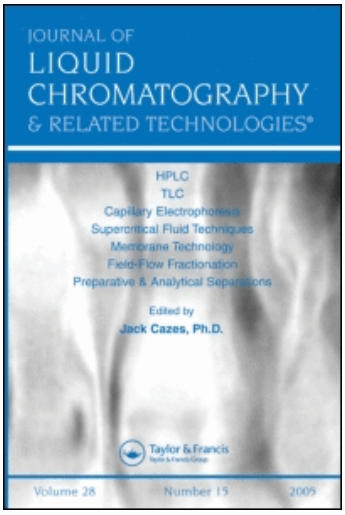

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Controlling the Structure of a Trifluoroacylated Hydrazino Pharmaceutical Intermediate for Improved HPLC and GC Analyses

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ABSTRACT

A process to generate an organofluorine pharmaceutical intermediate yields two main products, a *bis*- and mono-trifluoroacylated analog of the hydrazino starting material. Analytical methods are discussed that characterize solid and solution intermediate mixtures, as well as monitor their formation. In addition, quantitative high performance liquid chromatography (HPLC) assay method development and validation is presented. However, due to solution stability issues, a diluent system that forcibly, yet controllably, degraded the *bis*-trifluoroacylated product to the

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mono analog had to be employed. A more in depth discussion of the investigation into the appropriate diluent system is also presented.

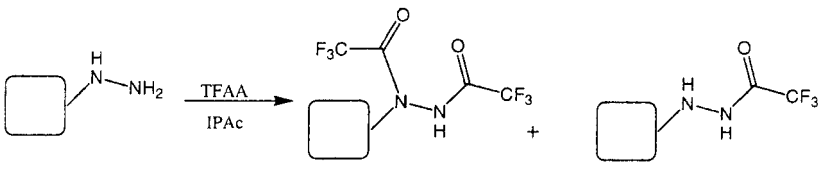
Key Words: HPLC; Controlled degradation; Organofluorine analysis; Pharmaceutical analysis.

INTRODUCTION

Organofluorine compounds have been found to possess unique chemical properties,[1,2] which has led to the observation of their interesting physiological profiles.[3] This has spurred a wide array of synthetic methods to introduce fluorine containing moieties into organic molecules.[4–6] During the development and scale-up of these and similar processes, it is necessary to monitor the reaction, as well as separate and quantitate the components of mixtures related to the process. This paper focuses on the challenges of monitoring and providing analytical data for the trifluoroacylation of a pharmaceutical intermediate.

In the process of interest, a hydrazine substituted starting material is treated with excess trifluoroacetic anhydride (TFAA) to form a mixture of acylated products (Sch. 1).

Both *bis*-acylated and mono-acylated intermediates (also referred to as *bis* and mono, respectively, throughout the remainder of this paper) are observed in the reaction samples. Continuation of the process has shown that the next step successfully proceeds independently of the ratio of *bis* to *mono* formed in Sch. 1. This suggests that this ratio does not have to be carefully controlled in the trifluoroacylation step. However, it is still desirable to have suitable analytical methods to characterize the process, as well as the product solids and liquors, in order to optimize the process during scale up and manufacturing. This paper discusses the development of a GC method for determining the *bis*/mono ratio and the development and validation of an high performance liquid chromatography (HPLC) method used to characterize this process step, and the solution and solid samples produced. In the course of developing these methods, it has been observed that the diluent used greatly influences the reliability of the analytical methods because of solution stability issues. It has



Scheme 1.

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been discovered that the chemical composition of the product can be manipulated via the inclusion of a diluent additive, to improve its stability and subsequent analysis. Previous analytical studies have shown that various compounds can be derivatized with TFAA to improve analytical methods.[7–9] In this discussion, this chemistry was exploited in order to forcibly, yet controllably, degrade the analyte of interest for improved quantization.

EXPERIMENTAL

Chemicals and Reagents

The HPLC grade methanol (MeOH), dimethyl acetimide (DMAC), acetonitrile, isopropyl acetate (IPAC), *ortho*-phosphoric acid, and sodium hydroxide were all obtained from Fisher Scientific (Fair Lawn, NJ). Benzylamine (BA) was obtained from Aldrich (Milwaukee, WI), and triethylamine (TEA) was obtained from Fluka (Buchs, Switzerland). All were used without further purification. The water was purified using a Hydro water filtration system. All trifluoroacylated solid products and reaction mixtures were provided by the Process Research and Development Department in Merck Research Laboratories (Rahway, NJ).

