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Title: Accelerated Forced Degradation of Pharmaceuticals in Levitated Microdroplet Reactors

Authors: Yangjie Li, Yong Liu, Hong Gao, Roy Helmy, W Peter Wuelfing, Christopher Welch, and R Graham Cooks

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Accelerated Forced Degradation of Pharmaceuticals in Levitated Microdroplet Reactors

Yangjie Li, Yong Liu, Hong Gao, Roy Helmy, W. Peter Wuelfing, Christopher J. Welch and R. Graham Cooks*

Abstract: Forced degradation is a method of studying the stability of pharmaceuticals in order to design stable formulations and predict drug product shelf life. Traditional methods of reaction and analysis usually take multiple days, and include LC–UV and LC–MS product analysis. In this study, the reaction/analysis sequence was accelerated to be completed within minutes using Leidenfrost droplets as reactors (acceleration factor: 23 – 188) and nanoelectrospray ionization MS analysis. The Leidenfrost droplets underwent the same reactions as seen in traditional bulk solution experiments for three chemical degradations studied. This combined method of accelerated reaction and analysis has the potential to be extended to forced degradation of other pharmaceuticals and to drug formulations. Control of reaction rate and yield is achieved by manipulating droplet size, levitation time and whether or not make-up solvent is added. Evidence is provided that interfacial effects contribute to rate acceleration.

Forced degradation studies have long been part of the pharmaceutical development process for active pharmaceutical ingredients (APIs). The aim of such studies is to selectively probe chemical reactivity of active compounds so as to understand the chemical degradation that is possible in drug formulations. Further, the rates of chemical reactions in these studies yield some insight into drug product shelf life. Once possible degradation mechanisms are understood, formulation strategies can be developed to minimize chemical degradation and enable design of a stable formulation product.[1]

Even the established forced degradation methods are time-consuming, typically being performed in solution over the course of 1 – 7 days for each API under each chosen condition, even when the extent of degradation is limited to 10% – 20%.[1d] A recent paper by Dow *et al.* describes forced stress workflows, including how and when structures are assessed in accordance with regulatory guidance.[2a] Acceleration of degradant formation is critical to understand potential liabilities in short time frames rather than waiting for several weeks for an actual formulation to degrade sufficiently for measurement.[2b] The study presented here explores new methods to rapidly assess degradation chemistry of APIs under dehydration, oxidation and hydrolysis conditions in solution.

It is known that the rates of chemical reactions are often accelerated in small droplets or thin films vs. the corresponding bulk or larger droplet rates.[3] Concentration and pH changes during solvent evaporation and incomplete solvation of molecules at the air-droplet interface are the major factors that contribute to such reaction acceleration.[3a] One method of droplet acceleration employs the Leidenfrost effect to create small levitated droplets with no net charge. By pouring a liquid onto a surface at a temperature significantly greater than the boiling point of the liquid, levitating droplets can be created from which solvent gradually evaporates. A previous study of several reactions showed acceleration in Leidenfrost droplets vs. bulk by factors of 2 – 50, as judged by the time required to reach a particular fractional conversion of the reactant to product.[4] The millimeter-sized Leidenfrost droplets last for some minutes and when used as reaction vessels for APIs they force degradation to occur much faster than would be the case under traditional 'forced degradation' conditions as is now shown.

Compared to the traditional forced degradation, not only is the reaction accelerated by a factor of one to two orders of magnitude but the subsequent analysis by non-accelerating[3a] nanoelectrospray ionization mass spectrometry (nESI–MS) is faster than traditionally used LC–UV and LC–MS methods (Fig. 1). The Leidenfrost experiment involves reactions in confined volumes under conditions similar to those encountered in ambient ionization.[5] Well-studied degradation chemistry was chosen to test this methodology: acid degradation of tetracycline (TCN) and hydrochlorothiazide (HCTZ) and oxidative degradation of trifluoperazine (TFP).[6] We followed the degradation in Leidenfrost droplets, while maintaining nearly constant volumes, and used nESI–MS for analysis. The data were compared with those for degradation in the corresponding bulk solution (traditional forced degradation) under similar conditions with respect to the concentration of APIs and degradation reagents, composition of the solvent, and reaction temperature.

