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BIOMEDICAL APPLICATIONS

High-performance liquid chromatographic method for the determination of an HIV-1 non-nucleoside reverse transcriptase inhibitor (L-696,229) in plasma samples from animals

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Abstract

A sensitive high-performance liquid chromatographic (HPLC) method was developed and validated to separate and quantitate the levels of L-696,229 (I), a novel human immunodeficiency virus type 1 non-nucleoside reverse transcriptase inhibitor, and its hydroxy metabolites (II and III) in plasma samples. The procedure involves the addition of a constant known quantity of internal standard to the biological specimen followed by extraction of the compounds of interest into methyl *tert.*-butyl ether. The organic phase is evaporated to dryness under a gentle stream of nitrogen. The residue is then dissolved in methanol and water and injected onto a reversed-phase HPLC column. A gradient HPLC method is used to elute the compounds which are monitored using UV detection at 319 nm. Absolute calibration factors (from the standard curve) were calculated by analyzing standards, and these factors were used to determine the concentration of drug (I) and its hydroxy metabolites (II and III) in the samples using the internal standard method. The method was linear using a standard concentration range of 50 to 20 000 ng/ml. The limit of quantitation was 50 ng/ml using 200 μl plasma. The procedure was utilized to monitor plasma levels of I, II and III in acute and chronic toxicity studies in several animal species.

Keywords: HIV-1 non-nucleoside reverse transcriptase inhibitor; Transcriptase

1. Introduction

L-696,229, 2-[2-(5-ethyl-6-methyl-2(1H)-pyridon-3-yl)ethyl]benzoxazole (I, Fig. 1), is a potent and specific inhibitor of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) [1,2]. It is one of a new series of non-nucleoside derivatives containing a 2-pyridino group and may prove to be clinically useful for the treatment of acquired immunodeficiency syndrome (AIDS) [3]. In vitro metabolism studies of I yielded several metabolites such as the hydroxyalkyl metabolites II and III (Fig. 1) which were identified and characterized by NMR and mass spectrometry. The HIV-1 RT inhibitory activity profiles in human plasma and urine showed that the hydroxyalkyl metabolites were RT inhibitors as well [4,5]. In order to determine whether II and III were present in plasma samples during the pre-clinical studies it was necessary to develop a sensitive and specific assay for I and its hydroxy metabolites (II and III). The development of a high-performance liquid chromatography (HPLC) method was used to support the acute and chronic toxicity studies for the

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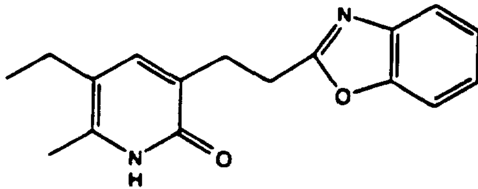
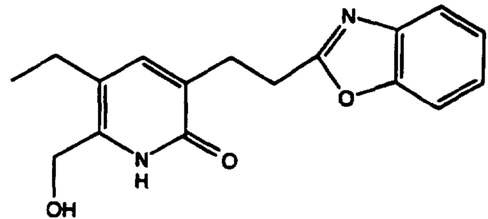
L-696,229,000X-004



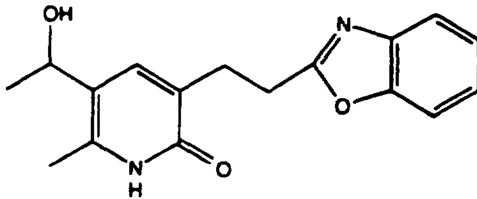
(I)

L-698,680-000B002



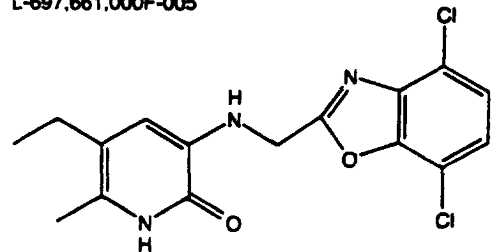
(II)

L-697,895-000M005



(III)

L-697,661,000F-005



(IV)

Fig. 1. Structures of compounds I, II, III and IV

quantitation of compounds I, II, and III in different species such as mouse, monkey, rats and dogs.

