

**CENTER FOR DRUG EVALUATION AND
RESEARCH**

APPLICATION NUMBER:

125293

IMMUNOGENICITY REVIEW(S)

Center for Drug Evaluation and Research
Office of Pharmaceutical Science
Office of Biotechnology Products
Division of Therapeutic Proteins
HFD-122

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7-16-09*

From: João Pedras-Vasconcelos, PhD, DTP/OBP/CDER

Subject: BLA 125293/IND 10122 SN 112 Immunogenicity assay validation

Through: Amy Rosenberg, M.D., Director, Division of Therapeutic Proteins

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7/14/09*

Product: Puricase (Pegloticase; PEG-uricase)

Proposed Use: Control of hyperuricemia in patients with symptomatic gout in whom conventional therapy is contraindicated or has been ineffective

Sponsor: Savient Pharmaceuticals Inc.

IND #: 10122

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Summary:

The anti-Puricase and anti-uricase IgM and IgG binding assays are suitable for clinical analyses. However, the anti-PEG binding assay, the anti-Puricase IgE assay and the assay to detect neutralizing antibodies will not provide reliable results (details below).

The presence of high titer anti-Puricase antibodies is associated with loss of efficacy and

increased chances of infusion reactions. High titer (>1:7290 dilution) was defined empirically by the Sponsor based on a threshold they observed for correlations between antibody titer and clinically observed responses. This is an appropriate way of classifying titers as it provides clinically meaningful information.

Patients were not followed to see if they reverted to seronegative status after drug withdrawal. We recommend that future safety and/or efficacy studies should incorporate following patients till they revert to sero-negative status.

Puricase is indicated for the treatment of allopurinol resistant gout. Many of the patients that participated in the clinical trials have a highly compromised quality of life due to their gout. Puricase is very effective in patients when it works, but 50 – 60% of patients fail therapy. The main reason for treatment failure is the development of antibodies that clinically neutralize drug efficacy, primarily by increasing the drug clearance rate.

Treatment failure due to immunogenicity has been observed for other therapeutic proteins, for example for Factor VIII and enzyme replacement therapies enzymes for the treatment of metabolic disorders. Although challenging, it has been possible for some patients to overcome their immune responses by intensive treatment regimens to induce immune tolerance to the drug. Due to the high level of disability and pain that many of these patients endure, we recommend that the Agency discuss the possibility of attempting immune tolerance induction regimens for appropriate patients.

The anti-PEG assay is very sensitive to the presence of drug in the serum and is extremely variable. Approximately 90% of subjects have anti-Puricase antibodies and a small subset of these have low titer anti-uricase antibodies. However, only 40% tested positive in the anti-PEG assay, indicating that the assay greatly underestimates the presence of anti-PEG antibodies.

Despite the inadequacy of the anti-PEG assay, it appears that the majority of antibodies to Puricase bind to the PEG component. This is a concern because it could impact patients' clinical responses to other PEGylated therapeutics. The Sponsor demonstrated that they could compete away the binding of anti-PEG antibodies in Puricase treated patients with other PEGylated proteins. Because the anti-PEG antibody assay is inadequate to portray the true incidence and strength of the responses, we recommend that the Sponsor develop a more suitable assay to measure anti-PEG antibodies. The assessment of anti-PEG antibodies should be incorporated into any future safety/efficacy studies until we have a more complete understanding of the naturally history of these antibodies.

The Sponsor was unable to develop a suitable positive control for the IgE assay. Therefore, they used two sub-optimal controls in the validation exercise, human IgE of unknown specificity and antigen specific IgG. The concentration of their IgE low positive control, which had a signal just above background was 30 ng/ml. For an antigen specific IgE assay to be useful it should have sensitivity in sub-nanogram to single digit nanogram range, as these concentrations of IgE are clinically relevant. Assays with such

sensitivity can be developed using technology that is currently widely available.

Per the immunogenicity consult from Dr. Susan Limb, approximately 5% of subjects had responses that the Agency considers to be anaphylactic. We do not know whether these were IgE mediated responses since the assay was not sensitive enough to provide reliable results. To develop appropriate strategies to manage patients' immune responses we recommend that the Sponsor redevelop the IgE assay using a technology that will allow them to detect antigen specific IgE antibodies.

The assay to detect neutralizing antibodies was not properly validated because the Sponsor could not develop positive control antibodies that neutralize puricase. This is likely due to the fact that the uricase component of Puricase is not accessible since it is surrounded by a large cloud of PEG. It is possible that the Sponsor could have used an anti-uricase neutralizing antibody to validate the assay, but they did not do so. Instead they used a small molecule inhibitor to validate the assay. Two subjects tested weakly positive for the presence of neutralizing antibodies. Since almost no subjects tested positive for anti-uricase antibodies it is unlikely that neutralization of catalytic activity contributes to clinical failure in subjects. Therefore there is no need to pursue further development of this assay at this time.

Abstract of Recommendations from Summary:

- Patients were not followed to see if they reverted to seronegative status after drug withdrawal. Future safety and/or efficacy studies should stipulate that patients' anti-Puricase and anti-PEG antibody status is tracked till they revert to seronegative status.
- Because of the lack of reliability of the assay in reporting results and of the potential of anti-PEG antibodies to impact patient responses to other PEGylated therapeutics, the Sponsor should develop a more suitable assay to measure anti-PEG antibodies.
- Due to the high level of disability and pain that many of the allopurinol resistant patients with tophi endure, the Agency ought to discuss with the company, the possibility of attempting immune tolerance induction regimens for appropriate patients.
- To develop appropriate strategies to manage patients' immune responses we recommend that the Sponsor redevelop the IgE assay using a technology that will allow them to detect antigen specific IgE antibodies.

Labeling

(b) (4)

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Immunogenicity Results

The Table below summarizes the anti-Puricase antibody results from the two identical phase 3 pivotal trails.

Text Table 1	Summary of the Results Obtained for the Anti-PEG-uricase IgG and IgM Antibody Detection in Human Serum	
	C0405	C0406
Screening:		
Subjects with at least 1 positive time	90/105	84/110
Positive study samples	461/744	403/771
Drug competition test:		
Subjects with at least 1 positive time	89/90	83/84
Study samples confirmed positive	439	369
False positive rate (study samples confirmed negative/overall study)	2.95 %	4.40 %
Titration (range of titers reported)	1:30 to 1:5314410	1:30 to 1:590490
Isotyping	IgG and IgM, predominantly IgM	IgG and IgM, predominantly IgM

The Sponsor reported that the plasma uric acid (PUA) levels were inversely related to plasma uricase levels and directly correlated with circulating anti-Puricase levels. They further reported that high titer anti-Puricase antibody levels was associated with failure to maintain PUA levels below 6 mg/dL for at least 80% of the time in months 3 and 6. The Tables below are the Sponsor’s summary of antibody titer vs efficacy or infusion reactions data. The Sponsor further found that subjects developed both IgM and IgG anti-Puricase and anti-PEG.