We performed traditional forced degradation by preparing a stock stress solution of hydrochloric acid or hydrogen peroxide, and a stock solution of the API of interest. Then the solutions were mixed and allowed to react at a set temperature (see Scheme 1 and Supporting Information, Sec.1 for details) for a set period of time. Likewise, the Leidenfrost reaction mixture was prepared the same way but instead of allowing the mixture to age at a fixed temperature, it was dropped into a concave ceramic well which was placed on a hotplate to form a single

[*] Y. Li, Prof. R. G. Cooks
Department of Chemistry, Purdue University
560 Oval Drive, West Lafayette, IN 47907 (USA)
E-mail: cooks@purdue.edu
Homepage: http://aston.chem.purdue.edu
Dr. Y. Liu, Dr. R. Helmy, Dr. W. P. Wuelfing,
Dept. of Analytical Sciences
MRL, Merck & Co., Inc.
West Point, PA 19446 (USA)
Dr. H. Gao, Dr. C. J. Welch,
Dept. of Analytical Research & Development
MRL, Merck & Co., Inc.
Rahway, NJ 07065 (USA)

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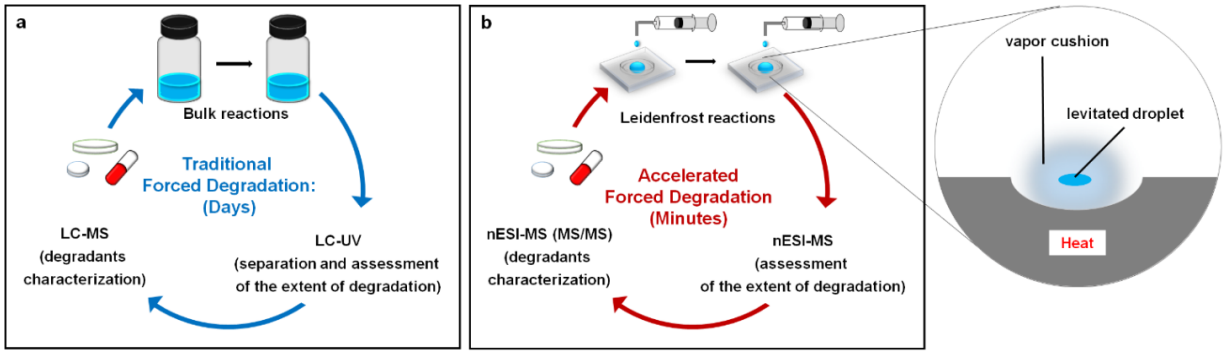
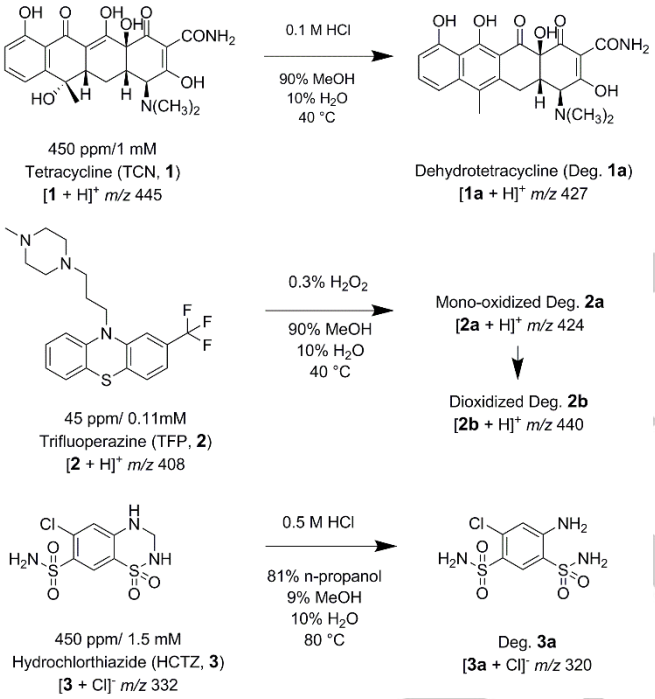


Figure 1. Comparison of (a) traditional and (b) accelerated reaction/analysis sequence. When make-up solvent is added continuously, the Leidenfrost acceleration is purely a result of the increased rate constant in the droplet reactions.



