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Automated high-performance liquid chromatographic analysis of (−)-2′-deoxy-3′-thiacytidine in biological fluids using the automated sequential trace enrichment of dialysate systems

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Abstract

A fully automated HPLC procedure was developed for the analysis of a small volume of perfusion solutions from an isolated perfused rat kidney study. The method involved separation of (−)-2′-deoxy-3′-thiacytidine (3TC) from the matrix by dialysis with 10 m*M* potassium phosphate buffer pH 3.0. 3TC was subsequently separated from the dialysate as it flowed through a SCX cation-exchange cartridge. The trapped 3TC was then eluted with a mobile phase of 50 m*M* ammonium acetate buffer (pH 5.5)–methanol (90.5:9.5, v/v) at a flow-rate of 1.0 ml/min for 2 min. The eluent was directed to the HPLC system and chromatographed with a BDS C_{18} analytical column at a temperature of 45°C. Detection of 3TC was carried out by UV absorption at 274 nm. The procedure was validated from 25 to 10 000 ng/ml. Coefficients of variance (C.V.) of 3TC quality control samples were less than 9%. C.V.s of the standard curve samples were also less than 10% except for the 25 ng/ml samples (11.5%). The mean interpolated concentrations were within 8% of the nominal concentrations for all samples. No interference from concurrent drugs was observed. Preliminary results suggested that this procedure may also be used for human serum and urine samples.

1. Introduction

(−)-2′-Deoxy-3′-thiacytidine(3TC,GR109714-X) is a novel synthetic cytidine analogue developed for the inhibition of HIV-1 and HIV-2 replication [1,2]. In common with other dideoxynucleosides analogues such as AZT, ddI, and ddC, the 5′-triphosphate derivative of 3TC inhibits the reversed transcriptase activity of the HIV-I virus [2]. Clinical studies so far suggested that 3TC might be less toxic than AZT [3,4]. Currently, 3TC is studied as a monotherapy or combination therapy with other nucleosides for the treatment of acquired immune deficiency syndrome (AIDS).

The major pathway of elimination of 3TC is via renal excretion [3,4]. The renal clearance of 3TC exceeded the glomerular filtration rate in rat and man. As patients with HIV infections tend to take several drugs concurrently, an *in vitro* study using isolated perfused rat kidney to

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study the interactions of 3TC with other drugs may offer some insight in the potential drug interactions in clinical situations. This report describes the validation of the bioanalytical procedure used to determine 3TC in the perfusion medium.

High-performance liquid chromatographic (HPLC) methods for 3TC in human serum [5] and human urine [6] have been published. The HPLC method for 3TC in serum involved a solid-phase extraction prior to reversed-phase chromatography. Using a 1-ml serum sample, the method was capable to accurate determine 3TC concentrations in the range of 10 to 5000 ng/ml. In the HPLC method for 3TC in urine the urine was directly injected onto the system with a two six-port column switching device prior to reversed-phase chromatography. Using 10 μl of urine, the method was capable to accurate determine 3TC concentrations in the range of 0.5 to 500 μg/ml. Unfortunately, because of the high osmotic content of the artificial perfusion medium [7], neither of the HPLC methods could be used for the analysis of 3TC samples from the isolated perfused kidney study. Therefore a method using an automated sequential trace enrichment of dialysates system (ASTED) coupled with HPLC was developed and validated for the determination of 3TC in the perfusion medium. The method used a dialysis block to separate 3TC from the matrix. 3TC was subsequently captured from the dialysate onto a SCX cation-exchange cartridge. The trapped 3TC was then eluted with the mobile phase for 2 min. The eluent was directed to the HPLC system and chromatographed on a BDS C_{18} analytical column. Detection of 3TC was carried out by UV absorption at 274 nm. This method was completely automated. No manual sample processing was required other than transferring the original samples into the injection vials. The method was suitable for small samples since only 130 μl was needed. It was validated from 25 to 10 000 ng/ml. Inter- and intra-day precision and accuracy were determined. In addition, preliminary work on the application of this method to human serum and urine samples is presented.

2. Experimental

2.1. Equipment and chromatographic conditions

The system consisted of a 231-401 autosampling injector coupled with a 100-μl dialyzer block (Gilson Medical Electronics, Middleton, WI, USA), a trace enrichment cartridge (Spherisorb SCX cartridge, 5 μm, 4.0 × 2.6 mm I.D., Keystone Scientific, Bellefonte, PA, USA), and a Waters 600E multiple solvent delivery system (Millipore, Milford, MA, USA). An analytical BDS Hypersil C_{18} column (250 × 4.6 mm I.D., 5 μm, Keystone Scientific) with a BDS Hypersil C_{18} precolumn was used for peak separation. The HPLC mobile phase consisted of 50 m*M* ammonium acetate buffer (pH 5.5)–methanol (90.5:9.5, v/v). The dialysis solution was a 10 m*M* potassium dihydrogen phosphate buffer (pH 3.0). The SCX cartridge and the BDS precolumn were replaced after every three runs. The flow-rate was 1.0 ml/min. The analytical column was maintained at a constant temperature of 45°C. The column eluent was monitored by a spectroflow 783 UV detector (Kratos Analytical Instruments, Ramsey, NJ, USA). The signal was collected with an HP A–D interface 35900A and analyzed by the HP 3550 LAS system Rev D00.01 (Hewlett-Packard, Palo Alto, CA, USA).