Table 57. PUA Treatment Responses by Highest Anti-Pegloticase Antibody Titer During Month 3 and 6 (ITT Population)

Anti-Pegloticase Antibody Level	8 mg Pegloticase			
	Every 2 Weeks (N = 82) n		Every 4 Weeks (N = 67) n	
	PUA less than 6 mg/dL for at least 80% of the time in Months 3 and 6 combined			
	Yes	No	Yes	No
No Increase	5	0	8	0
Low Titer	10	1	5	1
Moderate Titer	13	4	5	1
High Titer	2	17	1	23
Number of Subjects with Antibody Data	52		44	

“Months 3 and 6” interval includes intervals for Months 3 and 6 combined.
Source: Table 7.4, Section 14.2; and Listing 12.4A and Listing 28

Table 5. Seroconversion Categories for Anti-Pegloticase Antibodies

	Ratio of the highest of the Week 13 and Week 25 titers to the baseline titer:
No Increase	≤ 1 (or the Week 13 and Week 25 titers all equal zero)
Low Titer	>1 and the highest of the Week 13 and Week 25 titers is >0 and ≤ 810
Moderate Titer	>1 and the highest of the Week 13 and Week 25 titers is > 810 and ≤ 7290
High Titer	>1 and the highest of the Week 13 and Week 25 titers is > 7290

Note: Initial dilutions for ELISAs started at 1:30; results negative at this dilution were interpreted as 0.

Table 6. Seroconversion Categories for Anti-Peg Antibodies

Negative	The subject had negative screening results at baseline and had negative screening results at both Weeks 13 and 25
No Change	The subject had positive screening results at baseline and had a positive screening result at either Week 13 or 25
Positive	The subject had negative screening results at baseline and had a positive screening result at either Week 13 or 25.

Table 60. Incidence of Infusion Reactions at Any Time by Anti-PEG Antibody Category ITT Population)

	8 mg Pegloticase	
	Every 2 Weeks (N = 82) n (%) ¹	Every 4 Weeks (N = 67) n (%) ¹
Incidence of Any Infusion Reaction	19 (23.2)	26 (38.8)
Incidence of Infusion Reactions by Anti-PEG Antibody		
Negative	4/40 (10.0)	7/26 (26.9)
No Change	1/2 (50.0)	0/0 (0.0)
Positive	9/32 (28.1)	13/31 (41.9)

Note: Negative: The subject had a zero titer at baseline and had negative screening results at both Weeks 13 and 25. No Change: The subject had a positive titer at baseline and had a positive screening result at either Week 13 or 25. Positive: The subject had a zero titer at baseline and had a positive screening result at either Week 13 or 25.

¹ Percentages are based on the number of subjects with antibody findings within each seroconversion category.

Source: Table 31.3, Section 14.3.1; and Listing 19 and Listing 28.

The accuracy of the data (numbers of positive vs. negative patients) is being confirmed by the statistical and clinical groups. As the review below notes, the assay for the detection of anti-puricase binding antibodies is suitable for clinical analyses. The anti-PEG antibody assay is very sensitive to the presence of on-board drug. In addition the assay is highly variable and there is insufficient control of the negative cut-point, which may impact assay sensitivity. Therefore it is likely that the incidence of anti-PEG antibodies is under reported.

The presence of anti-PEG antibodies that modify PK has been reported previously (e.g. PEG-aspariginase). The high prevalence of anti-PEG and anti-Puricase antibodies is of concern because they are associated with treatment failure and infusion reactions. Tolerance induction regimens ought to be considered for this drug due to its efficacy in treating a debilitating and painful condition. The impact of anti-PEG antibodies on other PEGylated therapeutics patients' might be taking should be considered.

In a phase 2 study the presence of anti-PEG-uricase antibodies was found to be associated with more rapid drug clearance. A similar finding was reported in the literature for PEG-aspariginase. In study C0403 anti-Puricase IgE antibodies were detected in 2 subjects, one of whom had a delayed type hypersensitivity reaction but not anaphylactic responses. The DTH response was presumably related to the presence of IgG antibodies.

As noted in our review below, the IgE assay lacks the sensitivity necessary to reliably assert that subjects with anaphylactic responses are negative for antigen specific IgE.

Background

Indication: Treatment Resistant Gout

Gout is a very painful clinical condition that is characterized by persistent hyperuricemia, which results in the deposition of monosodium urate monohydrate (MSU) crystals in the joints and periarticular structures and of uric acid calculi in the urinary tract (“kidney stones”). When urate concentrations exceed the solubility limit of about 7 mg/dL at physiological pH, uric acid may nucleate to form crystals in tissues and joints. Shedding of MSU crystals into the synovial cavity induces acute inflammatory responses (IL1/Caspase 1/inflammasome dependent) known clinically as gout flares. The persistence of crystals in the synovial fluid and synovial membrane induces chronic inflammation.

In most mammalian species urate oxidase (E C1.7.3.3) catalyzes the enzymatic oxidation of uric acid to allantoin, H₂O₂ and CO₂. Allantoin is 5-10X more soluble in water than urate crystals and thus more readily excreted by the kidneys. However, the enzyme is absent in most primate species, and in humans, chimps and gorillas there are 3 mutations (2 nonsense mutations at codon 33 and 187, and a splice mutation in exon 3) which prevent translation of an active protein. These mutations are thought to have occurred during Miocene period, and to have been evolutionarily selected because the resulting hyperuricemia may have facilitated maintenance of blood pressure under the low-salt dietary conditions characteristic of most of hominoid history.

Puricase is a porcine-derived *recombinant* urate oxidase that according to the Sponsor is (b) (4) and enzymatically conjugated to PEG in order to reduce immunogenicity. Based on preclinical studies a conjugate containing an average of 9 ± 1 strands of 10-kDa PEG per subunit was selected for clinical development.

Puricase is intended for intravenous infusion (b) (4) with preservative-free normal saline for Injection, USP. Puricase has a relatively long plasma half-life (1-2 weeks).

Patients with treatment-failure gout were recruited in the United States, Canada and Mexico for two Phase 3 clinical trials of PEG-uricase starting in May 2006 and enrollment was completed in March 2007. The six-month, double-blind Phase 3 trials include patients receiving placebo at two-week intervals, patients receiving 8 mg of PEG-uricase at two-week intervals and patients receiving alternating infusions of 8 mg of PEG-uricase and placebo at two-week intervals. While the primary endpoint is reduced plasma urate concentration, these trials include assessments of gout symptoms, tophus resolution and quality of life. At the end of the six-month treatment period, all patients are given the option of continuing treatment in an open-label study, in which each patient

and his/her physician can select treatment with 8 mg of PEG-uricase at either two-week or four-week intervals. To date, nearly 100% of those patients who have completed the blinded phase of the study have elected to continue (or, for placebo recipients, to start) receiving treatment with 8 mg of PEG-uricase for an additional one or two years.

Puricase is contraindicated for people with Glucose-6-Phosphate-dehydrogenase deficiency due to their tendency to develop hemolytic anemia, resulting from lysis of red blood cells by the hydrogen peroxide, which is a byproduct of the enzymatic reaction.

Submission

Supplement 112 consists of Savient response to an FDA request during the End-of-phase 2 meeting on 7/26/05 that the Sponsor develop and validate a number of immunogenicity assays. The assays should detect responses against PEG-Uricase, Uricase or PEG, and determine if these responses are neutralizing to the drug product. The following studies are included in this supplement

1. Study 300572 Final Report of Method validation for the detection of IgG and IgM antibodies against puricase(PEG-uricase) in human serum by ELISA
2. Study 301138 Partial validation of an ELISA method for the detection of IgG and IgM against PEG-Uricase in human serum using a human anti-PEG-uricase positive control
3. Study 300910 Final report for validation of an ELISA for the detection of anti-PEG IgG and IgM antibodies in human serum
4. Study 301402 Draft research report for validation of an ELISA for the detection of anti-Uricase IgG and IgM antibodies in human serum
5. Study 300913 Final Protocol for the validation of an ELISA method for the detection of anti-PEG-Uricase IgE in human serum
6. Study 300914 Final validation Report on the detection of IgE antibodies against PEG-Uricase in human serum by an enzyme linked immunosorbent assay (ELISA)
7. Study 300569 Validation of a qualitative enzymatic assay for the detection of neutralizing anti-PEG-Uricase antibodies in human serum

Study 300572 Final report of method validation for the detection of IgG and IgM antibodies against puricase (PEG-uricase) in human serum by ELISA

The Sponsor provides a validation study for the qualitative detection of anti-puricase IgG and IgM antibodies in human serum. The Sponsor uses a rabbit anti-puricase antiserum (obtained 7 weeks post-immunization) as positive control antibody, which means that the secondary antibody to be used during routine analysis could not be validated with the PC antibody. To overcome this problem the study also uses human IgM (IPC-IgM, 8ng/ml) positive and human IgG positive (IPC-IgG, 10ng/ml) samples that were not puricase specific, but served to confirm the suitability of detection reagents (either goat anti-human IgM or goat anti-human-IgG, both HRP-conjugated). Plates were coated with puricase, while detection antibody was peroxidase-labeled anti-rabbit IgG.

