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Viral Clearance Issues Associated with the Use of an Animal-Derived Enzyme in the Synthesis of Emtricitabine

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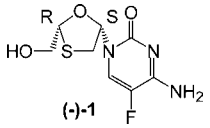
Abstract:

Emtricitabine, the active ingredient in the antiviral drug Coviracil, has been prepared by a process using pig liver esterase to resolve optical isomers of an intermediate. Since this enzyme was derived from an animal source, potential existed for viral contamination of the product and the multipurpose equipment in which the product was prepared. To address these issues, the potential risk of viral contamination was assessed, and the potential for the process to clear viruses from the process stream was determined.

Introduction

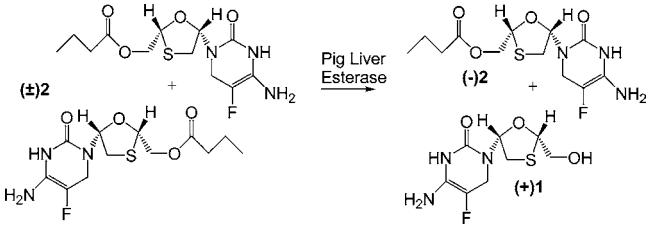
Emtricitabine is the active ingredient in the new antiviral drug coviracil (Emtriva), a nucleoside reverse transcriptase inhibitor targeted for the treatment of human immunodeficiency virus (HIV) and hepatitis B virus (HBV) infections.[1] Emtricitabine (2′,3′-dideoxy-5-fluoro-3′-thiacytidine, FTC, (−)-**1**) is an optically active drug, the active form being the (−), or [1′-*S*,4′-*R*] (numbering as a ribose analogue) form.



The process for preparing emtricitabine uses an enzymatic resolution in the penultimate step to generate the optically active form of (−)-**1** as its butyrate ester (−)-**2** (Scheme 1). The racemic butyrate ester (±)-**2** is treated with the enzyme, which results in cleavage of the unwanted isomer to the corresponding alcohol (+)-**1**. The desired isomer is left behind as the (−)-butyrate ester (−)-**2**, which undergoes nonspecific hydrolysis to optically active emtricitabine in a subsequent step.

Pig liver esterase (PLE) was chosen early in the development of the process due to its ready availability and superior selectivity over any of the other 13 enzymes surveyed.[2] We received a contract to scale up the synthesis and develop a validated manufacturing process at the time when the drug was already in clinical testing and on a tight timeline for filing; thus, development of an alternate process was not an option. Concerns were raised about the potential for PLE to introduce viruses into the process stream and thereby potentially to contaminate the product and the multipurpose equipment in which the process was carried out.[3]

Scheme 1



Methods

Detailed regulatory guidance exists for dealing with potential viral contamination in biological products used as pharmaceuticals,[4] but very little information is available on dealing with potential viral contamination of chemical products.[5] We carried out an extensive literature search, but no published work was found on the use of PLE or any other animal-derived enzyme in production of a pharmaceutical chemical. The following is the system developed for dealing with viruses in biological products[4] that has been adapted for our process:

1. potential viral load determined by testing representative lots of enzyme
2. scaled-down laboratory process developed and validated
3. viral clearance protocol developed and approved
4. viral clearance potential of a unit operation assessed
5. viral risk assessed

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(1) (a) Liotta, D. C.; Choi, W.-B. PCT Int. Appl. WO 91252418, 1991. (b) Liotta, D. C.; Schinazi, R. F.; Choi, W.-B. PCT Int. Appl. WO 9214743, 1992. (c) Painter, G. R.; Liotta, D. C.; Almond, M.; Cleary, D.; Soria, J. PCT Int. Appl. WO 2000009494, 2000.

(2) Hoong, L. K.; Strange, L. E.; Liotta, D. C.; Koszalka, G. W.; Burns, C. L.; Schinazi, R. F. *J. Org. Chem.* **1992**, *57*, 5563.

(3) While swine have not been identified as yet as carriers of TSE, potential contamination by TSE agents was also of concern. Specifications were put in place to require that enzyme vendors include certificates of origin stating that animals originated in countries where TSE risk was minimal, i.e., United States (Sigma) and Denmark (Roche). In addition, the organ (liver) is considered to be a "low infectivity" tissue. "Joint CPMP/CVMP Guidance on Minimizing the Risk of Transmitting Animal Spongiform Encephalopathy Agents via Human and Veterinary Medicinal Products," February, 2001; BP 2000 Vol. I, 1537; BP 2000 Vol. II, A435.

