Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 814 (2005) 225–232
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Automated online liquid chromatographic/mass spectrometric metabolic study for prodrug stability

Fran Lai*, Siamak Cyrus Khojasteh-Bakht

Drug Metabolism and Pharmacokinetics, Genentech Inc., 1 DNA Way, M/S 70, South San Francisco, CA 94080, USA

Received 4 June 2004; accepted 13 October 2004
Available online 11 November 2004

Abstract

In vitro metabolic stability studies are performed routinely in drug discovery to determine the rate of metabolism as well as the metabolic fate of compounds. These studies are labor intensive, involving incubation of the compound with a biological matrix, sampling at various time points, stopping the reaction, and sample preparation for analysis. All of these steps involve manual pipetting in the conventional method. An automated method for in vitro metabolism studies is reported here. The method reduces the time and manual labor required and has other advantages, such as better reproducibility and unattended operation. This method utilizes an autosampler custom configured with cooling and incubation capabilities. The autosampler is programmed to directly inject incubation samples at set time points onto an online extraction column. The extracted sample then enters an analytical column for separation and ultimately the mass spectrometer for detection. The injection has the dual function of stopping the reaction and starting the analysis on the LC–MS. This method was used for the metabolic stability study of a prodrug in plasma and liver S9 fractions of five different species. The stability data from the automated method were similar to those obtained using the conventional method. The potential for this method to increase throughput of metabolic stability studies in drug discovery is demonstrated.

Keywords: Liquid chromatography; Prodrug; Stability; LC/MS analysis; Automated online method; Metabolic study; Online incubation; Online analysis

1. Introduction

In drug discovery, in vitro metabolic studies in biological matrices are performed routinely to investigate the stability of drug candidates (compounds). One particular example is the conversion of prodrugs to active drugs [1]. For these studies, in vitro experiments are often conducted by using plasma, liver microsomes and S9 fractions prepared from a variety of species, and the half-lives are determined from the rate of conversion [2]. Conventional methods [3] involve sample incubation at 37 °C in a water bath, sampling at selected time points, reaction quenching and sample preparation [4] prior to analysis (e.g., LC/MS/MS) [5,6]. Attempts have been made to automate the process in order to increase throughput [7]. These attempts include multi-channel parallel LC–MS systems [8], automated data processing [9], and pulse ultrafiltration-mass spectrometry chambers in which the samples are directly introduced to the mass spectrometer [10]. Several methods have been developed to automate the sample preparation step prior to analysis, such as filtration of the protein-precipitated sample [11], solid phase extraction (SPE) in a 96-well format [12–15], or online SPE [16–18]. Direct injection of plasma samples has been made possible by online extraction columns that selectively remove protein, other macromolecules and salts prior to switching to an analytical column [19–23] and by mixed-phase columns that have both online extraction and analytical functionalities [24–28]. Recently, a new method was reported by Wang et al. on semi-automated direct plasma injection using a temperature-controlled autosampler [29,30].

An automated method, referred to as the "automated online method" for drug stability studies is reported here, which used a custom-configured autosampler (CTC HTS PAL) and LC–MS with online extraction. This automated online method was tested with a prodrug known to convert at differ-

* Corresponding author. Tel.: +1 650 225 5694; fax: +1 650 225 6452.
E-mail addresses: franlai@gene.com, laifran@AOL.com (F. Lai).

1570-0232/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2004.10.040

ent rates in different species and the results were compared to those obtained from the conventional method.

2. Experimental

2.1. Reagents and chemicals

Male beagle dog plasma was obtained from Lampire Biologicals Lab (Pipersville, PA). Plasma of male Sprague-Dawley rats and cynomolgous and rhesus monkeys were obtained from BioReclamation Inc. (East Meadow, NY). Human plasma was obtained from Occupational Health Services at Genentech Inc. (South San Francisco, CA). Liver S9 fractions of the above mentioned species were purchased from In Vitro Technologies (Baltimore, MD) at 20 mg/mL protein content.

