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Short communication

Semi-automated determination of plasma stability of drug discovery compounds using liquid chromatography–tandem mass spectrometry

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Abstract

A simple procedure for the measurement of stability of drug candidates in plasma was developed to eliminate the traditional labor-intensive and time-consuming sample preparation procedures that are typically used for these studies. The procedure makes use of a thermostatic autosampler as an incubator combined with the direct plasma injection method based on high-performance liquid chromatography (HPLC) coupled to tandem mass spectrometry (MS–MS). Untreated human, monkey, mouse and rat plasma containing the test compound was directly injected into a mixed-function column for on-line protein removal and chromatography. The test compound and its biotransformation product were separated via HPLC and monitored using the tandem mass spectrometer. The need for adequate chromatographic separation of the test compound (M) from its carboxylic acid metabolite (M+1) is demonstrated. Plasma samples from four different species at specified incubation temperatures were sequentially assayed in one analytical procedure. The injection-to-injection time was about 6 min. The peak responses of the test compound in individual plasma samples were repeatedly determined every 24 min. The retention times and peak shape of all analytes were found to be consistent throughout the experiments. The stability of the test compound in plasma was found to be a function of animal species, incubation time and temperature. The test compound was rapidly degraded in rat plasma at 37 °C, but it could be stabilized by adding sodium thiosulfate.

Keywords: Drug stability

1. Introduction

Drug stability in plasma is a concern in both drug discovery and development areas [1–3]. Except for ‘‘pro-drugs’’, drug candidates undergoing rapid degradation in plasma generally have undesirable pharmacokinetic parameters and pose analytical challenges. For drug development candidates, plasma instability will complicate validation of the bioanalytical assays for the unstable pharmaceutical components or their metabolites in plasma samples [4]. For these reasons, plasma stability data could be useful in drug discovery programs to avoid the selection of unstable drug candidates. Conventionally, the investigation of drug stability in plasma has required labor-intensive and time-consuming proce-

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dures, typically including sequential plasma extraction at each incubation time interval and each incubation temperature. Plasma samples from individual incubation time points were normally prepared by using one of several macromolecule removal techniques such as protein precipitation, solvent extraction or solid-phase extraction prior to HPLC analysis [5]. Furthermore, each plasma sample preparation method demands tedious analytical procedures. Therefore, it has not been realistic to evaluate a large number of potent discovery compounds for their plasma stability. These needs triggered the effort to develop a higher throughput plasma stability assay based on a semi-automated direct plasma injection methodology.

HPLC–MS–MS technology has significantly reduced the necessity for extensive sample clean-up procedures due to its inherent selectivity and sensitivity and has become the major bioanalytical tool for drug assay in most pharmaceutical companies [6,7]. However, the step to remove macromolecular compounds such as proteins from biological fluids is still essential to prevent column clogging from protein precipitation as a result of organic modifier in the mobile phase in reversed-phase chromatography. Many on-line extraction approaches for simplified sample preparation procedures have been explored to allow for direct analysis of pharmaceutical compounds in biological samples [8–10]. Normally, these direct plasma injection methods utilize dual-column HPLC systems that adapt one extraction column such as alkyl-diol silica [10] for on-line purification followed by an analytical column for chromatographic separation. Previously, we reported a simple and efficient direct HPLC–MS–MS method that used a single column to perform all the functions required for simultaneous direct plasma analysis of multi-components in various plasma samples [11–14]. The accuracy of the analytical results by the direct plasma injection method has been shown to be comparable with other approaches such as SPE or the protein precipitation technique [12–14]. In this work, we extend the utility of this direct single column HPLC–MS–MS system for drug plasma stability experiments. In the described example, we are able to continuously and sequentially monitor the responses of the test compound and its carboxylic acid metabolite (M+1) in rat, mouse, monkey and human plasma. Due to the 1 Da difference in the molecular mass of the test compound (M) and its carboxylic acid metabolite (M+1), the two compounds could not be distinguished solely on the basis of their MS–MS response characteristics. Therefore, it was important to obtain chromatographic separation of the carboxylic acid metabolite and the test compound in order to develop an assay for the two compounds. The stability results of the test compound in rat plasma at 37 °C obtained from the typical manual protein-precipitation method were compared to the proposed semi-automated direct plasma injection method using HPLC–MS–MS and the two methods produced results that are in good agreement. Finally, the stability of the test compound in the rat plasma was found to be controllable by addition of sodium thiosulfate under acidic conditions.

