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Monitoring and Control of Genotoxic Impurity Acetamide in the Synthesis of Zauritegrast Sulfate

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Abstract:

In this article we describe the strategy adopted to minimize the risk of acetamide presence in zauritegrast sulfate drug candidate. A risk of acetamide formation (a potential genotoxic impurity) was identified in the API formation step of the process during the early development phase. In order to keep the project development timelines unchanged and without having the appropriate analytical method ready developed, we chose to minimize the risk of acetamide impurity presence by applying an adequate chemical process design. The implementation of a workup sequence involving initially three aqueous washes was later proven to be successful when an appropriate analytical method to detect acetamide below ppm levels was available. Additionally the analytical tool gave us the opportunity to assess and fine-tune the designed process for acetamide elimination by spiking experiments. Data acquired during this evaluation showed that a single aqueous wash associated with two efficient crystallization steps were finally enough to deliver API with a content of acetamide below the level defined as the acceptance criterion.

Introduction to Genotoxic Impurities

The control of genotoxic impurities (GTI) during the development of drug substances is a growing concern in the pharmaceutical industry.[1] The European Medicines Agency (EMEA) issued in June 2006 a guideline for GTI limits that came into force in January 2007. This guideline included the concept of threshold of toxicological concern (TTC) to define an acceptable risk for new active substances. A TTC of 1.5 μg/day is given at a level at which exposure will not pose a significant carcinogenic risk.[2] Additionally, the CHMP Safety Working Party agreed with the use of a staged TTC concept during clinical development.[3]

The avoidance of potential GTI impurities[4] during the development of chemical processes may not only limit the efficiency (yield, selectivity, scalability) of some chemical transformations, but it may also entail increased development costs to perform a full chemical and analytical process assessment. A complementary approach to avoiding GTI impurity is therefore to assess and manage the risk through appropriate application of chemical process design and analytical testing. We share in this report an example of this strategy.

Introduction to Zauritegrast Sulfate. Zauritegrast sulfate **1** is a drug that had been under phase II clinical development and was indicated in the field of inflammation and more particularly for multiple sclerosis treatment.[5] The synthetic route to zauritegrast sulfate API **1** is a five cGMP step process in which the last two steps include a key bromination of the final intermediate UCB1193394 **2** leading to crude API **3** followed by a recrystallization from ethanol−water, giving rise to pure active pharmaceutical ingredient (API) **1** as shown in Scheme 1. The presence of the bromine atom was found essential for drug activity. Additionally, the ethyl ester is a prodrug of the corresponding carboxylic acid.

The bromination step as developed during early development phase raised three questions in terms of potential GTI impurity. First, the carry-over of methanesulfonic acid (MsOH) used during the bromination reaction may lead to the formation of the known GTI ethyl methanesulfonate in the presence of ethanol during the subsequent salt formation.[6] Second, the isolation of the required sulfate salt in ethanol may lead to the formation of monoethyl sulfate and/or diethyl sulfate impurity.[7] And finally, the use of aqueous acetonitrile (MeCN) associated to a strong acid may generate acetamide by acetonitrile hydrolysis.[8]

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(1) Pierson, D. A.; Olsen, B. A.; Robbins, D. K.; DeVries, K. M.; Varie, D. L. *Org. Process Res. Dev.* **2009**, *13*, 285–291. Teasdale, A.; Eyley, S. C.; Delaney, E.; Jacq, C.; Taylor-Worth, K.; Lipczynski, A.; Reif, V.; Elder, D. P.; Facchine, K. L.; Golec, S.; Schulte Oestrich, R.; Sandra, P.; David, F. *Org. Process Res. Dev.* **2009**, *13*, 429–433. Yang, Q.; Haney, B. P.; Vaux, A.; Riley, D. A.; Heidrich, L.; He, P.; Mason, P.; Tehim, A.; Fisher, L. E.; Maag, H.; Anderson, N. G. *Org. Process Res. Dev.* **2009**, *13*, 786–791.

(2) Guidelines on the Limits of Genotoxic Impurities. *CPMP/SWP/5199/02 EMEA/CHMP/QWP/251344/2006*; European Medicines Agency (EMEA), Committee for Medicinal Products for Human Use (CHMP): London, June 28, 2006.

(3) Questions & Answers on the CHMP Guideline on the Limits of Genotoxic Impurities. *EMA/CHMP/SWP/431994/2007*, revision 2; European Medicines Agency (EMEA), CHMP SAFETY WORKING PARTY (SWP): London, December 2009.

(4) GTI impurity may be a reactant, an intermediate, or a by-product.

(5) NTC00484536. Double-blind, Placebo-controlled, Randomized, Parallel-group Phase II Study in Subjects With Relapsing Forms of Multiple Sclerosis (MS) to Evaluate the Safety, Tolerability, and Effects of CDP323. http://clinicaltrials.gov/ct2/show/NCT00484536?term=CDP323&rank=1; accessed 12/11/2009

(6) Glowienke, S.; Frieauff, W.; Allmendinger, Th.; Martus, H.-J.; Sutter, W.; Mueller, L. *Mutat. Res.* **2005**, *581*, 23–34.

(7) *Summary and Evaluation Diethyl Sulfate*; International Agency for Research on Cancer (IARC): 1992; Vol. 54, p 213, http://www.inchem.org/documents/iarc/vol54/04-diethyl-sulfate.html.