(4) "International Conference on Harmonisation: Guidance on Viral Safety Evaluation of Biotechnology Products Derived From Cell Lines of Human or Animal Origin," *Federal Register* **1998**, *63*(185, 24 September), 51074−51084.

(5) "Draft Guidance for Industry on Monoclonal Antibodies Used as Reagents in Drug Manufacturing; Availability," *Federal Register* **1999**, *64*(121, 24 June), 33868−33869.

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Enzyme Testing. First, the extent of the viral load in the incoming enzyme[6] was assessed. Since it was not known which specific viruses might be present in the PLE, a nonspecific viral screening was carried out.[7] Screening of each lot of enzyme for viral contamination was continued as part of the quality control of the incoming reagent. This was done to ensure that the viral load of the enzyme used in the process would not exceed the capacity of the process to clear viruses. For all lots of the enzyme, no viruses were detected in the screening tests.

Scaled-Down Process. The ICH (International Conference on Harmonisation) guidance for biologics[4] requires that a scaled-down version of the manufacturing process be developed and validated and that the process material from this scaled-down manufacturing process be used to evaluate viral clearance. The use of a scaled-down process avoided the problem of scheduling viral clearance studies to match the manufacturing schedule. In this case the process was not yet operating at full manufacturing scale; thus, the pilot-plant process, at one-half to one-third of the planned full scale, was used as a starting point.

The scaled-down process was developed directly from the process used in the pilot-plant enzymatic resolution step. Process flow, equipment, and process parameters were compared for the pilot-plant and the scaled-down process. Acceptance criteria were established for the scaled-down lots based on interim specifications for the intermediate (−)-**2** (identity, assay, purity, impurity levels, etc.) The scaled-down protocol was pre-approved, and the scaled-down process was executed three times. Process parameters were controlled within the pre-approved ranges. The products of the three scaled-down runs were compared to the products from the three representative pilot-plant runs and shown to be equivalent. The data were reviewed and approved.

Procedure for Enzymatic Resolution and Viral Clearance of Racemic Ester (±)-2. A solution of 94.5 g of racemic ester (±)-**2** in 593.5 g of amyl alcohol,[8] and 595.4 g of phosphate buffer[9] was prepared in a 2 L flask equipped with a flat-blade agitator. The mixture was stirred and heated to 47°C until a clear solution was obtained. The solution was cooled to 27°C, and a solution of 1.9 g of copper (II) chloride dihydrate in 18.8 g of water was added. The pH of the solution was adjusted to 7.8−8.1 (pH meter) with dilute sodium hydroxide. To this mixture was added 198,000 units (49.7 g of @ 3992 units/g) Roche pig liver esterase,[10] and the mixture was stirred under nitrogen at 135−140 rpm. At this rate a thin layer of clear, amber amyl alcohol was visible on top of the blue emulsion. The reaction was sampled periodically for completion of reaction until the (+)-**2** ester was NMT 0.7 area % by chiral HPLC. When the reaction was complete, the entire reaction mixture was allowed to settle for 4 h, and the bottom aqueous layer was discarded. The upper layer was distilled at a bath temperature of ≤85°C to a volume of approximately 175 mL, whereupon 204 g of water was added and distillation continued until the volume was once again approximately 175 mL. This water charge and distillation was repeated, and 665 g of ethyl acetate and 338 g of water were added. In the normal process this mixture was heated to reflux for 1−2 h. Instead, the reaction mixture was cooled to ambient temperature, split into four portions, and then shipped to the contract lab for viral clearance studies.

Viral Clearance. There are a number of ways in which viruses can be cleared from a process stream.[11] Heat, solvent treatment, size filtration, affinity filtration, chromatography, and physical separation (crystallization of the product) can all result in either inactivation or removal of viral particles. The first candidate operation for viral clearance selected for study was an ethyl acetate reflux step. Not only was this operation judged to be the most likely to result in high levels of viral clearance, it also occurred fairly early in the process. Our plan was to evaluate the ethyl acetate reflux step first, and to determine what level of viral clearance could be attained. These results would be evaluated from the standpoint of viral risk, and further processing steps would be evaluated one at a time until adequate viral clearance was attained. As long as different clearance steps operated by independent mechanisms, the viral clearances attained in the steps (logarithmic scale) could be added together to give the viral clearance for the overall process.

