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Rapid quantitation of (−)-2′-deoxy-3′-thiacytidine in human serum by high-performance liquid chromatography with ultraviolet detection

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Abstract

A rapid, sensitive and specific high-performance liquid chromatographic (HPLC) assay was developed and validated for the measurement of (−)-2′-deoxy-3′-thiacytidine (3TC) in human serum. The method included precipitation of serum proteins by trichloroacetic acid (20%, w/v) treatment followed by centrifugation. The resulting supernatant was directly injected and 3TC was isocratically chromatographed on a reversed-phase C_{18} column using a mixture of phosphate buffer and methanol (88.3:11.7, v/v) and monitored at 280 nm. The limit of quantitation was 20 ng/ml using 100 μl of serum. The standard curve was linear within the range of 20–10 000 ng/ml. Replicate analysis of three quality control samples (40–1500 ng/ml) led to satisfactory intra- and inter-assay precision (coefficient of variation from 3.0 to 12.9%) and accuracy (deviation from −6.3 to 9.7%). Moreover, sample treatment processes including human immunodeficiency virus (HIV) heat-inactivation, exposure at room temperature and freezing-thawing cycles did not influence the stability of the analyte. This assay was successfully applied to the determination of 3TC serum levels in HIV-infected patients. In addition, preliminary results indicated that this procedure may also be extended to the measurement of 3TC in human plasma and urine.

Keywords: (−)-2′-Deoxy-3′-thiacytidine

1. Introduction

(−)-2′-Deoxy-3′-thiacytidine (3TC) (Fig. 1) is a novel synthetic dideoxynucleoside analog which has demonstrated potent in vitro antiretroviral activity against both human immunodeficiency virus (HIV) isolates [1] including zidovudine-resistant strains [2] and inhibition of human hepatitis B virus replication (HBV) [3]. 3TC has also been shown to be much

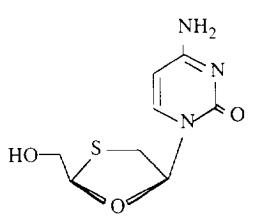

Fig. 1. Chemical structure of 3TC.

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less toxic towards human bone marrow cells as compared to nucleoside analogs currently used in AIDS therapy [4] and its high selectivity was also observed in liver cells, a critical target site for anti-hepatitis drugs [5]. Recent clinical trials evaluating the anti-HIV and anti-HBV effects of 3TC have confirmed the in vitro findings with an excellent safety profile without major dose-limiting toxicity at doses ranging from 0.5 to 20 mg/kg per day [6–10].

Pharmacokinetic studies of 3TC [11–13] have so far been performed using high-performance liquid chromatographic (HPLC) methods which exhibit different sensitivities for 3TC in human biological samples including serum and urine [14–16]. These assays either require a large sample volume (1 ml) [14] or special apparatus [15]. Herein, we have reported the development and validation of a rapid and sensitive HPLC assay for the quantitation of 3TC in human serum. This method is particularly desirable since only 100 μl of serum were required and therefore investigations on 3TC pharmacokinetic in pediatric AIDS can also be studied. Complete validation procedures were performed including the evaluation of sample treatment processes on the stability of 3TC and determination of intra- and inter-assay variability. This assay was successfully used in monitoring 3TC levels in serum of patients treated orally with the drug.

2. Experimental

2.1. Chemicals

3TC was purchased from Moravek Biochemicals (Brea, CA, USA). HPLC grade orthophosphoric acid (85%) and methanol, certified ACS grade TCA and potassium hydroxide were obtained from Fisher Scientific (Pittsburgh, PA, USA). Water was bidistilled from a glass apparatus. Helium was supplied by Airco (Murry Hill, NJ, USA).

2.2. Preparation of solutions and standards

The protein-precipitating solution (20% TCA, w/v) was prepared by dissolving 20.0 g of TCA in bisdistilled water to reach a final volume of 100 ml. This solution was kept under 4°C.