High Performance Liquid Chromatography

Agilent 1100 HPLC instruments were used for all analyses. The UV-VIS spectra were obtained with an Agilent 1100 HPLC system equipped with a diode array detector. An Aquasil C18 (100 × 4.6 mm; 3.5 μm dp) column was used for all analyses. The weak mobile phase (A) was 10 mM monobasic phosphate adjusted to pH = 6.5 with NaOH. The strong mobile phase (B) was pure MeOH. The mobile phase gradient went from 5% to 90% B from 0 to 15 min, followed by isocratic elution at 90% B for 5 min. A column re-equilibration time of 8 min was used between each injection for a total run time of approximately 30 min between injections. Other experimental conditions included a flow rate of 1.0 mL/min, a column temperature of 30°C, an injection volume of 5 μL, and a primary detection wavelength of 220 nm.

Gas Chromatography

Hewlett-Packard 5890 gas chromatographs were used for all GC analysis with flame ionization detection (FID). GC-MS data was obtained using an Agilent 6890 GC instrument equipped with a Agilent model 5973 MS detector. The column used was an RTX-5 Amine (30 m × 0.32 mm) with a

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nominal 1.0 μm film thickness. The temperature gradient consisted of holding the starting temperature of 45°C for 3 min, then proceeding to 135°C at 10°C/min. The temperature was then held at 135°C for 5 min prior to proceeding to 165°C at 5°C/min, and, finally, continuing to 280°C at 15°C/min. This final temperature was maintained for 10 min to complete the run. Other experimental conditions included split injection (25 : 1) at 275°C, an FID temperature of 280°C, and a column head pressure of approximately 15 psi. All quantitative GC analyses were performed under the same conditions, using an RTX-5 Amine column (30 m × 0.53 mm) with a nominal film thickness of 3.0 μm and splitless injection. The column head pressure was adjusted until the experimentally observed retention times were similar to those obtained with the smaller bore GC column.

RESULTS AND DISCUSSION

Process and Product Characterization

Both *bis* and mono have always been observed as intermediates. However, experiments show that the relative amount of each species can be roughly controlled by the number of TFAA equivalents used in the reaction (Table 1). In many cases, one form can be reduced to a minor component. These ratios were obtained by injecting the IPAc reaction mixture samples directly into a GC instrument with FID detection (Fig. 1). GC-MS data from the mother liquor samples support the two trifluoroacylated structures shown in Sch. 1. The products can then be isolated by adding hexane as an anti-solvent. GC analysis of the solids, after being dissolved in DMAc, shows that the *bis*/mono ratio is essentially preserved. Table 2 lists the *bis*/mono ratio in five reaction mixtures and their subsequent solid.

Table 1. Effect of the number of TFAA equivalents on the relative amounts of *bis*-acylated and mono-acylated intermediate in reaction mixtures.

Number of TFAA equivalents	*Bis*-acylated intermediate ratio	Mono-acylated intermediate ratio
1	0.1	99.9
2	97.9	2.1
3	99.0	1.0
4	98.7	1.3

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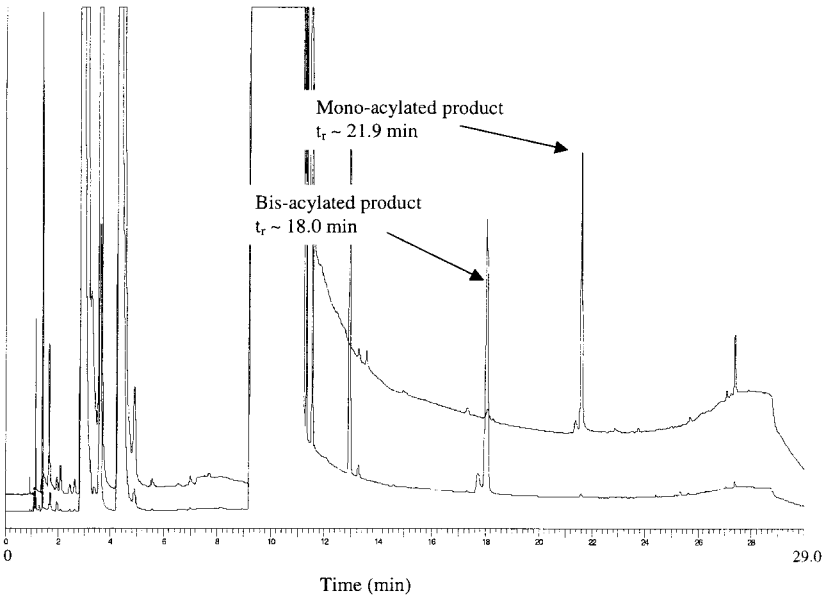

Figure 1. Gas chromatograms of reaction mixtures diluted in DMAc showing *bis*- and mono-acylated intermediates in Sch. 1. The column and conditions used are described in the Experimental section of the text.