The test compounds including active drug (A), its prodrug (P), and the internal standard were synthesized in the Medicinal Chemistry Department of Genentech Inc. *p*-Nitrophenyl butyrate (pNPB), magnesium chloride, D-nicotinamide adenine dinucleotide phosphate ($NADP^+$), D-glucose-6-phosphate (G6P), and D-glucose-6-phosphate dehydrogenase (G6PDH) were purchased from Sigma Chemical Co. (St. Louis, MO). Phosphate buffer saline (PBS; code A3300, lot #1R77MP10-RD) and 1 M potassium phosphate buffer (code A3108, lot #11L113MP5-RD) were provided by Media Preparation Facility at Genentech Inc. Ammonium hydroxide, trifluoroacetic acid (TFA) and acetonitrile, both "Baker analyzed" HPLC grade, were purchased from J.T. Baker (Phillipsburg, NJ). Glacial acetic acid (99.8%) was purchased from Mallinckrodt (Paris, KY). HPLC water was purified by MilliQ system from Millipore (Bedford, MA). Formic acid was purchased from EM Science (Darmstadt, Germany). Mobile phase A and B were 0.1% formic acid in water and 0.1% formic acid in acetonitrile, respectively.

2.2. Equipment

The HPLC system consisted of a HP1100 binary pump from Agilent Technologies (Palo Alto, CA), a CTC HTS PAL autosampler, a sample tray, a cooled tray holder, an agitator/incubator from LEAP Technologies (Carrboro, NC). The mass spectrometers were an API3000 from PE Sciex (Concord, Ontario, Canada) and an LCQ from Thermo-Finnigan (San Jose, CA).

2.3. Incubation and analysis

Samples of prodrug were incubated in plasma and liver S9 fractions of five different species (human, cynomolgous monkey, rhesus monkey, beagle dog, and Sprague-Dawley rat) and analyzed both by (1) the conventional method and (2) the new "automated online method" for comparison. The same was performed for samples of active drug but only the zero time point was sampled and analyzed. Since these metabolic stability studies are for high-throughput screening of prodrug for conversion study, quantitation was based on the amount of active drug or prodrug at the zero time point set as 100% in the calculations. Further explanation is in Section 3.

2.3.1. Conventional method

2.3.1.1. Plasma. The working solution of the test compounds (A and P) was 10 μM in 10% acetonitrile/90% water containing 0.1% DMSO. Plasma (10 μL, 0.8 mg total protein) and test compound working solution (20 μL) were added to 100 mM KPi prepared from 10-fold dilution of 1 M potassium phosphate buffer (170 μL, test compound final concentration was 1 μM). The sample was incubated at 37 °C in a water bath for 5, 15, and 45 min. The reaction was stopped by protein precipitation following the addition of 400 μL of acetonitrile containing an internal standard (5 μM final concentration). A zero time point sample (explained under Section 2.3) was prepared similarly except that the acetonitrile was added before the addition of the compound to prevent any conversion of the prodrug. The protein-precipitated sample was centrifuged at 3000 rpm for 10 min. The supernatant was lyophilized to dryness and reconstituted with 10% acetonitrile in water. Analysis of samples was performed using LC/MS/MS.

2.3.1.2. Liver S9 fraction. The procedure was similar to that for plasma (Section 2.3.1.1), except that 10 μL of liver S9 fraction (0.2 mg total protein) was used instead of plasma. NADPH-generating system (20 μL) was added, and the volume of KPi was 150 μL instead of 170 μL.

2.3.1.3. LC–MS conditions. The column used was a Luna C18, 3 μm, 50 mm × 2 mm from Phenomenex (Torrance, CA). The LC flow rate was constant at 0.25 mL/min. Injection volume was 10 μL. A gradient was started from 10% B to 90% B in 3 min, then it was held at 90% B for 1 min, back to 10% B in 0.1 min, and finally re-equilibrated for 2.9 min.

The MRM precursor ion to product ion transitions for the active drug and the prodrug were *m*/*z* 433–139, and *m*/*z* 461–139, respectively.

2.3.2. Automated online method

A temperature-controlled agitator and cooling tray were installed onto a CTC HTS PAL autosampler and set to 37 and 2 °C, respectively. The cooling tray was used for biological matrix and cofactor storage and the heated agitator was used for sample incubation. The regular sample tray at room temperature was used for the test compounds. Incubation vials (2 mL) containing the biological matrix (e.g., liver S9 fraction) diluted with buffer and vials containing cofactors (e.g., NADPH-regenerating system) were stored in the cooling tray. The autosampler was programmed to add the cofactor to the incubation vial and transport the latter to the heated agitator. It was then pre-incubated for 4.5 min. Finally, the test compound (e.g., prodrug) was introduced into the incubation vial to initiate the reaction and allowed to incubate at

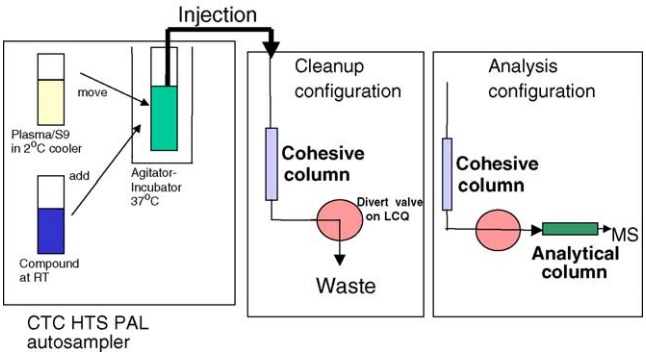


Fig. 1. Schematic of automated online method.

