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Development and Application of a User-Friendly Automated HPLC Dilution Macro for API Route Optimization

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ABSTRACT: Concentration determination by HPLC analysis is often utilized in pharmaceutical development activities. Traditionally, manual dilutions employing volumetric glassware have to be performed to obtain accurate concentration results. To circumvent this, an automated dilution method for the Agilent 1100 HPLC system has been developed to dilute and inject the samples online. After appropriate calibration, quantitative analysis can be achieved by placing the solution samples directly into HPLC vials without additional manipulation. This method is successfully applied to active pharmaceutical ingredient (API) solubility measurements and concentration determinations of routine mother liquor samples in the range of 0.1−100 mg/mL. It is particularly useful in crystallization development where obtaining accurate concentration information quickly drives down development time and material requirements.

INTRODUCTION

Monitoring reaction progress and impurity formation and identification of crystallization conditions are essential activities in active pharmaceutical ingredient (API) route development and optimization.[1,2] In recent years, these particular activities have been made easier by analytical HPLC, a now common tool in most industrial laboratories. In all cases, the act of preparing a sample for HPLC analysis necessitates sampling of the substrate from reaction mixture or crystallization and subsequent diluting with a large volume of an inert solvent. This dilution step is critical to ensure the sample concentration is within the linearity range of the analytical method. While effective, this process is laborious and solvent inefficient. Utilization of new technologies or modification of existing ones offers the opportunity to improve the efficiency of these key activities.[3−6]

Given our increasing emphasis on the use of green chemistries and technologies, we sought to develop a user-friendly technique for all synthetic chemists that would obviate the need for reaction sampling and dilution while at the same time reducing the overall amount of solvent used in the operation.[7−10] We realized that such a tool could facilitate dilution-intensive workflows such as rapid screening of crystallization conditions and facile monitoring of reaction kinetics. We herein report on a macro-driven approach to automated sample dilution that can be implemented on existing HPLC equipment. We highlight two case studies demonstrating the successful utilization of this user-friendly method for identification of crystallization conditions and reaction monitoring.[11,12]

EXPERIMENTAL SECTION

Reagents and Chemicals. Samples of compound A-I are experimental drugs which were supplied by Merck Process Chemistry (Merck Research Laboratories, Rahway, New Jersey, U.S.A.). HPLC grade acetonitrile, isopropyl acetate, dichloromethane, ethanol, *N*,*N*-dimethylformamide, *N*,*N*-dimethylacetamide, dimethyl sulfoxide, isopropanol, 2-methyltetrahydrofuran, toluene, ethyl acetate, methyl *tert*-butyl ether, and 99.99% phosphoric acid [85% (w/w) in H_2O] were used. All water used was distilled and purified by a HYDRO System (Garfield, NJ, U.S.A.).

Step	Command
1	Draw 89.0 μL from vial 11
2	Draw 1.0 μL from vial 1, 2.0 mm offset
3	Eject 90.0 μL into vial 21
4	Repeat 15 times
5	Draw 90.0 μL from vial 21, max. speed
6	Eject 90.0 μL into vial 21, max. speed
7	End repeat

Figure 1. Recommended injector program.

Sample Preparation. A range of up to 100 mg/mL of the samples in different solvents for concentration determination were supplied by chemists in the department of Merck Process Chemistry and Merck Chemical Process Development and Commercialization. Please note that the analyses described in this paper were done at ambient temperature. Appropriate calibration is required if sampling is done with the HPLC needle at nonambient temperatures.

