International Journal of Pharmaceutics, 34 (1986) 1–8
Elsevier

IJP 01126

Research Papers

Automated liquid chromatography for non-isothermal kinetic studies

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(Received 28 March 1986)
(Modified version received 13 June 1986)
(Accepted 16 June 1986)

Key words: Non-isothermal kinetics; Liquid chromatography; Automation; Cefotaxime; Cephalosporin; Degradation

Summary

An automated liquid chromatography workstation was developed to facilitate data collection in non-isothermal kinetics studies. Operation of this system was validated by studying the degradation of cefotaxime sodium (Claforan, Hoechst-Roussel Pharmaceuticals), a moderately stable cephalosporin antibiotic. As in a previous paper (Kipp, 1985), two data treatment methods were applied, a derivative and an integral approach, which provided similar results.

Introduction

Non-isothermal stability testing offers a way of rapidly yet accurately determining the shelf-life of a pharmaceutical formulation. In conventional accelerated studies, rates of drug degradation are determined at several constant temperatures. Regression of the derived rate constants on temperature according to the Arrhenius relationship:

$$k = Z \cdot \exp(-E/RT) \quad (1)$$

where k is the observed rate constant and R is the ideal gas constant, provides estimates of E, the experimentally observed activation energy, and Z, the pre-exponential factor. With these parameters, the rate constant at room temperature, and hence the shelf-life of the formulation, can be deduced.

In the non-isothermal method, the temperature of the reaction is continuously varied throughout the experiment. Temperature, time, and drug concentration are measured at successive intervals. By application of any of a number of data treatment methods, the Arrhenius parameters can be estimated.

The theoretical basis of non-isothermal testing is well documented (Tucker, 1985; and references therein). In these instances, the following general differential equation expressing drug loss is assumed:

$$dD/dt = -k(T) \cdot D^n \quad (2)$$

where D is the concentration of remaining drug, k(T) is the rate constant at temperature T, and n is

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the reaction order. Because temperature is a function of time, k can also be expressed by a time-dependent function, k(t). If temperature is fitted to a polynomial function of time, as in this study, then a solution of Eqn. 2 must be found by approximation. In this study, a derivative method (Waltersson and Lundgren, 1982; Hempenstall et al., 1983) and an integration method (Madsen et al., 1974) were used. In the derivative approach, Eqn. 2 is expressed as:

$$(dD/D^n)/dt = -k(t) \quad (3)$$

Letting $df(D) = dD/D^n$, function f(D) can be solved for different values of n (reaction order):

$$f(D) = D \quad \text{(zero order, } n = 0), \quad (4)$$

$$f(D) = \ln D \quad \text{(first order, } n = 1), \quad (5)$$

$$f(D) = -1/D \quad \text{(second order with respect to drug, } n = 2) \quad (6)$$

$$f(D) = [1/(C_0 - D_0)] \cdot \ln[(C_0 - D_0 + D)D_0/DC_0]$$

$$\text{(second order, } C + D \rightarrow \text{product)} \quad (7)$$

C_0 and D_0 are initial concentrations, where C_0 is the concentration of the species that is in excess.

If f(D) is fitted to a power series with time, then the negative of the derivative with respect to time affords a rate constant at each time, t. Thus,

$$f(D) = \sum_{i=0}^{m} a_i \cdot t^i \quad (8)$$

and

$$df(D)/dt = \sum_{i=1}^{m} i a_i \cdot t^{i-1} = -k, \quad (9)$$

where m is the number of terms used in the polynomial fit. Because temperature is measured at each interval, the value of k at each temperature is known. An Arrhenius plot (lnD versus 1/T) affords values of Z and E. Choice of the number of polynomial coefficients to employ in the series expansion is not arbitrary. As was discussed in an earlier study (Kipp, 1985), the polynomial fit which yields the lowest variance will not necessarily provide the best fit in the subsequent Arrhenius analysis. Differentiation of the power series may also produce negative rate constants (positive slopes).

