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Determination of lamivudine in plasma, amniotic fluid, and rat tissues by liquid chromatography

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Abstract

An HPLC method for the quantification of lamivudine (3TC) in rat plasma, amniotic fluid, placental and fetal tissues has been developed, validated and applied to the study of the placental transport of this drug in the pregnant rat. Placental and fetal tissues were processed using liquid-liquid extraction enhanced by salting out the sample using a saturated solution of ammonium sulfate. Plasma and amniotic fluid samples were processed by protein precipitation using 2 M perchloric acid. Reverse phase chromatography was performed using a phenyl column (5 μm, 150 mm × 2 mm i.d.) under a flow rate of 0.2 ml/min. The mobile phase consisted of 5% methanol in 20 mM dibasic phosphate buffer (pH 6). The method was validated over the range from 0.1 to 50 μg/ml for plasma and amniotic fluid and 0.2–50 μg/ml for the placental and fetal tissues.
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Keyword: Lamivudine

1. Introduction

Viral infections are the cause of significant morbidity and mortality in modern society. Human immunodeficiency virus (HIV), hepatitis B virus (HBV), herpes viruses (including herpes simplex virus [HSV]), varicella zoster virus (VZV) and cytomegalovirus (CMV) are of special socioeconomic importance because of their widespread prevalence in humans [1].

Lamivudine (3TC) is a synthetic dideoxynucleoside derivative that is active against HIV and HBV [2]. In common with other dideoxynucleosides, lamivudine should be intracellularly activated to its triphosphate derivative before it can inhibit the viral DNA polymerase enzyme [3]. Treatment of HIV infections with an antiviral regimen that includes 3TC is desirable since 3TC shows lower toxicity than other nucleoside derivatives [4]. The US Department of Health and Human Services current guidelines for the treatment of HIV infections strongly recommend 3TC in combination with other antiviral drugs [5].

Although much is known about the behavior of 3TC and other nucleoside antivirals in infected individuals, very little is known about their effects on pregnant women and their fetuses. Understanding the kinetics of these drugs in pregnant women and their transport profiles to the fetal compartment is crucial toward providing better protection to the fetus from the mother's infection. Mother to child vertical transmission accounts for 90% of global viral infections in children [6].

To investigate 3TC transport from the maternal to the fetal compartment, an appropriate model is needed where the kinetic profile of drug transport across the placenta can be extrapolated to humans. The pregnant rat model serves as an appropriate model to study drug distribution between the maternal and the fetal compartments. Rats and human placenta show anatomical resemblance because both placentas belong to the same hemochorial type and therefore, are expected to show similarity in drug transport profiles [7,8].

In order to build a kinetic profile with kinetic parameters that reliably describe the transport profile of the drug across the placenta, an accurate, sensitive and rugged analytical method is needed to analyze 3TC in the maternal plasma, amniotic fluid, placenta as well as the fetal tissues. Several HPLC methods for the quantification of 3TC in plasma, urine, saliva and cerebrospinal fluid are available

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in the literature [9–13]. All these methods use solid phase extraction techniques (SPE) with different cartridges for the extraction of 3TC out of the biological matrices. These sample clean-up techniques were not readily adapted to fetal and placental tissues. Fetal and placental tissues contain many more endogenous substances that need to be removed to enable a chromatographic separation of the analyte from the matrix components. Furthermore, SPE is generally an expensive and time-consuming process when compared with liquid-liquid extraction or protein precipitation based techniques.

This paper validates an analytical method using HPLC-UV for the quantification of 3TC in plasma, amniotic fluid, placental, and fetal matrices from the pregnant rat. This method utilizes liquid-liquid extraction and protein precipitation techniques for the extraction of 3TC from the four biological matrices. These extraction techniques provide faster and more convenient sample preparation procedures than SPE.