Viral Clearance Protocol. A panel of model viruses was chosen for the clearance studies, since no specific pathogens were shown to be present in the PLE. The viruses were selected to cover the range of RNA and DNA viruses, large, medium, and small viruses, and enveloped and nonenveloped viruses (Table 1). Sampling points were selected for each virus (Table 2). Short sample intervals were used for the enveloped viruses (BVDV and PrV), since the organic solvent was expected to rapidly deactivate these viruses, and longer time points were used for the more resistant viruses (REO-3 and particularly PPV). By selecting these time points, it was anticipated that deactivation as a function of time would be observed for each virus.

Prior to carrying out viral clearance studies, toxicity testing and interference testing were carried out. Toxicity testing determined the amount of process material that could be added to the host cells without causing damage to the cells. Similarly, interference testing determined if anything in the test article would prevent observation of viral infection of the host cells. It was determined that dilutions of 3- to 30-fold would be required to avoid damage to the host cells. These dilutions would figure into the maximum viral clearance that could be seen in the process step, since the amount of test article applied would determine the sensitivity

(6) Enzyme from two sources was used in the course of this work, a liquid suspension from Roche Bioscience and a lyophilized powder from Sigma.
(7) CFR Chapter 1, pp 113.53 (1 January, 1999 edition).
(8) The amyl alcohol used was a mixture of 1-pentanol and 3-methyl-1-butanol.
(9) Sodium dihydrogen phosphate monohydrate (2.2 g), dipotassium hydrogen phosphate (26.8 g), water (566.4 g).
(10) For Sigma lyophilized PLE a sample containing the same amount of protein was dissolved in sufficient amount of buffer to make the same volume as the that of the sample of Roche liquid suspension PLE.
(11) "Guide to Inspections of Viral Clearance Processes for Plama Derivatives", USFDA, Office of Regulatory Affairs, p 7; www.fda.gov/ora/inspect_ref/igs/viralcl.html.

Table 1. **Viral panel for clearance studies**

virus	name and family	enveloped	genome	approx. size (nm)	shape	comment
BVDV	bovine viral diarrhea virus (*Flaviviridae*)	yes	RNA	60−70	spherical	known to contaminate products of porcine origin
PPV	porcine parvovirus (*Parvoviridae*)	no	DNA	18−26	icosahedral	highly resistant to physicochemical reagents
PrV	psuedorabies virus (*Herpesviridae*)	yes	DNA	150−200	spherical	serves as model for other porcine herpesviruses such as cytomegalovirus
REO-3	reovirus type 3 (*Reoviridae*)	no	RNA	60−80	icosahedral	known in human and animal sources

Table 2. **Time points for viral clearance studies**

BVDV	PrV	PPV	REO-3
T_0	T_0	T_0	T_0
T_i	T_i	T_i	T_i
$T_{5\ min}$	$T_{5\ min}$	$T_{1\ h}$	$T_{15\ min}$
$T_{15\ min}$	$T_{15\ min}$	$T_{2\ h}$	$T_{1\ h}$
$T_{1\ h}$	$T_{1\ h}$	$T_{4\ h}$	$T_{2\ h}$
processing control	processing control	processing control	processing control

of the viral assay, and thereby the maximum viral clearance that could be assigned to the process step. Nothing in the process sample was found to interfere with the ability of viruses to infect the host cells.

In addition to the time point samples, processing controls were prepared by diluting the stock virus solution to the same extent as for the samples from the process step and holding at ambient conditions for the same time as the longest time point. The concentration of virus found in these control samples defined the maximum concentration of viral particles that could be cleared by the process step.

Viral Clearance Studies. For each viral clearance experiment, one portion of reaction mixture ≤72 h old was mixed well, and 27 mL of the mixture was removed to a 50 mL flask equipped with mechanical stirrer, thermocouple, and reflux condenser. The aliquot was spiked with 3 mL of the appropriate virus stock solution and a sample removed immediately (T_0). The mixture was heated to reflux over 39−57 min, and a sample was removed (T_i). Further samples were removed at indicated time points. All samples were diluted immediately to stop viral degradation and kept cold until assayed.