Two 1 mg/ml original stock solutions of 3TC, one for the preparation of calibration standards and another for the preparation of quality control samples (QCs) were prepared separately in bidistilled water. These stock solutions were then serially diluted to 100.0, 10.0 and 1.0 μg/ml and were stored at −20°C as 500-μl aliquots. The calibration curve standards (20–2000 ng/ml) and QCs (40, 700 and 1500 ng/ml) were prepared by combining appropriate 3TC stock solutions (from the two different batches) with normal human serum. Usually, 5 ml of each concentration of the QCs was prepared and divided into 100-μl aliquots. Calibration standards and the QCs were stored at −20°C until use.

2.3. Assay procedure

A 100 μl serum sample (blank serum, QCs, and patient serum) was transferred into a 1.5-ml plastic Eppendorf centrifuge tube. While the tube was being vortex mixed, 20 μl of the protein-precipitating solution (20% TCA) was added. The tube was vortexed for 10 s and then centrifuged on a model 5415C Eppendorf micro-centrifuge at 16 000 *g* for 5 min. After centrifugation, the resulting supernatant approximating 100 μl was transferred entirely into a 300-μl auto-sample vial and injected onto the HPLC system described in Section 2.4.

2.4. Instrumentation and HPLC conditions

The liquid chromatograph (Hewlett Packard 1090 M), was equipped with an automatic injector (250-μl sample loop), a diode array detector (detection wavelength 280 nm, reference wavelength 550 nm) and a Model HP79994A analytical workstation. Reversed-phase chromatography was performed on a Hypersil C_{18} 5 μm column, 4.6×250 mm I.D. (Rainin Instrument, Woburn, MA, USA) Elution was carried out isocratically at a flow-rate of 1 ml/min with a mobile phase of phosphate buffer (43 m*M* orthophosphoric acid and 10 m*M* of triethylammonium acetate, pH adjusted to 7.0 by 5 *M* potassium hydroxide solution)–methanol (88.3:11.7, v/v). Helium was continuously bubbled through the solvents to eliminate any dissolved air.

2.5. Calibration and calculation

Seven serum standards prepared by mixing 3TC stock solutions with normal serum (concentrations: 20, 50, 100, 200, 500, 100 and 2000 ng/ml), set for a calibration curve, were processed together with unknown samples and QCs. Standard curve parameters were obtained from an unweighted least-squares linear regression analysis of the standard concentrations (20–2000 ng/ml) and 3TC peak area. Unknown concentrations were calculated by interpolation using each observed 3TC peak area and standard curve parameters.

Complete quantitative HPLC analysis procedures were validated, including 3TC stability during sample treatment (HIV heat-deactivation at 58°C for 4 h, exposure at room temperature for 24 h and freezing–thawing cycles), selectivity towards endogenous substances and possible nucleoside analogs given in combination therapy, limit of detection and quantitation, range of linearity, precision and accuracy, and extraction recovery. Finally this novel assay was used to measure 3TC serum levels in patients after oral administration of the drug.

3. Results and discussion

3.1. Chromatographic system

Representative chromatograms of blank, spiked and patient serum samples are illustrated in Fig. 2. Under the specified chromatographic conditions, the mean retention time of 3TC was 9.5±0.3 min (n= 130). Since apolar endogenous substances were retained on the column and may interfere with the next injections, a 5-min column purge using 100% methanol was initiated at 10 min of each run followed by a 10-min postrun time allowing column re-equilibration. The total run time per sample was 25 min.

3.2. Analyte stability

Temperature changes during sample treatment processes may influence the stability of 3TC in serum. HIV-infected patient serum samples are usually heated at 57°C in order to inactivate that virus.

