Pharmacokinetics of 1-(2-Deoxy-2-fluoro-β-L-arabinofuranosyl)-5-methyluracil (L-FMAU) in Rats

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Purpose. The objective of this study was to characterize the pharmacokinetics of 1-(2-deoxy-2-fluoro-β-L-arabinofuranosyl)-5-methyluracil (L-FMAU), a nucleoside analogue with potent activity against the hepatitis B virus and the Epstein-Barr virus, in rats. ***Methods.*** Three doses of L-FMAU were administered intravenously (10, 25, and 50 mg/kg) to rats, and L-FMAU concentrations in plasma and urine were measured by HPLC. Pharmacokinetic parameters were generated by using area-moment analysis. ***Results.*** There were no significant differences in the pharmacokinetic parameters between the three doses ($\alpha < 0.05$). Thus, the disposition of L-FMAU was linear over the dosage of 10 to 50 mg/kg. Plasma concentrations of L-FMAU declined rapidly with a terminal phase half-life of 1.33 ± 0.45 h (mean ± SD). Total clearance of L-FMAU was moderate, averaging 1.15 ± 0.28 L/h/kg. The fraction of compound excreted unchanged in urine was 0.59 ± 0.13. No glucuronide metabolite was found in the urine. The steady-state volume of distribution was 1.12 ± 0.26 L/kg indicating intracellular distribution of the compound. The fraction of L-FMAU bound to plasma proteins was approximately 15% and was independent of nucleoside concentration. ***Conclusions.*** The pharmacokinetics of L-FMAU in rats were independent of dose over the dosage range of 10 to 50 mg/kg.

KEY WORDS: 1-(2-deoxy-2-fluoro-β-L-arabinofuranosyl)-5-methyluracil; L-FMAU; nucleoside; pharmacokinetics; hepatitis B virus.

INTRODUCTION

1-(2-deoxy-2-fluoro-β-L-arabinofuranosyl)-5-methyluracil (L-FMAU), the β-L isomer of the nucleoside analogue FMAU, has shown potent *in vitro* activity against the hepatitis B virus and the Epstein-Barr virus (1). The β-D isomer (D-FMAU) was also found to have potent antiviral activity as well as antineoplastic activity in murine leukemia lines resistant to cytosine arabinoside (2). The chemical structures of β-D-FMAU and β-L-FMAU are illustrated in Figure 1. D-FMAU has been previously studied in animal models (3) and has been investigated in human phase I clinical trials (4). However, D-FMAU has been shown to cause severe neurotoxic effects that may have a cumulative component (4). Two patients out of 8 patients in the phase I study who received multiple courses of therapy of 8 mg/m^2/day for 5 days developed severe neurologic toxic reactions leading to death. Abnormalities the patients experienced included encephalopathy, extrapyramidal dysfunction, cerebellar abnormalities, and possibly hypothalamic abnormalities (4).

Because stereoisomers can have different pharmacologic and pharmacokinetic profiles, L-FMAU may have a different toxicity profile than D-FMAU. Indeed, their antiviral activities are distinct. Therefore, the L-isomer of FMAU should be studied further. The purpose of this investigation was to characterize the preclinical pharmacokinetics of L-FMAU in an animal model, the rat. In addition, the effects of dose on the disposition of L-FMAU were assessed.

MATERIALS AND METHODS

Chemicals

L-FMAU (1,5) and 2′-fluoro-2′,3′-dideoxyinosine (6) (FDDI), used as an internal standard, were synthesized as previously described. Purity of the compounds, as determined by spectral and HPLC analysis, was greater than 99%. β-Glucuronidase was purchased from Sigma Chemical Co., St. Louis, MO. Acetonitrile, HPLC grade, and all other reagent grade chemicals were obtained from J.T. Baker, Phillipsburg, NJ.

