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High-performance liquid chromatographic assay for the determination of 2′-deoxy-3′-thiacytidine (lamivudine) in human plasma

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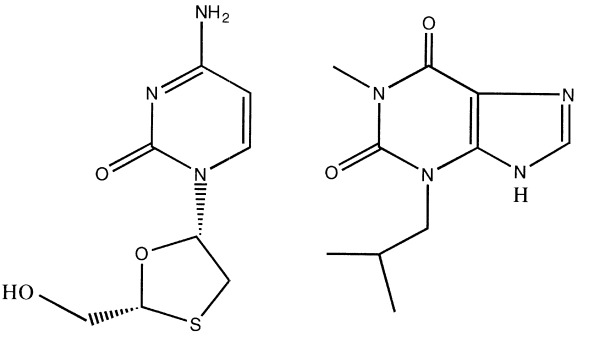
Abstract

A method for the quantification of 2′-deoxy-3′-thiacytidine (lamivudine, 3-TC), which incorporated the use of 3-isobutyl-methylxanthine as internal standard (I.S.) was developed and validated in human plasma, using HPLC with UV absorbance detection. Using solid-phase extraction, 3-TC and I.S. were selectively extracted from human plasma. Subsequently, chromatographic separation was performed using a YMC phenyl column with ion-pair chromatography and detection at 270 nm. The method was validated over a concentration range of 10 to 5000 ng/ml using 0.5 ml of human plasma. The extraction recovery for both 3-TC and I.S. was greater than 95%. The determination of inter- and intra-day precision (RSD) was less than 10% at all concentration levels, while the inter- and intra-day accuracy (% difference) was less than 6%.

Keywords: 2′-Deoxy-3′-thiacytidine; Lamivudine

1. Introduction

2′-Deoxy-3′-thiacytidine (lamivudine, 3-TC), a novel dideoxy-nucleoside analogue (Fig. 1), has potent activity against HIV-1 through inhibition of reversed transcriptase activity [1]. Since mono-therapy with nucleoside reverse transcriptase inhibitors (NRTIs) for treatment against HIV-1 results in rapid development of resistant HIV strains, co-administration of other anti-retroviral therapies is



3-TC **I.S.**

Fig. 1. Chemical structures of 3-TC and 3-isobutyl-methylxanthine (I.S.).

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necessary [2–4]. Treatment of HIV-1 infection with an antiviral regimen that includes 3-TC is desirable, since 3-TC has been shown to be somewhat less toxic than other NRTIs [5]. The US Department of Health and Human Services' current guideline for the treatment of established HIV infection strongly recommends 3-TC in combination with another NRTI and either a protease inhibitor or efavirenz [6].

Recently, the completion of a 1-year clinical trial has also demonstrated that 3-TC is an effective treatment for chronic hepatitis B infection [7]. Treatment with 3-TC resulted in the suppression of hepatitis B virus replication with a significant reduction in the progression of hepatic fibrosis.

Several high-performance liquid chromatographic (HPLC) methods for the determination of 3-TC in biological fluids have been published [8–11]. These assays utilize a variety of techniques including plasma protein precipitation [10], solid-phase extraction [9,11] and column switching [8]. While each of these HPLC methods achieves suitable assay sensitivity (10–25 ng/ml), none utilize an internal standard. However, Hoetelmans et al. [11] discussed the difficulty of finding a suitable internal standard. Since 3-TC is commonly administered with at least one other NRTI, none of the compounds in this class would be an acceptable internal standard for use with plasma obtained from patients. Kenney et al. [12] and Pereira et al. [13] resolved this issue by preparing a stable, isotopically labeled 3-TC for use as an internal standard with HPLC–tandem mass spectrometric (MS–MS) analysis. A method, which incorporates a commercially available internal standard, would be desirable, since it would correct for systemic errors and compensate unexpected sample loss during sample processing.

This report describes the development of a HPLC method that utilizes 3-isobutyl-methylxanthine, a commercially available compound, as internal standard (Fig. 1). The solid-phase extraction procedure we developed for the isolation of 3-TC from plasma provides a much cleaner background. Additionally, the application of isocratic elution with ion pair reagent strategically adjusts the analyte away from the endogenous interference peaks. By using this method, high sensitivity and clean plasma background can be achieved.