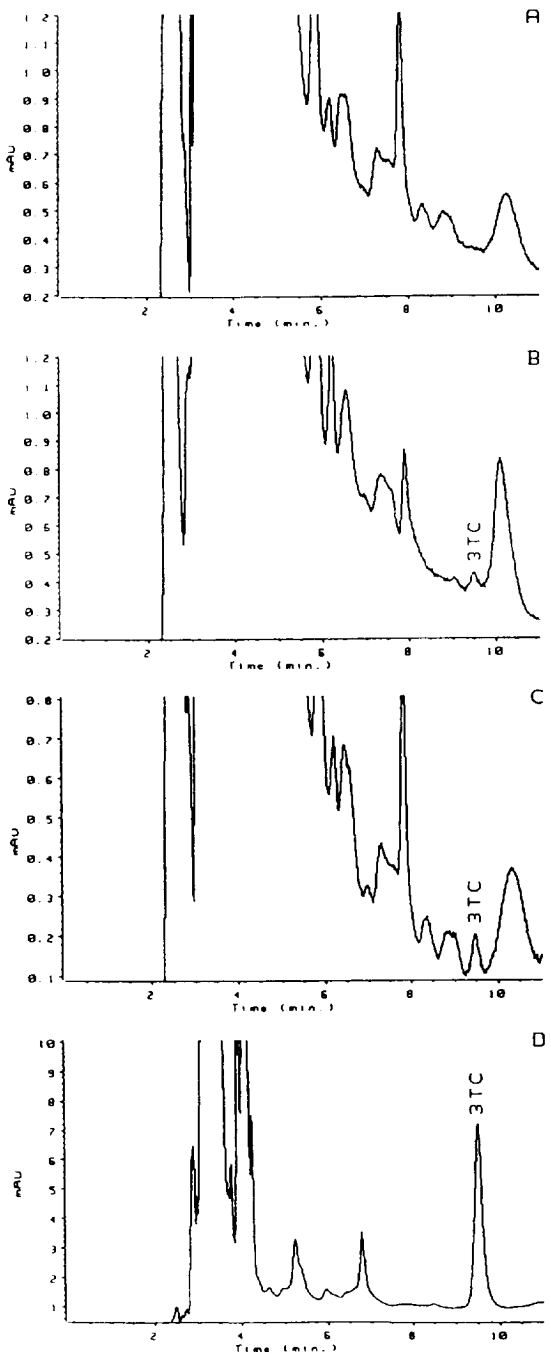

Fig. 2. Representative chromatograms of human serum samples. (A) blank serum; (B) blank serum spiked with 5 ng/ml of 3TC (detection limit); (C) blank serum spiked with 20 ng/ml of 3TC (quantitation limit); (D) a patient serum sample obtained 30 min after an oral dose of 150 mg of 3TC. Measured concentration: 940.1 ng/ml.

The stability of 3TC in serum under conditions selected for heat-inactivation of HIV was first investigated. One set of the QCs (40, 700 and 1500 ng/ml, six replicates per concentration) was incubated for 4 h at 58°C while the other set was kept at 4°C. The samples were then analyzed by the HPLC method described in Section 2.4. Results from Sstudent's *t*-test showed no statistically significant differences in 3TC concentrations between the two sets at 5% level. Following heat-inactivation, 3TC concentrations were 102.6% (95% confidence interval from 95.5 to 110.7%), 100.9% (98.3–101.0%), and 99.9% (97.1–102.6%) of corresponding levels at 40, 700 and 1500 ng/ml from non-heated samples, respectively.

Further stability was then assessed by leaving samples for 24 h at room temperature. One set of the three QCs (six replicates per concentration) was ascertained. Results from Student's *t* test indicated no statistically significant differences in 3TC concentrations between the two sets at 5%. Following this procedure, 3TC concentrations were 105.7% (95% confidence interval from 96.8 to 114.5%), 102.8% (100.9–104.7%), and 102.9% (101.6–104.2%) of the corresponding levels at 40, 700 and 1500 ng/ml from the control samples, respectively.