In page 54 of this study, sponsor mentions the existence of “selected positive phase II samples from study 300573”, which are used in long term stability study (up to 19 months) in addition to the surrogate (rabbit) positive control. No details are offered on these samples in this study, but they are discussed in study 301138 also provided in current submission.

Comments to the file:

It is frequently the case that human positive control sera are not available for assay validation. Therefore it is acceptable to use an animal derived surrogate. The validation should be confirmed by human positive controls if they are detected.

Cut-off determination:

Negative cut-off (NCO) determination was performed with 25 normal sera and 25 out-patient sera (obtained prior to treatment) using $NCO = \text{mean} + 1.645SD$ criteria. Initially, NCO appeared to be lower for patient sera (0.39 ± 0.029) compared to normal sera (1.17 ± 0.09), but the sponsor normalized the two values as global NCO at 0.86. After excluding 4 high background normal sera and one patient serum, the NCOs were very similar (normal= 0.22 ± 0.02 , patient 0.23 ± 0.004), with a global NCO=0.218.

Comments to the file:

The number of pre-immune sera used is on the lower end of the generally acceptable range (50 – 100), but this is acceptable since inter-subject variability was low. When there is no difference between normal sera and patient sera it is acceptable to use a mixed population to establish the NCO.

All subsequent validation testing on method related parameters (sensitivity, specificity and recovery, intra assay and inter-assay precision, drug interference and prozone effects) were done using the rabbit anti-puricase antisera diluted at low (1/100), medium (1/150) and high dilutions (1/350) in pooled normal human diluted 1/30 in assay buffer as a matrix.

Specificity:

The specificity of this rabbit ELISA was tested by examining the effect of puricase

pre-adsorption (with 20ug/ml of enzyme) on rabbit antisera (tested at LPC and HPC) which results in reduction of A450 of 70-72%.

Comment to the file:

Assay specificity has been successfully demonstrated

Recovery

Individual lots of patient sera (9) or normal sera (8) were spiked with rabbit positive control at both HPC and LPC levels. For HPC 90% and for LPC, 80% of the mean A450 values of the individual lots were within 25% of the mean A450 values of the rabbit positive control spiked in pooled normal human serum (1/30 dil).

Comment to the file:

Assay shows acceptable recovery levels.

Sensitivity:

The Sponsor stated that sensitivity could not be confirmed using the rabbit positive control because they could not successfully affinity-purify the antibodies. Instead The Sponsor used immunoglobulin positive controls. For IPC IgG, purified human IgG coated at 8ng/ml gave an OD value above NCO. The final calculated IgG concentration was 240ng/ml (30x8) of total IgG. For IPC IgM, 10 ng/ml of purified human IgM gave an OD value above NCO, and the final detectable IgM concentration was 300 ng/ml. Both of these sensitivities are higher recommended sensitivity for clinical studies of 500 ng/ml.

Comments to the file:

This assay sensitivity measurement is meant to refer to Ag specific Ig, not total Ig. In addition assay sensitivity can be assessed with polyclonal Ab. Sensitivity is generally lower under these circumstances. This issue is addressed more satisfactorily in study 301138 and therefore does not need to be pursued further.

Precision:

The intra and inter assay precision for the method was assessed for the blank TS, IPC-IgG and IgM and at 3 concentrations of the rabbit anti-puricase antiserum (LPC, MPC, HPC). Assay shows low intra-assay variability for both IPCs and rabbit PCs (%CV<10%), but high inter-assay variability (%CV 27-30% over 7 different days). Variability was attributed to one particular assay date out of 7, and upon removal of that data, %CV was 16-20% over 6 assays.

Comments to the file

Inter-assay variability is towards the high end for this type of assay (20 – 30% at the low and high ends of the dose-response curve). Nevertheless, because the high rate of variability is primarily driven by a single assay and the variability is still within industry standards the validation of this parameter is acceptable.

Stability:

Rabbit positive control was spiked in normal human serum at the LPC and shown to be stable at RT for 6h, at 4C for 24h, and after six freeze-thaw cycles at -20C. Long term stability assessment at -20C showed that the positive control remained stable up to 19 months.

Comments to the file

Rabbit positive control appears to be sufficiently stable for assay use.

Drug Interference

Rabbit positive control at LPC and HPC were spiked into pooled normal serum (1/30), and incubated with 7 different concentrations of puricase (at 0, 250, 500, 100, 200, 500, 1000 ng/ml). For LPC 500ng/ml reduced the response by ~25% (equivalent to 15ug/ml of drug/ml in neat sera) while for HPC, 1000ng/ml response was reduced by ~32% (equivalent to 30ug of drug/ml in neat sera).

Comments to the file:

These data indicate that on-board drug can interfere with the assay. This is expected for an antigen specific assay. The interference of on-board drug with the assay thus requires a detailed understanding of the optimal timing for obtaining samples in relationship to product administration, ie, at the time at which product concentration is at a level that does not interfere with the assay for relevant data interpretation.

Prozone Effect

To test prozone effect a spiked concentration of positive control was prepared at 1/20 (5xHQC), which was the highest concentration that could be prepared by keeping 95% of matrix. No prozone effect was observed.

Comments to the file

Prozone effect was NOT demonstrable given the conditions of the assay.

Study 301138 Partial validation of an ELISA method for the detection of IgG and IgM against PEG-Uricase in human serum using a human anti-PEG-uricase positive control

This study follows on the heels of study 300572 above. In a phase II clinical study, subject No. 8007 appears to have developed high dilution titers of IgG and IgM antibodies to PEG-uricase. A human positive control was prepared by pooling equal volumes of this patient's sera samples from time points d43, 57, 71, 99 and 126 and subsequently used in partial validation studies, including negative and positive control assessment (HPC 1/30, MPC 1/60 and LPC 1/120), immunodepletions, intra and inter-assay precision of positive control, sensitivity and drug interference. Parallelism between the human anti-PEG-uricase, purified mouse anti-PEG mAb and rabbit polyclonal anti-

uricase antibodies were also assessed for comparison.

Acceptance Criteria

- The positive control absorbance results must be above the negative cut-off value and the response must be proportional to the PC concentration.
- For the intra-assay to be acceptable the mean values (A450) of at least 2 out of 3 replicates of positive controls at each level should be above the negative cut off value (NCO) and the coefficient of variation should be within 20% for the MPC and HPC and 25% for the LPC.
- For the inter-assay to be acceptable, the mean values (A450) (global mean of the PC samples at each levels) should be within $\pm 25\%$ (% CV).