2. Experimental

2.1. Chemicals

Lamivudine (3-TC) was obtained from Glaxo Wellcome Research Institute, (Research Triangle Park, NC, USA). Internal standard (I.S.), 3-isobutyl-methylxanthine was purchased from Research Biochemicals International (Natick, MA, USA). 1-Octanesulfonic acid sodium salt monohydrate was purchased from Fluka (Ronkonkoma, NY, USA). Orthophosphoric acid (85%) was bought from J.T. Baker (Phillipsburg, NJ, USA). Acetonitrile (ACN) and methanol (HPLC grade) were purchased from EM Science (Gibbstown, NJ, USA). Disposable cartridges packed with cyclohexane-silica (Isolute CH, 100 mg/1 ml, endcapped) were purchased from Jones Chromatography (Lakewood, CO, USA). Human EDTA plasma was obtained from Biological Specialty (Colmar, PA, USA).

2.2. Instruments and HPLC conditions

HPLC instrumentation consisted of a Shimadzu (Columbia, MD, USA) SCL 10A controller, an SIL 10A autoinjector, two LC-10AD pumps and an SPD 10A UV detector. Data analysis was carried out using Water's Expert Ease data acquisition and analysis software.

A YMC (Wilmington, NC, USA) Phenyl column (100×4.6 mm, 3 μm) was used as the analytical column with a YMC RP Phenyl Newguard (15×3.2 mm, 7 μm) guard column. Both columns were thermostated at 35°C using a column heater. The isocratic mobile phase was composed of ACN–0.085% phosphoric acid (12:88, v/v), and 10 m*M* octanesulfonate. The UV absorbance was monitored at 270 nm. With a mobile phase flow-rate of 1.5 ml/min, the retention times of 3-TC and I.S. were 6.8 and 8.2 min, respectively. The analysis time was set at 17 min per sample to eliminate potential interference from late eluting peaks. A weighted least-squares regression analysis was performed using the peak height ratio versus the reciprocal of the squared drug concentration ($1/x^2$) as weight to derive a standard curve. The concentrations of

analytes in samples were determined from the slope and intercept obtained from a daily standard curve.

2.3. Reagent solutions

Phosphoric acid (0.085%) was prepared by dissolving 1 ml of orthophosphoric acid (~85%) in water and diluted to 1000 ml with distilled water. Internal standard stock solution was prepared by dissolving 3-isobutyl-methylxanthine in water–methanol (50:50, v/v), at a concentration of 1 mg/ml. Internal standard working solution was prepared by diluting the stock solution with water to give a 10 μg/ml concentration level. The 3-TC stock solution was prepared in water at a concentration of 0.1 mg/ml and was stored in glass at 4°C. Working standard solutions of 3-TC were made by serial dilution to desired concentrations with water. The working solutions were stored at 4°C and allowed to reach to room temperature before use.

2.4. Plasma sample preparation

Plasma calibration samples and quality cotrol (QC) samples were prepared by mixing 0.5 ml of blank plasma and 0.475 ml of water. Then, 25-μl aliquots of 3-TC working standard solutions and internal standard working solution were added, respectively. Study samples were prepared by mixing 0.5 ml of study sample plasma and 0.5 ml of water with 25 μl of internal standard working solution. The prepared plasma sample was vortex-mixed briefly. The plasma concentrations of standard curve were 0, 10, 25, 50, 100, 250, 500, 1000, 2500 and 5000 ng/ml.

2.5. Solid-phase extraction method

Extraction columns were prepared by sequential washing with 1 ml of methanol and 1 ml of water by gravity. The columns were not allowed to dry before loading plasma samples.

Each prepared plasma sample (1.025 ml) was transferred to a preconditioned extraction column. The sample was allowed to drain by gravity. The column was washed twice with 1 ml of water, and then dried in a vacuum manifold under 20 mmHg for 5 min (1 mmHg=133.322 Pa). The analytes were eluted with three 0.5-ml aliquots of acetonitrile; the column was drained completely before each addition. The eluates were combined and evaporated to dryness under a stream of nitrogen at 37°C. The residue was reconstituted with 200 μl of mobile phase. An aliquot of 100 μl was injected onto the HPLC column.

2.6. Inter-day precision, intra-day precision and accuracy

A single set of QC samples (five concentration levels) was prepared and analyzed together with an independent standard curve each day for 3 days. Inter-day precision was expressed as relative standard deviation (RSD).

Five sets of QC samples (five concentration levels) were prepared and analyzed on the same day, along with an independent standard curve for quantification. Intra-day precision was expressed as RSD.

The accuracy was expressed as the percent difference for each sample:

$$\%\ \text{difference} = \left(\frac{\text{determined concentration} - \text{nominal concentration}}{\text{nominal concentration}}\right) \cdot 100$$

2.7. Extraction recovery

The solid-phase extraction recovery of 3-TC was measured by comparing the peak height of the extracted 3-TC and I.S. with that of the unextracted samples. To prepare unextracted samples, the same amount of 3-TC and I.S. was transferred to a clean tube and dried with a stream of nitrogen. Both extracted and unextracted samples were reconstituted in the same volume of mobile phase and HPLC analysis was performed using identical injection volumes.