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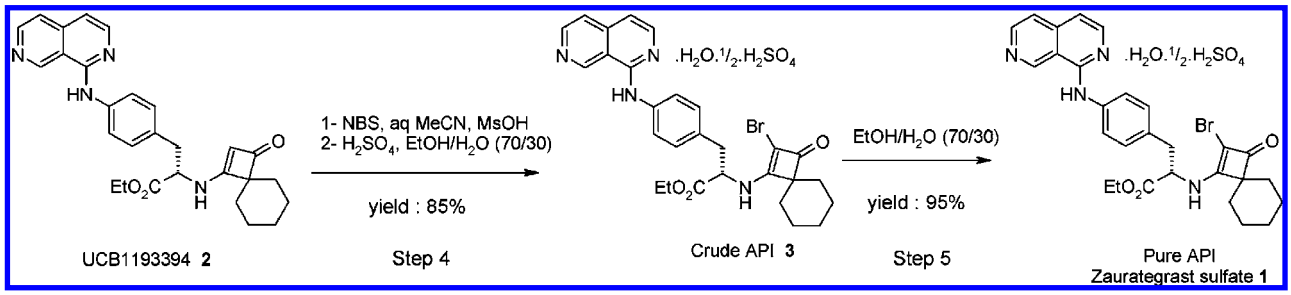
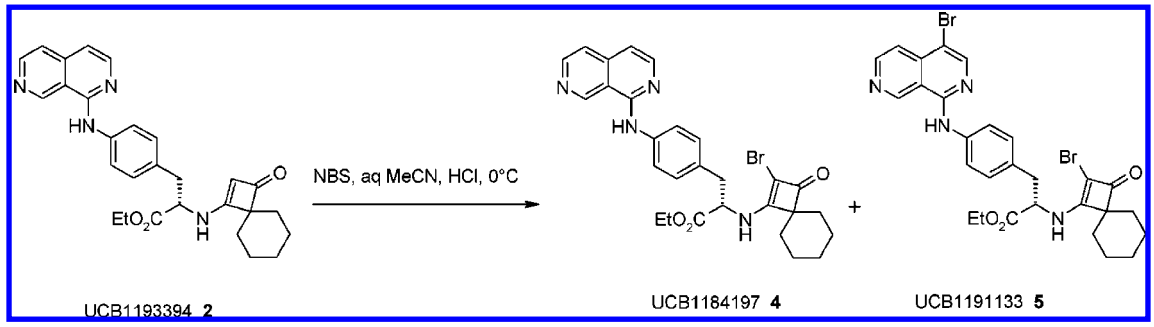
Scheme 1. **Last two steps of zauategrast sulfate synthesis**



Scheme 2. **Major impurity from the bromination process**



The potential risk of generating ethyl methanesulfonate was readily eliminated by changing the acid. A rapid screen showed that hydrochloric acid gave similar results and was therefore selected instead of methanesulfonic acid.

The choice of the sulfate as the counterion salt was driven by solid-state consideration: polymorphism and particle size control. The hydrate hemisulfate salt is formed by using half an equivalent of sulfuric acid in 11 volumes of EtOH/H_2O (70/30) mixture. The experimental protocol that we developed for zauategrast sulfate formation is very different from the experimental conditions that lead to formation of monoethylsulfate and diethylsulfate.[9] Additionally the monitoring of sulfate esters is not in the scope of this article.

The potential presence of acetamide in zauategrast sulfate **1** has received a detailed examination. Although the FDA lists acetamide as a food additive,[10] the International Agency for Research on Cancer (IARC) has classified acetamide as possibly carcinogenic to humans (Group 2B)[11] based on rodent toxicity data and thus is controlled to a threshold level of <5 μg/day. The compound is negative in the Ames test but shows genotoxicity *in vivo*.[12] For all these reasons it was decided to control the acetamide level in the drug candidate following guidelines for GTI control (<1.5 μg/day exposure for clinical trials with duration longer than 12 months).

Synthesis of Zauategrast Sulfate. The selective bromination of UCB1193394 **2** into crude API **3** appeared rapidly as the key step in the development of zauategrast sulfate **1**. In general, brominations of advanced pharmaceutical intermediates on industrial scale remain a challenge, more particularly in cases where overbromination can occur[13] and come to a head when the overbrominated product cannot be easily eliminated by conventional means (i.e., crystallization). This is the situation we faced in this bromination reaction which produced the overbrominated process impurity UCB1191133 **5** at a typical level of 0.10−0.30%-w/w (NMT 0.40%-w/w specified) as shown in Scheme 2. Developing a satisfactory industrial bromination process required in this case, simultaneous control of reaction conversion and overbromination. Amongst the brominating agents that were screened, NBS proved to be the

(8) Basu, M. K.; Luo, F.-T. Efficient Transformation of Nitrile into Amide under Mild Conditions. *Tetrahedron Lett.* **1998**, *39*, 3005–3006. Wilgus, C. P.; Downing, S.; Molitor, E.; Bains, S.; Pagni, R. M.; Kabalka, G. W. The Acid-Catalyzed and Uncatalyzed Hydrolysis of Nitriles on Unactivated Alumina. *Tetrahedron Lett.* **1995**, *36*, 3469–3472. Krieble, V. K.; Smellie, R. H. Process for hydrolyzing organic nitriles and dehydrating ethers. U.S. Patent 2,441,114, 1948. Liler, M.; Kosanovic, D. J. *J. Chem. Soc.* **1958**, 1084–1090. Travagli, G. *Gazz. Chim. Ital.* **1957**, *87*, 682–687 and 830–836. Barbosa, L. A. M. M.; Van Santen, R. A. Study of Hydrolysis of Acetonitrile Using Different Bronsted Acid Models: Zeolite-Type and HCl(H_2O)*x* Clusters. *J. Catal.* **2000**, *191*, 200–217. Lee, G. R.; Crayston, J. A. Hydrolysis of acetonitrile in the presence of $NbCl_5$. *Polyhedron* **1996**, *15* (11), 1817–1821.