37 °C. At programmed time points throughout the incubation period the autosampler withdrew a sample from the incubation vial and directly injected it onto the LC–MS system for analysis. The injection had the dual function of stopping the reaction immediately without any time lapse (as due to sample transfer in the conventional method) and starting analysis of the sample on the LC–MS. The LC–MS system consisted of an online extraction column and a reversed phase analytical column, with a diversion valve located between the two columns. Proteins and other macromolecules in the samples were passed through the extraction column and diverted to waste in the first part of the run while the test compound was selectively retained on the extraction column. The valve was then switched to divert the eluate to the analytical column and an organic gradient was started to elute the compound from the extraction column to the analytical column and onto the mass spectrometer. With this automated method, the conventional sample preparation steps, such as protein precipitation, dry down, and reconstitution, were eliminated (Fig. 1). To further increase throughput, two separate incubations were monitored simultaneously by alternating injections of two sample vials.

2.3.2.1. Plasma. Plasma sample (10 μL, 0.8 mg total protein, diluted with 350 μL of PBS buffer) and 40 μL of compound were added to the incubation vial with accurate timing using a time program on the CTC autosampler. Analysis cycle time was 5 min, so that samples could be injected at 5-min intervals. Two vials of incubation samples were monitored simultaneously by alternating injections. A typical sequence of events is shown in Table 1. Analysis was by LC–MS with online extraction. A zero time point sample was prepared similarly except that 360 μL of PBS buffer was used instead of plasma.

2.3.2.2. Liver S9 fraction. The procedure was similar to that for plasma (Section 2.3.2.1), except that 20 μL of liver S9 fraction (0.4 mg total protein, diluted with 300 μL of KPi buffer) was used instead of plasma. NADPH-generating system (40 μL) was added prior to addition of compound. A zero time point sample was prepared similarly except that 300 μL of 5% TFA instead of KPi buffer was used.

2.3.2.3. LC–MS conditions. The online extraction column used was a Cohesive Turbo Flow column (Franklin, MA) and the analytical column was a YMC C18 basic, 5 μm, 50 mm × 2 mm from Waters Chromatography (Milford, MA). The LC flow rate and gradient are tabulated in Table 2. Injection volume was 15 μL. The mass spectrometer was in full scan mode from 400 to 500 amu. The advantages of this mode were that prior tuning was unnecessary, and the data could also be used for metabolite searches that could not be pre-determined. Data analysis was performed By XIC of *m/z* 433 and 461 for the active drug and prodrug, respectively.

Table 1
Sequence of events for the automated online method

Time (min.)	Cooler	Compound Tray	Incubator	Injector	Action	Vial 1 incubation time point	Vial 2 incubation time point
0.0	Vial 1 →		→		Transfer. Shake. Preheat		
4.5	Vial 2 →		→		Transfer. Shake. Preheat		
		40 μL compound →			Add compound to Vial 1. Shake	0 min	
5.0			15 μL →		Inject 15 μL from Vial 1. Shake	0.5 min	
9.5		40 μL compound →			Add compound to Vial 2. Shake		0 min
10.0			15 μL →		Inject 15 μL from Vial 2. Shake		0.5 min
15.0			15 μL →		Inject 15 μL from Vail 1. Shake	10 min	
20.0			15 μL →		Inject 15 μL from Vial 2. Shake		10 min
25.0			15 μL →		Inject 15 μL from Vail 1. Shake	20 min	
30.0			15 μL →		Inject 15 μL from Vial 2. Shake		20 min
35.0			15 μL →		Inject 15 μL from Vail 1. Shake	30 min	
40.0			15 μL →		Inject 15 μL from Vial 2. Shake		30 min
45.0			15 μL →		Inject 15 μL from Vail 1. Shake	40 min	
50.0			15 μL →		Inject 15 μL from Vial 2. Shake		40 min
55.0	← Vial 1				Return Vial 1 to Cooler		
	← Vial 2				Return Vial 2 to Cooler		

Vial 1 containing matrix A and vial 2 containing matrix B were incubated and analyzed alternately, generating data for 0.5, 10, 20, 30, and 40-min time points.