Each timed sample from the process was diluted to the extent required by the toxicity and interference studies and applied to the test cells in a series of 10-fold dilutions. The dilution that showed the maximum number of plaque-forming units (PFUs, points on the monolayer of growing cells where virus particles had infected a cell and spread to the surrounding cells) without overlap of the plaques was chosen to determine the viral titer of the sample. Each isolated plaque represented infection of a cell by one virus in the test sample and subsequent replication and spread to surrounding cells. (Only in the case of the processing controls and the early time points of the nonenveloped viruses was it necessary to read diluted tests. For most of the tests the undiluted sample was read.) The clearance studies were run in duplicate for each virus in the panel.

Results and Discussion

The viral titers and reduction factors for each test virus over the five sampling points are given for the original and the duplicate study in Table 3, along with the 95% confidence intervals. These results are presented graphically in Figures 1−4.

Viral clearance for BVDV was extensive and immediate (Table 3a, Figure 1). No viruses were observed at any dilution starting with the T_0 sample taken within minutes of spiking virus, and before any heating was carried out. This result was expected, since the ethyl acetate present in the process mixture would destroy the protective lipid envelope of an enveloped virus. Most, if not all, of the viral clearance for this virus could be attributed to solvent effects, since maximum viral clearance was observed before heating.

A similar result was observed for PrV, another enveloped virus. In both the initial and duplicate studies, no viruses were observed with the T_0 sample, but low levels of virus were observed with some of the subsequent samples. The results were highly variable across replicates (one or two PFU in some wells and none in others) so that the confidence intervals were rather large. The original and duplicate studies agreed in that the error bars overlapped at each sample point (Table 3b, Figure 2); however, the viral reduction must have been exactly at the titration limit. Once again, solvent effects appeared to contribute most if not all of the viral clearance observed.

A time-dependent curve was observed for PPV (Table 3c, Figure 3). There was little or no reduction at T_0, some reduction during the heatup to T_i, and both studies reached maximum clearance (no virus detected) at T_{1h}. Application of heat contributed much, if not all, of the clearance for this virus.

For REO-3, there was some clearance due to solvent at T_0, and maximum reduction was reached during the heatup to T_i (Table 3d, Figure 4). This might have been due to longer exposure to solvent, or heating might have contributed some of the clearance.

Viral Risk Calculations. Since all lots of the enzyme showed no virus in testing, the viral risk was calculated statistically.[4] The virus testing on the enzyme samples used 2 mL of test article per flask, done in duplicate. The highest concentration that was not toxic to the cells was 1:5 dilution. This translated to 0.4 mL/flask, or a total of 0.8 mL tested.

Table 3. **Viral titers, reduction factors (log_{10}), and confidence intervals**