(9) Kazansky, V. B. *React. Kinet. Catal. Lett.* **1999**, *68* (1), 35–43. In this paper, the authors show that a 1:1 volume mixture of 70% H_2SO_4 with ethanol leads to the detection of the monoethylsulfate by ^{13}C NMR. In addition, diethylsulfate was only detected in harsher conditions when using a 10:1 volume mixture of 95% H_2SO_4 with ethanol.

(10) (a) According to FDA's EAFUS (Everything Added to Food in the US) database, "The EAFUS list of substances contains ingredients added directly to food that FDA has either approved as foods additives or listed or affirmed as GRAS (Generally Recognized as Safe)". In this table acetamide is listed in the EAF category, meaning "There is reported use of the substance, but it has not yet been assigned for toxicology literature search". (b) The Flavor and Extracts Manufacturers Association Expert Panel, an independent organization, has included acetamide in the GRAS (Generally Recognized as Safe) list with the average maximum use level of 5 ppm for baked goods.

(11) IARC (1999) Monogr. Eval. Carcinog. Risks Hum.: Acetamide, Vol 71, 1211−1220.

(12) Dybing, E.; Soderlung, E. G.; Gordon, W. P.; Holme, J. A.; Christensen, T.; Becher, G.; Rivedal, E.; Thorgeirsson, S. S. Studies on the mechanism of acetamide hepatocarcinogenicity. *Pharmacol. Toxicol.* **1987**, *60*, 9–16.

(13) Frutos, R. P.; Rodriguez, S.; Patel, N.; Johnson, J.; Saha, A.; Krisshnamurthy, D.; Senanayake, C. H. *Org. Process Res. Dev.* **2007**, *11*, 1076–1078. Li, B.; Buzon, R. A.; Zhang, Z. *Org. Process Res. Dev.* **2007**, *11*, 951–955. Li, X.; Jain, N.; Russell, R. K.; Ma, R.; Branum, S.; Xu, J.; Sui, Z. *Org. Process Res. Dev.* **2006**, *10*, 354–360. Zanka, A.; Kubota, A.; Hirabayashi, S.; Nakamura, H. *Org. Process Res. Dev.* **1988**, *2*, 71–77.

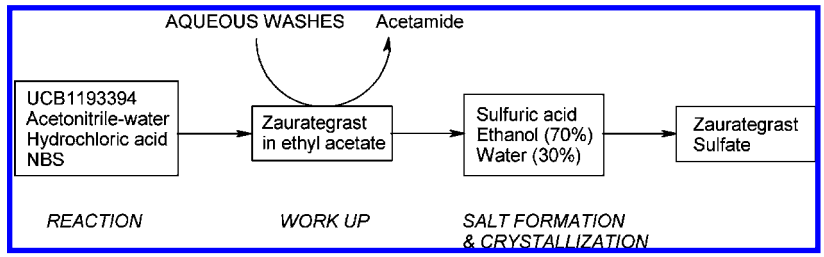


Figure 1. **Aqueous washes to eliminate acetamide.**

best for this reaction.[14] NBS is a common and cheap, stable, and relatively safe industrial brominating agent for scaling-up procedures.[15] It has, however, two major disadvantages, a low solubility in most solvents and a relatively high reactivity to most of the functional groups, that makes it incompatible with many solvents.[16]

Local high concentrations of NBS in the reaction mixture during the addition step have been hypothesized to be the cause of the overbromination reaction.[17] In order to avoid or minimize this overbromination side reaction, NBS was slowly added as a solution in acetonitrile instead of a solid addition. Moreover, water was added to acetonitrile to increase NBS solubility.[18] The NBS acetonitrile/water solution thus obtained proved to be stable for at least 12 h at 25 °C, which was sufficient for performing our slow addition on industrial scale.[19] The most robust reaction conditions identified to deliver API within the established UCB1191133 **5** specification was a mixture of acetonitrile/water/hydrochloric acid maintaining the reaction temperature below 0 °C. Since the clinical study plan was designed to cover up to 2 g of API as a daily dose, the level of acetamide tolerated in the API was adapted according to regulatory guidelines to <0.75 ppm.[2]

Once the risk of the presence of acetamide had been identified and assessed, we set up a risk management plan to reduce the probability of occurrence of this risk. The precise evaluation of the acetamide carry-over to the API by the chemical process in place was not possible at the time of the risk identification. The tight development timelines did not allow time to develop a sufficiently sensitive analytical method (with the ability to detect acetamide below the ppm level) before starting the planned production campaigns, nor to change the process solvent. These two options would have jeopardized the clinical program. The strategy adopted to reduce the risk of acetamide presence in the API was therefore a rapid design of this key step. The implementation of an aqueous workup between the bromination step and the sulfate salt isolation was selected as shown in Figure 1. This decision was motivated after having identified in the literature and verified in the lab the very high solubility of acetamide in water (>2 g/mL at r.t.).[20]