The second method applied in this study finds an iterative solution to differential Eqn. 2 in terms of E and Z. Substitution of k in Eqn. 3 by the Arrhenius expression, and substitution of (dD/D^n) by df(D) gives:

$$df(D)/dt = -Z \cdot \exp(-E/RT) \quad (10)$$

Integrating both sides:

$$f(D) = f(D)_0 - Z \cdot I_t \quad (11)$$

where $I_t = \int_0^t \exp(-E/RT)dt$

If an empirical relationship between temperature and time can be established, I_t can be solved numerically. Values of E and Z that best fit the data can be found by conventional non-linear least-squares methods. In this study, a polynomial regression of temperature on time was performed, and optimization of Eqn. 11 was carried out by the sequential simplex method (Deming and Parker, 1978). For details of the approach used in this study, the reader is directed to an earlier paper (Kipp, 1985).

Sensitivity of the derivative method to measurement errors has been noted (Tucker, 1985; Kipp, 1985). Despite this drawback, however, the derivative approach is more easily implemented on a microcomputer than the integration routine, which requires longer computation.

In an effort to minimize errors associated with sample withdrawal, as well as to decrease the effort required on the part of the experimentalist, an automated HPLC system was developed to make injections and record data. In this manner, longer experiments than have been previously attempted manually can be carried out. This is advantageous in the kinetic study of more stable pharmaceuticals. Moreover, a large number of injections can be made, providing better confidence in extrapolating the rate constant data to room temperature.

Experimental

Fig. 1 is a schematic representation of the automated sampling system. A one liter three-necked flask fitted with a water-jacketed condenser was used as the reaction vessel. In one neck of the flask, a two-hole rubber stopper was placed, through which a type T thermocouple and Teflon tubing (0.020 inch i.d.) was inserted. The thermocouple was attached to a digital thermometer (Sensortek, BAT-12), the analog output of which was sent to a 24-bit A/D converter (Hewlett-Packard 18652A). The digitalized signal (sampling rate: 1024 Hz, averaged every 0.5 s) was sent to a Hewlett-Packard 1000E Series minicomputer for storage and analysis.

Temperature was controlled by the heating system shown in Fig. 2. A digital temperature programmer (Honeywell Model 770111) with proportional-band control switched the line current flowing through a resistance heater immersed in the heating bath. The bath solution consisted of propylene glycol (20 liters). A platinum RTD sensor (100 Ω) monitored the bath temperature and relayed the information to the digital programmer.

Samples were periodically withdrawn by peristaltic pump (Gilson Minipuls 2), which ran continuously throughout an experiment. The sampled solution flowed first through the Teflon tubing and through a heat exchanger. This helped to minimize errors caused by variation of solution density with temperature. The heat exchanger consisted of a 30 cm section of stainless steel tubing (0.020 inch i.d.), wound into a coil (2 cm diameter). This was connected to an inlet port on valve no. 1. By determining the time required for blue dye (bromcresol blue) to travel from the reaction vessel through the outlet of the loop injector (valve no. 2), it was determined that 1 min was needed to completely load the sample loop. This facilitated the proper timing of relay events. To test the effectiveness in cooling sample prior to injection, water samples at 35°C and 85°C (the range of the temperature program) were run through the system, and the temperature of the effluent at the outlet of the sample loop was measured. The difference between the highest and lowest recorded temperatures was 14°C (42°C maximum, 28°C minimum), corresponding to a density change of approximately 0.5%. This is approximately equal to the expected change in concentration, ΔC, since $\Delta C = C_0[f/(1 + f)]$, where C_0 is the initial concentration, and f is the fractional change in volume. The expected measurement error due to changes in volume is therefore less than the expected relative error in the HPLC assay (approximately 1%).