2. Experimental

2.1. Reagents and chemicals

The test compound (M) and its biotransformation product (M+1) are new chemical entities made by Schering Plough Research Institute; their partial chemical structures are shown in Fig. 1. Acetonitrile, trifluoroacetic acid (TFA) (HPLC grade), sodium thiosulfate (Certified A.C.S.) were purchased from Fisher (Pittsburgh, PA, USA). Acetic acid and ammonium acetate were purchased from Aldrich (Milwaukee, WI, USA). Deionized water was generated from a Milli-Q water purifying system purchased from Millipore (Bedford, MA, USA) and house high-purity nitrogen (99.999%) was used. Drug-free human, monkey, mouse and rat plasma samples were purchased from Bioreclamation (Hicksville, NY, USA). Mobile phase A and B were 4 m*M* ammonium acetate and 0.015% TFA in water–acetonitrile (90:10) and 4 m*M* ammonium acetate in water–acetonitrile (10:90), respectively.

2.2. Equipment

LC–MS–MS analysis was performed using a PE Sciex (Concord, Ontario, Canada) Model API 3000 tandem triple quadrupole mass spectrometer

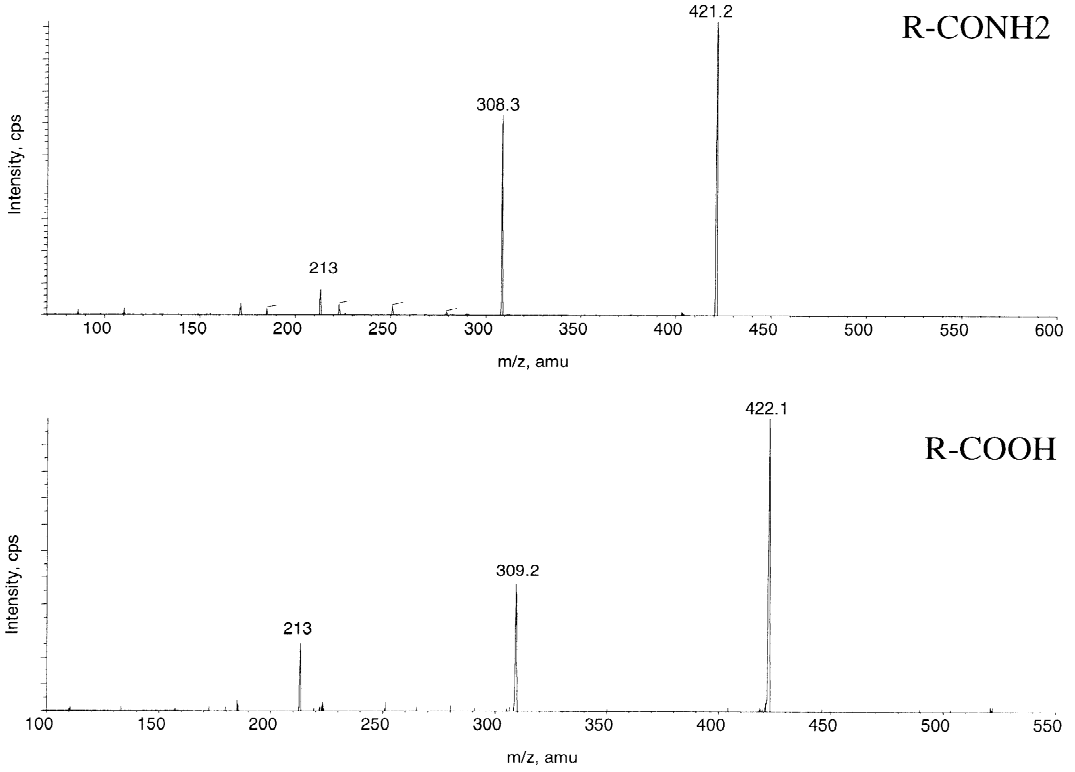

Fig. 1. Product ion spectra of (top) the test compound and (bottom) its carboxylic acid metabolite (M−1).

equipped with the atmospheric pressure chemical ionization (APCI) interface. The HPLC system consisted of a Leap autosampler (also used as the incubator) with a sample tray set at 4, 20 or 37 °C (LEAP Technologies, Carrboro, NC, USA), Shimadzu on-line degasser, LC-10AD VP pump and LC-10A VP controller (Columbia, MD, USA). For the single column direct injection method, a Capcell MF C_8 column (4.6×50 mm) from Phenomenex (Torrance, CA, USA) was used as both extraction and analytical column.

