Five separate sets of standard curve solutions, corresponding to plasma concentrations of 25, 50, 100, 200 and 400 n*M* were prepared and analysed by HPLC. Calculated concentrations were compared to nominal values and the slopes, intercepts, linear regression, and bias calculated. The mean coefficient of determination (r^2)±RSD was 0.9992 ±0.85%. The maximum bias was found to be less than 10%. Comparison of all slopes and *y*-intercepts showed no significant difference for both: mean slope = 1.00935 ($P = 0.8699$) and mean *y*-intercept = −1.88959 ($P = 0.895$).

To determine the accuracy and precision of the assay, 'simulated plasma' spiked with dUrd to final concentrations of 25, 50, 100, 200 and 400 n*M* ($n = 6$ for each) were prepared and analysed using the method described above. The dUrd concentrations were determined using the standard calibration curve and the calculated dUrd loss correction factor. The results were plotted as measured concentration vs. nominal values and the slope, RSD, bias and linear regression of the obtained line were calculated as before. This experiment was repeated using duplicate samples on three separate days to provide data on inter-day reproducibility of the method. The results of both experiments are shown in Table 1. Intra-batch RSD and bias were less than 13% and 8%, respectively, over the concentration range 25–400

Table 1
Variability of observed dUrd plasma values[a]

Nominal dUrd conc. (n*M*)	Mean concentration	RSD (%)	Bias (%)
Intra-batch ($n = 6$)			
25	23.1	12.1	−7.6
50	52.5	6.5	5.0
100	104.8	4.8	4.8
200	208.3	1.6	4.1
400	410.1	0.85	2.5
Inter-batch ($n = 3$)			
25	23.9	9.8	−4.4%
50	49.4	6.1	−1.2%
100	104.6	3.1	4.6%
200	206.3	4.6	3.2%
400	405.9	1.3	1.5%

[a] RSD = SD/mean × 100; bias = observed value − nominal value/nominal value × 100.

nM with a slope of 1.028±0.0083 and a coefficient of determination of 0.9998. The inter-batch RSD and bias were less than 10% and 5%, respectively, with a slope of 1.018±0.0087 and coefficient of determination of 0.9998.

3.1.3. Recovery

Recovery values for radiolabelled dUrd spiked into normal human plasma (approximate endogenous dUrd concentration = 60 nM) from 10 separate batch assays over a 3-month period showed a mean recovery of 70.9±3.8%. Further experiments were carried out using similarly pooled human plasma (endogenous dUrd concentration = 37 nM) spiked with unlabelled dUrd (final concentrations 50, 250 and 500 nM) to simulate the elevated levels produced following drug administration. As shown in Table 2 these showed no change in percentage dUrd recovery over the range investigated.

3.1.4. Sensitivity

The analytical limits of the assay were determined by preparing a halving dilution series of dUrd reference standard in 'simulated plasma' to yield concentrations of 25–0.8 nM ($n=6$ for each) which were prepared and chromatographed using the described method. An extended dUrd calibration curve was prepared to cover the sample range in this experiment and consisted of dUrd standards ranging from 0.8 nM to 400 nM. The limit of detection (LOD) was defined as the concentration that produced a peak signal height to baseline noise ratio of 10 or less and was found to be between 1.6 and 0.8 nM. The limit of quantification (LOQ) was defined as the concentration which produced assay results with an RSD equal or greater than 20% and was found to be approximately 6.2 nM.

Table 2
Percentage recovery of radiolabelled dUrd from plasma[a]

dUrd conc. in plasma (nM)	Batch1 ($n=2$)	Batch 2 ($n=2$)	Batch 3 ($n=2$)
50	67.5	69.5	71.3
250	72.4	68.4	70.1
500	68.4	72	70.9

[a] Mean recovery = 70.06±1.73%.

3.1.5. Stability

A stock of pooled plasma samples from normal volunteers was stored at −70°C and −20°C for up to 8 months and assayed at intervals to determine the long-term stability of the contained dUrd. Repeat assays of these samples showed a 4-month loss on storing of 4.5% at −20°C and no apparent loss after 8-months storage at −70°C (data not shown).