2.2. Sample preparation

Frozen samples (−30°C) were thawed and aliquots of 200 μl pipetted into plastic inserts. No other manual sample preparation other than pipetting the samples into sampling vials was required. The auto-sampling injector was programmed to take up 130 μl of the sample and load it into the upper chamber of the dialyzer. The sample was dialyzed with a constant flow of the dialysis solution (2.0 ml in approximately 25 min). The dialysate passed through a trace enrichment cartridge which selectively trapped the analyte. At the end of dialysis, the mobile phase flow was redirected through the trace enrichment cartridge for 2 min to elute the analyte onto the

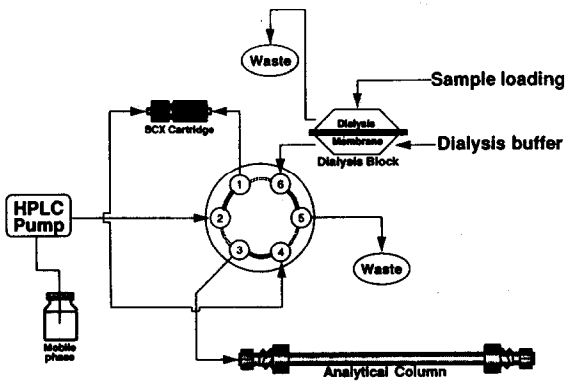

Fig. 1. Schematic configuration of the ASTED set-up during the dialysis phase (solid line in the 6-port switch) and elution phase (dotted line in the 6-port switch).

HPLC system. A 1-ml volume of the dialysis buffer was subsequently redirected to the trace enrichment cartridge to regenerate the cartridge. The sample introduction line and dialysis donor chamber were rinsed with 2.0 ml of deionized water while the dialysis recipient chamber was rinsed with 2.0 ml of the dialysis solution. A schematic set-up of the ASTED system is presented in Fig. 1. The program for this procedure is listed in Table 1.

3TC is very stable in aqueous and biological fluids. It is stable (>95% unchanged) for at least 24 days at room temperature in aqueous solution, serum, and urine (ref. 6 and unpublished data). Stored at −30°C, 3TC is stable for at least two years in serum and for at least 24 days in urine. Thus, no special procedure is needed for storage (other than freezing at −30°C for long-term storage) and handling. 3TC is also stable at 55°C for at least 5 h.

2.3. Regressional analysis

A weighted ($1/x^2$) least square linear regression was performed using the data of 3TC peak

Table 1
Program code for the analysis of 3TC

1. Sample volume (μl):	130	Sample volume used to fill donor (upper) chamber of dialysis block to excess
2. Sample height (mm):	0	Height of transfer needle tip above bottom of sample tube
3. Reagent number:	0	Sample not mixed with other reagents prior to dialysis
4. Air-gap volume (μl):	5	Volume of air to bracket liquids being transferred
5. Pulse mode:	0	Donor volume is held static (dilutor 0) Recipient volume flows continuously (dilutor 1)
6. Aspiration speed:	2	(dilutor 0) Code defining flow-rate during sample aspiration
	0	(dilutor 1) Flow-rate during dialysis and trace enrichment
7. Dispense speed:	2	(dilutor 0) Flow-rate of sample filling donor chamber
	0	(dilutor 1) Flow-rate during dialysis and trace enrichment
8. Load volume (μl):	200	Sample volume loaded into donor chamber + 100
9. Enrichment volume (μl):	2000	Volume of dialysate passed from recipient chamber through the trace enrichment cartridge
10. TEC elution time (1/100 min.):	200	Length of time the trace enrichment cartridge is Switched in-line with the HPLC system
11. Donor purge volume (μl):	2000	Rinse volume of donor chamber between samples
12. Recipient purge volume (μL):	2000	Rinse volume of recipient chamber between samples
13. Regenerate volume (l):	1000	Displaces organic component of mobile phase from elution of previous sample with 100% dialysis buffer in preparation for capturing drug in next sample

height from the analysis of the two sets of calibration standards from each run. 3TC concentration of each calibration standard sample was back calculated from the regression line. Any sample of which the calculated concentration deviated more than 20% from the nominal concentration was discarded. The regression was repeated until no calibration sample was excluded. No more than 4 calibration samples were allowed to be discarded for a given analytical run. The r value must be greater than 0.99 for the run to be accepted. Duplicate quality control (QC) samples of 100, 1000, and 5000 ng/ml were interspersed in the analytical run. At least two thirds of the QC samples and a minimum of one sample at each level had to pass the following acceptance criteria: deviation from the nominal concentration must be within 10%, 15%, and 20% for the 5000 ng/ml, 1000 ng/ml, and 100 ng/ml QC samples, respectively.

