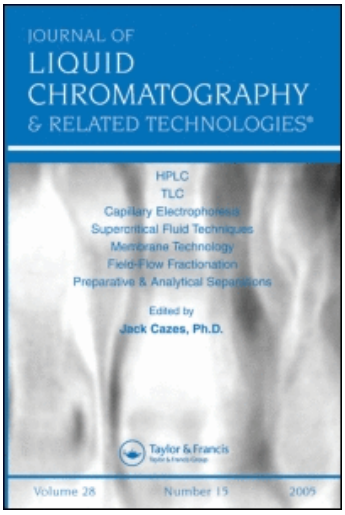

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HPLC ASSAY OF THEOPHYLLINE AND ZIDOVUDINE IN RAT SERUM

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ABSTRACT

A simple, sensitive, and reproducible HPLC method has been developed for the determination of theophylline and zidovudine (AZT) employing reverse phase high performance liquid chromatography with UV detection at 270 nm. The separation was performed on a Novapak C_{18}, 5 μm (3.9 x 150 mm) column. Acetonitrile (7.5%) in 0.2% acetic acid was used as the mobile phase and the run time was 8 min. Each drug was used as an internal standard for the other. The mean retention times of theophylline and zidovudine were 3.4 and 6.0 min, respectively. Linear response ($r > 0.998$) was observed over the range of 0.02 - 10 μg/ml for both drugs. There was no significant difference ($p < 0.05$) between inter- and intra-day studies for theophylline and zidovudine. The mean relative standard deviations (RSD%) of the results of within-day precision and accuracy of the two drugs were < 7%. The applicability of the assay was demonstrated in determining each drug concentrations in different groups of rats.

INTRODUCTION

Theophylline is widely employed as a bronchodilatant for the treatment of chronic obstructive airways diseases despite its low therapeutic index.[1-4]

Zidovudine (azidothymidine; AZT) is one of three approved drugs that are effective for treatment of neurological insult produced by HIV infection.[5-8] However, AZT remains the first-line agent in the treatment of patients with human immunodeficiency virus (HIV) infection.[9] It delays disease progression to acquired immunodeficiency syndrome (AIDS) and AIDS-related complex (ARC) disease.[10]

Several HPLC methods have been reported for theophylline and AZT.[11-18] However, these often consume more organic solvents, have longer run time, or use protein precipitation that resulted in many interfering peaks when tested in our laboratory. Some assays utilized solid-phase extraction which is time consuming and may lead to a lower drug recovery. Others have benefited of expensive detectors or equipment which are not on hand. The present study describes a sensitive, specific and rapid sample preparation assay for determining theophylline and zidovudine in rat serum with no interference from their metabolites. The assay was applied successfully to two different pilot studies for theophylline and AZT in three different groups of rats.

MATERIALS

Chemicals

Zidovudine (Lot number 8510567-177-W) was donated courtesy of M. Maguire, Burroughs Wellcome, Research Triangle Park, NC. Theophylline (Lot number 108F-0352) was purchased from Sigma Chemical Company, ST. Louis, MO. All other reagents and chemicals were analytical grade, and used as received.

Stock solutions of theophylline and AZT, 50 μg/ml, were prepared in methanol and stored in 15 ml amber glass vials at -4°C until used. Each drug was used as the assay internal standard (IS) for the other. Weekly dilutions were made in HPLC quality water to give AZT or theophylline concentrations of 0.02-10 μg/ml in rat serum, and a constant concentration of 0.5 μg/ml of the IS.

Instruments

Waters HPLC system was equipped with a Water 484 variable UV absorbance detector (set at 270 nm), and a Waters 717 autosampler. Waters 501 solvent delivery system was used to operate the gradient flow through a Novapak C18 column (3.9 x 150 mm) packed with 5 μm spherical particles. Flow rate was monitored by Waters automated gradient controller. The initial flow rate was 0.8 ml/min for 4 min and it was increased to 1.8 ml/min within 1 min. After 7 min flow rate was reduced to its initial value. Sample run time was 8 min. Chromatograms were recorded on a Waters 746 Data Module integrator chart. The HPLC system was operated at ambient temperature. Acetonitrile (7.5%) in

0.2% acetic acid solution was used as the mobile phase. Degassing was achieved by filtration through 0.22 μm filter. The injection volume was 50 μL.

METHODS

Drug Analysis

Two hundred microliters of blank rat serum was spiked with one of the two drugs and 10 μL of its IS (500 ng/ml) in 10 ml screw-capped test tube fitted with polyteflon-lined cap. Fifty microliter of isoamyl alcohol was added and the tube was vortexed for 30 seconds. The solution was mixed with 2 ml of chloroform, vortexed at high speed for 1 min, and centrifuged at 1000 rpm for 5 min. The aqueous layer was aspirated to a waste and the organic layer was transferred to a clean tube. The tube containing the organic layer was placed in a water bath (50°C) and evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 100 μL of mobile phase prior to injection into the chromatograph for analysis.