Prozone Effect

Prozone effect for human antiserum was not assessed according to the sponsor as “ the positive control was not concentrated enough to prepare it 5X more concentrated than the HPC (1/30) while keeping a 95% matrix”. Using the antisera at 1/6 would have resulted in a 67% matrix (normal human serum).

Comments to the file

Since a prozone effect was not seen with the PC antibody it is unlikely that it would be seen with the human antibody. Therefore it is acceptable not to re-validate this parameter.

Precision:

Precision was assessed by preparing human positive control at LPC, MPC and HPC Samples were assayed in replicates of three, each one in duplicate, on 6 occasions for the LPC and 7 occasions for the MPC and HPC. The group mean, standard deviation (SD) and coefficient of variation (% CV) of the obtained A450 mean values were calculated and were used to determine the inter- and intra-assay precision.

The intra-assay precision had a CV% < 15%, while the inter-assay precision was 16.8% for HPC, 19.7% for MPC and 24.9% for LPC.

Comments to the file:

Intra and inter-assay variability are acceptable for this type of assay.

Specificity:

Specificity was assessed by spiking Human positive control at the LPC (1/120) and the HPC (1/30) levels with PEG-uricase at 6 selected concentrations determined under Study No. 300572 (0, 250, 500, 1000, 2000, 5000 and 10000 ng/mL), but no significant interference was observed at these selected drug concentrations. The parameter was re-assessed using higher PEG-uricase concentrations (i.e. 0, 2000, 5000, 10000, 20000, 40000, 50000, 100000, 200000 and 400000 ng/mL). The spiked drug interference samples were incubated at RT for at least 1 hour prior to being loaded into the plate. Mean value A450 was reduced 72% for LPC and 82% for HPC when pre-incubated with

200ug/ml PEG-uricase, compared to non-immuno-depleted control. Pre-incubations with a negative control consisting of Lysozyme + polypropylene oxide (25%/75% w/w) resulted in 6-9% decreases in A450, confirming serum was specific for product. Spiking LPC with >10ug/ml and HPC with >20ug/ml of PEG-uricase also interfered with ELISA assay for this human standard.

Comment to the file:

Sponsor satisfactorily showed that the new human standard is sensitive to immuno-competition with high concentrations of puricase.

As complementary information, several pegylated proteins (i.e. PEG-asparaginase, PEG-catalase, PEG-chymotrypsin, PEG-subtilisin and PEG-SOD) were added at a final concentrations of 200 µg/mL and 500 µg/mL at the LPC and HPC level except for the PEG-asparaginase which was only added at 200 µg/mL due to insufficient volume available. Results (table 9) show substantial inhibition of binding by all pegylated-products (33-80%) suggesting that most of the antibodies in this human positive control cross-react with PEG.

Table 9 Immunodepletion of the Human Anti-PEG-Uricase IgG/IgM Antibodies Using Several Pegylated Proteins (R&D-37 Performed under Study No. 300909)

Sample ID	LPC (pooled 8907)		HPC (pooled 8907)	
	Mean A _{450nm}	Difference (%) ^a	Mean A _{450nm}	Difference (%) ^a
Not immunodepleted	0.349	-	0.959	-
Puricase 200 µg/mL	0.089	-74.5	0.254	-73.5
Lysozyme-Propylene oxide (negative control) 200 µg/mL	0.324	-7.2	0.913	-4.8
PEG-asparaginase 200 µg/mL	0.102	-70.8	0.326	-66.0
PEG-Catalase 200 µg/mL	0.129	-63.0	0.289	-69.9
PEG-Catalase 500 µg/mL	0.103	-70.5	0.294	-69.3
PEG-Chymotrypsin 200 µg/mL	0.159	-54.4	0.491	-48.8
PEG-Chymotrypsin 500 µg/mL	0.098	-71.9	0.190	-80.2
PEG-Subtilisin 200 µg/mL	0.158	-54.7	0.423	-55.9
PEG-Subtilisin 500 µg/mL	0.087	-75.1	0.186	-80.6
PEG-SOD 200 µg/mL	0.244	-30.1	0.646	-32.6
PEG-SOD 500 µg/mL	0.186	-46.7	0.502	-47.7

$$a = \% \text{ Difference} = \frac{(\text{Mean } A_{450} \text{ of Immunodepleted} - \text{Mean } A_{450} \text{ of Not Immunodepleted}) \times 100}{\text{Mean } A_{450} \text{ of Not Immunodepleted}}$$

Comments to the file:

New human positive control appears to be primarily specific for the PEG portion of the molecule. It would be useful for the Sponsor to perform a competition with un-PEGylated uricase.

Sensitivity:

Assay sensitivity was determined using purified rabbit **anti-uricase** (at 0.00, 15.63, 31.25, 62.50, 125, 250, 500 and 1000 ng/mL) and a cocktail of purified mouse anti-PEG IgG1 and IgM antibodies (0.00, 0.10, 0.20, 0.39, 0.78, 1.56, 3.13 and 6.25 ng/mL) in diluted human sera. The assay sensitivity was defined as the lowest concentration (corrected for the assay minimum required dilution, which was 30 fold) that produced a coefficient of variation (CV) value $\leq 25\%$. For unclear reasons, the sponsor did/could not purify the R35 rabbit anti-puricase polyclonal Ab. The assay sensitivity using 1/30 sera dilution was 250ng/ml for the protein portion and 0.78ng/ml for the pegylated portion.

Comment to the file

This study design doesn't address the overall sensitivity of the assay. As noted above the sensitivity can be assessed using the polyclonal positive control with the understanding that the sensitivity is likely to be much lower than would be expected using an affinity purified antibody. There is some debate in the industry regarding the utility of the sensitivity measure since it is so highly dependent on the Ab preparation used. Although the Agency still likes to have this parameter assessed the acceptability of the assay can still be assessed in the absence of those data. (see comment to Sponsor).

Dilutional Parallelism:

The Sponsor evaluated dilutional parallelism between the human anti-PEG-uricase antiserum, the mouse anti-PEG mAb and the rabbit anti-uricase polyclonal sera. The sera do not exhibit dilutional parallelism to each other.

The lack of dilutional parallelism indicates that quantitative analyses cannot be performed across species. It is common for there to be a lack of dilutional parallelism which is why the Agency generally dissuades companies from developing quantitative assays.

Summary:

The partial validation data demonstrates that new human anti-PEG-uricase control is suitable for detection of anti-PEG-uricase IgG and IgM antibodies in human sera and addresses some of this reviewer's criticisms of the earlier studies (300569 and 300572).

Comments to the sponsor:

Please assess the concentration of human anti-PEG-uricase IgG and IgM antibodies in your new human positive control.

Study 300910 Validation of an ELISA for the detection of anti-PEG IgG and IgM antibodies in human serum

This study provides results of validation of a qualitative ELISA for detection of anti-PEG IgG and IgM antibodies in human serum. The plates are coated with PEG, followed by

human sera samples, which are detected with HRP-conjugated goat anti-human IgG/M polyclonal. The new human positive control (HuPC) described in study 301138 is also used as a positive control for part of this validation study. However, most of the validation study was done using a mix of two surrogate PEG-specific mouse mAbs (an IgM and an IgG1), prepared at LPC (50ng/ml), MPC (100ng/ml) and HPC (250ng/ml) levels, since there was insufficient amounts of the human control to complete the entire validation exercise. Human IPC-IgG and IPC-IgM controls are also used (plate wells coated separately with human IgG and IgM for testing the binding anti-human detection reagents).

Comment to the file:

This assay format is common in industry and is acceptable. It is also generally both necessary and acceptable to use animal derived positive controls since human positive controls are frequently lacking.

