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Rapid Optimization of the Hydrolysis of *N*′-Trifluoroacetyl-*S*-*tert*-leucine-*N*-methylamide Using High-Throughput Chemical Development Techniques

Victor W. Rosso,* James L. Pazdan, and John J. Venit

Process Research and Development, Pharmaceutical Research Institute, Bristol-Myers Squibb Company, New Brunswick, New Jersey 08903, U.S.A.

Abstract:

Our efforts are focused on the application of automation to Process R&D. This article will describe the application of high throughput methods to rapidly investigate a development challenge. In this case we needed to study the deprotection of *N*′-trifluoroacetyl-*S*-*tert*-leucine-*N*-methylamide which afforded a lower than expected yield when subjected to standard deprotection reaction conditions. This chemistry was systematically investigated by a sequential series of high-throughput experiments using various automated and semi-automated systems. The studies included a combinatorial screen of discrete reaction conditions, a screening DOE to study a broad range of continuous factors, and a two-factor central composite design to optimize the important factors. By applying high-throughput methods we were able to optimize the yield of the reaction by performing a large number of experiments in a short period of time.

Introduction

Process automation efforts have focused on the introduction of new tools and techniques to Process R&D to enhance productivity throughout the pharmaceutical industry.[1] This includes not only the introduction of modular equipment such as reactor blocks and liquid handlers to automate the performance of experiments but also experimental strategies such as design of experiments[2] (DOE), and high-throughput synthetic techniques that allow process chemists to take full advantage of rapidly developing automation capabilities. One such application is to use a combination of screening and statistically designed experiments to simultaneously perform rapid and thorough investigations of chemical processes. These studies allow us to quickly develop the scientific understanding necessary for the implementation of processes at pilot scale.

The first step in our strategy for studying a chemical reaction is to systematically list all potential variables for the reaction. Since the typical chemical reaction contains both quantitative (continuous) factors such as temperature, equivalents of reagents, etc. and qualitative (discrete) factors such as solvents, identities of reagents, etc., a strategy is required to systematically study broad ranges of reaction conditions.

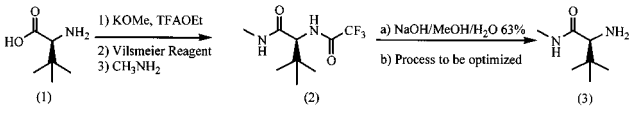

Figure 1. **Hydrolysis of trifluoroacetyl-L-*tert*-leucine-*N*-methyl amide.**

We generally use three types of designed experiments to accomplish this task. Combinatorial screens of discrete factors are used to identify the best performing reagent/solvent pairs for the chemical transformation. The screening DOE technique uses fractional factorial designs to assess a large number of discrete and continuous factors to identify which factors have the greatest impact on the desired results. Since the screening DOE measures multiple responses (e.g., yield) versus multiple factors, the resulting multidimensional analysis returns a massive amount of chemical knowledge for the time and material invested. The optimization DOE uses central composite designs to study the significant factors to arrive at the optimal reaction conditions. We have had many successful automated studies that use these techniques to rapidly optimize reactions for scale up.

An example of the application of these techniques to the chemistry in Figure 1 will be discussed. During the course of a study of an alternative synthetic strategy, we encountered a sluggish deprotection reaction that afforded lower than anticipated yields. The application of known[3] *N*-trifluoroacetyl deprotection conditions resulted in a slow reaction, and the use of more forcing conditions resulted in low yields (62−66%). Since the *tert*-leucine moiety accounted for much of the cost of the molecule, it was essential that the yield for this conversion be >90% for the route to be cost-effective. Therefore, we commenced a rapid automated study of this system.

The study consisted of the following phases: (1) a thorough listing of all reaction conditions, (2) a combinatorial screen of all discrete factor combinations, (3) a multivariable screening DOE of all the continuous factors, and (4) an optimization DOE on important factors identified in previous experiments. By applying this type of study, our objective was not only to identify the optimal reaction conditions in a short period of time but also to investigate the broadest range of reaction conditions so as to avoid the need to redevelop the reaction with "better" reagents in the future.