Experimental Design

Adult male Sprague-Dawley rats weighing 273 ± 24 g (mean ± SD) were allowed to acclimate for one week in a 12 h light-dark cycle at controlled temperature (22°C). External jugular vein cannulas were surgically implanted under ketamine/acepromazine/xylazine (50:3.3:3.4 mg/kg) anesthesia the day before the study. Food was withheld the night before the study, but water was freely available. The animals were placed in metabolism cages the morning of the study.

Three doses (10, 25, and 50 mg/kg) of L-FMAU were dissolved in 1 ml of normal saline and administered to rats ($n = 5$) via the cannula over a 1-min interval. The cannula was immediately flushed with saline. Preliminary studies demonstrated that L-FMAU did not adsorb to the cannulas. Blood samples (0.3 ml) were collected before and at 0.083, 0.25, 0.50, 0.75, 1.0, 1.5, 2, 3, 4, 5, 6, 7, 8, 10, 12, and 24 h after drug administration from the cannulas into heparinized tubes. The blood samples were immediately centrifuged and 100 μl of plasma was collected and stored at −20°C until analysis. Urine was collected for 24 h. Urine volume was recorded, and samples were stored at −20°C until analysis.

Protein Binding

Plasma protein binding of L-FMAU was determined by ultrafiltration. Blank plasma (1 ml) with L-FMAU (1, 10, 100, and 200 μg/ml) added was incubated for 1 h at 37°C, placed in a Centrifree® micropartition system (Amicon, Beverly, MA) and centrifuged at 1500 rpm for 5 min. This produced approximately 100 μl of ultrafiltrate which was stored at −20°C until analysis. Preliminary experiments demonstrated that L-FMAU did not adsorb to the membrane. Plasma samples containing the pre-filtration L-FMAU concentrations were also frozen until assayed.

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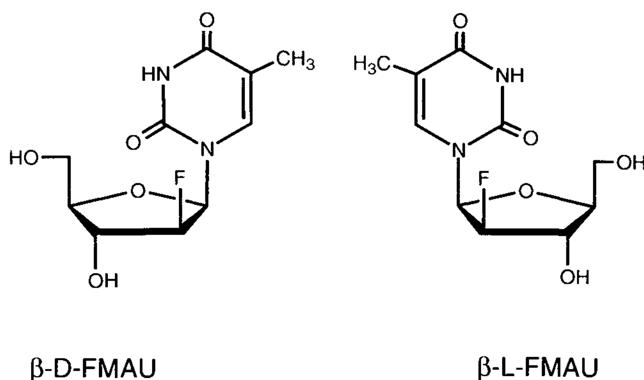

Fig. 1. Structures of β-L-FMAU and β-D-FMAU.

Drug Analysis

Concentrations of L-FMAU in plasma were determined by reversed phase high performance liquid chromatography (HPLC) using a Hypersil ODS (C_{18}) Column (150 mm × 4.6 mm, 5 μm particle size, Alltech Associates, Deerfield, IL). Plasma (100 μl) was added to 0.4 ml polypropylene microcentrifuge tubes followed by 50 μl internal standard, FDDI (10 μg/ml). Perchloric acid (50 μl of 2 M) was added as a protein precipitant and samples were mixed for 30 sec. Potassium hydroxide (50 μl of 2 M) was added to neutralize the perchloric acid and mixed for 30 sec. The samples were then centrifuged at 7000 rpm for 5 min and 20–200 μl supernatant was injected onto the HPLC (Waters Associates, Milford, MA). The mobile phase used was 3% acetonitrile in 0.05 M KH_2PO_4, pH 2.25 at a flow rate of 2.0 ml/min. The compounds were quantitated at a UV wavelength of 261 nm.