Assay Method Development

As mentioned previously, the form of the trifluoroacylated product does not affect the next step in the process. However, in order to better understand this step and aid in its optimization for scale up, methods were needed that

Table 2. Comparison of *bis* to mono in reaction mixtures and isolated solids in DMAc.

	Reaction mixtures		Isolated solids	
Sample	*Bis* area ratio	Mono area ratio	*Bis* area ratio	Mono area ratio
1	0.1	99.1	3.9	96.1
2	98.8	1.2	99.0	1.0
3	99.2	0.8	98.7	1.3
4	99.0	1.0	98.3	1.7
5	98.9	1.1	99.1	0.9

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could accurately assay the amount of the trifluoroacylated product isolated as a solid, as well as losses in mother liquors and wash solutions. GC and HPLC were both investigated for this purpose. The isolated solid product has been found to be primarily soluble in MeOH, DMAc, and dimethyl sulfoxide (DMSO). However, once dissolved in MeOH, *bis* slowly de-acylates to mono over the course of three hours. Since DMAc and DMSO are not suitable HPLC diluents, GC was investigated as an assay method. A solid *bis* sample of the acylated product mixture in Sch. 1 was dissolved in DMAc and injected repeatedly over a 20 hour period to test the solution stability in DMAc. A split injector configuration was utilized, so the peak area ratio of mono relative to the total of area of both *bis* and mono was used to observe any de-acylation. For the solid studied, the chromatographic area ratio of the mono-acylated product was found to remain at $3.9\% \pm 0.4\%$ for 24 injections over 20 hours. Gas chromatography with DMAc as the diluent was, therefore, used for all subsequent determinations of the relative amounts of *bis*- and mono-acylated analogs. This GC method was used to show that the relative amount of *bis* and mono is preserved upon isolation of the solid (Table 2).

Although solution stability is not an issue in DMAc, an HPLC assay method development was still pursued. Solubility limitations for the solid required MeOH to be used as the diluent. As mentioned previously, however, *bis* can be seen to slowly de-acylate to mono over the course of three hours in MeOH (Fig. 2). This degradation was confirmed by both GC-MS and HPLC-diode array detector (DAD) analysis. The GC-MS data also shows the formation of methyl trifluoroacetate when a *bis* sample is dissolved in MeOH. Investigation of the degradation kinetics reveals that it is first order with respect to *bis* with a rate constant of $0.0384\,s^{-1}$ (Fig. 3). Furthermore, there are signs of the complete de-acylation of the product in MeOH, which yields the original hydrazino starting material as early as two hours after the sample is prepared. As can be seen in Fig. 4, this occurs even before *bis* has fully de-acylated. Keeping the sample solution cool (5°C) during the analyses, did slow the de-acylation, but did not stop it (Fig. 5). The conversion from *bis* to mono at 5°C is still first order with respect to *bis* ($k = 0.0066\,s^{-1}$), however, there was no evidence of complete deacylation after 10 hours.

The de-acylation process, and the presence of both the *bis*- and mono-acylated intermediates in the product, causes difficulties when determining the purity of trifluoroacylated solids and concentrations in liquors by HPLC. Therefore, several different HPLC suitable diluents were investigated to improve the solution stability of the intermediates and the reliability of these calculations based on HPLC analyses. Acidic conditions were evaluated using a diluent composed of 50/50 MeOH/H_2O with 0.1% *ortho*-phosphoric acid. Using this diluent, the *bis*-acylated intermediate was converted to mono rapidly and was undetectable after 30 min. Unfortunately, it also hastened

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the complete de-acylation of the sample. The hydrazino starting material became evident after only 30 min and continued to increase over the 10 hours of the analysis. Complete de-acylation to the hydrazino starting material was observed to be first order with respect to mono, with a rate constant of $0.0004\,s^{-1}$ under these acidic diluent conditions. Figure 6 shows the effect of the acidic diluent on the presence of the mono-acylated analog in solution. Similar observations were also made with 0.1% *ortho*-phosphoric acid in 5% water in MeOH.