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Table 1. **List of all potential variables for the reaction**

a solvent	b base	c equiv of base	d % water	e temp	f concn (mL/g (M))
water	methylamine	1	0	30	5 (0.84 M)
methanol	K_2CO_3	2	50	45	10 (0.42 M)
ethanol	$KHCO_3$	3	100	60	15 (0.28 M)
THF	ethanolamine				
acetonitrile	LiOH				
ethyl acetate	K_2HPO_4				
2-propanol	DBU[a]				
	KOMe[a]				
	KOtBu[a]				
	DiPEA				

[a] Run without water as cosolvent.

Results and Discussion

Standard reaction conditions for this conversion were 1.5 equiv of sodium hydroxide in 50% aqueous methanol (0.3 M) at 45 °C for 2 h.[3] Application of these conditions resulted in only minimal conversion of starting material to product (<10% yield). The use of more vigorous (60 vs 45 °C) conditions drove the reaction to completion but resulted in poorer than expected yields (63−66%). To identify a system that would hydrolyze the *N*-trifluoroacetyl group in high yield without appreciable amide hydrolysis, a list of potential reaction conditions (Table 1) was assembled. Examination of all combinations of these factor settings would require a large number of reactions to be performed. Therefore we studied these experimental conditions in groups of statistically designed experiments to more efficiently cover the broad range of experimental conditions.

Screening Study

The first phase of this study was a combinatorial screen of all of the solvent/base pairs outlined in Table 1 using a general set of reaction conditions. The bases were selected to give a broad sampling of different types and strengths of base for the screen. For example, lithium hydroxide was chosen to represent the group of hydroxides. Since the more forcing conditions resulted in lower yields, we used the best known reaction conditions (2 equiv of base, 50% aqueous methanol, 45 °C) to study this array of reagents. More dilute reaction conditions (0.125 M) were used for the screen to obtain a manageable volume for the small-scale reactions. The reactions were performed in 50% aqueous methanol with the exception of the stronger bases KOMe, KOtBu, and DBU that were run in 100% primary solvent (0.125 M) instead of 50% aqueous to avoid decomposition of the base.

The reactions were performed by dispensing 100 μL of a 0.5 M stock solution of compound (**2**) in methylene chloride into each reactor. The solvent was evaporated in a Savant SpeedVac, and the appropriate reaction solvent (Table 1, column a, 200 μL) was dispensed into each reactor. The appropriate base (Table 1, column b, 2 equiv of, 200 μL of 0.5 M aqueous base) was then dispensed into each reaction vessel. The bases KOMe, KOtBu, and DBU were added neat, with an additional charge of the primary reaction solvent. The reactors were incubated at 45 °C for 3 h on a J-Kem shaker plate. The reactors were then cooled, and aliquots were taken for HPLC analysis. Due to the higher variability associated with quantitation of the small volumes used in the screening study, the apparent purity of the product as measured by HPLC was used to compare these experiments (Table 2).

Analysis of the full combinatorial screen allows us to compare mean values for each solvent and base in addition to the data for each solvent/base pair. The mean data for the solvents show that water is the best overall solvent for this reaction (Figure 2). Analysis of the mean data for the various bases as depicted in Figure 3 suggests that DBU is the best reagent for this transformation. However, when the solvent/base pair data is reviewed, we find the best overall combination is aqueous lithium hydroxide. This result is in agreement with the mean result for solvents. The mean value for bases does not agree, and this was caused by the poor performance of lithium hydroxide in the presence of several organic solvents. This is an example of strong two-way interactions that can occur between chemicals, underscoring the importance of including as many combinations of factors as possible in these screens to avoid missing unexpected results that occur with certain unique combinations of reagents.