Apparatus and Chromatographic Conditions. HPLC vials containing inserts were utilized for sample dilutions. A preassembled HPLC vial kit with a 2-mL HPLC vial, a 300-μL drop-in insert, and a cap with silicone/PTFE septa was purchased from Analytical Sales and Services, Inc. (Pompton

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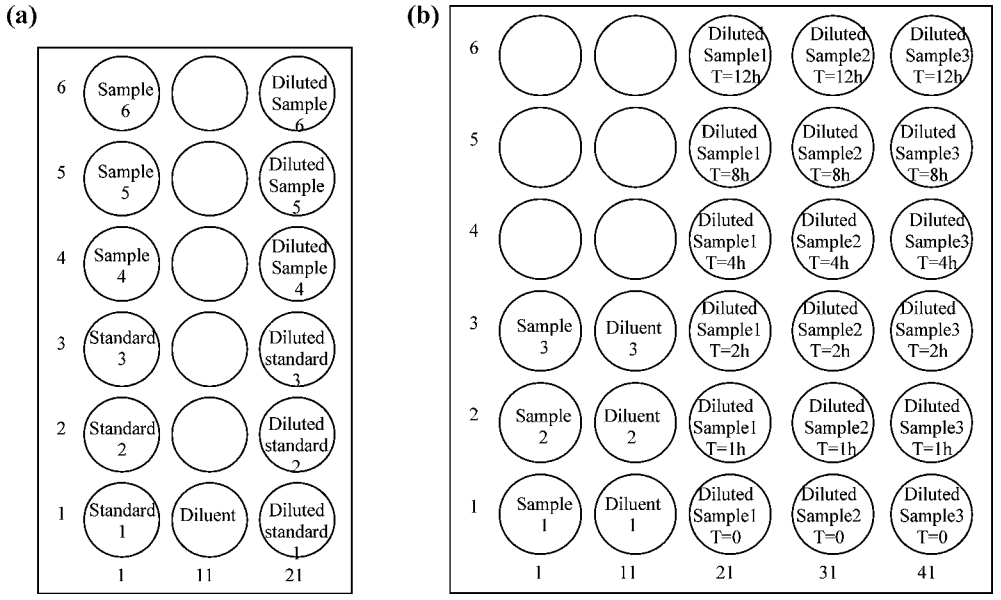

Figure 2. (a) HPLC vial layouts for concentration determination. For example, the injector draws the diluent from vial 11. The standard/sample is drawn from vial 1. The diluent and the standard/sample solution are mixed in an insert-contained HPLC vial at position 21. Then the diluted standard/sample solution is injected for HPLC analysis. The same procedure is performed for the remaining standards/samples at the positions illustrated. (b) HPLC vial layouts for reaction monitoring or solution stability determination. For example, at the initial time point, the injector draws the diluent from vial 11. The sample 1 is drawn from vial 1. The diluent and the sample 1 solution are mixed in an insert-contained HPLC vial at position 21. Then the diluted sample 1 solution is injected for HPLC analysis. The diluted solutions of sample 2 and sample 3 are prepared and injected for HPLC analysis in the same way at positions 31 and 41, respectively, at the initial time point. The same procedure is performed for all the samples at different time points at the positions illustrated in the figure.

Plains, NJ, U.S.A.). Mini-UniPrep syringeless filters were used to remove precipitates from sample solutions being prepared for automated HPLC dilutions.[13] The Mini-UniPrep filter was purchased from Whatman as a preassembled filtration device with a 0.4 mL capacity vial and a plunger containing a filtration membrane. The sample solution is filtered by pressing the plunger through the sample in the 2 mL outer HPLC vial and forcing the filtrate into the 0.4 mL inner vial.

The chromatographic experiments were performed using an Agilent 1100 series HPLC system equipped with an auto-injector, temperature-controlled sample tray, quaternary pump, temperature-controlled column compartment, and diode array detector. The column employed was an Agilent Eclipse Plus C18 with the following column dimensions: 50 mm × 4.6 mm, 1.8 μm. The mobile phase consisted of deionized water with phosphoric acid added in at 0.1% (v/v) [A] and HPLC-grade acetonitrile [B]. The mobile phase flow rate was 1.5 mL/min with the column temperature set at 40 °C. UV detection was set to 220 nm.