Scheme 1. Three degradation reactions studied, showing the compositions of the solutions used in both the bulk and Leidenfrost experiments. The major ionic forms of the APIs and their degradants and the nominal mass/charge ratios of products and reagents measured by MS are indicated. The structure of **2a** was elucidated by tandem mass spectrometry, Fig. S1, as a representative example.

levitated Leidenfrost droplet. Reaction occurred in the levitated droplet. Note that although a high temperature was used to heat the plate, the temperature of the levitating solution was very much lower and comparable to that used in the corresponding bulk experiment using traditional forced degradation. (The Leidenfrost droplet was levitated but not boiled and its temperature is roughly estimated as 20 °C less than the boiling point of the solvent.[4])

Considering the suggested mechanism of reaction acceleration in droplets,[3a] the microdroplet reactors were held at nearly constant volume, so that concentration and pH changes due to evaporation could be eliminated to allow direct comparison of the rate constants for Leidenfrost to traditional bulk experiments. This was realized by automatically adding make-up solvent to compensate for that lost during evaporation. The solvent was added via a syringe pump at a flow rate which depended on the size and evaporation rate of the levitating droplet (see Supporting Information, Table S1 for details).

In this study, the compositions of the final reaction mixtures in both bulk and Leidenfrost were analyzed by nESI–MS for direct comparison and the conversion ratio (*CR*), the ratio of the ion intensities of degradants to the sum of the intensities of degradants and the API, was used to infer the extent of reaction. The simple measurement of the time required to achieve the same conversion ratio is a good measure of the acceleration factor,[4] while kinetic measurements[7] were performed in this study for a more precise measure of the acceleration factor, as summarized in Table 1.

Table 1. Degradation of APIs under Leidenfrost vs. conventional forced degradation conditions and the reaction acceleration factor

API and degradation reagent [a]	Slope (bulk)	Slope (Leidenfrost) [b]	Reaction Acceleration factor [c]
TCN (**1**), HCl	0.000187 min^{-1}	0.0352 min^{-1}	188
TFP (**2**), H_2O_2	0.000323 min^{-1}	0.00762 min^{-1}	23.6
HCTZ (**3**), HCl	0.000318 min^{-1}	0.00725 min^{-1}	23.6

[a] Identical solutions were used for bulk and Leidenfrost experiments. [b] The slopes of the trend lines in both bulk and Leidenfrost kinetic profiles (*CR* vs time) have units of min^{-1} and the ratio of the slopes (Leidenfrost over bulk rate) was used to represent the reaction acceleration factor. [c] Leidenfrost reactions in acidic conditions were done in 500 µL droplets while oxidative degradation was carried out in 100 µL droplets. The acceleration factor value should not be used to compare these experiments since reaction rate in droplet reactor depends on the size (See Supporting Information, Sec. 4).

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Oxidative degradation of trifluoperazine (**2**) is discussed as a proof-of-concept experiment here. Comparison of the kinetic profiles in bulk solution and in Leidenfrost droplets confirms that the degradation occurs at very different rates (Fig. 2). Each data point represents information from at least three trials. The y-intercepts are slightly greater than zero since in both experiments timing started several minutes after the reactants were mixed.

$$Rate\ Acceleration\ Factor = \frac{Slope_{microdroplet}}{Slope_{bulk}} = \frac{0.00762\,min^{-1}}{0.000323\,min^{-1}} = 23.6$$

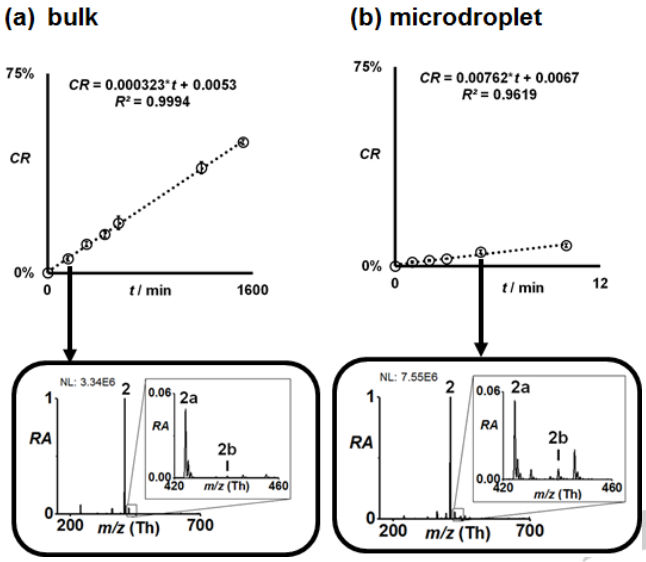

Figure 2. Conversion ratio (*CR*) of TFP (**2**) to **2a** and **2b** over time in (a) bulk and (b) constant volume 100 µL Leidenfrost droplet reactor. Mass spectra are for the same conversion ratio, calculated as the sum of the peak intensities of protonated **2a** and **2b** (the major oxidative degradants seen in MS) over the sum of the peak intensities for the ions of **2**, **2a** and **2b**.