Table 1. **Acetamide content in crude and pure APIs in the 250 kg campaign**

zauraterast sulfate crude API	acetamide content (ppm)	zauraterast sulfate pure API	acetamide content (ppm)
batch 1	BLQ	batch 4 (from batch 1)	0.18
batch 2	BLQ	batch 5 (from batch 2)	BLQ
batch 3	BLQ	batch 6 (from batch 3)	BLQ

Results and Discussion

The workup was readily implemented (Figure 1). After reaction completion, acetonitrile was efficiently removed by azeotropic distillation. The zauraterast hydrochloride salt in water was partitioned between ethyl acetate and potassium bicarbonate solution. The free base UCB1184197 **4** was then washed three times with 5%-w/w sodium chloride. A solvent swap from ethyl acetate to ethanol followed by addition of sulfuric acid finally led to the sulfate salt formation and isolation. The choice of three aqueous washes was balanced between expected wash efficiency to remove acetamide and manufacturing cost consideration (cycle time). The pilot-plant campaign proceeded successfully and afforded three batches of ∼80 kg of zauraterast sulfate **1** within the specifications for UCB1191133 **5** dibrominated impurity.

Meanwhile, the analytical department developed a suitable analytical method for detecting acetamide with a reported limit of 0.10 ppm on the isolated sulfate salt. The results obtained for both crude and pure APIs are reported in Table 1 (BLQ = below the limit of quantification).

All API batches obtained were well below the level of 0.75 ppm acetamide defined as the acceptance criterion. Moreover the results were mostly below the reported limit of 0.10 ppm associated to the analytical method. The discrepancy observed between batch 4 (0.18 ppm) and batch 1 (<BLQ) could be explained by analytical uncertainty of the method at the time of development.[21] Indeed the appearance of acetamide during the last recrystallization step by either a concentration effect or a simple formation is very unlikely due to the experimental conditions avoiding the presence of acetonitrile and acetamide. These results demonstrated that the appropriate design of both chemical process and analytical testing is very efficient to control the level of GTI in the API.

(14) The following bromination reagents were tested in various industrial pharmaceutical solvents: NBS, 1,3-dibromo-5,5-dimethylhydantoin, HBr/Br_2, and V_2O_5/H_2O_2/NH_4Br.
(15) NBS could be purchased at an approximate price of 15 €/kg at ton scale.
(16) The Merck Index, 14th ed.; https://themerckindex.cambridgesoft.com/TheMerckIndex (accessed 12/11/2009). *Synthetic Reagents*; John Wiley: New York, 1974; *2*, pp 1−63.
(17) A FTIR monitoring study comparison between NBS solution addition and portion-wise solid NBS addition showed that solid NBS addition favored the formation of the over-brominated impurity, UCB1191133.
(18) The NBS solution was prepared by dissolving 1 mol equiv of NBS in a mixture of acetonitrile/water (2.6 vol/0.4 vol) with respect to starting material UCB1193394.
(19) A batch size of 245 kg of UCB1193394 involved a NBS solution addition time of 3 h 38 min at a rate of 3.0 kg/min, maintaining the internal temperature at −2.3 °C to−2.6 °C.
(20) International Chemical Safety Cards, www.cdc.gov/niosh/ipcsnfrn/nfrn0233.html (accessed 12/01/2009).
(21) At the time these data were generated, the analytical method was neither fully developed nor qualified yet.

Table 2. **Acetamide content in crude and pure APIs in the 750 kg campaign**

zauraterast sulfate crude API	acetamide content (ppm)	zauraterast sulfate pure API	acetamide content (ppm)
batch 7	0.36	batch 10 (from batch 7)	BLQ
batch 8	0.22	batch 11 (from batch 8)	BLQ
batch 9	0.22	batch 12 (from batch 9)	BLQ

The robustness of the designed process (scalability and efficiency to supply API within the specification and acetamide acceptance criterion) was confirmed during a subsequent manufacturing campaign of 750 kg of API. The bromination step was run in three batches of ~230 kg. The analytical results for acetamide content in both crude and pure APIs are reported in Table 2.

Process Performance Understanding. The availability of an appropriate analytical method to track acetamide allowed performing an in-depth study on the capacity of the designed chemical process to eliminate acetamide potentially formed during the bromination step.

In the designed process used during the manufacturing campaigns, four operations were theoretically identified as efficient to eliminate the potential acetamide formed during the bromination. These four operations were (i) the potassium bicarbonate neutralization which can be considered as a very first aqueous wash, (ii) the three aqueous washes themselves, (iii) the sulfate salt formation, and (iv) the final recrystallization step. These substeps are depicted in Figure 2.

From an analytical point of view, the *in situ* monitoring of potential acetamide formed during the reaction itself was unfortunately not possible due to the low levels present and because of several analytical interferences (zauraterast itself and the inorganic salt content). An alternative way to proceed was to quantify the presence of acetamide in the aqueous and organic phases from the implemented aqueous washes. Once again, due to analytical interferences with the API free base in the organic layers and salt contents in the aqueous ones, it was impossible to generate reliable data on the acetamide content.

The evaluation of the capacity of the process to eliminate acetamide potentially formed during the bromination step was finally performed by spiking acetamide at key process points highlighted in Figure 2 (see spiking points 1, 2, and 3) and by analyzing the obtained crude and pure APIs.