A blank injection was made before each sample injection. The mobile phase gradient for the blank injection was as follows: 90:10 A/B to 5:95 A/B in 0.1 min, followed by a 0.9 min hold (the total run time was 1 min with 1 min for re-equilibration). For all the samples used for concentration or solution stability determination or for reaction monitoring, a linear mobile phase gradient was employed with an injection volume of 0.5 μL. The gradient was as follows: 90:10 A/B to 10:90 A/B in 5 min, followed by a 1 min hold (the total run time was 6 min with 2 min of re-equilibration). An injector program was carried out to dilute the sample for injection.

Injector Program. The injector program that was created to perform automated sample dilutions on the HPLC system is shown in Figure 1. The injector drew 89.0 μL of diluent from vial 11. The user has flexibility to select different diluents to dilute the samples. The autosampler is programmed to draw 1.0 μL of the sample from vial 1. The offset position of the needle was set at 2.0 mm to avoid picking up the precipitates in the sample solution. The total volume of 90.0 μL of the mixture of the diluent and the sample solution was ejected into the vial at position 21. Then the injector drew and ejected the mixture solution in the same vial at position 21 with maximum speed for 15 times.[14] In order to ensure adequate mixing for the low volume solution in position 21, an insert-contained HPLC vial was utilized.

HPLC Vial Layouts. As the injector program (Figure 1) shows, sample dilution was achieved by programming the injector to draw the sample, draw the diluent and then mix the two solutions into another insert-contained HPLC vial. Before starting the run, all the HPLC vials should be placed in the appropriate positions. The vials are placed in different layouts for concentration determination and reaction monitoring. For concentration determination, one empty insert-contained HPLC vial is positioned for each standard and sample. Up to 47 samples can be analyzed. For reaction monitoring, one empty insert-contained HPLC vial is positioned for each time point of the reaction sample. Multiple reaction samples can be monitored with different diluents. The same vial layout is also used for solution stability determination. Examples are shown in Figure 2.

Sequence Setup. A sequence file was created to perform automated sample dilutions and injections. The sequence was as follows: The first sample/standard was diluted into another HPLC vial with insert by injection program, followed by a blank injection to avoid the interference of the sample carried over from the sample dilution. The diluted sample/standard was then injected. The same procedure was performed for the remaining samples. For reaction monitoring and solution stability determination, the flow rate was reduced to 0.2 mL/

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min between each time point, after the sample injection for this time point, and before the sample dilution for next time point, to control the time points and conserve the mobile phase.

General Comments on HPLC Dilution System. Typically, a calibration curve of standard solutions is generated for concentration determination. The standard solutions are prepared in the concentration range used for HPLC injection. In contrast, for the HPLC automated dilution system described herein, it is recommended that the standard solutions are in the same concentration range as the samples. Furthermore, both the standard and sample solutions should be diluted using the same dilution process to eliminate systematic errors which could be introduced in the solution-transferring and the solution-mixing steps. Multiple-point calibration is recommended for more accurate results (Figures 3 and 4). Single-point calibration can also be utilized for certain applications (Figure 5).

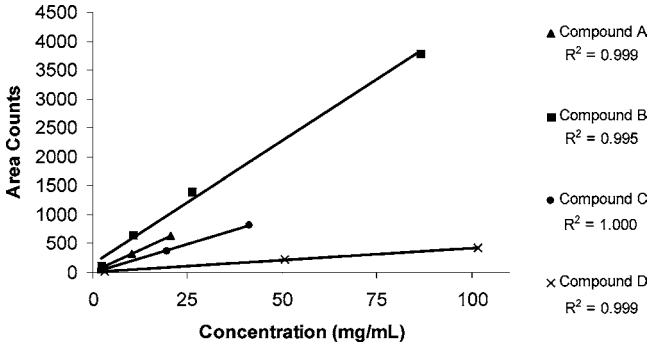

Figure 3. Calibration curves for compounds A–D by HPLC automated dilution system.