2.4. Validation

Validation of the assay in the range of 50 to 10 000 ng/ml of 3TC was performed on four different days to test the accuracy and precision of the procedure over time. Subsequently, a brief validation was carried out on two different days to validate the assay in the range of 25 to 10 000 ng/ml of 3TC.

2.5. Recovery

The relative recovery of 3TC from perfusion medium was tested by comparing the peak heights obtained from aqueous 3TC samples injected through the ASTED system with the peak heights obtained for 3TC in perfusion medium injected through the ASTED system. The absolute recovery of 3TC from perfusion medium was tested by comparing the peak heights resulting from aqueous 3TC samples directly injected onto the BDS analytical column with the peak heights resulting from 3TC in perfusion medium injected through the ASTED system.

2.6. Interference

Interference from the following compounds that may be used concurrently with 3TC was tested: AZT, ddI, ddC, trimethoprim, sulfamethoxazole, cimetidine, ranitidine, and probenecid. Aqueous solutions of these compounds were injected directly onto the HPLC system and eluted in the same way as the 3TC perfusate samples. Their retention time were determined to examine possible interference with the 3TC peak.

2.7. Human serum and urine samples

The same procedure (except that 100 mM instead of 10 mM potassium dihydrogen phosphate buffer was used) was applied to determine concentrations of two sets of spiked human serum samples containing 6, 10, 50, 200, 500, 2000, and 5000 ng/ml and one set of spiked human urine samples containing 500, 1000, 2000, 5000, and 10 000 ng/ml of 3TC. Serum and urine samples were obtained from a healthy volunteer. Samples were frozen at −30°C and stored in a freezer until assay.

3. Results and discussion

3TC is a very hydrophilic compound with a log partitioning coefficient of −0.93. Therefore, separation from endogenous compounds is essential for good analysis of 3TC in biological samples. In this report, we employed automated dialysis and trace enrichment procedures to clean up the samples using the ASTED system. Chromatography was carried out with a conventional reversed-phase analytical column. The procedure was demonstrated to be reproducible, precise, and accurate in the range 25–10 000 ng/ml. This method is particularly useful for small sample volumes as the system needs only 130 μl of sample. The total run time for each sample was approximately 30 min because of the long dialysis time employed. However, as the method is automated, no tedious manual sample processing is required.

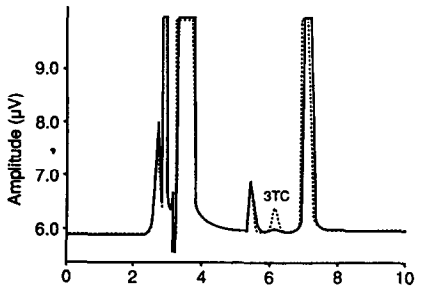

Fig. 2. Chromatograms of a blank perfusate sample (solid curve) and a perfusate sample containing 100 ng/ml of 3TC (dotted curve).

As shown in Fig. 2 3TC was eluted in this system in a fairly flat section of the baseline when compared with a blank perfusate sample. The retention time of 3TC was 6.2 min. A small peak was observed at approximately 6.1 min in the blank sample. Preliminary analysis of a 10 ng/ml perfusion solution produced a signal-to-noise ratio of 2 because of this interfering peak in the blank sample (Fig. 2). Thus, the range of 25 to 10 000 ng/ml was chosen for validation.

The standard curve was linear from 25 to 10 000 ng/ml ($y = 5.56 \times + 174$) with r values greater than 0.99. Coefficients of variation of 3TC QC samples were less than 9% (Table 2). Coefficients of variation of the standard curve samples were also less than 10% except for the 25 ng/ml samples (Table 3). The mean interpolated concentrations were within 8% of the nominal concentrations for all samples. These data suggest that the procedure is precise and accurate. This assay has been used in an isolated perfused rat kidney study. More than 350 perfusate samples were successfully analyzed. Except for a few samples, all perfusate samples had a 3TC concentration higher than 25 ng/ml. Thus, the validated concentration was adequate for the study.