	BVDV − initial test				BVDV − duplicate test			
time point	log_{10} adjusted titer (PFU)	95% confidence of log_{10} titer	log_{10} reduction	95% confidence of log_{10} reduction	log_{10} adjusted titer (PFU)	95% confidence of log_{10} titer	log_{10} reduction	95% confidence of log_{10} reduction
				A				
control	6.55	±0.10	N/A	N/A	6.46	±0.06	N/A	N/A
T_0	<1.87	N/A	>4.67	±0.10	<1.87	N/A	>4.58	±0.06
T_i	<1.87	N/A	>4.67	±0.10	<1.87	N/A	>4.58	±0.06
T_{5min}	<1.87	N/A	>4.67	±0.10	<1.87	N/A	>4.58	±0.06
T_{15min}	<1.87	N/A	>4.67	±0.10	<1.87	N/A	>4.58	±0.06
T_{1h}	<1.87	N/A	>4.67	±0.10	<1.87	N/A	>4.58	±0.06
				B				
	PrV − initial test				PrV − duplicate test			
time point	log_{10} adjusted titer (PFU)	95% confidence of log_{10} titer	log_{10} reduction	95% confidence of log_{10} reduction	log_{10} adjusted titer (PFU)	95% confidence of log_{10} titer	log_{10} reduction	95% confidence of log_{10} reduction
control	6.34	±0.14	N/A	N/A	6.46	±0.06	N/A	N/A
T_0	<1.87	N/A	>4.46	±0.14	<1.87	N/A	>4.59	±0.06
T_i	2.44	±0.31	3.90	±0.34	1.88	±0.33	4.58	±0.34
T_{5min}	1.88	±0.33	4.46	±0.36	<1.87	N/A	>4.59	±0.06
T_{15min}	2.18	±0.33	4.16	±0.36	<1.87	N/A	>4.59	±0.06
T_{1h}	2.10	±0.29	4.24	±0.32	2.30	±0.22	4.16	±0.23
				C				
	PPV − initial test				PPV − duplicate test			
time point	log_{10} adjusted titer (PFU)	95% confidence of log_{10} titer	log_{10} reduction	95% confidence of log_{10} reduction	log_{10} adjusted titer (PFU)	95% confidence of log_{10} titer	log_{10} reduction	95% confidence of log_{10} reduction
control	8.96	±0.30	N/A	N/A	8.46	±0.04	N/A	N/A
T_0	8.85	±0.17	0.11	±0.34	8.88	±0.33	None	N/A
Ti	4.26	±0.09	4.70	±0.31	6.80	±0.19	1.66	±0.19
T_{1h}	<2.87	N/A	>6.09	±0.30	<2.87	N/A	>5.59	±0.04
T_{2h}	<2.87	N/A	>6.09	±0.30	<2.87	N/A	>5.59	±0.04
T_{4h}	<2.87	N/A	>6.09	±0.30	<2.87	N/A	>5.59	±0.04
				D				
	REO-3 − initial test				REO-3 − duplicate test			
time point	log_{10} adjusted titer (PFU)	95% confidence of log_{10} titer	log_{10} reduction	95% confidence of log_{10} reduction	log_{10} adjusted titer (PFU)	95% confidence of log_{10} titer	log_{10} reduction	95% confidence of log_{10} reduction
control	7.60	±0.19	N/A	N/A	7.64	±0.22	N/A	N/A
T_0	4.50	±0.33	3.10	±0.38	5.62	±0.16	2.02	±0.27
T_i	<2.40	N/A	>5.20	±0.19	<2.40	N/A	>5.24	±0.22
T_{15min}	<2.40	N/A	>5.20	±0.19	<2.40	N/A	>5.24	±0.22
T_{1h}	<2.40	N/A	>5.20	±0.19	<2.40	N/A	>5.24	±0.22
T_{2h}	<2.40	N/A	>5.20	±0.19	<2.40	N/A	>5.24	±0.22

The probability that the test sample did not contain any infectious viruses was given by formula 1, where V (L) was the overall volume of the enzyme sampled, υ (L) was the volume of material tested, and n was the absolute number of infectious particles statistically distributed in V.

$$p = ((V - \upsilon)/V)^n \quad (1)$$

Setting p at 0.05 (95% confidence), V at 366 L (the volume of the manufacturer's master lot of enzyme tested), and υ at 0.0008 L, and solving for n gave eq 2:

$$n = 1.37 \times 10^6 \text{ viral particles} \quad (2)$$

Thus, there could be 1.37 million viral particles in the master batch, and there would be no viral particles in the sample 5% of the time. Similar calculations were carried out on enzyme from Sigma provided as a lyophilized powder. The powder was reconstituted in water for testing, and V and υ were determined on the basis of this reconstituted solution.

Product Risk Calculations. Different amounts of enzyme were used in various runs during development. The largest amount of enzyme used for one batch was 124 L, and this batch was used to prepare 31 kg of (−) **1**. The potential viral risk for this batch of product was calculated using eq 3:

$$(124 \text{ L}/366 \text{ L}) \times 1.37 \times 10^6 \text{ viral particles} = 4.64 \times 10^5 \text{ viral particles} \quad (3)$$

The viral clearance provided by the ethyl acetate reflux step reduced this risk. Since the nature of the viruses that might be present in the enzyme was unknown, the lowest