Besides the similarity in the kinetic profiles, the mass spectra of the reaction mixtures were recorded at particular times chosen to correspond to similar extents of degradation. These data show no significant differences, which confirms the reliability of using Leidenfrost droplet reactors to accelerate this particular degradation. Note that this method uses relative abundance (*RA*) ratios, i.e. peak height ratios in mass spectra, and does not account for matrix effects (which are similar in the two solutions) or for ionization efficiencies (intrinsic properties of the two species being measured). It also does not account for differences (if any) in by-products associated with the different experimental conditions. This quick analytical method provides insight into the extent of degradation without the need for any separation.

Tandem mass spectrometry (MS/MS) was used (Fig. S1) to elucidate the structure of mono-oxidized degradant **2a** as the *S*-oxide produced under the reaction conditions described in this study. A fragment ion *m/z* 141 indicates that the piperazine ring in **2a** was not oxidized, while the appearance of ions of *m/z* 296 and *m/z* 324, both shifted by 16 compared to the corresponding phenothiazine ring containing fragments in protonated **2**, confirms *S*-oxidation. The data show that nESI–MS can be adequate as a rapid and reliable method for both analyzing the extent of degradation and for characterizing the degradant.

The traditional and Leidenfrost accelerated reaction/analysis sequences of two other degradations were also compared. The kinetic profiles of acid degradation of tetracycline and hydrochlorothiazide, plotted in Fig. S2 and S3, were used to calculate reaction acceleration factors of 188 and 23.6, respectively. The MS recorded in both experiments showed no significant differences with respect to APIs and their major degradants in the Leidenfrost and bulk experiments except for the time required to achieve particular degradation levels.

To gain deeper insight into the mechanism of reaction acceleration, the oxidative degradation of trifluoperazine was carried out in a microdroplet reactor using different droplet sizes but maintaining constant sizes during the course of reaction. This procedure eliminates concentration effects as variables and allows the effects of interfacial factors to be studied directly. The data (Fig. 3) show that the reaction acceleration factor correlates inversely with the size of the Leidenfrost droplet and hence is positively correlated to the surface/volume ratio of the levitated droplets. The surface areas and volumes of the droplets are estimates only (see SI, Sec. 4) but the data show that the reaction acceleration factor for the smaller droplets (ca. 100 µL) is greater than that for the 500 µL droplets. The size-dependent reaction acceleration factor in constant volume levitated microdroplets is consistent with the proposal [3a] that the solution/air interface is key to reaction acceleration. The result also suggests that the ease of manipulation of reaction rate in the Leidenfrost acceleration experiment makes it an even more attractive acceleration method for forced degradation, where control of degradation within the range of 10% – 20% is usually needed.

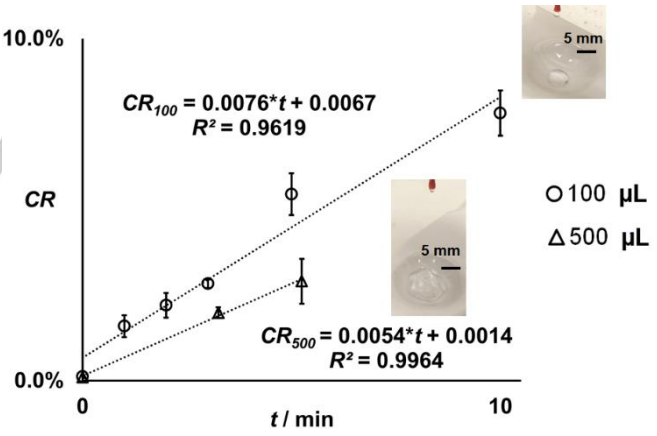

Figure 3. Conversion ratio (*CR*) of TFP (**2**) to **2a** and **2b** over time: (circles) constant volume 100 µL Leidenfrost droplet reactor; (triangles) constant volume 500 µL Leidenfrost droplet reactor.