The overall magnitude of acetamide purge was initially unknown. For that reason we started with the assessment of the two crystallizations (closest operations to the API). Acetamide was added in the aqueous ethanolic solution of the free base before the addition of sulfuric acid to form the required crude sulfate (spiking point 3, Figure 2). All amounts of acetamide spiked in our studies were related to the theoretical quantity of crude API obtained with an 85% yield for the bromination step obtained routinely in the laboratory and confirmed at larger scales.

An initial spike of 200 ppm of acetamide was performed. The results are described in Table 3. The experiment afforded the crude API containing 2.7 ppm acetamide only. Most of the spiked acetamide was eliminated in the mother liquors (81%) then during the first cake wash (11%). The mass balance for

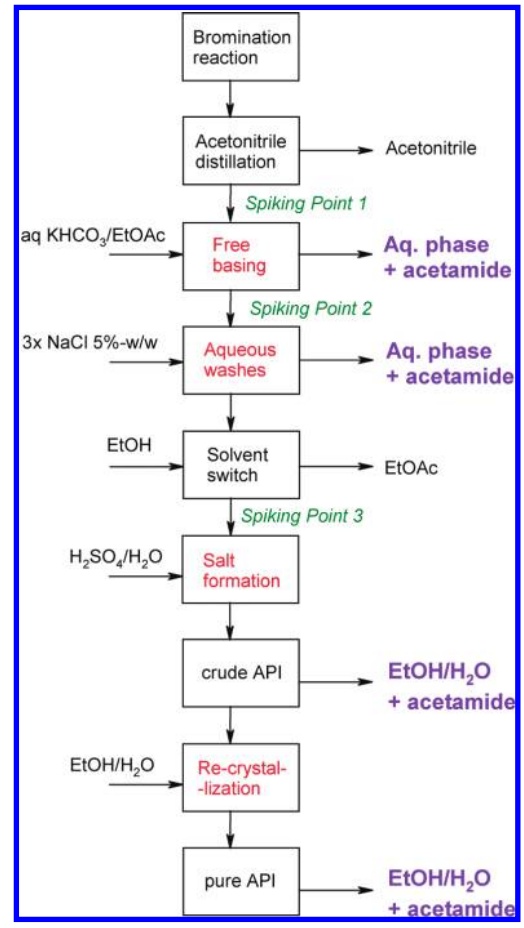


Figure 2. **Process flow diagram of zauraterast sulfate: steps 4 and 5.**

Table 3. **Purification effect of the isolation of crude and pure APIs: spiking experiment with 200 ppm acetamide**

step 4	acetamide content (mg/mL)	mass balance (mg)	percentage (%)
acetamide spiked	200 ppm	6.675 mg	100
mother liquors	0.0159	5.424 mg	81.3
1st cake wash (EtOH 70%)	0.0079	0.750 mg	11.2
2nd cake wash (H_2O 100%)	0.0002	0.005 mg	0.1
zauraterast sulfate crude **3**	2.7 ppm	0.090 mg	1.4
			Recovery 94%

step 5	acetamide content
mother liquors	not detected[22]
1st cake wash (EtOH 70%)	not detected
2nd cake wash (EtOH 15%)	not detected
zauraterast sulfate **1**	BLQ

acetamide indicated a recovery of 94%. The recrystallization of the corresponding crude API containing 2.7 ppm acetamide gave a pure API with acetamide below the reported limit (<0.10 ppm) and by consequence well below the level of 0.75 ppm defined as the acceptance criterion.

The good results obtained encouraged us to challenge the capacity of both crystallizations to eliminate acetamide. Larger amounts of acetamide—1000 ppm and 5000 ppm—were spiked,

Table 4. **Purification effect of the isolation of crude and pure APIs: spiking experiments with 1000 ppm and 5000 ppm acetamide**

	1000 ppm spike	5000 ppm spike
Step 4		
cake after filtration	155.64	*696.50*[a]
cake after EtOH/water (70/30) wash	113.21	*382.36*[a]
cake after water wash (**crude API**)	**54.00**	**158.83**
Step 5		
cake after filtration	4.32	31.17
cake after EtOH/water (70/30) wash	1.27	12.57
cake after EtOH/water (15/85) wash (**pure API**)	**0.77**	**5.88**

[a] Underestimated values due to detector saturation.

Table 5. **Purification effect of the bicarbonate neutralization and aqueous washes**

entry	10000 ppm acetamide spike	number of aqueous washes	acetamide content (ppm) in crude zauraterast sulfate
1	no spiking	3	0.21
2	no spiking	0	0.11
3	spiking point 2	3	0.31
4	spiking point 2	0	14.98
5	spiking point 3	1	20.31
6	spiking point 3	2	3.02
7	spiking point 3	3	0.65

and the results for sulfate salt samples are reported in Table 4. These experiments afforded crude APIs containing 54 ppm and 159 ppm acetamide, respectively. The corresponding pure APIs contained 0.77 ppm and 5.88 ppm acetamide, respectively. These results demonstrated the very high potential of acetamide elimination for both crystallization and recrystallization steps.

The second phase was the evaluation of the performance of the aqueous washes implemented. Depending on the operation studied (potassium bicarbonate neutralization or aqueous washes) the spiking point was set either before the bicarbonate neutralization (spiking point 1, Figure 2) or before the wash sequence (spiking point 2, Figure 2). The amount of acetamide selected for spiking experiments was 10000 ppm in order to take account of downstream crystallization efficiencies demonstrated previously.