Table 2
LC parameters and valve-switch timing for automated online method

Minutes	LC gradient: flow (mL/min)	A (%)	B (%)	Switching valve position on LCQ
0	0.3	100	0	Waste
0.05	2.5	100	0	
0.5	2.5	100	0	
0.6	0.3	100	0	Mass spectrometer
2.4	0.3	30	70	
2.8	0.3	2	98	
3.1	0.3	2	98	
3.6	0.3	100	0	
4.6	0.3	100	0	Waste

3. Results and discussion

Typical chromatograms from the conventional method and from the automated online method are shown in Figs. 2 and 3, respectively. The separation between the active drug and prodrug are similar in the chromatograms from both methods. Internal standard was used in the conventional method but not in the online method. The ion trap mass spectrometer in full scan mode had higher noise than the triple quadrupole mass spectrometer in MRM mode, but the sensitivity was sufficient for quantitation in the scope of this study.

Since there is an inherent time lapse between the addition of test compound and sampling of the first time point, a true "0 min" is practically not possible. In both the conventional and automated methods, a reasonable time lapse for the first time point following the initiation of the reaction is 0.5 min.

Since the active drug in this case is stable in plasma and liver S9 fraction, the 0.5 min sample in the automated method was theoretically the same in concentration as the 0 min sample, so it is used as the "0 time point" sample for the calculations. However, since the prodrug is convertible to the active drug in plasma or liver S9, the "0 time point" sample was prepared by inactivation of the enzyme using acetonitrile or 5% TFA prior to sampling.

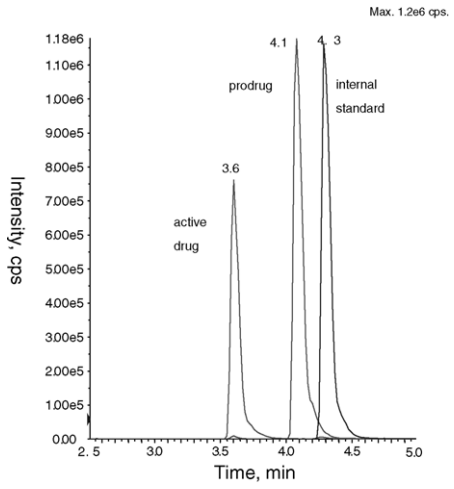


Fig. 2. Typical chromatogram (MRM) from conventional method: 45 min sample of prodrug incubated in dog liver S9 fraction.

For both the conventional and the automated online methods, percent active drug and percent prodrug at time point t were calculated as follows:

$$\%\,\text{Active drug} = \frac{\text{Peak area of active drug at } t \text{ minutes}}{\text{Peak area of active drug at 0 minute}} \times 100\%$$

$$\%\,\text{Prodrug} = \frac{\text{Peak area of prodrug at } t \text{ minutes}}{\text{Peak area of prodrug at 0 minute}} \times 100\%$$

The disappearance of the prodrug and the correspondent increase in active drug for the five species studied are shown in Tables 3 and 4. Data for one of the species, namely the rat, are plotted in Fig. 4 as an example. Comparing the data from the conventional method and the online method, the curve shapes are similar except in the rhesus monkey liver S9 and the dog plasma, where the conventional method had an apparent outlier point at 5 min in both cases. Comparing across species, all plasma studies showed slow conversion except for the rat. The liver S9 studies showed faster conversion, especially in the rat, and the slowest conversion was in the dog. Results from both methods were consistent.

Half-lives ($t_{1/2}$) were calculated based on the natural log of % prodrug curve using the equation [31]:

$$t_{1/2} = \ln 0.5/-k = -0.693/-k = 0.693/k$$

where $-k$ is the slope of the curve.

An example of the calculations is shown in Fig. 5 using the data from three liver S9 fraction samples by the conventional method. The slope of the curve is determined from the part of the curve before it starts to plateau off. The half-lives calculated from the incubation curves are tabulated with the percent active drug formed versus species in Table 5.