Solvent Examined	Concentration by manual dilution (mg/mL)	Concentration by HPLC auto-dilution (mg/mL)
Isopropyl acetate	4.1	4.1
Dichloromethane	0.2	0.3
Ethanol	2.1	2.2
Water	7.1	7.6
N,N-Dimethylacetamide	5.2	6.1

Figure 4. Comparison of traditional manual dilution vs HPLC automated dilution using compound A solubility samples.

Vol% DMSO in water	solubility (mg/mL) Manual dilution	solubility (mg/mL) Automated dilution
75	79.9	82.6
66	54.9	56.1
50	21.2	22.4
33	6.1	6.2
25	2.9	2.6
10	0.4	0.3

Figure 5. Comparison of traditional manual dilution vs HPLC automated dilution using compound B solubility samples (DMSO = dimethyl sulfoxide).

3. RESULTS AND DISCUSSION

As detailed in the Experimental Section, a macro for automated HPLC dilution has been created. To summarize the overall process: a solution of substrate is transferred to an HPLC vial, the HPLC injector samples a small amount of the substrate solution and the diluent, mixes the solutions, and injects the diluted sample on the HPLC automatically (Figure 2). To illustrate utility, we highlight application to solubility measurements (section 3.1) and then provide two case studies applying this technique to key activities in API route development: crystallization solvent screening and stability monitoring (section 3.2).

3.1. Validation and Application of the Automated HPLC Dilution Method for Concentration Determination. Linearity of the automated HPLC dilution method over a range of concentrations was evaluated using four different compounds, A, B, C, and D (Figure 3). To construct the calibration curve for each compound, a series of standard solutions were prepared and then diluted by the automated HPLC dilution system for HPLC injections. A linear regression equation was obtained for the calibration curve of each compound. The linearity of the automated HPLC dilution method was verified with determination coefficients (R^2) of >0.995 for all four compounds.

A comparison of manual dilution vs HPLC automated dilution was carried out by examining the solubilities of two substrates (compounds A and B) in solvents across a range of solubilities to ascertain the dynamic range of this technique. The solubilities of compound A in isopropyl acetate, dichloromethane, ethanol, water, and *N,N*-dimethylacetamide were examined within a solubility range <10 mg/mL. Mixtures of compound B in DMSO/water were examined for a much broader solubility range from 0.3 mg/mL to ~100 mg/mL.

As shown in Figure 3, a calibration curve for compound A was created by preparing standard solutions at 2 mg/mL, 10 mg/mL, and 20 mg/mL which were then diluted by the automated HPLC dilution system for HPLC injections.[15] A linear relationship with an excellent determination coefficient of 0.999 was established. A sufficient amount of compound A was added to the desired solvents to ensure that the solvent was saturated. These slurries of compound A were then filtered using the Mini-UniPrep filters (detailed in Experimental Section), and the filtrate was examined with this new technique. We were pleased to find, as illustrated in Figure 4, that the manual and automated methods gave similar values. In particular, measurements were found to deviate no more than 15% from the manual method (DMAc), a deviation that is acceptable for routine concentration determination and ranking.

To test the broader range of solubilities, we prepared saturated solutions of compound B in DMSO/water mixtures. Prior to execution, a four-point calibration was performed with compound B at 2, 10, 26, and 86 mg/mL again with an excellent determination coefficient of 0.995 (Figure 3). Compound B was added to varying ratios of dimethyl sulfoxide/water until solvent saturatation had been met. The resulting slurry was filtered with the Mini-UniPrep filter, and the mother liquor concentrations were then measured with the dilution macro on the HPLC and compared against the traditional volumetric flask dilution protocol (Figure 5). Comparable concentration results were achieved for the samples prepared traditionally and with this protocol. As shown in Figures 3, 4, and 5, the HPLC automated dilution method has been demonstrated to be applicable to solubility measurements and concentration determinations of samples in the range of 0.1–100 mg/mL—a range which covers the majority of our sample measurements. In addition, this HPLC automated dilution system reduces substrate and diluent usage and prep time (vide infra); 1 μL of the compound solution is used, and after automated dilution, the final volume of a diluted