3.2. Pharmacodynamic studies

Illustrative chromatograms of dUrd in patient plasma samples prepared and analysed under the conditions described are shown in Fig. 1. The chromatograms demonstrate the separation of dUrd from other endogenous components present in the sample, the lack of interference from the administered drug and show the rise in dUrd concentration following administration of the thymidylate synthase inhibitor ZD9331. The identities of the other endogenous peaks present have not been determined although spectral and chromatographic characteristics suggest a number may be other nucleoside species. Representative data from one patient treated with one cycle of ZD9331 (130 mg m^{-2} i.v., 30-min infusion on days 1 and 8, AstraZeneca ZD9331 Trial 0004) are given in Fig. 2. Plasma dUrd levels were measured in three normal volunteers over a 3-week period on days 1, 4, 6, 8, 15 and 22 to determine the degree of normal fluctuation in baseline dUrd concentration. The results of this experiment showed no significant rise or fall in dUrd levels over this period with mean±SD values of 52±10, 50±6 and 52±6 nM.

4. Discussion

Measurement of pyrimidine nucleosides and nucleobases in plasma or serum has presented many challenges to the investigator. The difficulties lie in the abundance of these compounds present, the wide range of concentration differences and the complex nature of the matrix in which they are found. Earlier workers have used radioimmunoassay [9], which, although sensitive, can lack specificity and are certainly lengthy to set-up and laborious to perform.

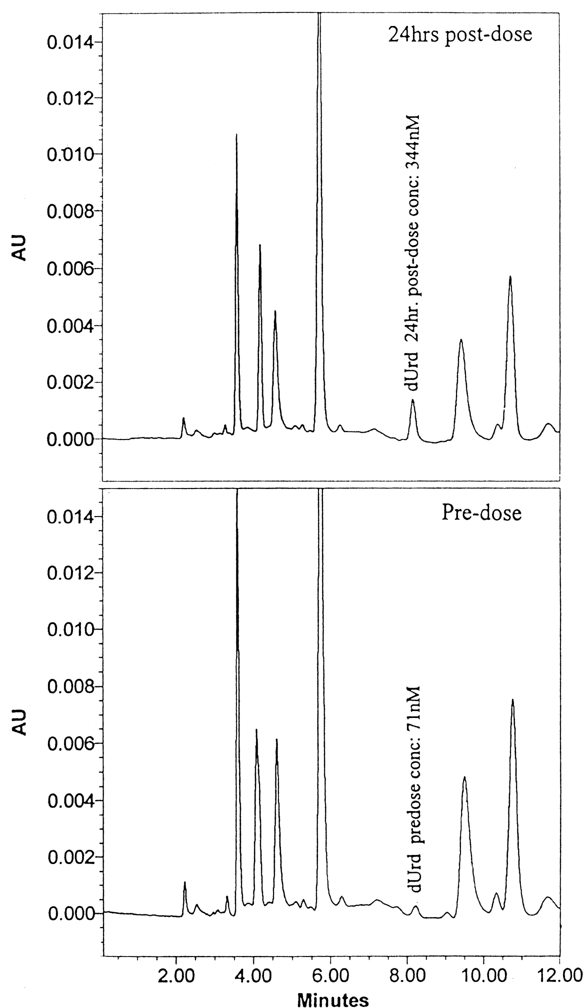

Fig. 1. Representative chromatograms showing rise in dUrd peak 24 h after administration of ZD9331.

HPLC is now widely used for the separation of these compounds. Ion exchange HPLC was used originally [10] but has now been largely replaced by reversed-phase HPLC [11–16], most often using direct UV detection and occasionally using precolumn fluorescent labelling [17–19]. This latter technique although sensitive, is limited in our experience by the inconvenience of carrying-out the fluorescent labelling step especially in the context of a large clinical trial. Earlier work using reversed-phase HPLC to measure changes in plasma dUrd as an indirect indicator of antifolate pharmacodynamic effect had overcome the problem of chromatographic interference by a lengthy two-stage chromatographic step [5]. Again it was felt that the complexity of such an assay procedure would preclude it from routine use in the analysis of large numbers of samples.

The analytical method described here relies on reversed-phase HPLC with direct UV detection as in earlier methods but incorporates two new novel features to lessen interference from other endogenous compounds present in plasma. The first is the use of solid-phase extraction following perchlorate precipitation of plasma proteins using a high-capacity polymeric phase. This has been claimed by the manufacturers to offer higher retention for polar organic compounds in an aqueous matrix than those achievable using conventional non-polar bonded-silica phases of the C_8 or C_{18} type [20]. Indeed, recovery experiments of our own comparing the polymeric phase vs. conventional silica-based C_8 and C_{18} SPE cartridges have shown this to be the case. This has allowed the use of more extensive washing-off of interfering plasma components without significant loss of column-bound dUrd. The second feature of the assay method has been the use of a trifluoroacetic acid containing mobile-phase without the addition of any organic modifier. The use of this ion-pairing reagent produces little if any change in retention time for dUrd, compared to similarly low pH acetate or phosphate containing mobile phases. However the use of trifluoroacetic acid results in a chromatogram noticeably freer of extraneous peaks. It is postulated that this occurs through the selective pairing with peptide and short protein fragments remaining in the sample after the clean-up steps. These are retained on the column until after the analytical run when they are eluted from the column using an acetonitrile gradient during the post-run wash sequence.