2.8. Stability

The stability of 3-TC in water at 4°C, and the freeze–thaw cycle stability in human plasma were examined. The stock solution stability was evaluated

by comparing separate HPLC injections of 100 and 1000 ng/ml concentration levels of a previous prepared stock solution and a stock solution prepared on the day of analysis.

To evaluate the freeze–thaw cycle stability, human plasma samples at concentrations of 50 and 500 ng/ml were prepared. A set of samples was assayed on the day of preparation as cycle 0. One set of samples was assayed after one freeze–thaw cycle and another set of samples was assayed after two freeze–thaw cycles. Each set of samples consisted of two concentration levels run in triplicate.

3. Results and discussion

3.1. Chromatography and detection

Since 3-TC is a relatively polar compound, solid-phase extraction (SPE) was chosen as it gave a better extraction recovery and minimal plasma background than liquid–liquid extraction. To select an SPE sorbent with adequate retention of 3-TC and I.S., different sorbents including C_8, C_2, C_{18}, CN, CH, PH, both end-capped and non end-capped were screened. Overall, the CH (cyclohexane, end-capped) cartridge showed the best results among the tested sorbents, in terms of extraction recovery of 3-TC and I.S., and the absence of interfering substances from plasma background. Using the procedures described in Section 2.5, the recovery of both 3-TC and I.S. was essentially quantitative (Table 4). To verify the robustness of the assay, two different lots of SPE columns were used. Plasma samples (500 ng/ml) which were assayed in triplicate on each lot of SPE columns showed similar recovery and peak height ratio.

Because of its hydrophilic property, 3-TC was difficult to retain on reversed-phase analytical columns. However, the free amino group of 3-TC formed an ion pair with octane sulfonate, which increased the retention of 3-TC on the stationary phase. 3-TC was resolved from endogenous peaks by adjusting the mobile phase pH and the concentration of octane sulfonate of the mobile phase. As shown in Fig. 2, 3-TC eluted at 6.8 min without any interference from plasma matrices. However, an endogenous peak that eluted late in the chromatogram was observed, and is partially shown in the chromato-

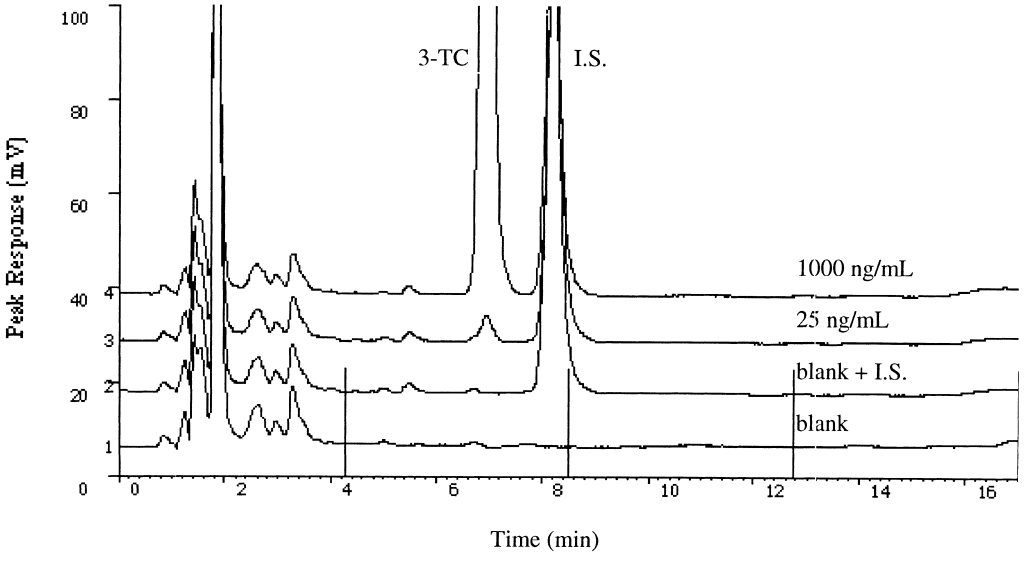

Fig. 2. The representative chromatograms of plasma blank, plasma spiked with I.S, plasma spiked with 25 and 1000 ng/ml 3-TC.