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Compound E		
Solvent	co-solvent	mg/mL
IPAc	36% heptane	29.4
IPAc	63% heptane	27.7
IPAc	90% heptane	6.9
MTBE	36% heptane	3.0*
MTBE	63% heptane	1.4
Toluene	10% heptane	50.6
Toluene	63% heptane	0
IPA	36% water	17.7
IPA	63% water	0
Acetonitrile	36% water	27.9
Acetonitrile	63% water	1.1
DMAc	36% water	2.5

Compound F		
Solvent	co-solvent	mg/mL
IPAc	36% heptane	43.7
IPAc	63% heptane	11.3
IPAc	90% heptane	0.2
MeTHF	36% heptane	65.5
MeTHF	63% heptane	11.5
MeTHF	90% heptane	0.2
DCM	36% heptane	116.5
DCM	63% heptane	119
DCM	90% heptane	10.5
EtOAc	36% heptane	90.8
EtOAc	63% heptane	28.1
EtOAc	90% heptane	2.1
Toluene	36% heptane	59.2
Toluene	63% heptane	11.1
Toluene	90% heptane	2.1
Ethanol	36% water	3.9*

Compound G		
Solvent	co-solvent	mg/mL
IPAc	63% heptane	45.3
IPAc	90% heptane	6.7
MeTHF	63% heptane	65.3
MeTHF	90% heptane	6.3
Toluene	63% heptane	28.4
Toluene	90% heptane	5
DMF	36% water	14.1
Acetonitrile	36% water	31.8
DMAc	36% water	11.5

Figure 6. Selected results from solubility screenings of compounds E−G using the HPLC automated dilution protocol. * = verified upon scale-up.

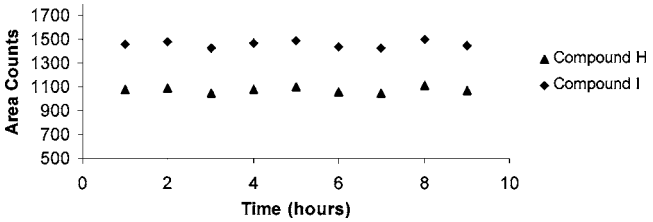

Figure 7. Stability hold point case study. Percent relative standard deviation (%RSD) for both measurements <3%.

sample is less than 100 μL without any need for manual dilution.

3.2. Application of the HPLC Automated Dilution System to API Route Development Workflow. *Case Study 1. Solvent Screening to Identify Crystallization Conditions.* Identifying crystallization conditions to improve substrate purity and maximize recovery is a critical, yet tedious, task requiring multiple sampling and dilution steps. This system reduces this task by effectively removing dilution steps. To illustrate, we examined the solubility of three compounds (E−G) in different solvents. A Symyx automated dilution system was employed to dispense 48 different solvent/cosolvent mixtures into HPLC vials containing ∼5−10 mg of substrate. After heating with agitation, the solutions were cooled to ambient temperature and filtered through Mini-UniPrep filters. These vials were then placed inside the HPLC sample tray and substrate mother liquor concentrations were determined using the dilution macro. Results of solubility screenings for these different compounds (E−G) are shown in Figure 6. Based on the concentration information and analysis of the mother liquor profile, the condition which gave optimum impurity rejection with minimal loss of desired substrate was used for implementation. Note that successful solvent hits were verified by repeating the crystallization on >0.5-g scale of substrates using the conditions identified in Figure 6. In each case, minimal manipulation allows the chemist to map a large solubility space in a quantitative manner.