The method validation included negative cut-off and cut-point factor determination, intra and inter-assay precision, sensitivity, specificity and recovery (using HuPC at 1/30 dil), drug interference, prozone effect and drug competition test (using HuPC at 1/30 dil). Stability studies were done with mouse positive control mix (MuPC) at 4°C (for 6 and 24h) and following 6 freeze-thaw cycles from -20 °C and MuPC proved stable under these conditions.

Negative cut-off and cut-point factor determination

The negative cut-off (NCO), which is normalized for each plate using a cut-point factor, is used to determine the threshold for identifying samples as negative (equal to or below the NCO) or positive (above the NCO) for the presence of anti-PEG IgG and IgM antibodies. Twenty-five lots of normal human serum (normal population) and 25 predose clinical samples were diluted 1/10 with Blocker Casein in PBS. The individual lots and predose samples were tested on six occasions over 3 different days by 3 different analysts. On each occasion the individual lots and predose samples were analyzed in a single well. Since the volume of predose samples was insufficient to create a pool, individual lots of normal serum were also included in the cut-point determination. The absorbance response for each serum sample within each plate was extracted from Softmax Pro. To ease the presentation of the following section, let k be the number of plates and assume that there are n subjects who each has one sample of his/her serum lot analyzed in each of these k plates.

For each plate, the average absorbance of these n values was computed and used to calculate the plate-adjusted absorbance response of each sample. More precisely, the average absorbance for a given plate was subtracted from the absorbance response of each sample within the considered plate, yielding n absorbance differences for each plate. For each serum lot, the obtained absorbance differences were averaged across all plates, generating then an averaged absorbance difference value for each serum lot. The absorbance data consisting of all averaged absorbance difference values (n values) was statistically analyzed, and outliers identified. All serum lots considered as outliers were assayed in the drug competition test, and excluded from cut point calculations.

The cut-point factor was set at 1.954. During the validation exercise this generally led to a cut-point between .11 and .2 but during the in-study phase of the assay the NCO ranged from ~0.2 - ~0.66.

Comments to the file:

The number of lots of normal sera and outpatient sera used is on the lower end of the acceptable range, particularly in light of the variability observed. During the in-phase portion of the study, the NCO was always higher than during the assay validation. Assessment of the data indicate that the variability in the assay may lead to underestimation of the incidence of anti-PEG antibodies in the population.

Precision:

Intra-assay and inter-assay precision were assessed by preparing a low, mid and high mouse positive control levels (LPC, MPC, HPC, respectively) and testing them on 7 different occasions in 3 replicates per occasion. Each of the replicates comprised of 2 duplicate wells. The occasions were performed by 4 different analysts. The data were reported as the mean A450 value of the duplicate wells. The group means, standard deviation (SD) and coefficient of variation (%CV) of the mean A450 value were calculated and used to determine the intra- and inter-assay precision. For the intra-assay to be acceptable, the coefficient of variation of the mean A450 should be $\leq 25\%$. For the inter-assay to be acceptable, the coefficient of variation (%CV) of the intra-assay mean A450 should be $\leq 25\%$.

Intra-assay precision: Using the mouse positive control intra-assay variability had a CV% $<25\%$ for 5/7 assay repeats. Two of the repeats had CV% $>26\%$ and data was not included in calculation

Comments to the file:

Intra-assay variability is towards the high end; ideally acceptance criteria should be $<20\%$ for this type of assay.

Inter-assay precision: CV% ranged between 43% for the LPC, 33.9% for MPC and 27% for the HPC which is higher than recommended. To address this problem, the sponsor intends to calculate plate specific cut-points using a pre-determined cut-point factor and argues that the ratio between LPC/MPC (11.4%) and HPC/MPC (8.6%) indicates that “the assay had an acceptable inter-assay precision”. In addition, false positive rate was 8.6%, higher than the recommended 5%. For the NCO determination sponsor used normal human sera and gout patient sera (prior to treatment), 10 of each type.

Comments to the file:

Inter-assay variability was high so the sponsor was forced to use a floating cut-point calculation. Problem may lie in coating of plate with PEG or potentially with the affinity

of the anti-PEG antibodies since according to the literature anti-PEG-Ab tend to be low affinity. A different approach to assessing the epitope specificity of the Ab is recommended. For example, the Sponsor could use unlabeled PEG to compete for Ab binding in the anti-drug Ab assay.

Assay sensitivity

The sensitivity of the assay, which is relative to the characteristics of the positive control used in the assay, was determined by testing the mouse anti-PEG IgG1 and the mouse anti-PEG IgM antibodies in cocktail at 10, 20, 30, 40 and 50 ng/mL in 1/10 diluted serum during the precision assessment (n = 1 in duplicate for each occasion). The sensitivity relative to the positive control was 500ng/ml in pooled neat human serum.

Comments to the file:

Given the reagents and assay format, assays with a sensitivity of 250 – 500 ng/ml should be able to detect potentially clinically relevant Ab.

Drug Interference:

The drug interference with PEG-uricase was assessed using the HuPC. In the initial analysis) most of the samples, including the reference sample without drug, had a % difference between duplicate wells $\geq \pm 25\%$. In the repeat analysis three of the samples (0.50, 5.00 and 100.00 ng/mL) still had a % difference between duplicate wells $\geq \pm 25\%$ (between 29.1 and 37.3%). Due to the limited volume of the HuPC, no additional repeat analysis could be performed. Nevertheless, results indicate that PEG-uricase concentrations as low as 0.5 ng/mL in serum 1/10 (5 ng/mL in neat serum) interfered with the assay results (a % difference between PEG-uricase spiked HuPC and unspiked HuPC $> 25\%$). Since the lower limit of quantitation (LLOQ) of the PK assay detecting PEG-uricase levels in clinical samples (600 ng/mL) is greater than the drug interference level, any sample analyzed in the assay will be suspected as potentially containing PEG-uricase levels that could interfere with the assay results.

Comment to the file:

Although the levels of PEG and PEG-uricase interference are relative to the positive control used and will depend on antibody characteristics (e.g. affinity), nevertheless the assay may have limited usefulness as the presence of puricase in sera at concentrations below PK assay detection point ($< 600\text{ng/ml}$) interferes with anti-PEG Ab detection. This problem can in part be addressed by looking at samples obtained after a sufficient washout period (generally at least 5 half lives).

Prozone Effect:

To investigate the possibility of assay deficiency at a very high concentration of the target analyte (anti-PEG antibodies), the prozone effect was assessed. For the evaluation of the prozone effect one PC concentration (2500 ng/mL), 10 times higher than HPC (250 ng/mL), was analyzed on 1 occasion (n = 3). No prozone effect was observed even when using MuPC at 10X HPC levels (2500ng/ml).

Comment to the file:

Prozone effect could not be demonstrated for the assay.

Drug competition test:

The drug competition test was used to confirm the presence of anti-PEG antibodies in human serum samples considered potential positives during validation and will be used during sample analysis. Since this assay detects antibodies targeted against the PEG portion of the drug (puricase), the drug competition test was performed with PEG and not with PEG-uricase. The blank TS, LPC, HPC and potentially positive serum samples found during the NCO determination and specificity assessment, were spiked with a final PEG concentration of 150 µg/mL or with a final Dextran concentration of 150 µg/mL (drug competition test negative control). The drug competition samples were incubated for 1 hour ±5 minutes on a plate shaker set at 100 rpm, in an incubator set at 20.5°C prior to analysis. The mean A450 of the positive control mouse anti-PEG prepared at the LPC and the HPC levels was reduced by more than 30% in the presence of 150 µg/mL of PEG when compared to the corresponding control prepared in Blocker Casein in PBS. In addition, the mean A450 of the LPC and the HPC was reduced by less than 30% in the presence of 150 µg/mL of Dextran (drug competition test negative control).