Table 1
Back calculated concentration of 3-TC from the standard curves in human plasma

Concentration (ng/ml)	Day 1		Day 2		Day 3	
	Calculated concentration		Calculated concentration		Calculated concentration	
	ng/ml	% Difference	ng/ml	% Difference	ng/ml	% Difference
10	10.00	0.00	10.02	0.19	10.04	0.36
25	25.44	1.75	24.78	−0.86	24.70	−1.19
50	48.08	−3.84	49.86	−0.29	49.30	−1.40
100	99.82	−0.18	102.0	1.99	104.3	4.27
250	253.6	1.45	249.1	−0.35	249.7	−0.12
500	503.0	0.60	504.7	0.94	492.7	−1.45
1000	1023	2.29	1016	1.65	1007	0.71
2500	2438	−2.47	2449	−2.06	2472	−1.13
5000	5020	0.40	4940	−1.20	4998	−0.04
R^2	0.9997		0.9995		0.9999	

grams (Fig. 2) eluting at 16 min (~30 min from previous injection). While these peaks do not obliterate peaks of interest, their presence in chromatograms of samples containing <50 ng/ml 3-TC cause inconsistency in peak integration. By setting the total run time at 17 min, the interfering peak was effectively eliminated.

3.2. Assay validation

The method was validated over a concentration range of 10–5000 ng/ml using 0.5 ml of plasma. Three day inter- and intra-day validation results are shown in Tables 1–3. The RSDs of precision for inter- and intra-days were less than 10% at all concentration levels. The accuracy ranged from 94 to 99%.

3.3. Internal standard selection

Since 3-TC is typically co-administered with other HIV reverse transcriptase inhibitors, it is unacceptable to use an NRTI as internal standard. Screening of commercially available compounds was initially evaluated based on relative chromatographic retention time, followed by evaluation of the extraction recovery of the potential internal standards. 3-Isobutyl-methylxanthine was selected as the I.S., since it eluted within 2 min after 3-TC elution and its extraction recovery was also quantitative under the optimal conditions for 3-TC (see Table 4).

Table 2
Intra-day precision and accuracy of 3-TC from human plasma

Spiked concentration (ng/ml)	Found concentration (mean±SD, $n=5$) (ng/ml)	RSD (%)	Mean absolute difference (%)
10	10.6±0.3	2.3	6.2
25	26.1±0.6	2.4	4.3
500	496±2.2	0.4	0.9
2500	2513±23.4	0.9	0.9
5000	5079±76.8	1.5	1.6

Table 3
Inter-day precision and accuracy of 3-TC in human plasma

Spiked concentration (ng/ml)	Found concentration (mean±SD, $n=5$) (ng/ml)	RSD (%)	Mean absolute difference (%)
10	10.1±0.1	9.9	7.9
25	23.9±2.5	10.3	6.6
500	490±8.0	1.6	2.0
2500	2468±61.3	2.5	2.3
5000	5015±28.4	0.6	0.4

3.4. Specificity and selectivity

The specificity of the assay was evaluated by analysis of six different lots of pooled human blank plasma samples. None of the six samples yielded peaks that would interfere with the quantification of either 3-TC or internal standard. Additional plasma samples, which were spiked with other NRTIs (non-nucleoside reverse transcriptase inhibitors); AZT and AMT (the metabolite of AZT), DDC, and d4T, protease inhibitors; indinavir, ritonavir, nelfinavir or saquinavir, or NNRTIs: efavirenz and DPC 083, were subjected to the same solid-phase extraction procedures and HPLC analysis. None of these compounds caused interference with 3-TC or the I.S.

3.5. Stability

A comparison of the responses obtained from HPLC injection of stock solutions resulted in no more than 5% difference in the potency of stock solutions. The stock solution of 3-TC in water was stable for at least 1 month, stored at 4°C. Following two freeze–thaw cycles, no more than 6% difference was observed compared to freshly prepared samples. Therefore, 3-TC was stable in human plasma over two cycles of freeze–thaw.

4. Conclusions

A simple and reproducible method for the determination of 3-TC has been developed and validated. The specificity of this procedure has been demonstrated against representative examples of commonly co-administered anti-virals including protease inhibitors (indinavir), NRTIs (AZT), and NNRTIs (efavirenz). Additionally, the linearity over a wide range from 10 to 5000 ng/ml makes this method applicable to clinical pharmacokinetic studies as well as toxicokinetic and preclinical studies.

Table 4
Extraction recovery of 3-TC and I.S. from human plasma

Concentration (ng/ml)	Unextracted, peak height (mean±SD, $n=3$)	RSD (%)	Extracted, peak height (mean±SD, $n=5$)	RSD (%)	Recovery (%)
3-TC					
10	272.0±33.2	12.2	289.4±10.6	3.7	106
25	722.0±30.6	4.2	722.8±19.2	2.7	100
500	14 507±763.8	5.3	14 147±164.3	1.2	98
2500	73 710±2106	2.9	72 111±1027	1.4	98
5000	151 783±6473	4.3	144 592±3199	2.2	95
I.S.					
500	11 586± 119.9	1.0	11 473±193.2	1.7	99

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