2. Experimental

2.1. Chemicals and reagents

Stavudine (d4T) was purchased from Sigma (St. Louis, MO, USA). HPLC-grade acetonitrile, methanol and sodium phosphate dibasic were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Ammonium sulfate was obtained from J.T Baker Inc. (Philipsburg, NJ, USA). Syringe filters (0.22 μm) were obtained from XPERTEK (St. Louis, MO, USA). Syringes (1 ml) were purchased from Becton Dickinson Co. (Franklin Lakes, NJ, USA). BOND ELUT C2, silica, cyano and phenyl cartridges were purchased from Varian (Harbor City, CA, USA). C8, C18 and Oasis HLB cartridges were obtained from Waters Corporation (Milford, MA, USA).

2.2. Instrumentation

The chromatographic analyses were performed using an HPLC system consisting of Waters (Milford, MA, USA) 510 pump, 717 autosampler and 486 UV detector operated with Millennium 2010 data system.

A YMC phenyl column (5 μm, 150 mm × 2 mm i.d., Waters, Milford, MA, USA) equipped with a Phenomenex C18 guard column (Torrance, CA, USA) was used to achieve all the chromatographic separations. The mobile phase consisted of 5% methanol in 20 mM dibasic sodium phosphate (pH 6), pH was adjusted using phosphoric acid and NaOH concentrated solutions. The flow rate was 0.2 ml/min and the detection wavelength was 256 nm. This low flow rate would facilitate the transfer of this method to LC–MS, if it became necessary. The injection volume of plasma and amniotic fluid samples was 20 μl, while the injection volume of placental and fetal samples was 15 μl.

2.3. Preparation of standard solutions

One mg/ml stock solutions of 3TC and D4T were individually prepared in distilled water. Lamivudine standard solutions with concentrations of 500, 400, 250, 100, 10, 7.5, 5, 2.5, 2 and 1 μg/ml were prepared from the 1 mg/ml stock solution by serial dilution with distilled water. D4T standard solution at the concentration 50 μg/ml was prepared by dilution from the stock solution with distilled water.

2.4. Calibration curves

Blank plasma, amniotic fluid, placenta and fetal tissues were collected from untreated animals. The placental and fetal tissues were homogenized with two volumes of distilled water (v/w). Plasma, placental and fetal calibration points were prepared by spiking 100 μl of the biological matrices with 10 μl of each lamivudine standard solution and 10 μl of the 50 μg/ml D4T solution. Amniotic fluid calibration points were prepared by spiking 50 μl of the biological matrices with 5 μl of each lamivudine standard solution and 5 μl of the 50 μg/ml D4T standard solution.

The calibration curves of placental and fetal homogenates were in the range of 0.2–50 μg/ml with individual calibration points of 50, 25, 10, 1, 0.75, 0.25 and 0.2 μg/ml. The calibration curves of the amniotic fluid and plasma were in the range of 0.1–50 μg/ml with individual calibration points of 50, 25, 10, 1, 0.75, 0.25 and 0.1 μg/ml. The internal standard concentration was 5 μg/ml for all samples.

2.5. Precision and accuracy

This method was validated using four QC points for each calibration curve. Five replicates of each QC point were analyzed each day to determine the intra-day accuracy and precision. This process was repeated 3 times in 3 days to determine the inter-day accuracy and precision. The QC points for plasma and amniotic fluid were 0.1, 0.5, 5 and 40 μg/ml while for the fetal and placental homogenates the QC points were 0.2, 0.5, 5 and 40 μg/ml.

2.6. Sample preparation

Solid phase extraction (SPE) using different cartridges was investigated for samples clean up. The cartridges included C18, C8, silica, phenyl, cyano and Oasis mixed bed cartridges. Each cartridge was first conditioned with 1 ml of methanol and 1 ml of distilled water followed by loading a 100 μl sample. The cartridge was then washed with 1 ml of water and finally the analytes were eluted with 1 ml of methanol. The eluent was then dried under vacuum and reconstituted in 100 μl of distilled water.

Protein precipitation was another approach attempted to extract the analytes. Acid precipitation with 2 M perchloric acid was tried. Fifteeen microliters of the acid was added to 100 μl of the sample. Samples were then vortexed and

centrifuged at 13,000 rpm for 10 min. The supernatant was aspirated and neutralized with 2 M NH_4OH. Finally, the samples were filtered through 0.2 μm nylon filters.