The observed acid sensitivity of these acylated intermediates led to the investigation of basic diluent additives. Using a 0.4% (v/v) TEA in MeOH, the de-acylation of *bis* to mono was rapid, taking less than 10 min. However, unlike acidic diluents, there was no evidence of complete de-acylation to the hydrazino starting material after 24 hours. To investigate the role the amine plays in the de-acylation process, a 0.4% (v/v) BA in MeOH diluent was studied. It

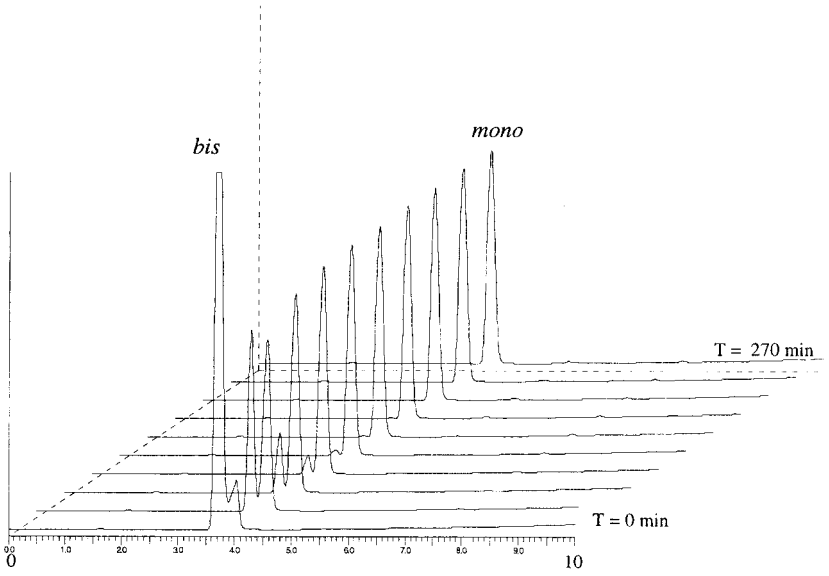

Figure 2. (A) Monitoring *bis* to mono conversion by HPLC in MeOH; (B) *bis*-acylated intermediate (◆) and mono-acylated intermediate (▲) peak area vs. time using pure MeOH as the diluent. Peak areas are those obtained using HPLC-UV. Column and conditions are described in the Experimental section of the text.

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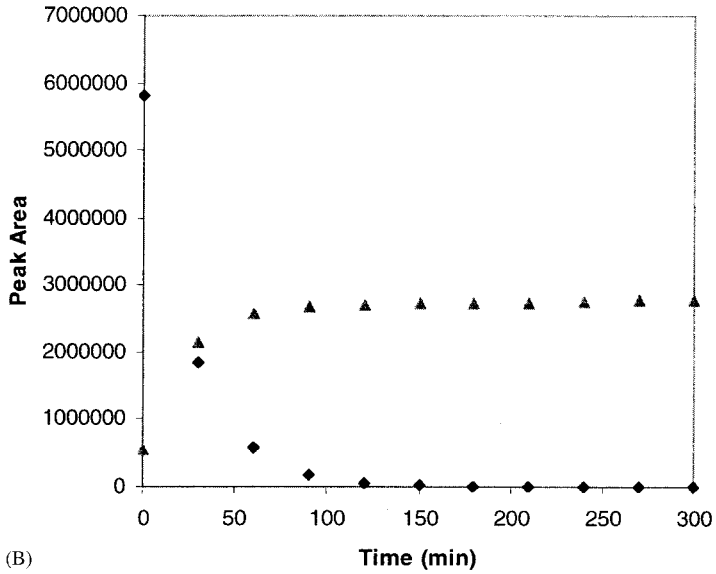

(B)

Figure 2. Continued.

was found by both HPLC-DAD and GC-MS analysis, that the amines effectively remove the more weakly bound trifluoroacylated substituent of the *bis* product, forming mono and a new triflouroacylated amine. In the case of the benzylamine diluent, trifluoroacylated benzylamine is formed.