The results obtained are described in Table 5. Entry 1 was the reference-designed process without any acetamide spike and with the implemented neutralization and three aqueous washes. The amount of acetamide contained in the crude API (0.21 ppm) was in the expected range and well below the limit of 0.75 ppm acetamide defined as the acceptance criterion.

(22) The 2.7 ppm acetamide contained in the crude API is likely to be removed in the mother liquors of the step 5 final recrystallization based on data obtained for the crude API isolation at step 4. In that case 80% of acetamide spiked was removed in the mother liquors (11 vol). For step 5, 2.7 ppm (1.4% acetamide spiked) diluted in approximately the same volume of solvent (9 vol) makes its quantification not possible by the analytical method. This explains why the amount of 2.7 ppm acetamide is neither detected nor reported.

The set of experiments entries 2−4 dealt with the potassium bicarbonate neutralization influence. Entry 2 gave a significant result concerning the usefulness of the aqueous-wash sequence when the bromination reaction was run as developed. When no acetamide spike was done, skipping all three aqueous washes led to crude API containing 0.11 ppm acetamide which was already in the expected range. It could retrospectively be concluded that in the designed chemical process, the introduction of three aqueous washes did not bring any measurable improvement and was therefore unnecessary. If some acetamide is generated during the reaction, it will be removed by the single or combined actions of the neutralization and the crystallization steps.

Entry 3 dealt with the 10000 ppm spiking before neutralization in the reference process. The combined effects of the neutralization, washes, and crystallizations removed all the initially spiked amount of acetamide to afford a crude API containing 0.31 ppm acetamide. Entry 4 showed that in these engineered conditions, skipping all three aqueous washes gave a crude API containing 15 ppm acetamide. In such a case, when a larger amount of acetamide is present in the reaction mixture, the combined actions of the neutralization and the crystallization steps are not enough to ensure a complete elimination of acetamide.

The second set of experiments (entries 5−7) dealt with the evaluation of aqueous washes for which the spike was done before the wash sequence. Entry 5 involved only one aqueous wash and afforded a crude API containing 20 ppm acetamide only. Considering the crystallization evaluation where a 1000 ppm spike gave a crude API containing 54 ppm acetamide, it can be concluded that a single aqueous wash is able to eliminate more than 9000 ppm of acetamide in the conditions used.

Two aqueous washes (entry 6) gave a crude API containing 3 ppm acetamide, and three aqueous washes (entry 7) afforded a crude API whose acetamide content was 0.65 ppm. These results showed that for the removal of a large quantity of acetamide the first aqueous wash is the most effective.

The purification process implemented (potassium bicarbonate neutralization and three aqueous washes) proved to be highly efficient for acetamide elimination. Moreover, these purification steps are complemented by two very effective crystallization and recrystallization steps as demonstrated in Tables 3 and 4. In that respect, a recommendation to optimize the cycle times of the modified chemical process on the plant was to skip the unnecessary second and third aqueous washes. It was, however, recommended to keep only one aqueous wash to ensure the elimination of all salts brought during the workup (sodium sulfite and potassium bicarbonate).

Acetamide Input from Industrial Acetonitrile. The acetonitrile used during the pilot-plant campaign was analyzed to evaluate its acetamide content and the potential influence on API acetamide level. The analysis showed a concentration of 0.018 mg/L of acetamide in the acetonitrile used in the bromination step. The significance of this result can be easily translated for a bromination batch run during the campaign. Even if no acetamide was removed during the process, the acetamide level within industrial acetonitrile is such that only 64 ppm would remain in the API.

The hypothesis that this amount of acetamide present in the solvent would pass through the entire process is unlikely as demonstrated in this paper with the huge capability of the neutralization, the aqueous washes, and both crystallizations to purge out acetamide. Therefore, the acetamide content in industrial acetonitrile is marginal and can be considered totally removed by the designed bromination process put in place.

Conclusion

The synthesis of zauritegrast sulfate **1** is an example of a process for which fulfilling the specifications involved conditions that potentially lead to GTI presence in the API. At an early stage of development, the management of such a potential risk is often in conflict with the tight timelines allocated to the drug development program. In such cases, the development of an appropriate analytical method in order to track ppm levels of impurities, where there is also a high degree of interferences, takes time and resources.

A valuable solution (when applicable) is therefore to manage the risk through the appropriate application of chemical process design as presented in this paper. Based on a strong rationale the implementation of aqueous washes to eliminate acetamide proved retrospectively to be successful when an appropriate analytical method was available. Two manufacturing campaigns of 250 kg and 750 kg of zauritegrast sulfate **1** were successfully accomplished, giving API within the required specification of the overbrominated impurity and below the acceptance criterion for acetamide content.

The availability of the analytical method gave us then the opportunity to assess and challenge the designed process. This evaluation unambiguously demonstrated its high performance to eliminate acetamide potentially formed during the bromination reaction. Additionally, this evaluation allowed the adjustment of the designed process to a high level of confidence by implementing a single aqueous wash instead of the three ones initially introduced. This process adjustment was unfortunately not performed at industrial scale due to the discontinuation of the project at the end of phase II clinical trials.

Finally, from a commercial API production perspective, the validated, sensitive GC/MS method used to detect acetamide at ppm levels was not suitable for QC routine analysis. The foreseen strategy therefore was to combine a less sensitive detection at the crude stage with our knowledge of the acetamide rejection capacity of the recrystallization. An initial spike of 1000 ppm acetamide (see Table 4) resulted in a level of 54 ppm in crude API which in turn gave 0.77 ppm in the recrystallized product. Given that the acceptance criterion for acetamide in API was 0.75 ppm, a level of 25−30 ppm acetamide could have been proposed for the crude API. Details of the overall chemical process for zauritegrast sulfate **1** synthesis will be presented in a separate communication.