Comments to the file:

The assay appears to show a high degree of intra-assay variability, which the sponsor tries to rationalize/bypass by resorting to floating cut point. While this approach is sometimes necessary, it is only recommended when other approaches are not feasible. In this case, the Sponsor may be able to explore the antigen specificity of patient Ab by competitive inhibition studies using the anti-drug Ab assay.

Comments to sponsor:

Your anti-PEG antibody ELISA shows a very high degree of intra-and inter-assay variability possibly related to the PEG coating of your ELISA plate. This indicates either that the assay is not sufficiently optimized or that the format is unsuitable. Please address these concerns by developing alternative acceptable approaches for assessing the antigen specificity of the anti-drug antibodies: e.g. assess inhibition of binding of the anti-drug antibodies in the presence of excess (b) (4) in the anti-drug antibody assay.

Summary

This assay does not provide reliable data. The Sponsor should be asked to redevelop the assay.

Study 301402 Draft research report for validation of an ELISA for the detection of anti-Uricase IgG and IgM antibodies in human serum

This study provides results of validation of qualitative ELISA for detection of anti-uricase IgG and IgM antibodies in human serum. The plates are coated with uricase, followed by human sera samples, which are detected with HRP-conjugated goat anti-human IgG/M polyclonal. As sponsor had no available human-anti-uricase antisera, an affinity purified rabbit anti-uricase antibody (primarily IgG since the animal was hyper-immunized) is used as surrogate positive control (RbPC) in assay, at LPC(1ng/ml), MPC(2.5ng/ml) and HPC (5ng/ml). Purified human IPC-IgG and IPC-IgM immunoglobulin positive controls are used to test for binding of goat anti-human Ig secondary antibodies. The method validation included negative cut-off and cut-point factor determination, intra- and inter-assay precision, sensitivity, specificity and recovery, drug interference, prozone effect and drug competition test. Similar stability studies were performed for this PC as for the MuPC in anti-PEG ab ELISA validation, with the RbPC proving equally stable. Unlike the anti-PEG Ab ELISA, the current ELISA shows low intra- and inter-assay variability (CV%<10% for intra-assay, and <16% for inter-assay). The assay sensitivity was established at 22.5 ng/ml of RbPC in pooled neat human sera. The false positive rate was 11%, which is again higher than the recommended. For the NCO determination the Sponsor used normal human sera and gout patient sera (prior to treatment), 10 of each type. Interference of (b) (4) with detection of rabbit anti-uricase antibody was seen at dose for LPC equivalent to 600 ug/ml in neat human sera, and for HPCat a dose equivalent to 3000 ug/ml in neat human sera. Puricase at 2ug/ml was able to reduce the LPC and HPC >30% while (b) (4) at an analogous concentration failed to do so.

Comments to the file:

The high dose of puricase required to interfere with anti-uricase rabbit positive control indicates how effective PEG is at masking antigenic epitopes when covalently attached to the uricase enzyme

Comments to the sponsor:

Please determine if there are anti-uricase antibodies in your new human anti-PEG-uricase positive control Ab, and evaluate its possible use as control in the anti-uricase ELISA.

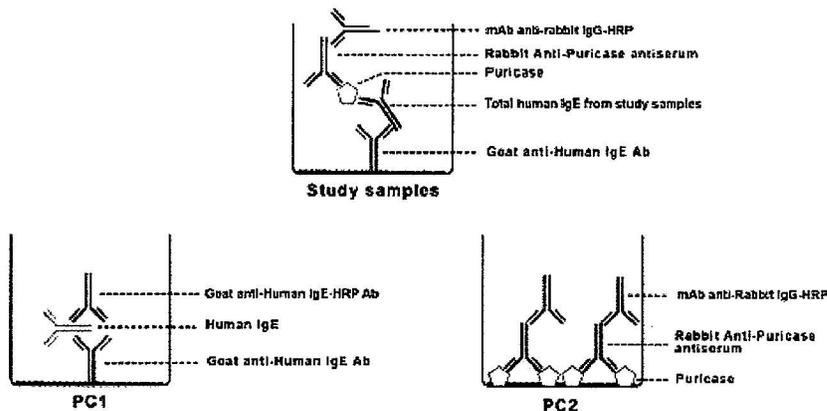
Study 300913 Final Protocol for the validation of an ELISA method for the detection of anti-PEG-Uricase IgE in human serum

Sponsor provides a validation study for the qualitative detection of anti-puricase IgE antibodies in human serum. The ELISA has an interesting format for the study samples: coating with affinity purified goat anti-human IgE, followed by human test sera; detection involves 3 steps- puricase drug product, rabbit anti-puricase sera (from the previous study), and HRP-conjugated goat anti-rabbit IgG. This complicated set up necessitates two positive controls: PC1- coating with same goat anti-human IgE ,

followed by purified total human IgE (at low, medium and high concentrations) and detected with HRP conjugated goat anti-human IgE polyclonal; PC2- coating with puricase drug product, followed by rabbit anti-puricase antisera, and HRP-conjugated goat anti-rabbit IgG. The sponsor plans to determine negative cut off point and cut point factor (using 25 normal sera and 25 pre-dose gout patient samples), specificity and intra and inter-assay precision and stability (RT for 24h, 4°C for 24h, long-term storage of unspecified duration at -20°C, and after 3 freeze thaw cycles) using set up for PC1 [LPC (15-30ng/ml) MPC (125ng/ml) and HPC(500ng/ml) of purified total human IgE].

If positive clinical samples are found in any of the studies, and there are suitable volumes available following a confirmatory assay, Sponsor plans to test intra-assay precision, recovery, drug product interference, titration and prozone effect and stability of these samples. Sensitivity will be assessed on purified IgE from clinical samples.

Figure 1 Detection of Anti-PEG-uricase IgE Antibodies in Human Sera - ELISA Format (Study Samples, PC1 and PC2)



For PC1 control samples and Study Samples:

NCO and CPF determination:

The negative cut-off (NCO), which was normalized for each plate using the cut-point factor (CPF), was used to determine the threshold for identifying samples as negative (equal or below the NCO) or positive (above the NCO) for the presence of anti-PEG-uricase IgE antibodies. Twenty-five lots of normal human serum (normal population) and 25 predose samples (gout disease population) were diluted 1/30 with assay buffer and assayed on 6 occasions over 3 different days by two different analysts.

During validation, the CPF was established at 1.123 using selected A450nm values from serum from healthy donors and gout disease patients. However, due to insufficient

volume of gout disease serum, blank TS was prepared from only selected serum from healthy donors. A pool of blank human serum was prepared using the 23 lots of human serum from healthy donors and was used as matrix in all validation assays, except the assays for the NCO and CPF determination where a preliminary pool was used.

Comments to the file:

Number of normal sera used is on the lower end of the acceptable range. Since there appears to be no difference between healthy subject sera and target population sera it is acceptable to set the NCO using healthy subject sera.

Specificity

To assess specificity, 8 individual lots of human serum (other than the lots used for the CP determination) and 10 lots from predose samples from C0403 (Savient Pharmaceuticals phase II clinical study; gout disease patients) were analyzed. In addition, the blank TS was analyzed as the reference sample. All samples were analyzed on one occasion, except sample 41911267, which was analyzed a second time due to repeat analysis using the study samples format. All lots of serum from healthy donors were < CP, except lots BRH145506 (A450nm = 0.197) and BRH145526 (A450nm = 0.191), which were slightly higher than the CP (0.1892), and all lots from gout disease patients were < CP.