Urine samples were diluted 1:50 or 1:100 with deionized distilled water and 50 μl of internal standard was added to 100 μl of diluted urine and injected onto the HPLC. Urine samples were also treated with β-glucuronidase to detect any glucuronide metabolites in the urine (7). To 100 μl of urine, 15 μl of 0.12 N acetic acid, 100 μl β-glucuronidase (1000 U/ml in water), and 35 μl phosphate buffer pH 5.8 were added. Tubes were mixed and incubated at 37°C overnight in a shaking water bath. After incubating, samples were diluted 1:10 and 50 μl FDDI was added to 100 μl diluted urine and injected onto the HPLC. AZT-glucuronide, treated using the same method, served as a positive control to ensure enzyme activity.

Standard curves of L-FMAU ranging from 0.05 μg/ml to 200 μg/ml were prepared in blank rat plasma and water. Sample L-FMAU concentrations were determined from the slope of standard curves of the peak area ratio of drug:internal standard v. standard L-FMAU concentrations. A weighting factor of $1/x^2$ yielded a normal distribution of residuals around the fitted standard curves. The analytical recovery, precision and accuracy were assessed at 0.2 μg/ml and 100 μg/ml L-FMAU. The peak areas of six extracted plasma samples and six direct injections of the same amount of drug were determined, and analytical recovery was calculated from peak $\text{area}_{\text{extracted drug}}$/mean peak $\text{area}_{\text{direct injection}}$ × 100%. The intra- and inter-day precision of the assay was determined by the analysis of six quality control samples. The accuracy of the assay was assessed by comparing the results of the precision study to the known concentrations.

Data Analysis

Pharmacokinetic parameters were determined by area-moment analysis. The area under the plasma concentration v. time curve (AUC) and first moment (AUMC) were calculated by Lagrange polynomial interpolation from time zero to the last sample time with extrapolation to time infinity using the least-squares terminal slope (λ_z) (8,9). Total clearance (CL_T) was calculated from Dose/AUC. The fraction excreted in the urine (f_e) was calculated from Au^∞/Dose, where Au^∞ is the total amount of unchanged L-FMAU excreted in the urine. Renal clearance (CL_R) was calculated from $CL_T \times f_e$, and non-renal clearance (CL_{NR}) was calculated from $CL_T - CL_R$. Mean residence time (MRT) was calculated from AUMC/AUC, and steady-state volume of distribution (V_{ss}) was calculated from CL_T × MRT. Half-life ($t_{1/2}$) was calculated from $0.693/\lambda_z$. A one-way analysis of variance ($\alpha < 0.05$) was performed to determine if there were statistically significant differences in the pharmacokinetic parameters between the three doses studied.

RESULTS AND DISCUSSION

Figure 2 shows typical chromatograms for extracts of blank plasma, plasma spiked with L-FMAU and internal standard (FDDI), and a plasma sample taken after intravenous administration of 10 mg/kg of L-FMAU to a rat. No interfering endogenous peaks were observed in the blank rat plasma. L-FMAU and FDDI had retention times of 9.0 and 7.8 min, respectively. The peaks with retention times of 15 and 16 min were endogenous peaks, however, these peaks did not interfere with either L-FMAU or FDDI. The assay was linear over the range of 0.05 to 200 μg/ml with a lower limit of quantitation of 0.05 μg/ml. The intra- and inter-day coefficients of variation were less than 10%, and the accuracy of the assay was 95%. The analytical recovery of L-FMAU and FDDI was greater than 95%.