2. Experimental

2.1. Reagents and materials

2-[2-(5-Ethyl-6-methyl-2(1H)-pyridon-3-yl)ethyl]-benzoxazole (L-696,229, I), its 5-α-hydroxy metabolite 3-[2-(benzoxazol-2-yl)ethyl]-5-(1-hydroxyethyl)-6-methylpyridin-2(1H)-one (L-697,895, II), its 6-hydroxy metabolite 3-[2(benzoxazol-2- yl)ethyl]-5-ethyl-6-hydroxymethyl-pyridin-2-(1H)-one (L-698,680, III) and the internal standard 3-[2-(4,7-di-chlorobenzoxazolyl)methylamino] -5-ethyl-6-methyl-(1H)-pyridin-2-one (L-697,661, IV) were synthesized at Merck Research Laboratories (West Point, PA, USA). All organic solvents and chemicals were HPLC grade; methyl *tert.*-butyl ether (MTBE) was purchased from Burdick and Jackson; acetonitrile, methanol, and ortho-phosphoric acid (85%, v/v) were purchased from Fisher Scientific (Springfield, NJ, USA); triethylamine was purchased from Pierce (Rockford, IL, USA).

2.2. Preparation of standards

Stock solutions of compounds I, II, III, and internal standards were prepared in methanol. The stock solutions were diluted with MeOH–H_2O (50:50, v/v) to obtain the appropriate concentrations of the working standards. Known concentrations of the mixed working standards were consequently spiked into the control plasma for the standard curve preparation.

2.3. Assay procedure

Plasma samples were isolated from heparinized whole blood specimens following centrifugation at 15°C. An aliquot of plasma (usually 200 μl) was added to a 15-ml polypropylene centrifuge tube followed by the additions of 25 μl working standard

of IV, 200 μl of water and 6 ml MTBE. The tubes were capped and mixed for approximately 10 min. After brief centrifugation, the organic layer was transferred to a clean 15-ml polypropylene centrifuge tube. The organic solvent was evaporated to dryness under nitrogen at a temperature of about 40°C. The residue was then dissolved in 200 μl of MeOH followed by 100 μl of H_2O. The tubes were capped, vortexed, and centrifuged before the solution was transferred to an auto-sampler vial for HPLC analysis.

2.4. HPLC instrumentation and chromatographic conditions

Analyses were performed on an HPLC system consisting of a Perkin-Elmer (Norwalk, CT, USA) Series 410 pump, Waters Model 715 autosampler and Applied Biosystems Model 785A UV detector. The detector output signals were interfaced to a Perkin-Elmer Nelson ACCESS*CHROM data system.

The HPLC peaks were identified by retention times with reference to standard compounds and quantitated using peak height of the compound of interest to internal standard. Separations were achieved using a Zorbax Rx-C_8 base-deactivated reversed-phase column (150×4.6 mm I.D.) from MAC MOD (Chadds Ford, PA, USA). The mobile phase consisted of solvent A [0.1% H_3PO_4 containing 0.2% triethylamine (v/v, pH 3.0±0.1)] and solvent B (acetonitrile). In order to achieve optimum separation, a gradient system was employed. The conditions of the solvent gradient involved a linear gradient change of solvents A and B with a constant 1 ml/min flow-rate. At time 0 min, the ratio was solvent A 70% and solvent B 30%. This proportion of solvent mixture was changed in a linear fashion to solvent A 45% and solvent B 55% in the next 20 min. At the end of the 20-min cycle, the proportion of solvent mixture was adjusted again in a linear fashion to solvent A 15% and solvent B 85% in the next 5 min. At the end of the 25-min cycle, the solvent proportions changed back to the original concentrations, i.e. solvent A 70% and solvent 30% for 10 min before the next injection. The retention of compounds I, II, III, and internal standard were approximately 16.0, 4.3, 8.4, and 21.0 min, respectively.

2.5. Assay validation

The absolute recoveries of I and the hydroxy metabolites from monkey plasma were estimated in the range of 50 to 5000 ng/ml by comparing the detector response from the extracted spiked plasma samples to those of unextracted standards.

The linearity of the method was based on evaluation of correlation coefficient and variability of slope for intra- and inter-day analyses. The accuracy and precision of the method was determined from the quality control (QC) samples which were processed the same time with the test samples. The lower limit of quantitation (LOQ) was defined as the concentration which could be quantified with acceptable accuracy (difference<15%) and precision (C.V.< 15%).

2.6. Animal studies

The utility of this method is demonstrated by measurement of the plasma concentration of I in rhesus monkeys after repeated oral doses.

A total of 32 rhesus monkeys (*Macaca mulatta*) were used in this study. These monkeys were approximately 1 to 3 years old and weighed between 2.0 to 3.4 kg at the initiation of the study. Animals were assigned to the vehicle control (0.5% methylcellulose solution), 10, 40, and 160 mg/kg/day dose groups. There were 4 animals/sex/dose in each group. During drug day 1 and drug week 13, a blood sample was collected into heparinized tubes from each non-fasted monkey at 1, 2, 4, 6, 8, 12, and 24 h post dose. The heparinized blood samples were centrifuged and plasma was separated and stored frozen until analyzed for I and metabolite content. The control blood samples were treated the same manner as the study samples and used for the standard curve preparation.