The relative recovery of 3TC was 100 ± 16% suggesting that perfusion samples had the same recovery as aqueous samples in the ASTED system. The absolute recovery of 3TC was 27 ± 5%. This low recovery may have resulted from inefficient dialysis, incomplete trapping of 3TC

Table 2
Precision and accuracy data of the QC samples of 3TC

Concentration (ng/ml)		n	C.V. (%)	Percentage of nominal
Added	Measured (mean ± S.D.)			
100	92 ± 8	16	9	92
1000	1037 ± 59	17	6	104
5000	5186 ± 371	15	7	104

Table 3
Precision and accuracy data of calibration samples of 3TC

Concentration (ng/ml)		n	C.V. (%)	Percentage of nominal
Added	Measured (mean ± S.D.)			
25.0	24.8 ± 2.9	4	11.5	99
50	50 ± 2	5	5	100
100	98 ± 6	11	6	98
300	310 ± 29	8	9	104
1000	993 ± 66	12	7	99
3000	2940 ± 191	9	7	98
10 000	9798 ± 737	11	8	98

by the SCX cartridge, and/or incomplete elution of 3TC from the SCX cartridge by the mobile phase. No direct recovery experiment was performed for 3TC in human serum or urine. However, based on the slope values of the calibration curves, it seemed that the recoveries of 3TC in perfusion solution, human serum, and human urine samples were similar. It was noticed that replacement of the SCX cartridges produced significantly different slope values for perfusion samples (ranging from 4.91 to 7.82) probably due to variations between the different cartridges. In contrast, the %C.V. of the slopes obtained with the same cartridge was less than 5%. However, frequent replacement of the cartridges was necessary to maintain the quality of the chromatography. It is likely that the albumin in the perfusion medium leaked through the dialysis membrane and affected the integrity of the SCX cartridge. The aqueous solution and human urine did not appear to affect the SCX cartridge as quickly. Moreover, the dialysis membrane was only replaced after processing at least 1000 samples.

Because this procedure was specifically developed for drug interactions in the isolated perfused rat kidney, it was important to demonstrate that the drugs of interest did not interfere with the measurement of 3TC. None of the compounds studied had a retention time near that of 3TC. The only metabolite of 3TC, the sulfoxide of 3TC, was also unlikely to interfere with 3TC as it was eluted in the solvent front. Actually, only ddC eluted (retention time = 5.0 min) within the time between the solvent front and 10 min after injection onto the HPLC system. 3TC and ddC are very similar in chemical structure. Thus, it was not surprising to observe ddC to be dialyzed, trapped, and chromatographed in a similar fashion as 3TC. This suggested that this method could also be easily modified for the analysis of ddC in biological samples.

Representative chromatograms of human serum and urine samples are shown in Fig. 2. The standard curves of both the human serum and urine samples were linear ($y = 7.55 \times + 1.4$, and $y = 6.95 \times + 767$ for the serum and urine curves, respectively) with r values greater than 0.99. The interpolated concentrations were all within 8% of the nominal concentrations. The preliminary results from the human and serum urine samples indicated that this analytical method may be used for the analysis of 3TC in human serum and urine. Urine samples (Fig. 3) tended to have a higher noise level which limited the sensitivity of the method. Interfering peaks tended to become larger for samples injected later in the same analytical run. However, this did not significantly affect the area around the 3TC peak. It appeared that 500 ng/ml of 3TC in human urine could be accurately determined. This should be adequate for clinical studies where urine concentrations of 3TC are usually higher than 500 ng/ml. Further experiments are needed to validate the application of this method for 3TC determination in human serum or urine.

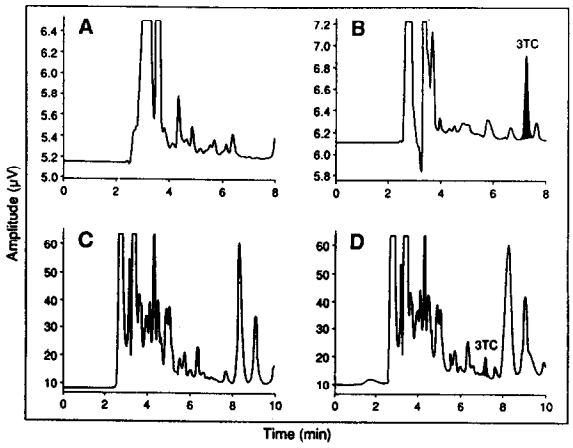

Fig. 3. Chromatograms of a blank human serum sample (A), a human serum sample containing 200 ng/ml of 3TC (B), a blank human urine sample (C), and a human urine sample containing 2000 ng/ml of 3TC (D).

In conclusion, we have developed and validated a specific, sensitive analytical method for 3TC in a biological perfusion medium. The method is reproducible, precise, and accurate. The method also showed potential for the analysis of 3TC in other matrices and may be modified for the analysis of ddC.

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