Protein precipitation using acetonitrile was also investigated. In this procedure, 400 μl of cold acetonitrile was added to 100 μl samples, centrifuged at 13,000 rpm for 10 min and the supernatant dried under vacuum. The samples were then reconstituted in 100 μl of distilled water.

Finally, 3TC and D4T extraction from the different biological matrices were attempted exploiting the salting out effect. One hundred and eighty microliters of saturated ammonium sulfate solution and 360 μl of cold acetonitrile were added to 100 μl samples, vortexed and centrifuged at 13,000 rpm for 10 min. The upper organic layer was aspirated and dried under vacuum. Samples were then reconstituted in 100 μl of distilled water.

2.7. Sample collection

The use of animals was approved by the UGA Animal Use and Care Committee. A pregnant female Sprague-Dawley rat (Harlan, Indianapolis, IN, USA) weighting 330 g was anesthetized with ketamine:acepromazine:xylazine (50:3.3:3.4 mg/kg IM) and dosed with 25 mg/kg of lamivudine administered as an IV bolus on day 19th of gestation. For blood sampling a cannula was placed in the right jugular vein. For sampling of the amniotic fluid, placenta and fetus, a laparotamy was performed. Blood samples were collected at 5, 15, 30, 45, 60, 90, 120 and 180 min into heparinized tubes. Samples were then centrifuged at 5000 rpm for 10 min to collect plasma. Amniotic fluid, fetuses and placentas were collected at the same time points as plasma. Fetal and placental tissues were homogenized in two volumes of distilled water. Samples from all the matrices were spiked with the internal standard (D4T) solution to yield a concentration 5 μg/ml.

3. Results and discussion

3.1. Development of HPLC assay

Structures of 3TC and D4T are shown in Fig. 1. Lamivudine is a hydrophilic weak base with a $pK_a = 4.3$ [14]. To increase the retention time of such a base in reverse phase chromatography and therefore separate it from the polar early eluting endogenous peaks, the analyte should be in the unionized form. Therefore, the pH of the mobile phase was an important factor in the successful separation of 3TC from endogenous peaks. At pH 3, 3TC was in the ionized form, and eluted at 7 min. At pH 6, 3TC was in the unionized form, and eluted at 13 min. pH was adjusted using concentrated phosphoric acid or NaOH solutions.

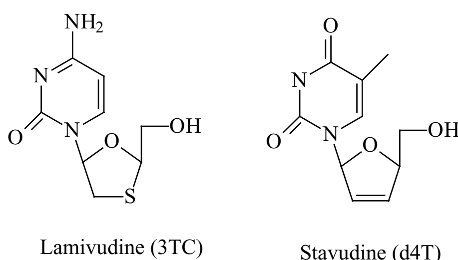

Fig. 1. Chemical structures of lamivudine and stavudine.

The organic content of the mobile phase was also investigated to optimize the separation of 3TC from the endogenous peaks. Early on, acetonitrile was excluded as an organic modifier because of its high eluotropic strength, which led to fast elution of 3TC and poor resolution from endogenous peaks. Methanol was a better choice due to its weaker strength. The addition of 5% methanol as the organic modifier achieved satisfactory resolution of 3TC and D4T from endogenous peaks in all the biological matrices. Representative chromatograms of 3TC and D4T in the four biological matrices are shown in Fig. 2. The within day variation in the elution time of 3TC and D4T was less than 3.5%.

3.2. Extraction procedure

Several liquid and solid phase extraction procedures were investigated to extract 3TC and the internal standard from the different biological matrices. Despite the availability of several extraction techniques for 3TC, we were not able to adapt these for our application. The literature techniques mainly focussed on the extraction of 3TC from human or animal plasma. In our case, however, we had to deal with fetal and placental tissues, which carry a wider variety of endogenous substances. Therefore, sample clean up played a critical role in generating chromatograms with no peaks from endogenous substances overlapping with the peaks of interest.