Comments to the file:

No specificity was tested in this assay as sponsor has no true puricase specific IgE positive control.

Intra- and Inter-assay Precision

Three levels of total IgE - low, medium and high from a commercial source of human serum (with sodium azide) that contain a high level of IgE (PC1A) spiked in assay buffer and a pool preparation from individual lots of human serum from healthy donors containing IgE (PC1B) diluted 1/30 in assay buffer were analyzed in replicates of 3 on 6 occasions for all PC except on 7 occasions for PC1B. The precision was not assessed with the PC2 format because the coating used for this control does not mimic the coating conditions of the study samples.

Acceptance Criteria:

For the intra-assay to be acceptable, the mean absorbance (A450nm) values of 2 or more replicates at each level should have been above the negative control (i.e. Blank PC1A) mean absorbance (A450nm) value, and the coefficient of variation should have been < 25%. In addition, the global mean absorbance (A450nm) value of LPC1A should have been less than the global mean absorbance (A450nm) value of MPC1A, which should have been less than the global mean absorbance (A450nm) value of HPC1A. For the inter-assay to be acceptable, the coefficient of variation (% CV) of the intra-assay mean A450nm values should have been < 25%.

The intra- and inter-assay precision of the assay was demonstrated using total IgE positive controls (LPC1A 15.6 ng/ml, MPC1A 62.5 ng/ml, HPC1A 250ng/ml and PC1B pooled healthy serum). The intra-assay precision ranged from 0.7% to 11.7%, and the inter-assay precision ranged from 17.7% to 21.8%. The overall results of HPC1A, PC1B (24 July 2007), blank TS and PC2 precision (% CV) were below 25%, except for LPC1A, MPC1A and PC1B (10 July 2007) where % CV = 38.1%, 32.1% and 27.7%, respectively due to higher A450nm values observed in assay VAL-09. The assay VAL-09 successfully met the acceptance criteria, but higher A450nm were observed in PC1A, which affected the overall % CV of LPC1A, MPC1A and PC1B (10 July 2007). This assay was performed to assess the NCO, and the higher A450nm values observed in PC1A did not affect the values observed in individual samples and blank TS (i.e. study sample format) as they were comparable to the five other NCO occasions. The intra-assay precision ranged from 0.7% to 11.7%, and the inter-assay precision ranged from 17.7% to 21.8%.

Comments to the file:

Intra-assay and inter-assay precisions fall within the acceptable ranges for this type of assay.

Stability

The stability of the anti-PEG-uricase IgE antibodies in human serum was performed by confirming the stability of total IgE in human serum (i.e. unpurified human serum; PC1 format) since no suitable positive samples were available. The stability of human serum from healthy donors was assessed at room temperature (RT) for approximately 6 hours and 24 hours (i.e. combined with long-term storage at approximately -20°C after 121 days), at approximately 4°C for approximately 24 hours and after six freeze and thaw cycles and up to 121 days at approximately -20°C. Additional long-term stability evaluation at approximately -20°C is ongoing.

Four lots of human serum from healthy donors containing total IgE were shown to be stable at room temperature for approximately 6 and 24 hours, at approximately 4°C for approximately 24 hours and after 6 freeze-thaw cycles at approximately -20°C and up to 121 days at approximately -20°C. The % difference of the overall mean concentration of the stability samples was within ±25% for all samples tested when compared to the overall mean concentration of the control sample (i.e. Day 0) within the same plate.

Comment to the file:

Total IgE containing samples appear to maintain their stability under the conditions tested.

Summary:

The ELISA format chosen by the Sponsor is not suitable for an IgE assay because it is not capable of sufficient sensitivity. The assay could not be validated due to a lack of positive control antibody. It is not clear what approaches the Sponsor tried for developing a positive control but minimally they should have been able to cross-link the

rabbit polyclonal to a human IgE to create a more satisfactory control. The low positive control for total IgE, which gave a signal near their cut-point, was ~30 ng/ml. For an antigen specific IgE assay to be useful it should have sensitivity in the nanogram to sub-nanogram range, and there are technologies currently available that can meet these criteria. Any IgE data should not be considered reliable.

Study 300914 Detection of IgE antibodies against PEG-Uricase in human serum by an enzyme linked immunosorbent assay (ELISA)

The purpose of this study was to detect Anti-PEG-uricase IgE antibodies in human serum Study Samples generated from the Savient Pharmaceuticals Clinical Studies: C0405 and C0406 using a qualitative ELISA. This study follows in the heels of the previous one (300913), where the qualitative ELISA assay used for Study Sample analysis was partially validated to GLP. Due to the unavailability of a suitable positive control sample, the intra-assay precision, recovery, confirmatory assay, drug interference, titration, prozone effect and stability using positive Clinical Study Samples (i.e. Anti-PEG-uricase IgE antibodies) could not be assessed during validation (Study No. 300913) but could be assessed at a later time if a suitable positive Study Sample with sufficient volume becomes available. Although potential positive Study Samples were detected in Study No. 300914, they were not considered suitable as their A450nm values were only slightly above the CP (cut-point). The assay's sensitivity could not be validated and will be assessed only if an appropriate study sample from clinical studies can be confirmed a true positive and can be affinity purified. An assessment of the above-mentioned parameters will be documented in a final report amendment under Study No. 300913 (validation), if applicable. The assay's parameters were established in the absence of a suitable antibody positive control. Therefore, assay parameters will need to be re-optimized if a suitable positive control becomes available. The assay's ability and sensitivity to accurately detect Anti-PEG-uricase IgE antibodies could not be fully demonstrated during the validation (Study No.: 300913).

Study samples were collected from a total of 215 Subjects: 105 from the sponsor's Phase 3 Clinical Study (Ref. No. C0405); and 110 from the sponsor's Phase 3 clinical study (Ref. No. C0406). The Phase 3 Study Samples were stored at approximately -20°C until analysis (up to 8 time points were collected for each Subject: Weeks 1, 3, 5, 9, 13, 17, 21 and 23 and Subject early termination time points, where applicable). A total of 737 (C0405) and 756 (C0406) Study Samples were analyzed for screening. All serum study samples that have an A450nm value > CP were considered potential positive because no confirmatory assay could be validated. All Study Samples that were considered potential positive were titrated. The screening and titration results are presented in this report.

A cut-point factor (CPF) of 1.123 was determined during validation (Study No. 300913) and was established from 45 individual serum lots (i.e. 22 lots from healthy donors received from a commercial supplier and 23 predose gout disease study samples from Study No. C0403). This CPF value was used to calculate the cut-point (i.e. CP = blank TS (Test Sample) x CPF) for each ELISA plate. A total of 126 study samples from 48 clinical study C0405 subjects and 90 study samples from 37 clinical study C0406

subjects were screened potential positives. (i.e. mean A450nm > CP) and titrated. The Study samples' titers ranged from 30 to 960.

Stability of human serum from healthy donors (i.e. total IgE measurement) was validated at RT for up to 24 hours, at approximately 4°C for approximately 24 hours and after six freeze and thaw cycles. The long-term stability at approximately -20°C for up to 545 days is ongoing.

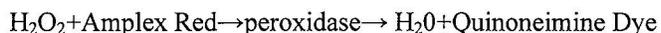
Comments to the file:

The lack of a suitable positive control severely constraints the extent to which this assay can be validated. Sponsor should be encouraged to develop a humanized puricase specific IgE antibody.