Valve no. 1, a six-port Rheodyne injector (see

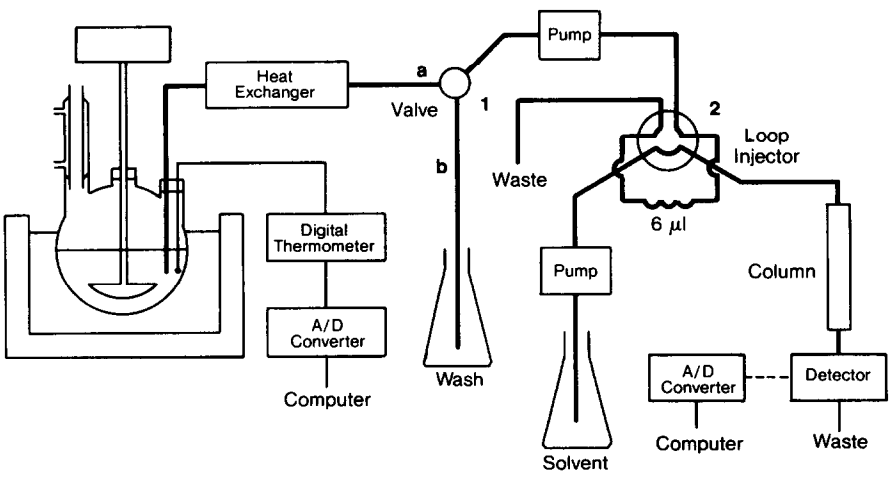

Fig. 1. Automated sampling system.

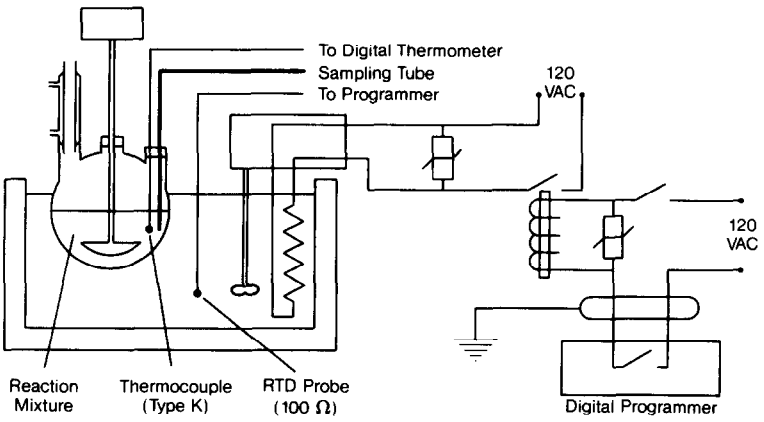

Fig. 2. Heating system.

Fig. 1), was switched by solenoid relays either to position *a* (sample withdrawal), or to position *b* (withdrawal of wash solution for rinsing out sample loop between injections). Valve no. 2, another six-port Rheodyne injector, was also switched by relay. Switching of both valves was done pneumatically, and the air flow was controlled by 110 V, 15 A solenoids (not shown in Fig. 1). The solenoid relays were in turn switched by a bank of optically-isolated solid-state relays (Hewlett-Packard Event Control Module, 18653). (These relays are omitted from Fig. 1 for the sake of clarity.)

The assay of cefotaxime used in this study is similar to that of Berge and coworkers (Berge et al., 1983). Samples were injected using a 6 μl sample loop. A Zorbax C-8 column (DuPont) was used. An Altex Model 110A HPLC pump (flow rate: 1.5 ml/min) and a variable wavelength detector (Kratos Spectroflow 757) were employed. A detector wavelength of 254 nm (sensitivity: 0.5 AUFS) was used. The retention time of cefotaxime was approximately 8.0 min. The analog signal from the detector was fed into an A/D converter (HP-18653A). An acquisition rate of 1024 Hz (averaged over 0.5-s intervals) was used.