The method presented is both reproducible and sensitive and is appropriate for use in clinical studies. A single analyst can usually process about 35 samples per day with the HPLC analysis requiring a further 24 h. The limit of quantitation of 6 nM is adequate for the analysis of human plasma dUrd which have pretreatment concentrations ranging from approximately 20 to100 nM between individuals (mean concentration$\pm$SD found in 100 cancer

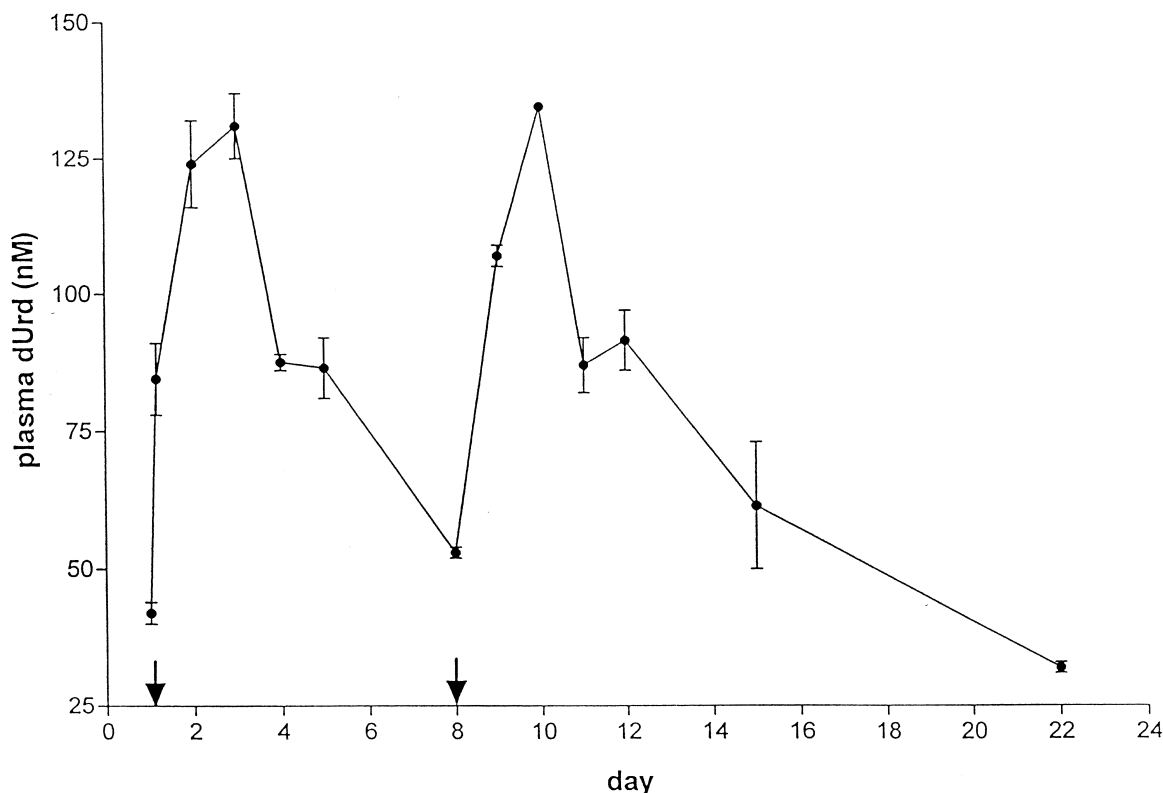

Fig. 2. Plasma dUrd in one patient treated with one cycle of ZD9331 (130 mg m^{-2} i.v., days 1 and 8). Error bars show the range of duplicate plasma extractions and chromatography.

patients = 60±23 n*M*). We have demonstrated that this method can form the basis for pharmacodynamic monitoring of TS inhibition within an antifolate drug development program. The method with slight adjustments has been used to measure plasma dUrd and thymidine in mice and ongoing work suggests it may also be applied to the determination of thymidine in human plasma. Similar modifications of the method could permit a broad applicability to the analysis of other nucleoside species.

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