The stability of 3TC during sample treatment processes was also evaluated by submitting samples three times to freezing–thawing cycles. One set of the QCs (six replicates per concentration) was frozen to −20°C and then thawed at room temperature. The statistical analyses showed no significant differences in 3TC concentrations between the two sets at 5%. 3TC concentrations were 93.2% (95% confidence interval from 90.0 to 96.4%), 97.6% (93.5–101.7%), and 98.5% (95.6–101.5%) of the corresponding levels from the control samples, respectively.

These results demonstrated that 3TC was stable under various sample storage and treatment conditions.

3.3. Selectivity

Selectivity is often a major issue for direct injection without conventional liquid or solid extraction procedures. Human serum samples of 3TC from more than twenty different individuals, including serum from patients treated with other anti-HIV nucleoside analogs were analyzed. No interference with endogenous substances was observed (Fig. 2A). In on-going clinical trials, combination of 3TC with other nucleoside analogs also approved for AIDS therapy such as zidovudine (ZDV), didanosine (ddI) and stavudine (d4T) is being studied. Under the specified chromatographic conditions, these nucleoside analogs and their in vivo metabolites did not interfere with 3TC detection. The retention times of 3′-amino-3′-deoxythymidine, ddI and ZDV glucuronide were 4.6, 11.9 and 13.2 min, respectively, while both d4T and ZDV were retained and only eluted during the column purging period.

3.4. Limit of detection and quantitation

The limit of detection as defined by a signal-to-noise ratio of 3:1 was 5 ng/ml using a 100 μl serum sample. At this concentration, however, the coefficient of variation on 3TC peak area was not acceptable (29.5%, $n=6$). The limit of quantitation was 20 ng/ml with a coefficient of variation of 12.9% ($n=6$). Chromatograms of blank human serum without and with 3TC spiked at a concentration of 15 ng/ml (limit of detection), and at a concentration of 20 ng/ml (limit of quantitation) are shown in Fig. 2A, Fig. 2B and Fig. 2C, respectively.

3.5. Linearity

The relationship between peak area and the concentrations of 3TC was linear over the range of 20–10 000 ng/ml using 100 μl serum ($r\geq 0.99$). A calibration curve from 20 to 2000 ng/ml was validated and routinely used.

3.6. Recovery

No conventional extraction (liquid–liquid or solid-phase) was used in this assay. Samples containing 3TC were directly analyzed after protein removal using diluted TCA. Therefore, recovery should be virtually complete. Recovery of 3TC from serum was determined at three concentrations identical to those of the QCs. Aqueous solutions of 3TC were prepared from the QC stock solutions to achieve final concentrations of 40, 700 and 1500 ng/ml. Portions of 100 μl (six replicates per concentration) of these

Table 1
Intra-assay reproducibility from quality control samples ($n=6$)

Nominal concentration (ng/ml)	Measured concentration (ng/ml)	Deviation (%)	Coefficient of variation (%)
40	42.2	+5.4	9.0
700	672.2	−4.0	4.0
1500	1572.4	+4.8	4.0

solutions were then analyzed by HPLC and the resulting peak area were considered to represent 100% recovery. One set of the QCs (six replicates per concentration) was processed according to the normal sample preparation procedure and the peak area from these samples was compared to that of the aqueous samples to estimate recovery. As expected, a high recovery was obtained, being 88.6% (95% confidence interval: 84.6–90.6%), 87.6% (85.9–89.3%) and 93.5% (91.7–95.3) at 40, 700 and 1500 ng/ml, respectively.

3.7. Precision and accuracy

The intra-assay precision (coefficient of variation) and accuracy (deviation from nominal values) were measured by simultaneously assessing the QCs (40, 700 and 1500 ng/ml) in replicates of six. Results are presented in Table 1. The intra-assay coefficient of variation and deviation ranged from 4.0 to 9.0% and from −4.0 to +5.4%, respectively.