To assess the accuracy and precision of the within-day assay, six extraction of serum sample of AZT or theophylline, at the following concentrations 0.05, 0.5 and 5 μg/ml, were performed on a single day. The reproducibility of the assay (within-day and between-day) was evaluated by comparing the linear regression analysis of three standard plots obtained from spiked rat serum samples at three different days over a two month period for each drug. The recovery of each drug was assessed by extracting plasma specimens spiked to contain each drug concentrations from 0.02 to 10 mg/ml. The peak area ratio (AZT/theophylline or theophylline/AZT) was then compared with the peak area ratio for aqueous standards containing equivalent amounts of the drug and IS without extraction.

Animals

Eighteen male Sprague- Dawely rats (262 - 314 g) were used in the pilot studies of both drugs. For AZT, 12 rats were divided into 2 groups (6 rats /group) and each group was housed in one cage. One group was used as a control and the other group was subjected to acute liver damage which was induced by a single oral dose of CCl_4 solution in paraffin oil. AZT (3 mg/kg) was IP injected after 24 hr of CCl_4 applications to both groups. Theophylline was given to 6 rats as 4 mg/kg IP doses. Food and water were available *at Libitum* at all times during the experiment. Rats were lightly anesthetized with ether only during blood sampling. Blood samples were collected from the orbital venous plexus 30 min, 1, and 2 hr after each drug administrations. Therefore, each data point is the mean of 6 replicates and only three blood samples were collected from each rat per day to avoid any damage to the eye. Serum samples were separated by centrifugation at 6,000 rpm for 15 min and stored at -4°C till assayed as described above.

Statistical Analysis[19]

All results are expressed as mean ± SD. The relative standard deviation RSD% was calculated for all values. The *t*-test was used to examine the concentration difference at each day, and one-way analysis of variance (ANOVA) was employed to evaluate the reproducibility of the assay. The level of confidence was 95%.

RESULTS and DISCUSSION

Figure 1 shows representative chromatograms of extracted drug-free serum (1A), serum samples taken from rats after IP administration of 3 mg/kg AZT (1B), and serum samples withdrawn from rats after IP administration of 4 mg/kg theophylline (1C). A comparison of Figure 1A with 1B and 1C indicates that theophylline and AZT peaks are free from matrix interference. Using the chromatographic conditions described, AZT and theophylline were well resolved with mean retention times of 6.0 and 3.4 min, respectively.

Least-squares regression calibration curves were found to be linear at serum concentrations between 0.02 to 10 μg/ml for AZT and theophylline. The mean

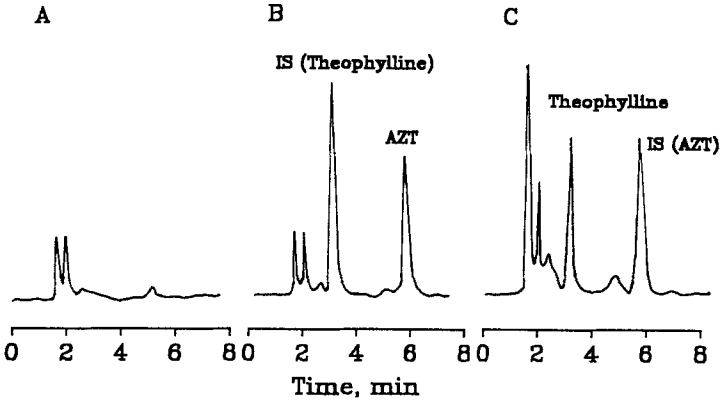

FIGURE 1

Chromatograms of extracted drug-free serum (A), serum samples (spiked with 0.5μg/ml of theophylline) taken from rat after IP administration of 3 mg/kg AZT (B), and serum samples (spiked with 0.5μg/ml of AZT) withdrawn from rat after IP administration of 4 mg/kg theophylline (C).

linear regression equations of the peak area ratios (Y) *vs.* drug concentrations (X) of AZT and theophylline were typically of the form Y = 0.858 X - 0.014 and Y = 1.55 X - 0.038, respectively. The mean correlation coefficients, r, were generally > 0.998. The detection limit of the assay, based on extraction of 0.2 ml of serum, was 20 ng/ml at a signal to noise ratio of >3.

Within-day precision and accuracy of the method were determined from replicate analysis (n=6) of 3 spiked serum test standards at concentrations within the linear range of the assay for each drug (Table 1). The mean percentage recovery of 0.02 to 10 μg/ml (n = 6) of theophylline and AZT were 97 ± 5% (RSD% = 5.3) and 94 ± 3.5% (RSD% = 3.7), respectively. Table 1 shows the recoveries of 3 different concentration of the tested drugs. Extraction efficacy was found not to significantly vary among different concentrations of AZT and theophylline.