These studies show that the stability and subsequent HPLC analyses of the acylated products in Sch. 1 are quite dependent on the diluent used. Under neutral MeOH conditions, the de-acylation is slow, yet complete, and ultimately yields the hydrazino starting material and methyl trifluoroacetate. This process is faster under acidic conditions, and cannot be controlled or stopped in either diluent. The GC-MS data shows the formation of methyl trifluoroacetate when a *bis* sample is dissolved in MeOH. From these observations, it is believed that the slow loss of the first trifluoroacylated substituent of *bis* in MeOH eventually causes the diluent to become increasingly acidic, and therefore, promotes complete de-acylation similar to that observed in the acidic diluent. However, by adding an amine to the diluent, the degradation of *bis* to mono is rapid and controllable. The complete de-acylation is likely impeded because the amine additive scavenges the trifluoroacylated substituent and maintains the diluent at an alkaline pH. Controlling the deacylation of product in solution in this manner, leads to more reliable HPLC assay results for the total amount of trifluoroacylated product in solids and solutions.

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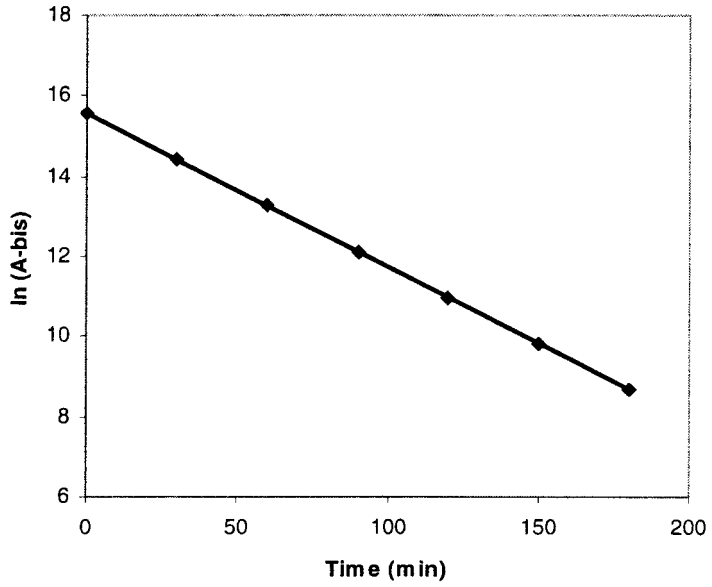

Figure 3. Natural logarithm (ln) of the *bis*-acylated intermediate peak area in MeOH vs. time using HPLC-DAD. $\ln(A) = -0.0384(t) + 15.58$; $R^2 = 1.000$.

The process of forcibly degrading the entire sample to the mono-acylated analog for the HPLC method is believed to be more advantageous when compared to the GC method. Using the GC method to quantify the total amount of trifluoroacylated intermediates would require the characterization and use of both a mono and a *bis* standard. The controlled degradation of *bis* to mono in the HPLC method, however, reduces the requirement to a single standard. Furthermore, any sample can be used as a standard regardless of its *bis*/mono ratio. It is only necessary to characterize the standard and determine its *bis*/mono ratio. It is worth noting again, that the degradation of the sample is made possible because the next step in the process proceeds independently of the *bis*/mono ratio produced in this step. It is only necessary to know the number of equivalents of the primary compound moiety (denoted by the empty box in Sch. 1), and not whether it is *bis*- or mono-acylated. Finally, the injection reproducibility of the HPLC (0.8 % RSD) method was found to be slightly better than the GC method using splitless injection (2.1 % RSD). Therefore, it has been determined that using a combination of the methods is the fastest and most reliable way to monitor the process, as well as the effects of changes to the process for scale up and optimization. The GC method is best used to determine the relative amount of *bis* and mono as well as overall purity