The inter-assay precision and accuracy were determined over a period of three months by using both the calibration standards and the QCs. Results are summarized in Tables 2 and 3. The inter-assay coefficient of variation and deviation varied from 3.0 to 12.9% and −6.3 to 9.7%, and from 4.2 to 9.7% and −3.1 to +2.6% for calibration standards and QCs, respectively.

3.8. Application to biological samples

Using this novel HPLC methodology, serum 3TC levels were determined following oral administration of the drug to HIV-infected patients. A representative chromatogram of a patient serum sample is shown in Fig. 2D. A 3TC serum concentration–time course after oral administration of a single dose of 150 mg

Table 2
Inter-assay reproducibility from calibration standards ($n=13$)

Calibration concentration (ng/ml)	Calculated concentration (ng/ml)	Deviation (%)	Coefficient of variation (%)
20	21.9	+9.7	12.9
50	47.2	−5.6	9.5
100	93.7	−6.3	5.5
200	191.5	−4.2	3.5
500	511.9	+2.4	3.7
1000	988.1	−1.2	4.6
2000	2057.2	+2.9	3.0

Table 3
Inter-assay reproducibility from quality control samples ($n=12$)

Nominal concentration (ng/ml)	Measured concentration (ng/ml)	Deviation (%)	Coefficient of variation (%)
40	41.0	+2.5	9.7
700	678.5	−3.1	6.3
1500	1539.3	+2.6	4.2

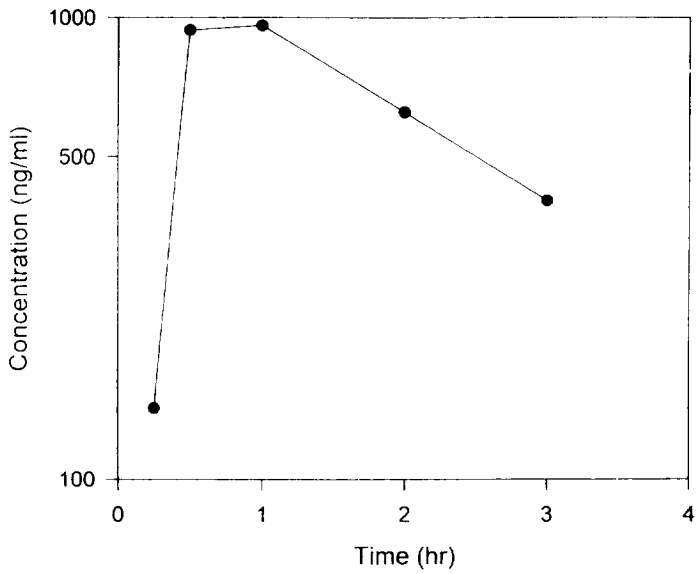

Fig. 3. Time-course of serum 3TC concentration in a patient receiving an oral dose of 150 mg of 3TC.

of 3TC to a naive patient is depicted in Fig. 3. In that patient, 3TC exhibited an apparent elimination half-life of 1.62 h, a serum clearance (Cl/F) of 51.9 l/kg and an apparent total volume of distribution (V_d/F) of 121.2 l, as estimated by a non-compartmental pharmacokinetic analysis of the above kinetics [17].

Though no validation was performed, this novel assay should be applicable to the determination of 3TC in human plasma and urine. For plasma, identical sample treatment procedure was used. Because of the substantial content of heparin in plasma, the noise was slightly greater than that observed with serum. Chromatograms of blank and spiked human plasma samples are shown in Fig. 4A and Fig. 4B, respectively. In man, 3TC is largely, approximately 70%, excreted unchanged in urine after an oral dose [13]. Concomitant medications and renal failure may therefore significantly affect the renal clearance of 3TC, resulting in unexpected pharmacological and/or toxicological interactions further demonstrating the interest of dosing 3TC in urine. Urine samples were diluted to 1:5 with 4% TCA (w/v) and filtered through 0.2 μm filters prior to analysis. No additional sample treatment was required. Because of the high levels of 3TC in urine, a small sample volume (50 μl of the diluted urine) was injected. Chromatograms of blank and spiked human urine samples are shown in Fig. 4C and Fig. 4D, respectively. Further experiments are, however, needed to validate the application of this procedure to the measurement of 3TC in human plasma and urine.