Software control was provided in part by proprietary programs (Hewlett-Packard Laboratory Automation System). The duration of the chromatographic run, peak integration parameters, and post-run analysis programs were listed in a method file, used by a program associated with A/D channel no. 17. Channel no. 26 was used for collection of temperature data. The method file associated with channel no. 26 contained a list of control commands, which instructed the computer to switch valves no. 1 and no. 2 in the proper sequence. Each A/D channel could be started either manually, by a "start button" on the face of the A/D panels, or by an internal trigger through software control. This internal control was accomplished by an executive program, STIME, which was written in BASIC. Program STIME was started by entering from a terminal the program name followed by a list of parameters. The parameters passed to the program included the interval between the start of each run, the total number of runs, and the output device to which a report was sent. Program STIME started both A/D channels simultaneously, added the interval from the parameter list to the current system clock time, and stored this adduct in memory. When the system time equalled this preset value, the next run was initiated. This process loop was continued until the total number of runs had been reached.

The programs associated with channels no. 17 and no. 26 directed execution of several post-run analysis subprograms. The program for channel no. 17, for example, ran subprograms after completion of data acquisition to convert raw data to peak areas. The program associated with channel no. 26 initiated execution of a routine that aver-

aged an adjustable number of acquired data. Another post-run routine converted this time-averaged signal in volts into a temperature reading in degrees Celsius by polynomial calibration. A thermometer calibration had to be done because the analog output of the thermometer was non-linear over the temperature range of the experiment. The calibration was performed as follows. A Dewar flask was filled with heated water, an insulated lid was placed on top, and the thermocouple was inserted through a hole in the lid so that the entire thermocouple element was immersed. After allowing for thermal equilibration, a voltage was recorded (average of 20 points). Voltage readings were obtained at different temperatures between 20 and 100°C. These limits were outside the expected experimental range. The corresponding temperatures were read from the digital readout on the thermometer, which had a specified accuracy of ±0.1°C. A quadratic fit (voltage versus measured temperature) produced coefficients that were entered into the post-run analysis program. Temperature values obtained from the program differed from actual values by no more than 0.1°C.

An experiment was started as follows. Initially, valve no. 1 was set to position *b*, and the loop injector was set in the "load" position (as shown in Fig. 1). The temperature programmer was started so that the temperature in the heating bath was linearly increased from 35 to 85°C. The command STIME with the associated parameter list was entered from the keyboard, starting data acquisition from both channels. The relay control sequence stored in the method file for channel no. 26 initiated the following event sequence. Valve no. 2 (loop injector) was activated to inject the wash solution so that an external standard (optional) could be injected prior to injecting sample. After 5 s, valve no. 2 was switched back to "load". One minute later, valve no. 1 was switched to position *a*, and sample was removed from the reaction mixture. An additional minute was allowed to flush the previous contents from the loop. Valve no. 1 was then switched back to position *b*, and immediately valve no. 2 was activated to inject the sample. Minimizing the length of time in which valve no. 1 was set to position *a* conserved sample.

Prior to the kinetic experiments, the linearity of the detector response at 254 nm was verified over the expected drug concentration range. A correlation coefficient of 0.999 was obtained. Reproducibility was checked by a series of injections (100 μg/ml). The relative standard deviation was less than 1%.

In a typical experiment, the flask was charged with 780 ml of 0.1 N acetate buffer at pH 5.52, adjusted to an ionic strength of 0.5 with potassium chloride. Mobile phase was prepared by mixing 120 ml of methanol and 880 ml of phosphate buffer for each liter. Buffer was prepared by adding 60 mg of potassium dihydrogen phosphate, and 31.8 mg of anhydrous sodium monohydrogen phosphate to 120 ml of distilled water. The flask was immersed in the heating bath, as shown in Figs. 1 and 2, and the temperature programmer was set to allow for thermal equilibration at 35°C, followed by an approximately linear temperature increase of 4°C per hour. After equilibration for 0.5 h, 80 mg of cefotaxime sodium in 20 ml of the acetate buffer was quickly added, producing an initial concentration of approximately 100 μg/ml. The temperature increase was started, and automated sampling was initiated. The system was then allowed to run overnight.