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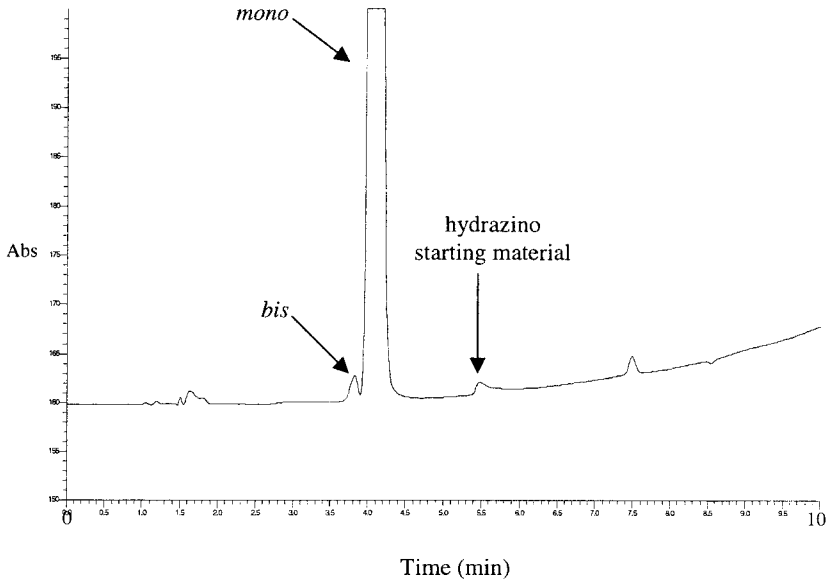

Figure 4. High performance liquid chromatography chromatogram of acylated intermediate in MeOH after 120 min.

when desired. The HPLC assay method can then be used to quantify solid and liquid trifluoroacylated samples.

Trifluoroacylated Product Method Validation

Triethylamine and BA behave similarly in their ability to quickly convert the product to mono and impede further de-acylation of the intermediate. Changing the concentration of both of these diluent additives did not influence the observed conversion and solution stability of mono provided that they were present in excess. Therefore, either additive would be an adequate selection, but 0.2% (v/v) BA was chosen for validation because of its ability to track and quantify amount of trifluoro-acylated substituent liberated.

Linearity

The linearity of the HPLC method using 0.2% (v/v) BA in MeOH was tested in two different analyte concentration ranges. One concentration

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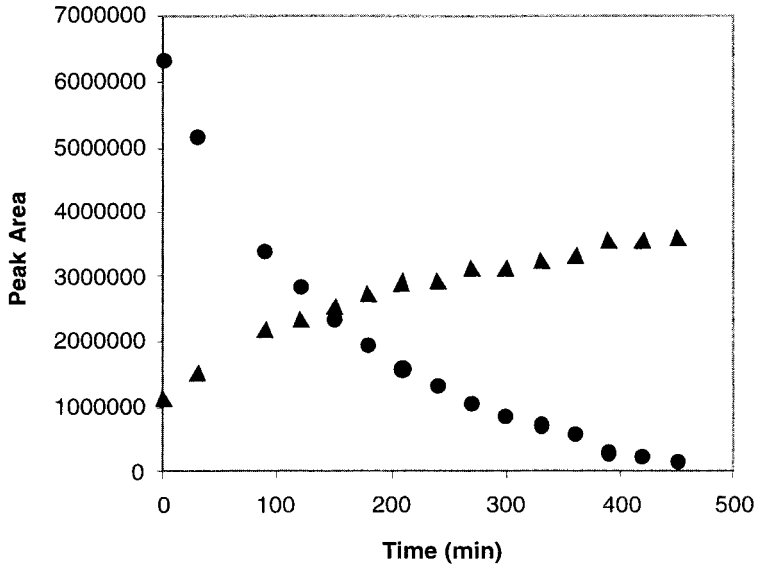

Figure 5. *Bis*-acylated intermediate (◆) and mono-acylated intermediate (▲) peak area vs. time using pure MeOH as the diluent at 5°C. Peak areas are those obtained using HPLC-UV. Column and conditions are described in the Experimental section of the text.

range was 1.2 mg/mL (±25%) because of the analyte's low UV response at 220 nm. A solid precipitate formed in these solutions, so a second set of sample solutions that were 0.35 mg/mL (±25%) were prepared and evaluated simultaneously. The higher concentration samples were analyzed both before and after being filtered through a 0.2 μm nylon syringe filter. The results presented in Table 3 show that both concentration ranges have a linear response. There is, also, essentially no difference between response factors of the filtered and unfiltered solutions at the high concentrations range. Furthermore, these concentrations do not represent a saturation point for the mono-acylated product, suggesting that the precipitate formed may be another triflouroacylated salt or solid unrelated to the acylated intermediate.