Case Study 2. Reaction Monitoring and Solution Stability Determination. Knowledge of substrate stability in solution informs route liabilities and key hold points for processing. This information is typically gained via periodic, manual sampling of reaction solutions. Application of the HPLC automated dilution system allows for controlled, systematic reaction sampling and dilution using a minimal amount of solvent in an HPLC vial. To illustrate this, we highlight the monitoring of two compounds H and I in solution at approximately 100 mg/mL (Figure 7) over the course of 10 h. A nine-point data sampling from the same vial over 10 h was collected, showing that the substrates are stable for at least 10 h. The HPLC automated dilution system can be programmed to monitor multiple samples across many solvents for stability determination with different diluents at user-selected time points, providing a quick survey of potential solvents. It should also be noted that this

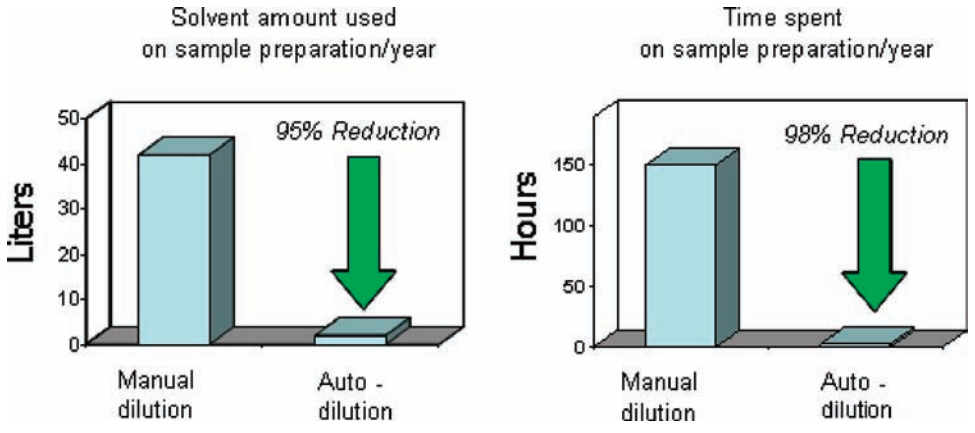

Figure 8. Comparison of solvent amount used and time spent on sample preparation with the use of the HPLC automated dilution system vs volumetric flasks.

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technique can potentially serve as a convenient method for monitoring homogeneous reaction kinetics by running the reaction in the HPLC vial in the temperature range allowed by HPLC thermostatted autosampler.

SUMMARY AND CONCLUSIONS

With the use of the HPLC automated dilution system, in which the HPLC injector is programmed to execute the entire sample preparation process, routine sample preparation efforts are reduced significantly. With this new protocol, assuming that 400 samples/year are examined with a single HPLC instrument to perform solubility/stability studies, the solvent and time requirements for sample preparation are reduced compared to those using traditional method (Figure 8). Solvent use is reduced by approximately 100, and the time spent on sample preparation, by a factor of 50. For the bench chemist, this HPLC automated dilution protocol uses familiar equipment and provides an opportunity to improve efficiency and minimize environmental impact.

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(11) The methodology applied in this paper can also be applied to HPLC instrumentation currently offered by additional vendors.
(12) Please note that this method was developed internally at Merck. In addition to the Experimental Section described herein, please consult internal analytical support or an automation engineer to fine-tune this method for specific application.
(13) In cases where the solid cleanly settled from solution, sampling of the supernatant could be performed instead of filtration, since the offset position of the HPLC injector needle was set at 2.0 mm to avoid picking up the precipitates in the sample solution.
(14) The mixing function that is available in the injector program was evaluated. The injector executes the mixing inside the injector loop. Solutions of different volumes and diluents were mixed. The procedure was repeated; however, the results achieved were not reproducible.
(15) In general, single-point calibrations are sufficient to determine the substrate response factor; however, for validation purposes, multiple points were obtained.