After approximately 10 h, the processed data (concentration, time, and temperature) were printed out. A time stamp was also printed out for each sample injection. The data was transferred to a floppy diskette, and analyzed by the derivative program, NISO, described in an earlier paper (Kipp, 1985). The derivative analysis was run on a Compaq portable computer. This program, originally written in FORTRAN, was transcribed into PASCAL for execution on microcomputers that utilize MS-DOS. A copy of source code is available on request. The data was also analyzed by the integration routine NIS03 (Kipp, 1985), which is written in FORTRAN and runs on the HP-1000.

Results and Discussion

The degradation of cefotaxime is discussed elsewhere (Berge et al., 1983). Three experiments were conducted, the results of which are listed in

Table 1. Applying the derivative program to the data from experiment no. 1, the best rate model was found in terms of reaction order (zero, first, or second with respect to drug). The first-order model produced, as expected, the highest negative correlation in the Arrhenius plot when seven terms were used (Table 2). Magnitudes of r were, in general, highest when the order was 1. When using the derivative program, the same criteria for choosing the correct polynomial were employed as in a previous study (Kipp, 1985). All functions giving negative rate constants were rejected, and the function that yielded the lowest variance in the Arrhenius plot was used to determine definitive results. As seen in Table 1, the number of terms yielding the lowest error in the linear plot was consistently smaller than the number corresponding to minimum error in the polynomial least-squares fit. This was due to error transformation associated with differentiation of the polynomial.

The integration routine used in this study is described in further detail in the earlier paper

TABLE 1

DEGRADATION OF CEFOTAXIME IN 0.1 N ACETATE BUFFER AT pH 5.5 (IONIC STRENGTH = 0.5)

Experiment	No. of terms [a]	Derivative method			Integration method		
		E [b]	log Z [c]	k_{25} (h^{-1}) ($\times 10^3$)	E [b]	log Z [c]	k_{25} (h^{-1}) ($\times 10^3$)
1	4	18.95 (±4.73) [d]	11.83 (±3.15)	8.7 (±24.8)	–	–	–
	5 [e]	–	–	–	25.5	16.15	2.8
	6	23.36 (±3.22)	14.74 (±2.14)	4.1 (±6.8)	–	–	–
	7 [f]	25.27 (±1.26)	16.00 (±0.84)	3.0 (±1.6)	25.5	16.14	2.8
	8	23.86 (±2.99)	15.07 (±1.99)	3.8 (±5.7)	–	–	–
	9	–	–	–	25.5	16.15	2.8
	10 [g]	23.77 (±2.12)	15.02 (±1.41)	3.9 (±3.8)	–	–	–
	12	23.68 (±2.45)	14.96 (±1.63)	4.0 (±2.3)	–	–	–
2	4	20.00 (±4.00)	12.52 (±2.68)	7.2 (±16.1)	–	–	–
	5	–	–	–	26.1	16.49	2.5
	6 [f]	25.76 (±0.58)	16.30 (±0.38)	2.6 (±0.6)	–	–	–
	7 [e,g]	28.14 (±1.70)	17.85 (±1.13)	1.7 (±1.2)	26.1	16.50	2.5
	8	29.27 (±2.88)	18.59 (±1.92)	1.3 (±1.9)	–	–	–
	9	–	–	–	26.1	16.50	2.5
3	3	–	–	–	26.5	16.80	2.3
	4	20.52 (±4.18)	12.86 (±2.77)	6.6 (±15.7)	–	–	–
	5	–	–	–	26.4	16.71	2.3
	6	26.31 (±0.36)	16.66 (±0.24)	2.3 (±0.3)	–	–	–
	7 [e,f]	26.68 (±0.30)	16.90 (±0.20)	2.2 (±0.3)	26.4	16.72	2.3
	8 [g]	29.06 (±2.67)	18.45 (±1.77)	1.4 (±1.8)	–	–	–
	9	–	–	–	26.4	16.72	2.3
Averages [h]:		25.9 (±3.1)	16.4 (±2.0)	2.6 (±1.7)	26.0 (±2.0)	16.5 (±1.2)	2.5 (±1.1)

[a] In the derivative model, "number of terms" refers to number in the polynomial fit of log (concentration) versus time. In the integration model, it refers to the number of terms in the polynomial fit of temperature versus time.