The reproducibility of the assay was evaluated by comparing the linear regressions of three standard plots prepared at three different days over a three month period for each drug. The results of this evaluation are summarized in Table 2. The mean correlation coefficient was > 0.998 for both drugs with RSD% of the slopes of the three lines were 8 % and 6% for AZT and theophylline, respectively. Analysis of variance (Table 3) of the data indicated no significant

TABLE 1

Within-day Precision and Accuracy of Theophylline and Zidovudine Recoveries From Spiked Rat Serum Samples

Spiked Concentration (μg/ml)	Mean Measured Concentration (μg/ml), n = 6	Standard Deviation	Recovery %	Relative Standard Deviation %
Theophylline				
0.05	0.051	0.005	102	10.0
0.50	0.490	0.030	98	6.0
5.00	4.850	0.230	97	4.7
Zidovudine				
0.05	0.046	0.0037	92.0	8.0
0.50	0.458	0.0230	91.6	5.0
5.00	4.750	0.3100	95.0	6.5

TABLE 2
Reproducibility of Data from Standard Plots In Rat serum
n = 6

Standard Plot[a]	Slope[b]	Intercept[b]	Correlation Coefficient[b]
Theophylline			
1	1.64	- 0.056	0.999
2	1.57	- 0.034	0.999
3	1.45	-0.023	0.999
Zidovudine			
1	0.918	-0.0046	0.999
2	0.870	-0.0020	0.998
3	0.785	-0.0350	0.999

a Obtained from assays on three different days.
b The mean of six determinations.

TABLE 3

One-Way ANOVA for the Reproducibility of the Assay
(Within-Day and Between-Day)

Source of Variation	Sum of Squares	Degree of Freedom	Mean Square	F value
Theophylline:				
Total	0.9065	17		
Between	0.0748	2	0.0371	0.27
Within	0.8317	15	0.1386	
Zidovudine:				
Total	0.0466	17		
Between	0.0146	2	0.0073	1.37
Within	0.0320	15	0.0053	

F(95%) tabulated = 3.68, No significant difference at $p<0.05$

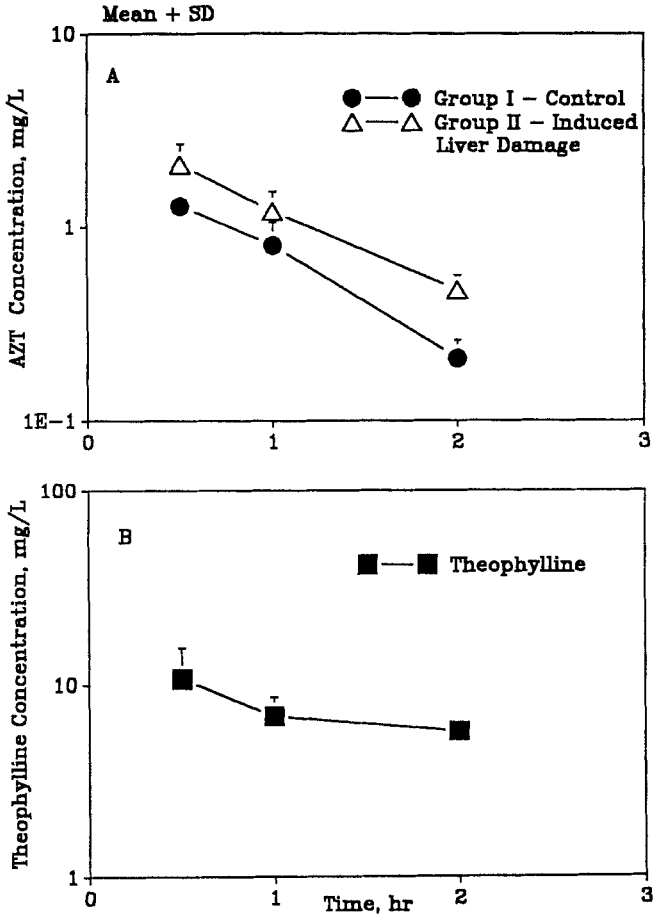

FIGURE 2

A plot of serum drug concentration - time profiles after IP administrations of AZT (3 mg/kg) given to 2 groups of rats (A); and theophylline (4 mg/kg) given to a group of rats (B).

difference in the slopes, within-day and between-day, of the calibration curves of the two drugs. The results confirmed the reproducibility of the assay method.

Figure 2A depicts a typical plot of AZT concentrations after IP administrations (3 mg/kg) to two groups of rats. Group I was the control and group II was with induced acute liver damage. While Figure 2B represents theophylline concentration time profile after 4 mg/kg IP dose to 6 rats. Figure 2 demonstrates the usefulness of the method for analysis of theophylline and AZT in serum. Re-analysis of samples several weeks after the initial analysis showed no loss in both drugs.

CONCLUSION

The HPLC method described herein has sufficient sensitivity to determine the pharmacokinetics of theophylline and AZT following a single IP dose. The assay is simple, accurate, and reproducible, and has been employed on a routine basis over the last two years for the analysis of samples of both drugs in different studies.

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