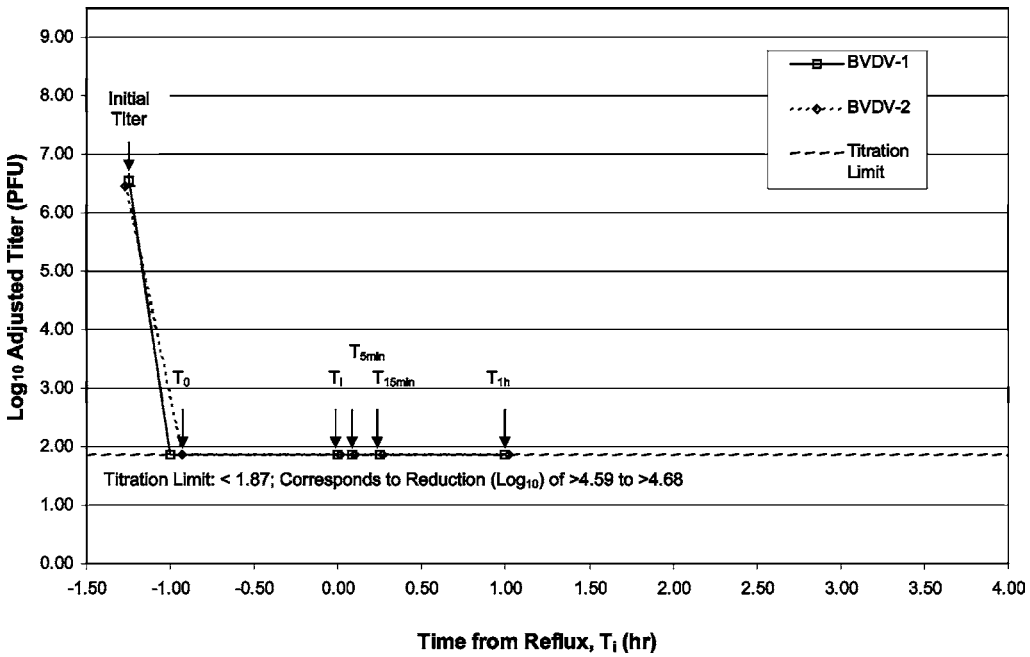

Figure 1. **BVDV. Reduction of viral titers during viral clearance studies. Error bars represent 95% confidence interval on nonzero data points.**

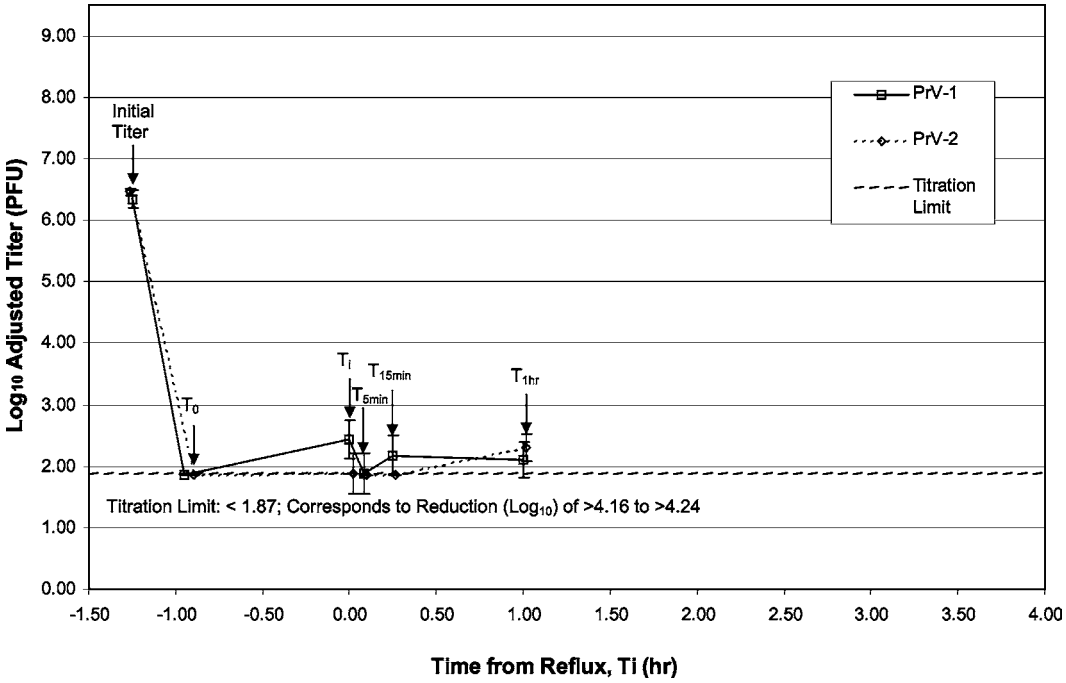

Figure 2. **PrV. Reduction of viral titers during viral clearance studies. Error bars represent 95% confidence interval on nonzero data points.**