On the basis of the data generated by this screening study, we decided to pursue aqueous hydroxide reactions for the next round of experimentation based on the following criteria: the strong performance of LiOH in water, the environmental friendliness of aqueous reaction conditions,

Table 2. **HPLC area percent (AP) results of screening solvents vs bases**

cosolvent	base	water	THF	acetonitrile	ethyl acetate	methanol	ethanol	2-propanol	mean
50% aq	methylamine	34.1	4.0	5.2	14.7	10.8	3.8	5.6	11.2
50% aq	potassium bicarbonate	14.2	3.1	8.6	3.6	4.0	2.8	1.6	5.4
50% aq	potassium carbonate	54.1	26.2	52.8	4.0	24.3	30.8	2.8	27.9
50% aq	ethanolamine	12.5	0.8	1.5	8.5	2.9	1.7	2.0	4.3
50% aq	lithium hydride	82.8	9.7	8.3	3.1	3.4	70.9	2.1	25.8
50% aq	potassium phosphate dibasic	15.2	1.0	x	1.7	4.7	1.9	0.6	4.2
organic	potassium *tert* butoxide	48.6	2.5	x	4.9	3.8	3.1	2.1	10.8
organic	DBU	72.8	54.8	x	38.6	49.0	52.3	56.6	54.0
organic	potassium methoxide	47.6	2.7	x	3.4	4.1	3.3	2.9	10.7
50% aq	DiPEA	12.5	0.7	0.7	0.4	0.7	0.7	0.6	2.3
	mean	39.4	10.6	12.9	8.3	10.8	17.1	7.7	15.2