Experimental Section

Analytical Method for the Determination of Acetamide Content. The analytical method for the determination of acetamide content in crude and pure APIs consists of a GC/MS determination using single ion monitoring (SIM) for mass detection. The GC/MS system is an Agilent 6890 GC system with 5975B mass spectrometer operating under Chemstation software control. The column is a Phenomenex ZB-Wax Plus GC column, 30 m × 0.25 mm with 0.25 μm film thickness, fitted with 2−3 m retention gap of deactivated silica tubing. The column selection has been highly influenced by the possible presence of significant amount of residual water in the final samples injected. Carrier gas: helium; initial head pressure: 200 kPa, constant mode pressure; injector: temperature 300 °C, pulsed splitless mode, pulse pressure 250 kPa, pulse time 1.0 min, purge time 1.0 min; injection linear: Agilent 5185-8818 or equivalent (straight, 2 mm, ID, 250 μL); injection volume: 1 μL, (10 μL syringe); injection program: slow plunger speed, post injection dwell = 1.00 min; injector wash: ethanol/water 95/5 v/v; purge flow: 100 mL/min; oven temperature: initial temperature = 100 °C, hold 1 min, then 10 °C/min until 180 °C is reached. Solid samples are typically dissolved in dichloromethane, and the acetamide is separated from the matrix by liquid/liquid extraction principle toward an alkaline aqueous layer. The use of ultrapure NH_3 is required since NH_3 of poor quality has been identified as primary source of acetamide in blanks. Additional solid-phase extraction and concentration steps have been added to increase sensitivity of the method. Samples are quantified against an acetamide external standard, but the complexity of sample preparation has required the use of an additional internal standard. D3-Acetamide has been naturally selected as internal standard considering the detection technique used. In terms of qualification, the method has been validated for specificity, precision, sample stability, linearity, and accuracy from 0.10 ppm to 1.50 ppm.

The detailed sample preparation procedure is described hereafter: 3.00 g of sample is accurately weighed into a 40 mL vial. Three milliliters of internal standard solution (3 μg (1 ppm) D3-acetamide) and 6 mL of dichloromethane are added followed by the addition of 3 mL of 7% aqueous NH_3 solution. The vial is thoroughly shaken to extract analytes. A sample of the aqueous layer, 1.5−2 mL, is placed into a 20 mL vial; 1.5 mL of clean aqueous solution is eluted onto a conditioned HLB cartridge. One milliliter of eluent is diluted with 19 mL of ethanol and left to stand for a few minutes. The solution is filtered through a 25 mm, 0.45 μm preconditioned GDX-XP (PVDF) filter into a 50 mL round-bottom flask. The solution is evaporated under moderate vacuum (30 mbar/40 °C) until a volume of approximately 1 mL remains. If volume is less than 1 mL, make up to 1 mL with ethanol/water 95/5 v/v. The solution is transferred into a 2 mL autosampler vial and left for 1 h. The 1 mL solution is filtered through a preconditioned 0.2 mm PTFE filter into a fresh vial for injection.

NMR spectra were recorded on an Oxford AS400 instrument. Elemental analyses were performed on an Elementar Vario EL3 apparatus. All reagent quantities quoted are relative to starting UCB1193394, assuming 100% purity, unless otherwise stated.

Zauritegrast Sulfate 1 Formation. A double-jacketed glass reactor equipped with anchor is charged with UCB1193394 (1 equiv), acetonitrile (1 vol), and water (1 vol). The suspension is stirred at 210 rpm at 20 °C. To that suspension is added hydrochloric acid (1.1 equiv) maintaining $T \leq 25$ °C, and the resulting mixture is stirred until complete dissolution. The

solution is cooled to 0 °C, and a cold solution of NBS (1 equiv) in acetonitrile/water (2.6/0.4 vol) at 8−10 °C (prepared in a separate vessel equipped with stirring) is added, maintaining the reaction temperature at 0 °C. IPC is performed 30 min after the end of the addition. UCB1193394 IPC specification is <0.5% PAR left. The reaction is quenched with Na_2SO_3 (0.04 equiv) in water (1 vol). The temperature is increased to 40 °C. Acetonitrile is then removed by azeotropic distillation (40 °C/vacuum) to end up with protonated free base in water (1.6 vol theor.). EtOAc (4 vol) is added at 40 °C and maintained for the following workup: a solution of $KHCO_3$ (1.1 equiv) in water (1 vol) is added. Phases are separated and the aqueous one discarded. The organic layer is washed with 5% w/w aqueous NaCl solution (3 vol) three times. Ethyl acetate is switched to EtOH (8 vol)[a] by azeotropic distillation. The temperature is set to 45 °C, and water (2.2 vol)[a] is added followed by a solution of H_2SO_4 (0.48 equiv) in water (1 vol)[a] maintaining *T* at 45 °C. The solution is seeded with **1** (0.32% w/w). The crystallization appears within 5 min. The suspension is stirred vigorously an extra 10 min and cooled to 0 °C in 4 h. The product is isolated by filtration, washed with cold EtOH/water (70/30, 2.4 vol)[a] and cold water (2 vol)[a] to obtain zauategrast sulfate **1** wet considered at 35% LoD (water).