For the online method, the half-lives were calculated both with and without the 0.5 min time point. In most cases, the results are comparable to those obtained with the conventional method. There is significant difference, however, in the rat plasma and liver S9 samples where the conversion was fast before 10 min. The missing 0.5 min data from the conventional method and the missing 5 min data from the online method apparently caused the difference in the half-lives calculated. In the rhesus monkey and dog plasma samples, when the half-lives were calculated without the 0.5 min for the online method, the results were closer to those from the conventional method. The 0.5 min data drastically changes the initial slope, and at the same time this time point is not available from the conventional method.

Overall, the automated online method yielded data similar to those from the conventional method, although there was some trade-off between sensitivity and speed since full scan mode was used. In the rat samples (Fig. 4), the prodrug probably converted so fast that the amount of active drug formed was already significant by 0.5 min. If this were anticipated ahead of time, a 0.5 min time point could have been added in the conventional method. It is often difficult, though, to manually collect sample as early as 0.5 min especially when the op-

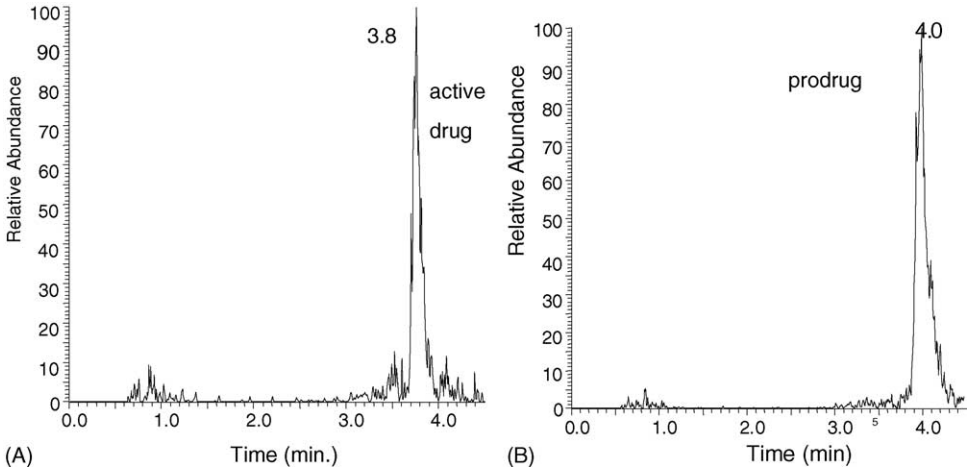


Fig. 3. Typical chromatograms from automated online method: 45 min sample of prodrug incubated in dog liver S9. (A) XIC for active drug, 100% = 1.8×10^6 cps. (B) XIC for prodrug, 100% = 5.0×10^6 cps.

Table 3
% Active drug and % prodrug vs. time in plasma study by (A) conventional method and (B) automated online method

Minutes	Species									
	Human		Cynomolgous monkey		Rhesus monkey		Rat		Dog	
	Active drug[a]	Prodrug[a]	Active drug[a]	Prodrug[a]	Active drug[a]	Prodrug[a]	Active drug[a]	Prodrug[a]	Active drug[a]	Prodrug[a]
(A)										
0	3	100	1	100	1	100	7	100	1	100
5	7	100	2	100	2	84	80	0	1	118
15	10	81	3	87	2	86	90	0	1	77
45	11	76	3	82	5	85	88	0	4	77
(B)										
0.0	1	100	1	100	1	100	1	100	1	100
0.5	4	119	4	96	3	83	57	56	2	57
10.0	4	122	5	115	4	82	87	0	2	60
20.0	4	113	6	118	5	84	83	0	3	68
30.0	5	122	7	118	4	80	87	0	3	71
40.0	5	111	8	121	5	86	87	0	3	72

[a] % Present.

Table 4
% Active drug and % prodrug vs. time in liver S9 fraction study by (A) conventional method and (B) automated online method

Minutes	Species									
	Human		Cynomolgous monkey		Rhesus monkey		Rat		Dog	
	Active drug[a]	Prodrug[a]	Active drug[a]	Prodrug[a]	Active drug[a]	Prodrug[a]	Active drug[a]	Prodrug[a]	Active drug[a]	Prodrug[a]
(A)										
0	0	100	0	100	2	100	0	100	2	104
5	71	38	19	66	42	85	80	15	21	92
15	88	1	74	9	44	54	100	0	29	80
45	100	0	102	0	96	0	100	0	72	67
(B)										
0.0	8	100	13	100	54	100	10	100	2	100
0.5	24	86	21	109	66	90	45	40	23	94
10.0	85	9	80	24	126	28	100	8	49	72
20.0	94	6	96	15	126	7	102	8	55	70
30.0	94	5	94	14	131	4	106	6	63	70
40.0	98	5	96	9	128	4	102	4	70	72

[a] % Present.