3. Results and discussion

3.1. Chromatography

As mentioned in the Section 2, the HPLC chromatographic condition required a shallow gradient

pump method in order to separate the four compounds of interest (including internal standard) with the retention times ranging from approximately 4 to 21 min. The peak retention times were very consistent during the one-month period of analysis. The C.V.s of peak retention time were 0.58% ($n=144$), 1.02% ($n=144$), and 1.13% ($n=142$), respectively, for I, II, and III.

Blank plasma obtained from species including dog, rat, monkey, and mouse were tested and found to show no significant endogenous peaks that would interfere with the analysis of I and its metabolites (Fig. 2). There was an unidentified drug related peak shown in the III region for most of the study samples. This peak could be separated either by increasing the aqueous composition in the mobile phase or using the peak height for quantitation (Fig. 3).

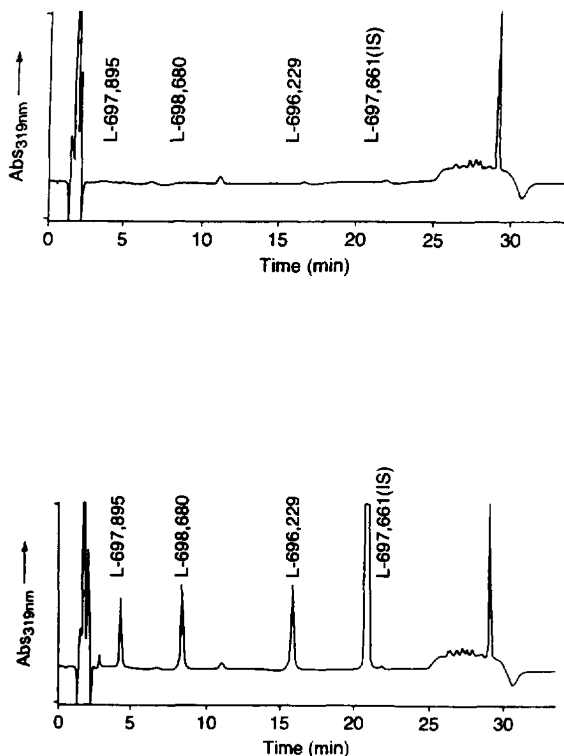


Fig. 2. Control monkey plasma spiked with 100 ng of L-697,895, L-698,680 and L-696,229 and 500 ng of L-697,661 (I.S.) per 200 μl plasma.

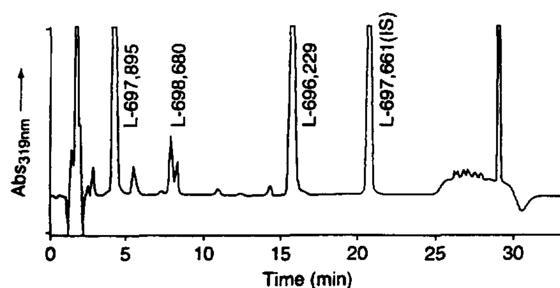


Fig. 3. Monkey plasma 1 h after dosing at 40 mg/kg/day.

3.2. Validation

The mean percent absolute recoveries for I, II and III are presented in Table 1. There was no evidence for concentration dependence within the test range of 50 to 5000 ng/ml. The slightly lower recovery of II was also observed in rat plasma matrix (data not shown) and probably is attributed to its greater polarity.

Triplicates of the standard curve containing seven concentrations over the range from 50 to 5000 ng/ml were prepared within one day. The intra-day calculated concentrations, precision and accuracy of low, medium and high concentrations, specifically 50, 500 and 5000 ng/ml plasma, are reported in Table 2. As indicated, the precision was from 0.2 to 7.7% and the accuracy was from 97.3 to 103%. The standard curves using $1/y$ weighed least-squares regression analysis were found to be linear in this range. The correlation coefficients were >0.993 of compounds I, II, and III for all curves. The slopes were quite reproducible with C.V. of 2.04, 2.43, and 3.16% for compounds I, II, and III, respectively. A concen-

Table 1
Mean percent absolute recovery data for I, II and III in monkey plasma

Compound	Absolute recovery (mean, $n=3$) (%)						
	50[a]	100	300	500	1000	3000	5000
I	94.3	91.3	93.7	93.0	87.7	88.3	96.3
II	79.7	75.7	80.0	75.7	75.3	79.7	90.0
III	91.7	84.3	91.0	86.7	86.7	90.3	99.7

[a] Nominal concentrations (ng/ml).