4. Conclusion

A simple, rapid, specific and sensitive HPLC assay was successfully developed and validated for the quantitation of 3TC in human serum with direct injection following protein removal. The procedure required only 100 μl of serum and was precise and accurate. The run time per sample (25 min) was reasonably short and allowed automated sample analysis of as many as 40 specimens being performed daily. In addition, this procedure may also be extended to the measurement of 3TC in other biological fluids such as human plasma and urine and the need of a small volume meets the requirement of analysis in pediatric patients. Therefore, this method appears suitable for the therapeutic drug monitoring in forthcoming large scale clinical trials of 3TC in

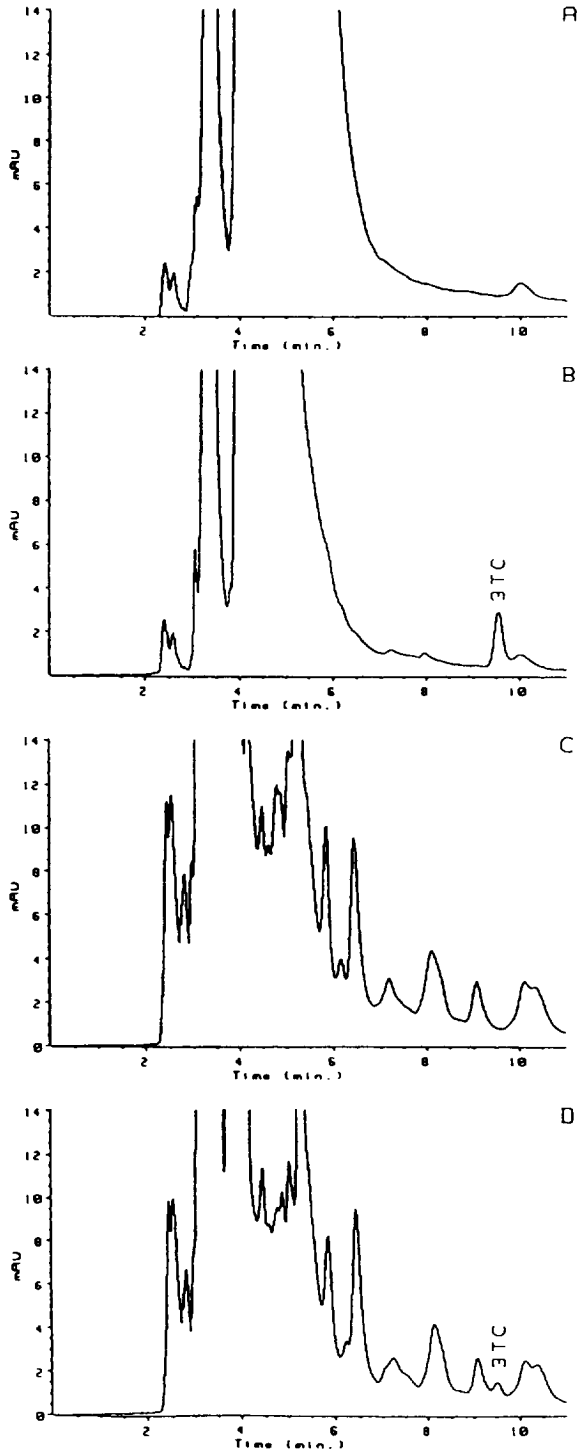

Fig. 4. Representative chromatograms of human plasma and urine samples. (A) blank plasma; (B) blank plasma spiked with 600 ng/ml of 3TC; (C) blank urine; (D) blank urine spiked with 1300 ng/ml of 3TC.

adult and pediatric patients infected with HBV and HIV.

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