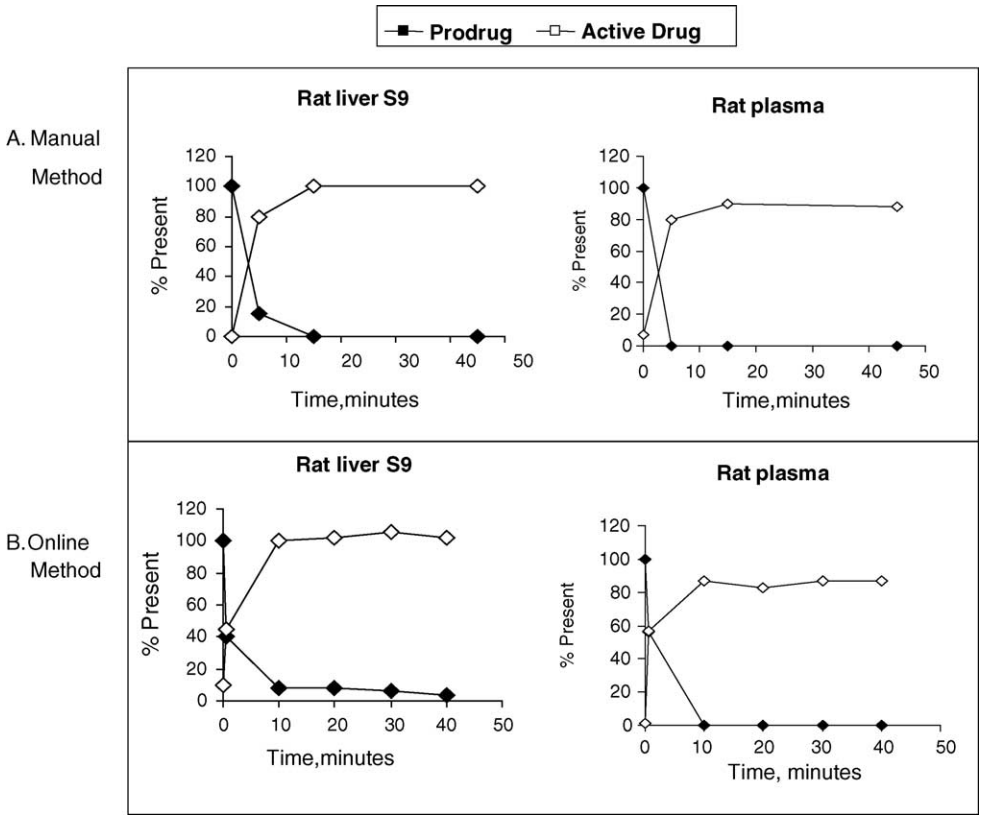
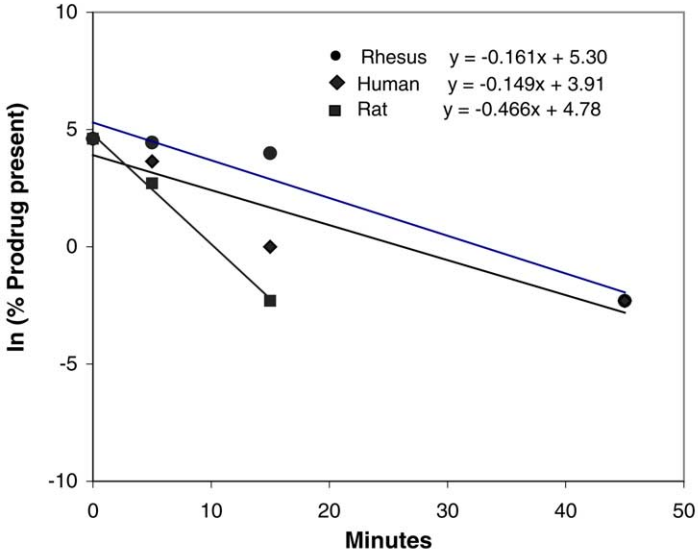


Fig. 4. In vitro conversion of prodrug to active drug in rat liver S9 fraction and plasma using: (A) conventional manual method and (B) automated online method.