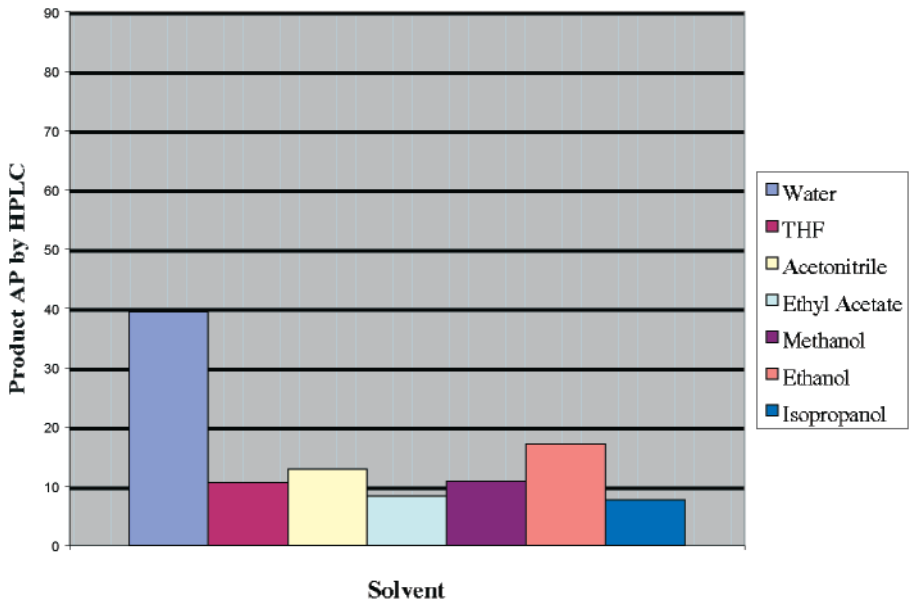

Figure 2. **Average AP product by HPLC in various solvents.**

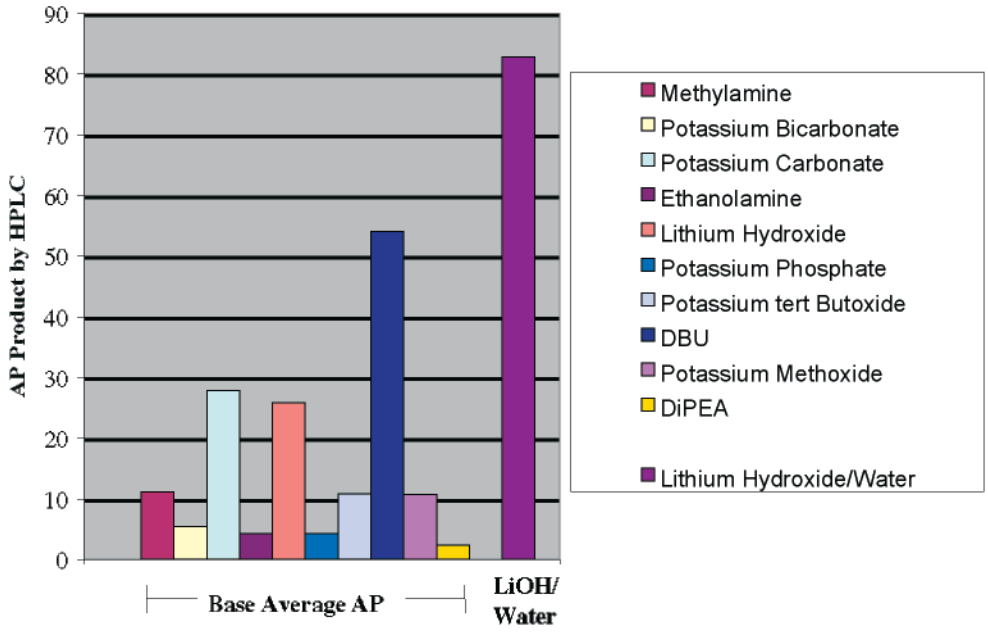

Figure 3. **Average AP product by HPLC in various bases.**

and the low cost of aqueous hydroxides as opposed to that of DBU.

Screening DOE

With the selection of aqueous alkoxide solutions for the screening DOE, we were left with the remaining variables to study: the hydroxide for the reaction (lithium, sodium, or potassium hydroxide), the overall concentration of the reaction (0.28 to 0.84 M), reaction temperature (30 to 60 °C), and equivalents of base (1 to 3 equiv). We performed a full factorial 2^3 experiment with each base, with an additional six center points distributed among the three bases to measure experimental error. The experiments were performed by a Zymark Robot that dispensed the appropriate amount of water and dilute aqueous hydroxide solution to each reaction. After the reactions were heated and stirred for 3 h, samples were taken for HPLC assay. The yields of the reaction were then calculated based upon HPLC quantitation.

The control experiments (2 equiv of base, 0.42 M, 45 °C) resulted in 97% conversion of starting material to product and an average yield of 87%. Of the four factors, only equivalents of base had a statistically significant impact on conversion of starting material to product. With 1 equiv of base, we had an average of 19% unreacted starting material, whereas with 2−3 equiv of base, the reactions proceeded to completion. These data suggested we needed to further investigate the range between 1 and 2 equiv of base to determine the necessary amount of base for this conversion.

Table 3. **Yields for hydrolysis reaction under various reaction conditions by HPLC**

		equiv of base				
		1 equiv		2 equiv	3 equiv	
base	temp, °C	0.28 M	0.84 M	0.42 M	0.28 M	0.84 M
LiOH	30	60.7	58.0		92.2	49.7
	45			92.3, 85.7		
	60	98.6	68.9		92.3	55.3
NaOH	30	83.4	50.9		97.5	57.4
	45			84.6, 88.9		
	60	97.2	58.4		98.8	56.8
KOH	30	78.1	51.7		95.5	50.4
	45			87.1, 83.5		
	60	90.1	53.7		105.8[a]	58.9

[a] Yields of >100% are caused by experimental error during sample preparation (determined to be ±4.2%).

Table 4. **Two-way interaction of equivalents vs concentration**

concentration	1 equiv	2 equiv	3 equiv
0.28 M	84.7		97.0
0.42 M		87.0	
0.84 M	56.9		54.8

The quantitated yields of the reactions with of various factor settings are outlined in Table 3. The main effects that each factor had on the yield of the reaction are detailed below in order of importance:

- Concentration: most significant yield improvement occurred with more dilute reactions.
- Temperature: higher temperatures afforded better yields.
- Equiv of base: there was an increase in yield when excess base was used with more dilute reaction conditions.
- Identity of base: no significant effect was observed.

Of the experimental factors, the concentration was the most significant factor affecting the yield. Additionally, there was also a significant two-way interaction between the concentration and the equivalents of base used (Table 4). When 3 equiv of base was used, the concentration had a greater influence on yield than when 1 equivalent of base was used.

The conclusions from the screening DOE were: concentration and equivalents of base would be the key factors for an optimization DOE; the identity of the hydroxide was not significant, justifying the selection of sodium hydroxide for further optimization; and the temperature of the reaction did not have a major impact upon yield (with dilute concentrations, and excess base), and thus we selected the center point, 45 °C, for further optimization.