For fetal and placental homogenates, a salting out technique using saturated ammonium sulfate solution and acetonitrile provided the best extraction technique. The resulting chromatograms showed base line resolution of 3TC and D4T from all endogenous peaks. The percentage of acetonitrile relative to the amount of saturated ammonium solution was investigated. Increasing the percentage of acetonitrile relative to the ammonium sulfate increased the extraction efficiency of 3TC, but also yielded more endogenous peaks in the chromatogram. At high percentages of acetonitrile endogenous peaks were observed to overlap with the 3TC peak. One hundred and eighty microliters of saturated ammonium sulfate solution and 400 μl of acetonitrile resulted in the optimum extraction efficiency of the analytes and yet showed no unwanted endogenous peaks.

For plasma and amniotic fluid, acid precipitation using 15 μl of 2 M perchloric acid achieved satisfactory separation of the analytes from the biological content. From our experience, the high acidity of the injected sample can significantly shorten the lifetime of the analytical column, in

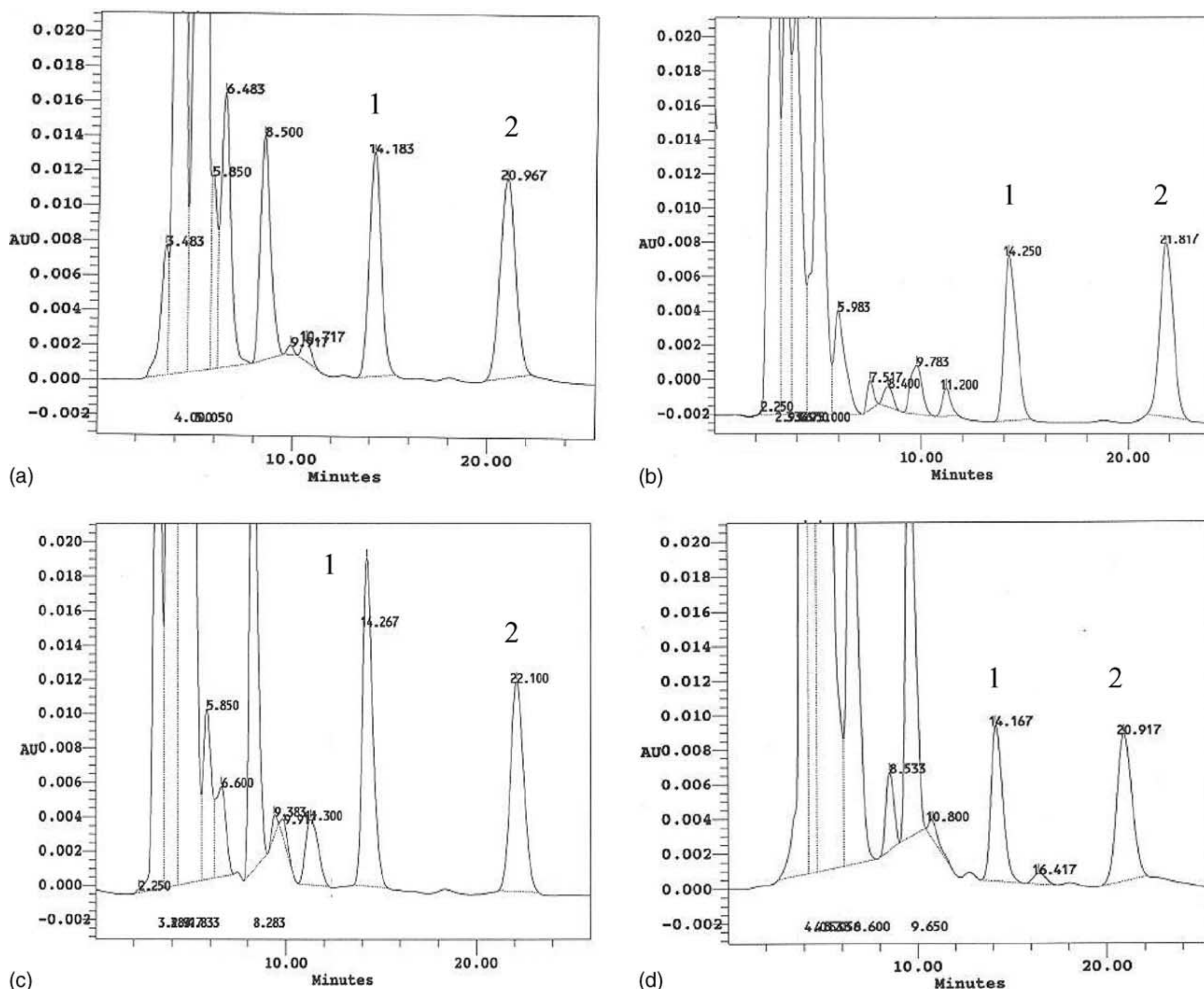

Fig. 2. Representative chromatograms of 3TC (peaks labeled '1') and D4T (peaks labeled '2') in (a) plasma, (b) placenta, (c) amniotic fluid and (d) fetus. The concentration of 3TC and D4T is 5 μg/ml in all chromatograms.

spite of the small injection volume. Therefore, the samples were neutralized with 2 M NH_4OH of equal volume prior to their injection. Fig. 3 shows representative chromatograms of plasma, amniotic fluid, placenta and fetus blank matrices.

The recoveries of 3TC and D4T from the four biological matrices are shown in Table 1. The absolute recoveries were calculated by comparing the peak areas of spiked plasma, amniotic fluid, fetal and placental homogenate samples to the corresponding peak areas of the untreated stock solutions. Absolute recoveries of 3TC and D4T ($n = 15$) in plasma and amniotic fluid ranged from 72 to 79%, while the range was from 61 to 71% in fetal and placental tissues.

Table 1
Absolute recoveries of 3TC and D4T from plasma, amniotic fluid, placenta and fetus ($n = 15$)

Analyte	Concentration	Plasma	Amniotic fluid	Placenta	Fetus