Table 5
Half life ($t_{1/2}$) and % active drug formed vs. species in (A) plasma and (B) liver S9 studies by conventional and automated online methods

	Conventional method			Online method				
	k	$t_{1/2}$ (min)	% Drug formed[a]	k[b]	$t_{1/2}$ (min)	k[c] (without 0.5 min)	$t_{1/2}$ (min)	% Drug formed[a]
(A) Plasma								
Human		N/A	11		N/A		N/A	5
Cynomolgous		N/A	3		N/A		N/A	8
Rhesus	0.008	83	5	0.012	60	0.009	80	5
Rat	1.382	0.5	88	0.680	1.0	0.691	1.0	87
Dog	0.019	36	4	0.026	27	0.019	36	3
	Conventional method			Online method				
	k[d]	$t_{1/2}$ (min)	% Drug formed[a]	k[e]	$t_{1/2}$ (min)	k[c] (without 0.5 min)	$t_{1/2}$ (min)	% Drug formed[a]
(B) Liver S9								
Human	0.149	4.6	100	0.147	4.7	0.141	4.9	98
Cynomolgous	0.157	4.4	102	0.103	6.7	0.095	7.3	96
Rhesus	0.161	4.3	96	0.131	5.3	0.133	5.2	128
Rat	0.466	1.5	100	0.215	3.2	0.253	2.7	102
Dog	0.017	41	72	0.018	39	0.018	39	70

[a] After 45 min.
[b] From curve of 0, 0.5, 10 min.
[c] From curve of 0, 10, 20 min.
[d] For rat and dog, 45 min time point is not used for determination of k due to already plateauing.
[e] From curve of 0, 0.5, 10, 20 min.



	ln (% prodrug present)		
Time point (min.)	Human	Rhesus	Rat
0	4.61	4.61	4.61
5	3.64	4.44	2.71
15	0.00	3.99	-2.30
45	-2.30	-2.30	-2.30
k*	0.149	0.161	0.466
t½ (min.)	4.6	4.3	1.5

* For rat, 45 min timepoint is not used for determination of k since it has plateaued.

Fig. 5. Example of calculations of half-life ($t_{1/2}$): human, rhesus monkey, and rat liver S9 fractions from conventional method.

erator is still busy with multi pipetting steps prior to 0.5 min. Whereas, with the online method, the 0.5 min time point can be easily programmed. Subsequent time points can be as close as the analytical cycle time will allow, which, in this case, is 5 min. That means time points could be every 5 min if desired.

The dog samples show a slow conversion compared to other species, but the curves are similar in both methods. In some cases (rhesus liver S9 fraction and dog plasma), the curves from the automated method are smoother than from the manual method.

Alternating vial injections doubled the throughput, yielding incubation curves of 0.5, 10, 20, 30, 40 min for each. Ten such incubation curves were performed in 10 h unattended versus 4 days by the conventional method.

Since there were no transfer steps after the incubation was started, internal standard was not used in the online method. Results turned out to be comparable with those from the conventional method.

4. Conclusions

The automated online method was tested with a prodrug conversion study of a test compound using five different species and two different biological matrices. The results give a good approximation of the prodrug conversion, as compared to the conventional method.

The results also show multiple advantages of the automated method versus the conventional method. These advantages include immediate sampling and analysis of the incubation sample, eliminating pipetting, deproteination, dry-down, and reconstitution steps (as in the conventional method), and saving time, space and cost of equipment and solvents.

This online method should also provide more accurate internal timing and minimize errors from multi-step transfers in sample preparation that sometimes lead to compound degradation. The method also generates real-time chromatographic results during incubation such that the operator can extend time points on the spot as desired by simply adding a line in the sequence.

This automated online method should be very useful for high-throughput prodrug stability studies in drug discovery.

Acknowledgements

The test compounds used in this study were provided by David Jackson and Mark Reynolds at Medicinal Chemistry, Genentech Inc. We gratefully acknowledge Miryam

Kadkhodayan at Bioanalytical R&D, Genentech Inc., for developing the parameters used in the Cohesive turbo flow method and John Celani of LEAP Technologies for customizing the LEAP part of the system for this work. We also acknowledge Jason Halladay, Matthew Baumgardner, Douglas Leipold, and Sara Kenkare for their technical input.