Optimization Designed Experiment

To optimize the reaction for equivalents of base and for concentration, we used a two-factor central composite design with four center points. This study was centered near the best concentration (0.28 M) identified in the screening DOE and was varied over a range of 0.17−0.65 M. The experimental parameters for concentration were expressed in terms of mL of water/g of starting material for the purposes of the design and analysis of this experiment. The equivalents of base was centered at 1.5 to investigate the range of 1.075−1.925 equiv of base. The reactions were prepared on a Gilson 215 liquid handler, heated for 3 h at 45 °C in a reactor block, and sampled for HPLC analysis. Quantitative analysis of the samples by HPLC affords yield data shown in Table 5.

Table 5. **Experimental Data for Optimization DOE**

expt	vol (mL water/g)	concentration [M]	equiv of base	yield
1	15	0.28	1.925	88.3
2	20	0.21	1.75	89.6
3	15	0.28	1.5	92.0[a]
4	20	0.21	1.25	93.3
5	10	0.42	1.25	85.8
6	6.5	0.65	1.5	78.8
7	10	0.42	1.75	89.4
8	15	0.28	1.5	99.3[a]
9	20	0.21	1.75	97.8
10	20	0.21	1.25	98.9
11	15	0.28	1.5	95.6[a]
12	10	0.42	1.75	93.6
13	23.5	0.18	1.5	94.5
14	15	0.28	1.5	100.1[a]
15	10	0.42	1.25	86.8
16	15	0.28	1.075	95.0

[a] Replicate center points to assess experimental variability.

Table 6. **Significance of the constants for the model equation**

constant	parameter	*p*-value	significance
k_2	vol	0.0039	high
k_3	equiv of base	0.6889	
k_4	vol × (equiv of base)	0.1725	
k_5	vol^2	0.0092	high
k_6	equiv of base^2	0.1415	

A total of four replicates were performed at the center points to measure experimental variability which is used to determine if the observed effects are statistically significant. The *p*-values assessing the statistical significance of each potential term in the quadratic equation are listed in Table 6. Evaluation of the yield of product showed statistically significant improvements caused by more dilute reaction conditions, but no significant impact was observed for the equivalents of base used. After removal of the insignificant terms ($p > 0.05$) we obtain the following model for the yield of the reaction:

$$\text{yield} = k_1 + k_2(\text{vol}) + k_5(\text{vol})^2$$

The relationship between yield and volume is plotted in Figure 4 and is given by the equation:

$$\text{yield} = 54.74 + 4.877(\text{vol}) - 0.1381(\text{vol})^2$$

The optimization DOE shows good yield at water volumes of 15 mL/g and greater with the model predicting a localized maximum at 17.5 mL/g. The true optimal conditions would be arrived at by factoring the cost of reagents versus the