Figure 3 shows median plasma L-FMAU concentration

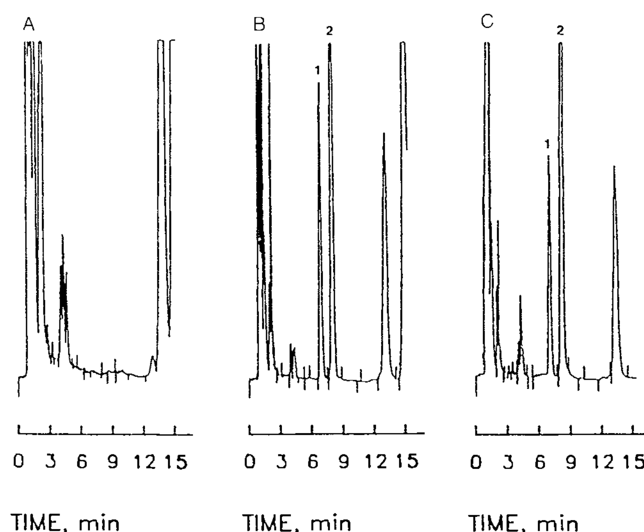

Fig. 2. Typical chromatograms for extracts of (A) blank plasma, (B) plasma spiked with (1) internal standard (FDDI), and (2) L-FMAU, and (C) a plasma sample taken after intravenous administration of 10 mg/kg L-FMAU to a rat.

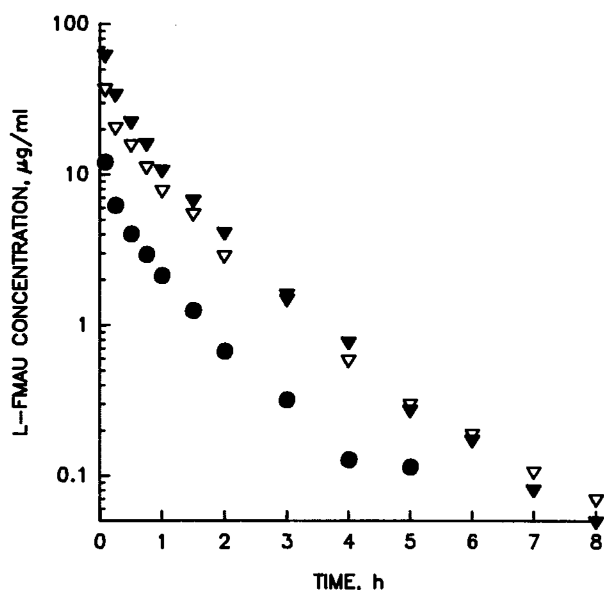

Fig. 3. Median plasma concentrations of L-FMAU as a function of time following intravenous administration of (●) 10, (▽) 25, and (▼) 50 mg/kg L-FMAU to rats.

versus time curves following intravenous administration of 10, 25, and 50 mg/kg to rats. After intravenous administration, plasma levels declined rapidly in a biexponential manner. Pharmacokinetic parameters generated by area-moment analysis are presented in Table I. There were no statistically significant differences ($\alpha < 0.05$) in any of the parameters between the three doses. Therefore, L-FMAU displayed linear pharmacokinetics over the dose range of 10 to 50 mg/kg, and overall mean values of the pharmacokinetic parameters for the three doses are also presented in Table I. The total clearance of L-FMAU was moderate, averaging 1.15 ± 0.28 L/h/kg. The fraction of the dose excreted unchanged in the urine averaged 0.59 ± 0.13, and the renal clearance was 0.70

Table I. Mean Pharmacokinetic Parameters[a] After Single Intravenous Administration of 10, 25, and 50 mg/kg L-FMAU to Rats

	Doses, mg/kg			
	10	25	50	Overall
AUC, mg * h/L	8.42 (2.96)	28.93 (7.58)	40.74 (3.96)	
CL_T, L/h/kg	1.26 (0.32)	0.95 (0.26)	1.23 (0.16)	1.15 (.28)
CL_R, L/h/kg	0.85 (0.36)	0.57 (0.26)	0.68 (0.10)	0.70 (0.27)
CL_{NR}, L/h/kg	0.41 (0.20)	0.37 (0.20)	0.56 (0.19)	0.45 (0.20)
f_e	0.66 (0.17)	0.55 (0.11)	0.55 (0.10)	0.59 (0.13)
V_{ss}, L/kg	1.24 (0.13)	0.97 (0.39)	1.14 (0.13)	1.12 (0.26)
$t_{1/2}$, h	1.13 (0.45)	1.45 (0.20)	1.42 (0.62)	1.33 (0.45)
MRT, h	1.06 (0.40)	1.25 (0.35)	0.93 (0.13)	1.08 (0.32)

[a] Parameters values are expressed as mean (SD); $n = 5$.