3TC	40	76 ± 2.1	78 ± 4.1	61 ± 4.1	69 ± 2.1
	5	73 ± 3.2	79 ± 3.6	62 ± 3.7	68 ± 4.4
	0.5	72 ± 1.9	76 ± 2.4	63 ± 3.3	71 ± 4.9
	0.2	–	–	64 ± 2.8	68 ± 2.4
	0.1	74 ± 3.3	76 ± 1.6	–	–
D4T	5	74 ± 1.5	75 ± 2.1	70 ± 3.5	67 ± 2.9

3.3. Accuracy and precision

Assay precision and accuracy were calculated for each matrix over 3 days. Precision, as expressed by % R.S.D., and accuracy as expressed by % error for 3TC and D4T in the four biological matrices are shown in Table 2. Intra-day ($n = 5$) precision and accuracy were calculated from the measurement of five samples at each QC point on three separate days. Inter-day ($n = 15$) precision and accuracy were calculated from pooled data over 3 days. Four QC points of concentrations 40, 5, 0.5 μg/ml and the lowest concentration in the calibration curve (0.1 μg/ml for plasma and amniotic fluid, 0.2 μg/ml for fetus and placenta) were used for

Fig. 3. Representative chromatograms of blank matrices (a) plasma, (b) placenta, (c) amniotic fluid and (d) fetus.

these calculations. Intra-day precision (% R.S.D.) and accuracy (% error) of 3TC ranged from 0.82 to 14.7 and 0.99 to 16.9%, respectively. Inter-day precision and accuracy of 3TC ranged from 1.36 to 12.1 and 3.51 to 12.6%, respectively. Results are shown in Table 2.

The calibration curves showed acceptable linearity ($R^2 \sim 0.99$) over the range 0.1–50 μg/ml for plasma and amniotic fluid and 0.2–50 μg/ml for placental and fetal homogenates.

3.4. Animal study

To demonstrate the application of this method in animal studies, a female rat received an IV bolus dose (25 mg/kg) of 3TC. This dose was chosen because of its comparability to human dosing and to allow comparison to previous animal studies involving antiviral agents in the pregnant rat [15–21]. Plasma, amniotic fluid, placental and fetal tissues were processed and analyzed as mentioned. Fig. 4 shows the concentration versus time profile for 3TC in all matrices. Noncompartmental analysis was used to obtain pharmacokinetic parameters using WinNonlin (Pharsight, Mountain View, CA, USA). The half-life, volume of distribution at steady state, and clearance were 88.5 min, 1.4 l/kg, and 1 l/(h kg), respectively. This data is consistent with earlier reported pharmacokinetic data on 3TC [15].