viral clearance observed in the clearance studies was used for this calculation. This was the >3.90 log of clearance encountered in the initial PrV study. Applying this viral clearance to the viral load from eq 3 gave the result in eq 4:

$$\text{viral load before clearance}/10^{(\text{reduction (log)})} = \text{viral load after clearance}$$

$$4.64 \times 10^5/10^{(>3.90)} = <58.4 \text{ viral particles after clearance} \quad (4)$$

For emtricitabine, with a daily dose of 200 mg and a worst-case batch size of 31 kg, the single patient dose is 6.45×10^{-6} of the batch. Applying this fraction of the total batch to the viral load from eq 5 gives a viral risk of $<3.77 \times 10^{-4}$ viral particles per daily dose. Thus, a patient being treated with emtricitabine would have to take the drug for about 7.5 years to receive even one viral particle from the enzyme used to make the drug.

Similar calculations may be applied to products subsequently prepared in the same equipment. These calculations must take into account the batch size and dose of the subsequent product and any reduction in viral load that can be obtained from any specific cleaning procedures involved.

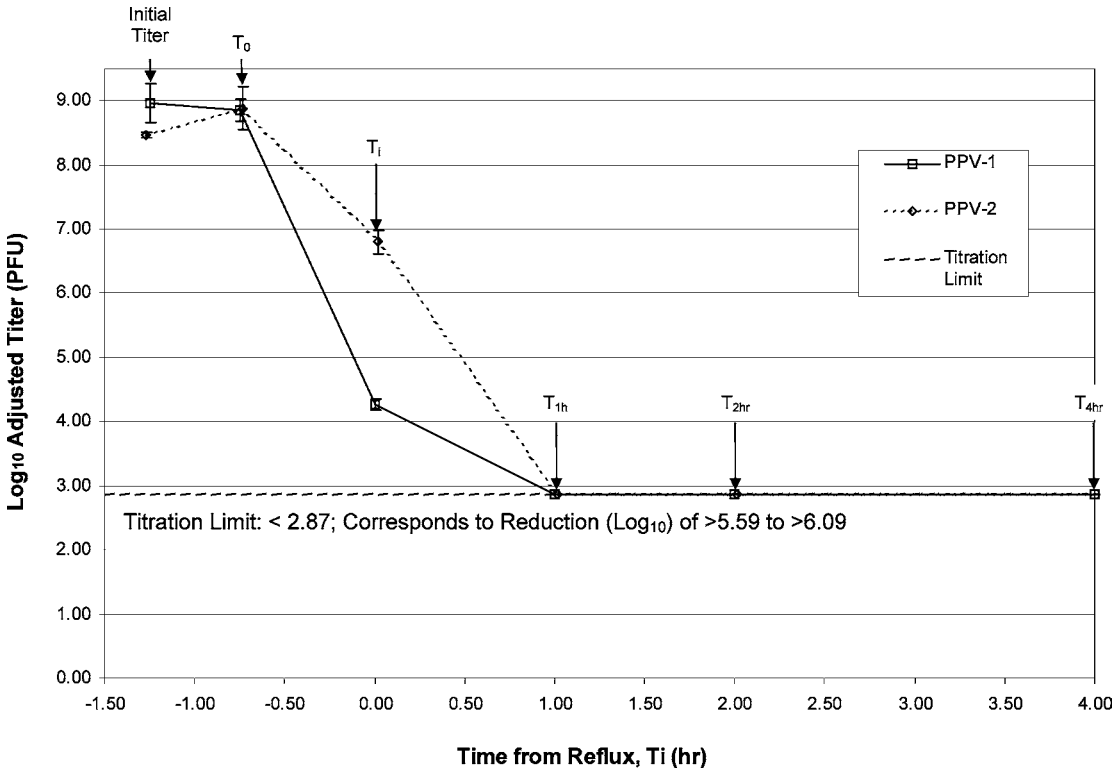

Figure 3. **PPV. Reduction of viral titers during viral clearance studies. Error bars represent 95% confidence interval on nonzero data points.**