± 0.27 L/h/kg. Renal clearance was greater than glomerular filtration rate (0.27 L/h/kg) in the rat (10), indicating that L-FMAU underwent active tubular secretion. L-FMAU concentrations in urine samples treated with β-glucuronidase were identical to those of untreated urine. Therefore, no glucuronide metabolite was detected in the urine. Non-renal clearance was moderate averaging 0.45 ± 0.20 L/h/kg. The steady state volume of distribution was 1.12 ± 0.26 L/kg, which indicates intracellular distribution of the compound. The fraction of L-FMAU bound to plasma proteins was approximately 15% and was independent of nucleoside concentration.

L-FMAU showed potent antiviral activity against hepatitis B virus *in vitro* with an EC_{50} value of 0.1 μM (0.026 μg/ml) (1). The compound was also active against the Epstein-Barr virus with an *in vitro* EC_{90} of 5 μM (1.3 μg/ml) (1). Thus, plasma concentrations of L-FMAU achieved in this study were well above the range of the *in vitro* effective antiviral concentrations for L-FMAU.

Two rats exhibited distinctly different pharmacokinetic profiles from the other 15 rats that were studied. One rat which was administered 10 mg/kg of L-FMAU had significant plasma concentrations (0.41 μg/ml) 10 h following nucleoside administration, while another rat which was administered 50 mg/kg of L-FMAU had significant blood levels (3.16 μg/ml) 24 h following administration. Both of these rats had markedly lower total (0.40 L/h/kg and 0.16 L/h/kg), renal (0.24 L/h/kg and 0.08 L/h/kg) and non-renal (0.16 L/h/kg and 0.08 L/h/kg) clearance values in comparison to the other rats studied. Interestingly, the fraction of the dose of L-FMAU excreted unchanged in the urine for both rats was consistent with the other rats. Dixon's test for extreme values (11) was applied to the clearance values of these two rats and showed that these two rats were considered outliers ($\alpha < 0.05$). Therefore, data from these outliers were not included in the statistical analysis. These two rats also appeared to be quite lethargic and unresponsive. Further studies are planned to determine if these were drug-related toxicities associated with L-FMAU. Certainly, in light of the severe toxicities of D-FMAU, extensive preclinical research should be done on L-FMAU before clinical trials are warranted.

Concentration versus time data following intravenous administration of 10 mg/kg D-FMAU to rats from a previous study (3) were evaluated using area/moment analysis to generate pharmacokinetic parameters in order to compare the disposition of this isomer with that of L-FMAU. The D-FMAU data yielded a CL_T value of 0.50 L/h/kg, which is 2-fold lower than the CL_T of L-FMAU (1.15 L/h/kg). The D-FMAU study, using radiolabeled nucleoside, showed that unchanged D-FMAU accounted for 95 to 97% of the radioactivity in rat urine. This demonstrated that essentially all of an administered dose of D-FMAU was excreted unchanged in urine. Thus, renal clearance accounted for virtually all of the CL_T of D-FMAU, with only minimal non-renal clearance. Renal clearance values for D-FMAU (0.50 L/h/kg) were similar to those for L-FMAU (0.70 L/h/kg). Thus, the difference in total clearance values between the isomers can be explained by the greater non-renal clearance of L-FMAU. The V_{ss} for the D-FMAU was 1.0 L/kg, which was similar to that of L-FMAU (1.12 L/kg). The half-lives for the two isomers were also comparable (1.3 h and 1.5 h). Thus, the phar-

macokinetics of L-FMAU differs from that of D-FMAU primarily by the existence of non-renal clearance of the former.

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