Selectivity

Figure 7 shows that there is no interference from the BA additive in the diluent, as well as the trifluoroacylated BA for the *bis*- or mono-acylated

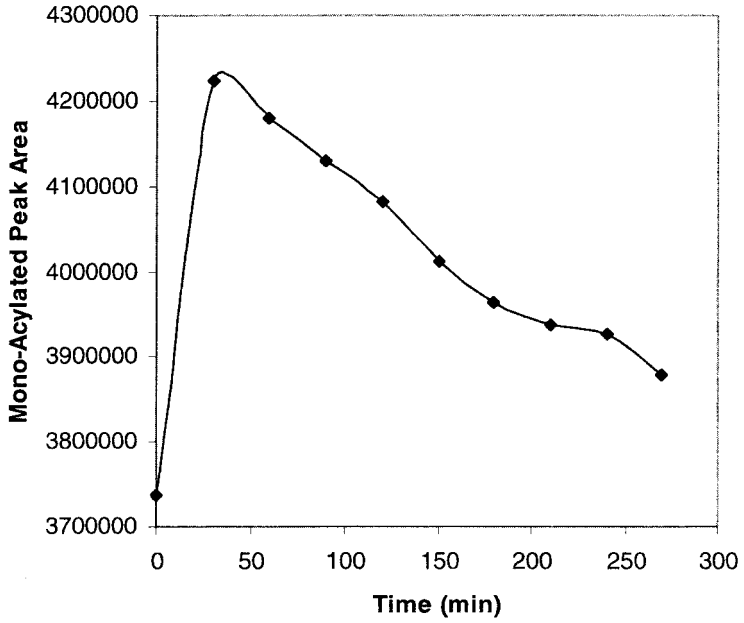

Figure 6. Mono-acylated intermediate peak area vs. time using 0.1% *ortho*-phosphoric acid in 50/50 MeOH/H_2O as the diluent. Peak areas are obtained from HPLC-UV experiments. Column and conditions are described in the Experimental section of the text.

intermediates in both the HPLC and GC methods. Peaks were confirmed by MS for the GC analysis and by UV for the HPLC analysis. It should also be noted that the formation of the hydrazino starting material, through complete deacylation of the product does not interfere with either acylated analog peak (Fig. 4).

Table 3. Linearity parameters for the mono-trifluoroacylated intermediate in 0.2% (v/v) BA in MeOH.

Sample	b_0	b_1	R^2
$0.35 \pm$ mg/mL	2972	2.122×10^6	0.9994
$1.2 \pm$ mg/mL (filtered)	982	1.897×10^6	0.9999
$1.2 \pm$ mg/mL (unfiltered)	7204	1.877×10^6	0.9992

Note: Peak area $= b_0 + b_1$[acylated intermediate].

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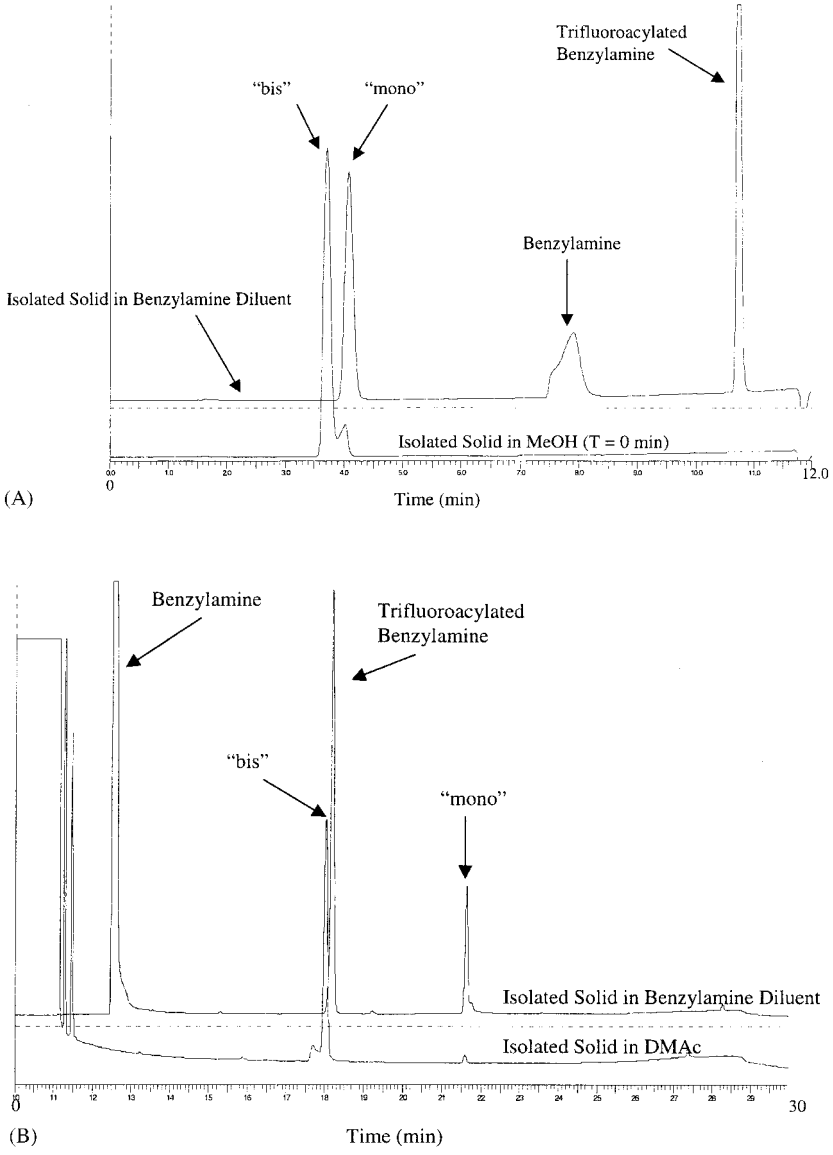