[a]Volumes relative to 100% theoretical yield of zauategrast from this point.

The wet zauategrast sulfate **1** crude considered at 35% LoD is suspended in a mixture of EtOH (6.4 vol[b]) and water (2.5 vol[b]). The suspension is heated to 70 °C to obtain a solution, and polish-filtered to a clean vessel. The temperature is adjusted to 53 °C in −5 °C/h and seed material added (0.2% w/w[b]). The temperature is held at 53 °C for one hour, allowing crystallization to set, then cooled according to the following controlled temperature profile: 53 °C to 50 °C in −5 °C/h, temperature held at 50 °C for one hour, 50 °C to 45 °C in −5 °C/h, temperature held at 45 °C for one hour, 45 °C to 40 °C in −5 °C/h, temperature held at 40 °C for one hour, 40 °C to −10 °C in 8 h. The suspension is filtered. The cake is washed with cold EtOH/water (70/30, 1 vol[b]), then with EtOH/water (15/85, 1 vol[b]) so as to reduce the EtOH content of the wet cake. The product is then dried under vacuum at 40 °C until the LOD is ∼4−5% (in the lab: dried then rehydrated in a 58% RH chamber overnight using saturated NaBr solution at 25 °C).

[b]Calculated on theoretical zauategrast sulfate **1** crude content with the stated 35% LoD.

UCB1193394 **2**: ^{1}H NMR (400 MHz, DMSO-d_6): δ = 9.84 (s, 1H), 9.55 (s, 1H), 8.65 (d, J = 5.5, 1H), 8.42 (d, J = 8.6, 1H), 8.16 (d, J = 5.8, 1H), 7.78 (d, J = 8.6, 2H), 7.68 (d, J = 5.8, 1H), 7.22 (d, J = 8.6, 2H), 7.13 (d, J = 5.8, 1H), 4.36 (s, 1H), 4.21−4.12 (m, 3H), 3.13 (dd, J = 13.9, J = 5.3, 1H), 2.98 (dd, J = 13.6, J = 9.3, 1H), 1.75−1.44 (m, 9H), 1.19 (t, J = 7.1, 3H), 1.17 (m, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 190.8, 177.1, 170.6, 153.2, 148.1, 147.0, 145.4, 140.3, 139.0, 130.5, 129.2, 120.8, 119.1, 113.7, 110.3, 94.7, 61.7, 61.0, 59.1, 36.1, 30.9, 25.3, 23.6, 14.0. Anal. Calcd for $C_{28}H_{30}N_4O_3$: C, 71.47; H, 6.43; N, 11.91. Found: C, 71.62; H, 6.55; N, 11.85.

UCB1184197 **4**: ^{1}H NMR (400 MHz, DMSO-d_6): δ = 9.84 (s, 1H), 9.57 (s, 1H), 8.98 (d, J = 9.1, 1H), 8.66 (d, J = 5.5, 1H), 8.16 (d, J = 5.5, 1H), 7.81 (d, J = 8.6, 2H), 7.69 (d, J = 5.8, 1H), 7.23 (d, J = 8.6, 2H), 7.14 (d, J = 5.5, 1H), 4.80 (m, 1H), 4.20 (q, J = 7.1, 2H), 3.20 (dd, J = 13.8, J = 4.5, 1H), 3.00 (dd, J = 13.8, J = 9.9, 1H), 1.70−1.65 (m, 2H), 1.65−1.48 (m, 6H), 1.40 (m, 1H), 1.23 (t, J = 7.1, 3H), 1.13 (m, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 186.5, 173.2, 170.1, 153.1, 148.1, 147.0, 145.3, 140.2, 139.0, 130.0, 129.2, 120.8, 119.1, 113.7, 110.3, 67.3, 61.6, 61.2, 57.4, 37.0, 30.3, 24.9, 23.4, 13.9. Anal. Calcd for $C_{28}H_{29}BrN_4O_3$: C, 61.21; H, 5.32; N, 10.02. Found: C, 61.16; H, 5.42; N, 10.11.

UCB1191133 **5**: ^{1}H NMR (400 MHz, DMSO-d_6): δ = 9.85 (s, 1H), 9.77 (s, 1H), 8.98 (d, J = 9.1, 1H), 8.81 (d, J = 5.5, 1H), 8.34 (s, 1H), 7.73 (d_{app}, J = 7.1, 3H), 7.24 (d, J = 8.6, 2H), 4.80 (m, 1H), 4.20 (q, J = 7.1, 2H), 3.21 (dd, J = 13.8, J = 4.5, 1H), 3.01 (dd, J = 13.9, J = 10.1, 1H), 1.75−1.66 (m, 2H), 1.66−1.48 (m, 6H), 1.40 (m, 1H), 1.23 (t, J = 7.1, 3H), 1.13 (m, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 186.4, 173.9, 170.2, 152.8, 148.7, 146.5, 138.6, 138.5, 130.8, 129.4, 117.5, 115.0, 105.1, 67.4, 61.7, 61.3, 57.4, 37.0, 30.5, 25.0, 23.5, 14.0. Anal. Calcd for $C_{28}H_{28}Br_2N_4O_3$: C, 53.52; H, 4.49; N, 8.92. Found: C, 53.15; H, 4.53; N, 8.89.

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Supporting Information Available

^{1}H and ^{13}C NMR spectra are available for UCB1193394 (**2**), zauategrast (**4**) and UCB1191133 (**5**). This material is available free of charge via the Internet at http://pubs.acs.org.

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