Study 300569 Validation of a qualitative enzymatic assay for the detection of neutralizing anti-PEG-Uricase antibodies in human serum

The neutralization assay will be performed using commercial reagents assembled as a kit to assess urolytic activity. Briefly, puricase catalyzes the oxidation of uric acid to allantoin, CO₂ and H₂O₂. Unlike uric acid, allantoin is soluble and can be eliminated. The assay utilized measures the amount of H₂O₂ produced, which breaks down Amplex Red (Molecular Probes) to form a quinoneimine dye. Briefly, undisclosed dilutions of different lots of human sera (no study samples will be used during validation study) are spiked with undisclosed concentrations of puricase. The reaction begins with the addition of the start reagent (HRP, uric acid and Amplex Red in 0.1M tris buffer, pH7.5) After incubation for 45 min at 37°C, fluorescence is measured using a plate reader with excitation λ of 540nm and emission λ of 590nm. The measured response is proportional to the concentration of PEG-uricase present in the sample and inversely proportional to the levels of neutralizing activity in the sera. According to the Sponsor, no suitable neutralizing antibody was available when the assay validation protocol was developed so they opted to use a competitive inhibitor of puricase (8-azaxanthine monohydrate) instead.

The Sponsor might have had more success in generating a positive control Ab using uricase as the antigen but they did not try this approach.



(Detect fluorescence at 540nm/590nm, intensity of dye colour is proportional to ureate oxidase activity)

The assay is qualitative in nature as it is based on determination of negative cut-off (NCO) and cut point factor. Samples above NCO will be considered positive for neutralizing activity, and below it negative for neutralizing activity.

The method validation included negative cut-off (NCO) and cut-point factor (CPF) determination, intra- and inter assay precision, and specificity. Due to the unavailability of a suitable neutralizing antibody positive control, recovery, drug interference, prozone, titration, drug competition test and stability of neutralizing anti-PEG-uricase antibodies could not be assessed during validation but might be assessed at a later stage if a suitable positive study sample with sufficient volume becomes available. The sensitivity will be assessed only if a study sample from the clinical studies can be confirmed as true positive and can be affinity purified. Assessment of the above-mentioned parameters will be documented in a final report amendment if suitable positive study samples become available. During the method development the optimal dilution of human serum was established at 1/25 with Tris-buffer pH 7.5, and the final PEG-uricase spiking concentration in the diluted samples was established at 500 ng/mL.

Negative Cut-off (NCO) and Cut-point Factor (CPF) Determination

For NCO and CPF determination, 25 lots of normal human serum (normal population) and 25 predose samples from Savient Pharmaceuticals Phase II clinical study C0403 (disease population) were diluted 1/25 with Tris buffer pH 7.5. The individual lots and predose samples were tested on six occasions over 3 different days by two different analysts. Due to differences observed between normal sera and patient sera, the sponsor opted to use only results from 25 patient sera for NCO and CPF determination. Four individual lots of gout serum (predose samples) were further flagged as outliers during the NCO and CPF determination, and excluded from calculations. Based on the results obtained from the remaining 21 lots, the CPF was set at 0.701.

A pool of human serum, to be used as a source of blank human serum during the remaining validation assays and during sample analysis, was to be prepared by combining equal volumes of the individual lots of normal human serum selected for the NCO calculation. Given the difference observed between normal and gout sera, it would have been inappropriate to prepare the pool using normal human serum since the pool would not have been representative of the study sample tested. Since the volumes of predose samples (gout serum) were insufficient to create a pool, 3 individual lots of gout serum were purchased in sufficient volume from Bioreclamation. These lots were not tested to establish the NCO/CPF. These lots were the only source of gout serum available in sufficient volume to create a pool. During validation, the NC prepared with the pool produced mean RFU values between 2058.118 and 3374.992, which was similar the mean RFU of the individual predose sera used for NCO calculation (between 2103.50 RFU and 2702.45 RFU).

Considering the reagents available, this was an appropriate alternative to obtain a representative pool of human serum.

Given the substantial inter-lot variability observed among the individual gout sera tested, it was decided to calculate the ratio predose/post dose samples in addition to using the plate NCO to define samples as negative or positive. Any post dose samples below the NCO and with a mean

fluorescence intensity reduction greater than 50% when compared to its corresponding predose sample would be considered as potentially containing neutralizing anti-PEG antibodies. The rationale for adopting a 50% reduction in signal was based on Savient Phase II C0403 clinical study results, which showed that a substantial decrease in circulating drug levels correlated with the detection of anti-drug antibodies. Assuming that this decrease was due to the presence of neutralizing antibodies, it was hypothesized that they were present at very high concentration and could therefore generate at least a 50% reduction in the assay signal. In the absence of a confirmatory assay, this comparison predose/post dose would allow for a better characterization of the samples that are below the NCO. Samples will not only be defined as positive based on a cut-off point but also based on the decrease of signal observed for a particular subject upon treatment.

The assumption that the decrease in circulating drug and the presence of antibodies that can interfere with uricase activity is not valid. However, to neutralize activity in an in vitro assay the antibodies should recognize uricase. Since no anti-uricase antibodies were detected it is not necessary to pursue the cut-point at this time. It is possible that occasionally anti-PEG antibodies might prove neutralizing in this assay. However, this is unlikely to be an important mechanism clinically since the Sponsor tried and failed to identify anti-Puricase antibodies that neutralized activity.

During sample analysis study a cut-point factor the Sponsor intended to re-calculated using the predose samples if a sufficient number of samples is available. The predose CPF will be compared with the validation CPF. Based on scientific judgment and upon discussion with the Sponsor, the predose CPF might be used for sample analysis assays. This information will be reported in each sample analysis report, if applicable.

No suitable samples were available the confirmatory analysis could not be performed

Precision of the Negative Controls

The precision of the assay was demonstrated using the negative control (NC) and for information purposes was also demonstrated using the background samples (BKG). The negative controls were tested on 6 different occasions in 12 replicates per occasion. The intra-assay precision of the NC ranged between 1.9% and 7.9% whereas for the BKG it ranged between 1.0% and 6.3%. The inter-assay precision of the NC and BKG were 20.1% and 10.9%, respectively.

The assay precision using the negative control sample was well within industry standards for this type of assay and is therefore acceptable.

Specificity

Ten individual lots of normal human serum and 10 individual lots of serum from gout donors (predose samples), different from the lots tested for the negative cut off and cut point factor determination, were used to assess the method specificity. Considering that the NCO was established using gout serum only, the specificity with normal and gout serum was evaluated separately. All lots of normal serum were > NCO, which met the acceptance criteria. Seven lots

of gout serum out of 10 (70%) were > NCO which is below the 80% acceptance criteria. Nevertheless, among the 3 lots of gout serum not meeting acceptance criteria, one was just slightly below the NCO. In addition, the NCO will not be the only acceptance criteria for defining samples as positive or negative, predose/post dose ratio will also be calculated. Thus, the specificity was considered acceptable for the gout sera.

Comment to the file:

During validation, the cut-point factor was established at 0.701, the intra-assay and inter-assay precision of the NC and BKG was demonstrated (< 25% CV) and the specificity of the assay was considered acceptable. However, due to the unavailability of a suitable neutralizing antibody positive control, recovery, drug interference, sensitivity, prozone, titration, drug competition test and stability of neutralizing anti-PEG-uricase antibodies could not be assessed. Therefore, the assay's ability to accurately detect neutralizing anti-PEG-uricase antibodies could not be fully demonstrated. However, as noted above, since anti-uracase antibodies were not detected in patients, and neutralizing anti-Puricase antibodies were not detected, the vast majority of anti-Puricase antibody positive patient sera are expected to be negative in this assay. Therefore we do not recommend further assay development and validation at this time.