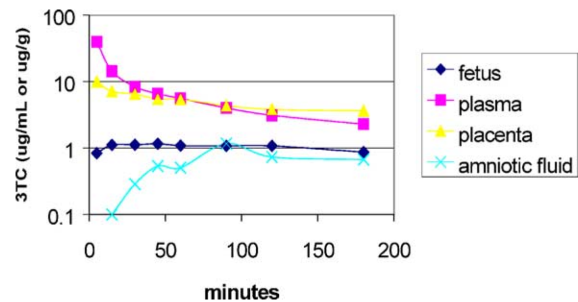

Fig. 4. Concentration-time profile of 3TC in plasma, amniotic fluid, placental and fetal homogenates.

Table 2
Inter-day ($n = 5$) and intra-day ($n = 15$) precision (% R.S.D.) and accuracy (% error) measured for four QC points for 3TC from plasma, amniotic fluid, placental and fetal tissues

T.C	Day 1			Day 2			Day 3			Inter-day		
	E.C	R.S.D.	Error	E.C	R.S.D.	Error	E.C	R.S.D.	Error	E.C	R.S.D.	Error
Plasma												
40	43.2	8.71	7.51	44.2	6.13	6.25	44.2	8.67	9.37	43.9	6.96	7.71
5	5.17	2.16	0.99	5.26	3.54	3.61	5.14	2.17	7.34	5.19	2.28	3.98
0.5	0.54	9.43	7.53	0.47	9.30	8.91	0.49	12.6	9.36	0.50	10.2	8.60
0.1	0.11	10.9	9.41	0.12	14.7	16.9	0.09	11.4	7.40	0.11	12.1	11.3
Amniotic fluid												
40	42.1	6.71	6.51	39.2	7.54	5.43	37.4	8.12	8.11	39.6	7.14	6.68
5	4.75	3.64	5.12	4.73	2.63	8.65	5.26	1.13	3.16	4.91	1.98	5.64
0.5	0.48	0.91	9.44	0.46	8.97	9.53	0.45	9.42	12.4	0.46	9.16	10.5
0.1	0.10	13.4	12.9	0.09	7.86	10.3	0.11	12.14	14.0	0.10	8.89	12.4
Placenta												
40	45.6	9.40	6.71	45.2	9.31	9.36	39.6	6.37	7.77	43.5	7.11	7.95
5	4.86	0.82	2.46	4.93	4.65	3.65	5.21	1.56	4.41	5.0	1.36	3.51
0.5	0.51	6.50	7.85	0.54	7.31	6.43	0.53	9.87	13.7	0.53	7.02	9.34
0.2	0.23	9.40	13.34	0.21	13.1	9.87	0.23	12.1	14.6	0.23	11.9	12.6
Fetus												
40	42.1	4.54	5.36	45.1	10.4	8.14	38.4	6.43	4.49	41.9	6.14	6.00
5	5.46	6.13	4.88	5.51	2.17	3.76	5.32	6.55	5.21	5.43	3.65	4.62
0.5	0.55	8.30	9.33	0.53	6.51	8.76	0.48	9.35	11.2	0.52	7.55	9.76
0.2	0.19	7.50	12.16	0.17	14.4	8.47	0.21	7.51	9.52	0.19	9.43	10.1

Note: T.C stands for theoretical concentration and E.C stands for the experimental concentration.

4. Conclusion

A simple method was developed and validated for the quantification of lamivudine (3TC) in rat plasma, amniotic fluid, placental, and fetal tissues. Samples were processed by acid protein precipitation and salting out techniques as opposed to traditional solid phase extraction. The liquid-liquid extraction method used here is much less expensive, faster, and more convenient. This method is suitable for pharmacokinetic studies to investigate the 3TC distribution profile between the maternal and fetal compartments in rats.

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