[b] Activation energy in kcal/mol.

[c] The pre-exponential factor, Z, is expressed in h^{-1}.

[d] 95% confidence limits (37 degrees of freedom, $t = 2.03$).

[e] Minimum error in the fitted data (integration method).

[f] Minimum error in the linear-least squares fit (Arrhenius plot, derivative method).

[g] Minimum error in the polynomial fit (derivative method).

[h] Obtained either by averaging values producing the best fit in the Arrhenius plot (derivative method), or by averaging those values which provided the lowest error in the concentration–time regression (integration method).

TABLE 2

EFFECT OF RATE MODEL ON CORRELATION IN THE ARRHENIUS PLOT

Reaction order	No. of terms	Correlation coefficient (r)	E (kcal/mol)	k ($\times 10^3$ h^{-1})
0	3	−0.9319	15.33	7.52
	4 [a]	−0.7518	16.51	5.85
	5	−0.8160	10.65	15.30
	6	−0.8206	10.75	14.94
	7	−0.8028	10.55	15.39
	8	−0.7970	10.63	15.36
	9	−0.8377	11.13	14.20
	10	−0.8370	11.45	13.26
	12	−0.8144	10.88	14.90
1	3 [a]	−0.8928	22.62	4.87
	4	−0.7992	18.95	8.68
	5 [a]	−0.9659	27.68	1.89
	6	−0.9238	23.36	4.11
	7	−0.9889	25.27	2.95
	8	−0.9356	23.87	3.80
	9 [a]	−0.9679	24.91	3.14
	10	−0.9658	23.77	3.90
	12	−0.9552	23.68	3.96
2	3 [a]	−0.9195	23.49	20.82
	4 [a]	−0.4034	8.82	434.36
	5 [a]	−0.6698	22.41	26.86
	6 [a]	−0.5167	15.65	79.78
	7 [a]	−0.7342	21.72	24.08
	8 [a]	−0.6578	20.35	30.87
	9 [a]	−0.7324	27.98	5.36
	10 [a]	−0.7600	33.11	2.00
	12 [a]	−0.8437	31.47	2.90

[a] Negative rate constants were encountered upon differentiation of the polynomial and were excluded.

(Kipp, 1985). The same tolerance limit, 10^{-6}, which determines the precision of the integration, was used as before. Using smaller tolerances led to excessive computation and resulted in insignificant changes in the results. A first-order model was used, based on results from the derivative approach. When zero- or second-order kinetics were assumed (with five terms in the temperature–time regression), higher regression errors were found.

Averages of the rate parameters obtained from the three experiments compare favorably with results from an earlier investigation on cefotaxime under similar conditions (Berge et al., 1983). This comparision is shown in Table 3.

TABLE 3

COMPARISON OF EXPERIMENTAL RESULTS WITH LITERATURE VALUES

Method of data analysis	E (kcal/mol)	Log Z (h^{-1})	k (@ 25°C) ($h^{-1} \times 10^3$)
Derivative	25.9 ± 3.1 [a]	16.4 ± 2.0	2.6 ± 1.7
Integration	26.0 ± 2.0	16.5 ± 1.2	2.5 ± 1.1
Literature values			
Berge et al., 1983 [b]	24.7	15.6	3.24

[a] 95% confidence limits.

[b] Confidence intervals were not reported.

Conclusion

Use of an automated liquid chromatography workstation greatly facilitates the acquisition of non-isothermal rate data, thereby enabling unattended overnight runs. It has been shown that reliable rate estimates can be obtained with this technique. It is hoped that studies that would normally be tedious to carry out manually, can now be conducted. Studies of highly stable pharmaceuticals are an example. Such investigations are currently planned.

Acknowledgements

We wish to thank Mr. Alok Krishen for his assistance in the statistical interpretation of the results. We also wish to thank Hoechst-Roussel Pharmaceuticals, Inc., for supplying drug used in this study.

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