The kinetic profile of the constant volume Leidenfrost experiments shows that reaction yield can be increased by levitating a Leidenfrost droplet for a longer time. In comparison, Leidenfrost experiments examined without adding make-up solvent (Fig. 4) showed very rapid product formation. In one such case, the 500 µL oblate spheroidal droplet was collected when it had evaporated to ca. 30 µL (just before complete evaporation), diluted and analyzed. Oxidative degradation assessed by MS was found to be extensive during the evaporation and shrinking of the droplet. After reacting for ca. 60

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s, the major axis of the oblate spheroidal droplet had shrunk from ca. 13 mm to 5 mm (about 100 µL, see Supporting Information Sec. 4 for estimation method), and the conversion ratio was measured to be 5%. In a shrinkage experiment of 90 s, the major axis of the droplet had shrunk to ca. 3 mm (about 30 µL), and the mass spectrum showed that 68% of TFP has been degraded (Fig. 4b). These results suggest that letting the droplet shrink without adding make-up solvent and collecting the droplet just before all solvent has evaporated is potentially a quick, very rough synthetic method (compare ref. 3, 4). Parenthetically we note that the use of shrinking levitated droplets for synthesis was seen for acid dehydration of TCN. About 70 s after generating the 500 uL droplet and letting it evaporate without adding make-up solvent, the final ca. 10 uL droplet was collected, diluted, and analyzed. The spectrum (Fig. 4d) showed a 99% conversion ratio to give a pure product.

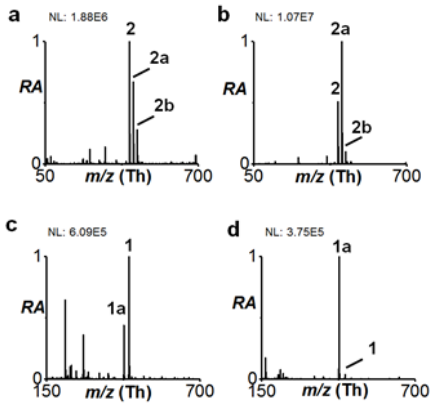

Figure 4. Full MS (positive mode) showing (a) conversion of TFP (**2**) to **2a** and **2b** in bulk after reacting at 40 °C for 1523 min, achieving 50% conversion ratio; (b) conversion of TFP (**2**) to **2a** and **2b** in ca. 30 µL Leidenfrost reactor alllowed to shrink from 500 µL on the hotplate over ca. 90 s, achieving 68% conversion ratio; (c) conversion of TCN (**1**) to **1a** in bulk after reacting at 40 °C for 1548 min, achieving 29% conversion ratio; (d) conversion of TCN (**1**) to **1a** in tiny (ca. 10 µL) Leidenfrost reactor allowed to shrink from 500 µL on the hotplate for ca. 70 s, achieving 99% conversion ratio.

In conclusion, the reaction/analysis sequence of forced degradation was achieved within minutes, in the three degradation reactions studied. The nESI–MS and MS/MS analysis show reproducible data when using Leidenfrost droplets of constant volume for accelerated forced degradation and the capability to rapidly assess the extent of degradation and characterize degradant structure without using any separation method. The experiments with droplets of different but constant size also demonstrate an effect of surface/volume ratio on reaction acceleration, and support the suggestion that interface is a significant factor. The levitated droplet shrinking experiments suggested a quick, rough synthetic method. Future prospects include further automation and multiplexing of the reactions to make the method even more efficient. Given the reliability and speed of the accelerated reaction/analysis and the small quantity of API needed, this method is potentially valuable in both drug discovery and development to (1) investigate the stability of other APIs including biomolecules; (2) investigate the stability of APIs in complex drug formulations, especially in sterile solution formulations; and (3) speed up polymorph screening.[8] The methodology developed here is potentially applicable to accelerate processes in many other fields.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: forced degradation • kinetics • Leidenfrost effect • mass spectrometry • reaction acceleration

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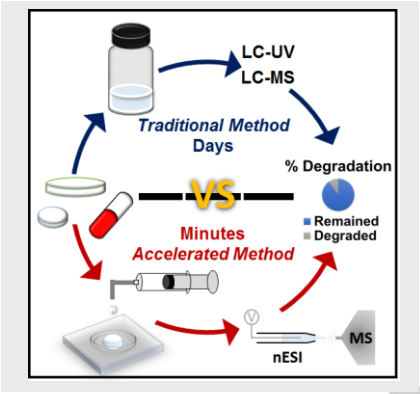
Traditional forced degradation of drug substances and drug products can take days to weeks but accelerated reaction/analysis of active pharmaceutical ingredients was achieved within minutes using Leidenfrost droplets and MS analysis. Lifetimes and sizes of the droplets and degree of solvent evaporation control reaction rate and product yield. Interfacial effects increase reaction rates in small droplets.



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