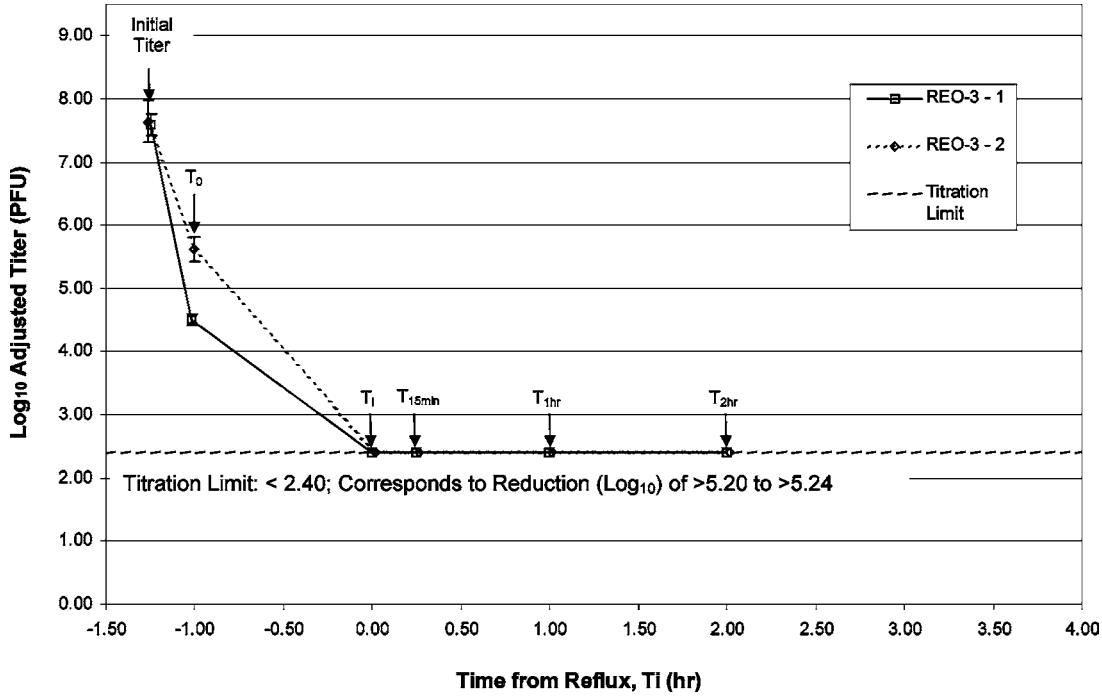

Figure 4. **REO-3. Reduction of viral titers during viral clearance studies. Error bars represent 95% confidence interval on nonzero data points.**

In our case, data were available from other viral clearance studies that showed further clearance of virus by heat treatment in the absence of any product. The equipment was cleaned, verified to be free of residual product and residual protein by analytical testing,[12] and thereafter subjected to the heat treatment.

Conclusions

One step of the emtricitabine process was found to give substantial reduction in viral load from solvent and heat treatment. The temperatures achieved during reflux, and the

(12) Residual product was analyzed by HPLC. It was necessary to determine that the equipment was free of product prior to testing for residual protein since the product cross-reacted with the protein reagent. Residual protein was analyzed by rinse or swab test followed by reaction with Bio-Rad protein assay dye reagent. PLE of known protein content was used as standard and to validate recovery from process surfaces.

duration of the reflux step, were placed in the manufacturing directions as required parameters to ensure that adequate viral clearance would be attained. Applying this reduction to the level of virus that could potentially be present gave a very low level of calculated viral risk for a patient taking emtricitabine. The question of the acceptability of such viral risk was a more difficult problem. The regulatory document[4] required that the production process "be able to eliminate substantially more virus than is estimated to be present in a single-dose-equivalent of unprocessed bulk". With a viral risk per daily dose of about 10^{-4} viral particles, the step that was evaluated fulfilled this requirement.

The use of an animal-derived enzyme in processing an active pharmaceutical ingredient should be approached with great caution. Only in the case where no alternative exists to carry out the required transformation should a reagent of this sort be considered. Expert advice should be sought on the regulatory implications of such a choice, and adequate time and resources should be set aside to carry out the necessary studies during process development.

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