Figure 7. Analysis of de-acylation process using BA in MeOH. (A) HPLC; (B) GC.

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Solution Stability and Repeatability

The solution stability using both TEA and BA was also investigated. The response factor of mono after conversion, using 0.2% (v/v) BA in MeOH as the diluent, was found to be stable for a minimum of 24 hours, with a percent relative standard deviation (%RSD) of less than 1.5% over 48 injections during that time. For 12 injections over a 6 hour period, the %RSD was less than 1.0%. Using TEA in MeOH gave a similar solution stability with a %RSD of less than 1.0% for repetitive injections over a 24 hour period. In addition, there was never any chromatographic evidence of the hydrazino starting material forming prior to the termination of the solution stability analysis.

Precision

Recovery experiments were performed to test the reliability of the HPLC method. A trifluoroacylated sample was mixed with thiourea in known amounts. Thiourea was chosen because it is inert, does not interfere with the analyte peaks in the chromatograms, and is soluble in the diluent. A separate trifluoroacylated product was used as the standard. GC analysis was used to determine the *bis*/mono ratio in both the intermediate used as the standard and the one used in the sample mixture. The standard was determined to be 85.1% *bis* and 12.5% mono and the sample was found to be 94.1% *bis* and 4.1% mono prior to mixing it with thiourea. The less pure *bis* solid was deliberately chosen as the standard to show that the HPLC method is robust and precise regardless of the standard's purity. Both the standard and the sample solution concentrations were converted to solely the mono concentration using the following equation.

$$(\text{g/L mono}) = \left[\left(\frac{\text{g sample}}{\text{L soln}}\right) \times \left(\frac{\text{GCA}\varphi\, bis}{100}\right) \times \left(\frac{1\ \text{g mono}}{1.466\ \text{g}\ bis}\right) + \left(\frac{\text{g sample}}{\text{L soln}}\right) \times \left(\frac{\text{GCA}\ \varphi\text{mono}}{100}\right)\right] \times \varphi$$

In the equation, GCAP is the gas chromatographically determined area percent, and φ is the known weight percent of the product in the product/thiourea mixture ($\varphi = 1$ for the standard). The results of this recovery experiment are listed in Table 4. As is evident in the table, no product information is lost during the degradation in the HPLC diluent. Even though the standard composition is significantly different from that of the sample, the results agree well with those expected. This shows that it is not necessary to have both a *bis* and mono standard. It is only necessary to know the composition of the standard used, in order to obtain reliable concentrations or weight percent results using this method.

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Table 4. Expected and HPLC assay measured weight percent mono results for recovery experiments.

Theoretical	Observed
55.9	56.2
53.3	53.9
63.0	63.6
59.5	61.1

CONCLUSION

Methods have been developed to characterize a mixture of pharmaceutical intermediates and the process in which they are made. Gas chromatography has been found to be best used as a method to determine the relative amount of *bis*-acylated and mono-acylated intermediate in samples. A forced, yet controllable, degradation of the product mixture in a suitable diluent, then allows for HPLC to be used as an assay method for solution samples, as well as isolated solids. Validation of the HPLC method shows it to be a reliable way to quantitate the number of equivalents of the primary structure of the intermediate in solids and solutions, and confirms that no information is lost during the degradation.

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