Table 2
Standard curve summary for the determination of I, II and III in monkey plasma (50–20 000 ng/ml plasma range)

	Nominal concentration (ng/ml)								
	I			II			III		
	50	500	5000	0	500	5000	50	500	5000
Intra-day									
Calculated concentration (ng/ml)	50.4	497	4996	50.6	487	5052	51.5	491	5066
Precision (C.V., %)	2.5	1.6	4.7	0.2	2.0	6.7	2.4	2.2	7.7
Accuracy (%)	101	99.4	99.9	101	97.3	101	103	98.2	101
n	3	3	3	3	3	3	3	3	3
Inter-day									
Calculated concentration	53.1	487	4787	53.6	490	4802	53.4	493	4752
Precision (C.V., %)	8.8	4.2	4.8	10.4	5.2	4.8	9.9	5.0	5.4
Accuracy (%)	106	97.4	95.7	107	97.9	96.0	107	98.7	95.0
n	16	16	15	16	16	15	16	16	15

tration of 50 ng/ml was established as the LOQ based on acceptable estimates obtained for precision (C.V.< 15%) and accuracy (% difference< 15%) at this level.

The inter-day variation of the standard curves which were generated in the period of one month are presented in Table 2. The standard curves using $1/y$ weighed least-squares regression analysis were found to be linear in the range of 50 to 20 000 ng/ml plasma. The slopes were reproducible with C.V. ($n=13$) of less than 13% for all three compounds of interest. The accuracy of the assay was demonstrated in Table 2; C.V. was less than 6.6% for all three compounds at all concentrations except at the concentration of LOQ where C.V. was less than 10%.

The accuracy and precision of this method was estimated from the QC samples. The QC samples were prepared by spiking the known amount of the test compounds into the control monkey plasma and stored at the same condition as the study samples. They were prepared within a week of when the study plasma samples were received and analyzed as unknowns. Table 3 summarizes the mean accuracies and precision (% of C.V.) for the QC monkey plasma samples for I, II and III. The C.V. was <10% for I and II and was <15% for III.

3.3. Toxicity/toxicokinetic study in monkeys

The typical mean plasma concentration data ($n=4$) obtained following various treatments of I in male monkeys are shown in Fig. 4. As indicated, maximum concentration (C_{max}) occurred about one hour after dosing on both drug day 1 (DD 1) and drug week 13 (DW 13). Once C_{max} was reached, the drug concentration declined rapidly and no drug was

Table 3
Mean accuracy and precision data of the quality control monkey plasma samples for I, II and III

	Nominal concentration (ng/ml)								
	I			II			III		
	100	1000	5000	100	1000	5000	100	1000	5000
Calculated concentration (ng/ml)	109	994	5102	107	1021	5241	104	938	4784
Precision (C.V., %)	7.8	5.3	7.8	7.2	7.1	8.6	12.4	8.6	11.5
Accuracy (%)	109	99.4	102	107	102	105	104	93.8	95.7
n	17	20	20	17	20	20	17	20	20

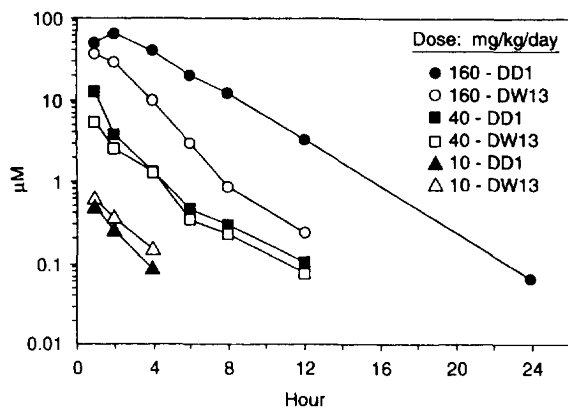


Fig. 4. Mean L-696,229 concentration vs. time following various doses of L-696,229 (male monkeys).

detected in most animals by 24 h post dose. The AUC values (not shown) gave, in general, non-linear responses with increasing dose. The maximum systemic exposure of I was not attained at the dose levels studies. When DD 1 data were compared with DW 13 data, the plasma profiles at 10 and 40 mg/kg/day agree closely, whereas there is a divergence in response at the 160 mg/kg/day dose (i.e. the DW 13 curve is lower).

4. Conclusion

This HPLC method for the determination of I and its hydroxy metabolites has been demonstrated to be accurate and reliable. Thousands of plasma samples from the preclinical studies on different species had been analyzed using the aforementioned method.

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