References

[1] E. Pop, S. Rachwal, J. Vlasak, A. Zharikova, L. Prokai, J. Pharm. Sci. 88 (1999) 1156.
[2] R.S. Obach, Curr. Opin. Drug Discovery Dev. 4 (2001) 36.
[3] K.K. Hong, C.C. Lin, D. Parker, J. Veals, J. Lim, P. Likhari, P. Statkevich, A. Marco, A.A. Nomeir, J. Chromatogr. B 703 (1–2) (1997) 225.
[4] J. Henion, S.J. Prosser, T.N. Corso, G.A. Schultz, Am. Pharm. Rev. 3 (2000) 19.
[5] K.A. Cox, R.E. White, W.A. Korfmacher, Comb. Chem. High Throughput Screening 5 (2002) 29.
[6] W.A. Korfmacher, K.A. Cox, M.S. Bryant, J. Veals, K. Ng, R. Watkins, C.C. Lin, Drug Discovery Today 2 (1997) 532.
[7] R.E. White, Annu. Rev. Pharmacol. Toxicol. 40 (2000) 133.
[8] R. Xu, C. Nemes, K.M. Jenkins, R.A. Rourick, D.B. Kassel, C.Z.C. Liu, J. ASMS 13 (2) (2002) 155.
[9] W.A. Korfmacher, C.A. Palmer, C. Nardo, K. Dunn-Meynell, D. Grotz, K. Cox, C. Lin, C. Elicone, C. Liu, E. Duchoslav, Rapid Commun. Mass Spectrom. 13 (1999) 901.
[10] R.B. Van Breemen, D. Nikolic, J.L. Bolton, Drug Metab. Dispos. 26 (1998) 85.
[11] R. Riedel, P.M. Ueland, A.M. Svardal, Clin. Chem. 41 (1995) 1164.
[12] L. Yang, R.P. Clement, B. Kantesaria, L. Reyderman, F. Beaudry, C. Grandmaison, L. Di Donato, R. Masse, P. Rudewicz, J. Chromatogr. B 792 (2003) 229.
[13] J. Hempenius, R.J.J.M. Steenvoorden, F.M. Lagerwerf, J. Wieling, J.H.G. Jonkman, J. Pharm. Biomed. Anal. 20 (1999) 889.
[14] I.D. Davies, J.P. Allanson, R.C. Causon, Chromatographia 52 (Suppl.) (2000) S-92.
[15] J. Hempenius, J. Wieling, J.P.G. Brakenhoff, F.A. Maris, J.H.G. Jonkman, J. Chromatogr. B 714 (1998) 361.
[16] A. Pruvost, I. Ragueneau, A. Ferry, P. Jaillon, J.M. Grognet, H. Benech, J. Mass Spectrom. 35 (2000) 625.
[17] S. Calderoli, E. Colombo, E. Frigerio, C.A. James, M. Sibum, J. Crommen, H. Lingeman, J. Pharm. Biomed. Anal. 32 (2003) 601.
[18] S.R. Needham, M.J. Cole, H.G. Fouda, J. Chromatogr. B 718 (1998) 87.
[19] M. Breda, G. Basileo, G. Fonte, J. Long, C.A. James, J. Chromatogr. A 854 (1999) 81.
[20] H.K. Lim, K.W. Chan, S. Sisenwine, J.A. Scatina, Anal. Chem. 73 (2001) 2140.
[21] M. Jemal, Rapid Commun. Mass Spectrom. 13 (1999) 2125.
[22] J.T. Wu, H. Zeng, M. Qian, B.L. Brogdon, S.E. Unger, Anal. Chem. 72 (2000) 61.
[23] E. Hogendoorn, P.V. Zoonen, A. Polettini, G.M. Bouland, M. Montagna, Anal. Chem. 70 (1998) 1362.
[24] Y. Hsieh, M. Bryant, G. Gruela, J. Brisson, W.A. Korfmacher, Rapid Commun. Mass Spectrom. 14 (2000) 1384.
[25] Y. Hsieh, J. Brisson, K. Ng, R. White, W.A. Korfmacher, Analyst 126 (2001) 2139.
[26] Y. Hsieh, J. Brisson, K. Ng, R. White, W.A. Korfmacher, J. Pharm. Biomed. Anal. 27 (2002) 285.
[27] Y. Hsieh, M. Bryant, J. Brisson, K. Ng, W.A. Korfmacher, J. Chromatogr. B 767 (2002) 353.
[28] O. Shirota, J. Microcol. 7 (1995) 29.
[29] G. Wang, Y. Hsieh, Y. Lau, K.C. Cheng, K. Ng, W.A. Korgmacher, R.E. White, J. Chromatogr. B 780 (2002) 451.
[30] G. Wang, Y. Hsieh, Am. Lab. 12 (2002) 24.
[31] C.D. Smith, A.M. Reynard, Essentials of Pharmacology, W.B. Saunders Co., Philadelphia, 1995.