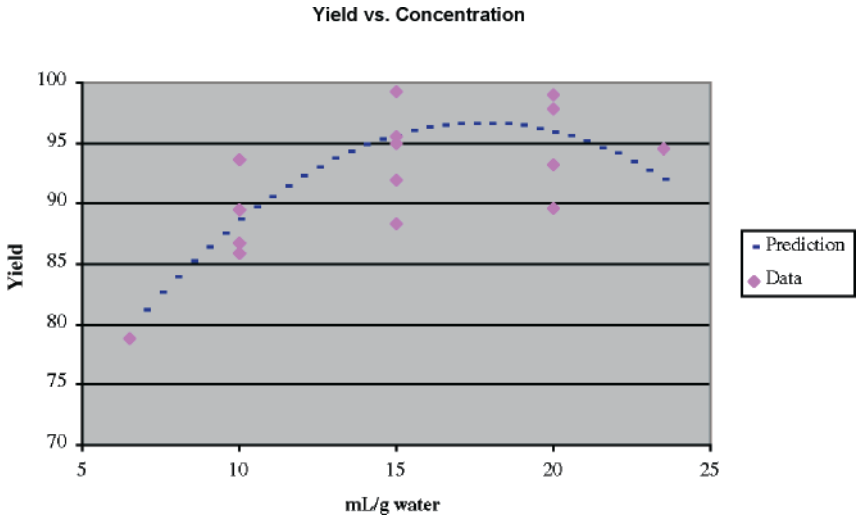

Figure 4. **Optimization DOE model of yield vs mL/g concentration.**

savings of performing the reactions at lower volumes using the information from this study. With respect to equivalents of base, there were no significant effects detected in the range investigated. Therefore, conditions we further studied were 0.24 M (17.5 mL/g starting material) in water with 1.25 equiv of aqueous sodium hydroxide at 45 °C. A scale up of these conditions resulted in an isolated yield of 95% of product that is ready to be coupled in the next reaction in the synthetic sequence.

Conclusions

We were able to rapidly and thoroughly optimize a typical chemical reaction using combinatorial screens of discrete variables, screening DOEs of multiple factors, and optimization DOE. Important results from this study are the scientific approach and automated execution of a study that covered a broad range of reaction conditions in a systematic manner. Specifically, we performed 116 reactions in 4 days to optimize the yield from 63 to >95%. This study not only is a model for what can be accomplished when these techniques are applied to more challenging chemical reactions but also helps us to define the capabilities of automated equipment required to accelerate the pace of chemical process development.

Methods and Materials

***N′*-Trifluoroacetyl-*S*-*tert*-leucine-*N*-methylamide.** *tert*-Leucine (23.96 g, 187 mmol) was charged to a 500-mL, round-bottomed flask with methanol (48 mL), and 4.4 M methanolic potassium methoxide (46 mL, 202.4 mmol, 1.1 equiv). After dissolution, ethyl trifluoroacetate (25 mL, 210 mmol 1.12 equiv) was charged, and the reaction mixture was maintained at 40 °C for 2 h. The solution was then quenched into 2 N aqueous HCl (113 mL, 226 mmol, 1.21 equiv). Butyl acetate was charged (200 mL), and the phases were separated. The organic phase was washed twice with water (50 mL) and concentrated to an oil.

The oil was suspended in ethyl acetate (250 mL) and cooled to −15 °C. (Chloromethylene)dimethylammonium chloride, Vilsmeier reagent, (28.7 g, 224 mmol, 1.2 equiv) was then charged to the reaction while maintaining the temperature below −15 °C for 2 h. The reaction slurry was then quenched into excess aqueous methylamine (60 mL, 800 mmol, 4.3 equiv). The phases were separated, and the organic phase was washed twice with water and concentrated to minimal volume. The product was crystallized by the addition of MTBE to afford 39.0 g (162 mmol, 87.4%). HPLC AP 100, data: white solid, mp 160−162 °C, IR: KBr 3385, 2970, 1711, 1654, 1572, 1220, 1188, 1160, 738 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.98 (s, 9H), 1.52 (s, NH_2), 2.82 (S, CH_3), 3.15 (s, CH), 6.83 (Br, NH) ppm; EA Theory 45.00% C, 6.29% H, 11.66% N, 23.73% F; Found 45.15% C, 6.45% H, 11.63% N, 23.53% F.

***S*-*tert*-Leucine-*N*-methylamide.** *N′*-Trifluoroacetyl-*S*-*tert*-leucine-*N*-methylamide (1.66 g, 6.9 mmol) was charged to a 50-mL reaction flask. Water (24.2 mL) and 10 N aqueous NaOH (0.86 mL, 1.25 equiv) were charged, and the slurry was heated to 45 C for 3 h. The solution was assayed to contain 95.6 mol % *S*-*tert*-leucine-*N*-methylamide by in-process HPLC versus a reference standard supplied by Great Lakes Fine Chemicals.

HPLC analysis was performed on a Shimadzu Discovery VP Walk Up System. A volume of 10 μL was injected on a YMC ODS-A S3μ 4.6 × 50 mm column. A gradient elution from 10 to 50% CH_3CN in 0.05 M aqueous NH_4OAc over 4 min at a flow of 1.5 mL/minute and detection at λ 210. Retention times: TFA-*N*-*t*-Leu-NHMe, 1.4 min; NH_2-*t*-Leu-NHMe, 0.5 min.

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