2.3. Plasma preparation

A stock solution of the test compound was prepared as a 1 mg/ml solution in methanol. For the semi-automated procedure, plasma stability samples were made by spiking the test compound into the blank plasma from the stock solution to yield a concentration of 30 μ*M*. The spiked plasma samples were then immediately placed into a 96-well plate in the thermostatic autosampler at various incubation temperatures for direct and sequential injection into the HPLC–MS–MS system. For the traditional protein precipitation method, the stability plasma samples were incubated in a thermostatic incubator. A 50-μl plasma sample from the incubation mixture was placed into a test-tube approximately at 15, 30, 40 and 100 min and then mixed with 150 μl acetonitrile solution for protein precipitation. After centrifugation, the supernatant was then transferred into a 96-well plate for HPLC–MS–MS analysis. Stabilization of the test compound was achieved by addition of 40 μl of 20% sodium thiosulfate solution and 10 μl of 20% acetic acid into 1 ml rat plasma (pH≈5.5).

2.4. Chromatographic conditions

The instrumental configurations for the single column direct plasma injection method were reported previously [11]. The LC flow-rate was constant at 1

ml/min. A 10-μl portion of the plasma stability sample was injected by the autosampler directly onto the CAPCELL MF C_8 column with a mobile phase A and the divert valve (post-column) switched to waste to remove the macromolecules from the plasma matrix. Macromolecules such as protein passed quickly through the column due to the restricted access to the surface of the packing while the column retained the drug molecules on the bonded hydrophobic phase. After 2 min, the column effluent was diverted from waste to the mass spectrometer for analyte detection and the LC pump was switched from mobile phase A to mobile phase B over 2.5 min to elute and separate all analytes. The retention times for all compounds of interest were all less than 4 min. The experimental procedure to explore apparent recovery yield of the test compound through the CAPCELL column was described elsewhere [12].

2.5. Mass spectrometric conditions

The mass spectrometer was operated in the positive ion mode. The heated pneumatic nebulizer probe conditions were as follows: 450 °C temperature setting, 80 p.s.i. nebulizing gas pressure, 1.0 l min^{-1} auxiliary gas flow, 0.9 l min^{-1} curtain gas flow-rate. The MS–MS reactions selected to monitor the test compound and its carboxylic acid metabolite (M+1) were the transition from m/z 520 and 521 (MH^+ ions), to product ions at m/z 421 and 422, respectively. The protonated molecules were fragmented by collision-activated dissociation with nitrogen as collision gas at a pressure of instrument setting 6. The collision-offset voltage was set at 40 V.

3. Results and discussion

The principal objective of this study was to semi-automate the experimental procedure for stability evaluation of the drug discovery compounds in plasma by combination of an autosampler (also used as the incubator) and the direct plasma injection approach. This new procedure is much easier than the time-consuming and labor-intensive sample preparation procedures traditionally used for drug stability studies. The role of PCMF column for direct plasma injection is to exclude macromolecules such as protein quickly due to the restricted access to the surface by large bonded hydrophilic groups and to separate small drug molecules by interaction with the small bonded hydrophobic group [15]. For the proposed automated method, individual rat, mouse, monkey and human plasma samples were first placed in the thermostatic autosampler which was programmed for sequential injection into the HPLC–APCI-MS–MS system. The peak responses of the test compound and its metabolite (M+1) were simultaneously monitored from plasma of one of the four species (rat, mouse, monkey and human) in sequential order every 6 min (the run cycle time of the assay). As shown in Table 1, these plasma samples were assayed again for seven repetitive injections after first injection period following the same injection order with a constant extended reaction time for each plasma sample. No significant change in sample concentration due to evaporation was observed.

Fig. 2 shows the mass chromatograms of the test compound in rat plasma following different incubation times at 37 °C; these chromatograms show that the peak area of the test compound decreased as the incubation time increased. Fig. 3 displays the change in the peak responses of the test compound in each plasma sample obtained at different incubation times. These data indicate that the test compound is stable in mouse, monkey and human plasma within the 2-h incubation time at 37 °C, but rapidly degraded in rat plasma (20% remaining relative to the peak response from first injection in the 2-h incubation time) at 37 °C. Fig. 4 shows the mass chromatograms of the carboxylic acid metabolite (M+1) in the rat plasma following different incubation times at 37 °C. The growth of the first peak areas of the degradant (M+1) in the rat plasma as a function of incubation times corresponds well to the disappearance of the

Table 1
Arrangement and sequential injection of plasma samples in the 96-well plate

Well	Plasma species	Injection sequence
1	Rat	1 5 9 13 17 21 25
2	Mouse	2 6 10 14 18 22 26
3	Monkey	3 7 11 15 19 23 27
4	Human	4 8 12 16 20 24 28

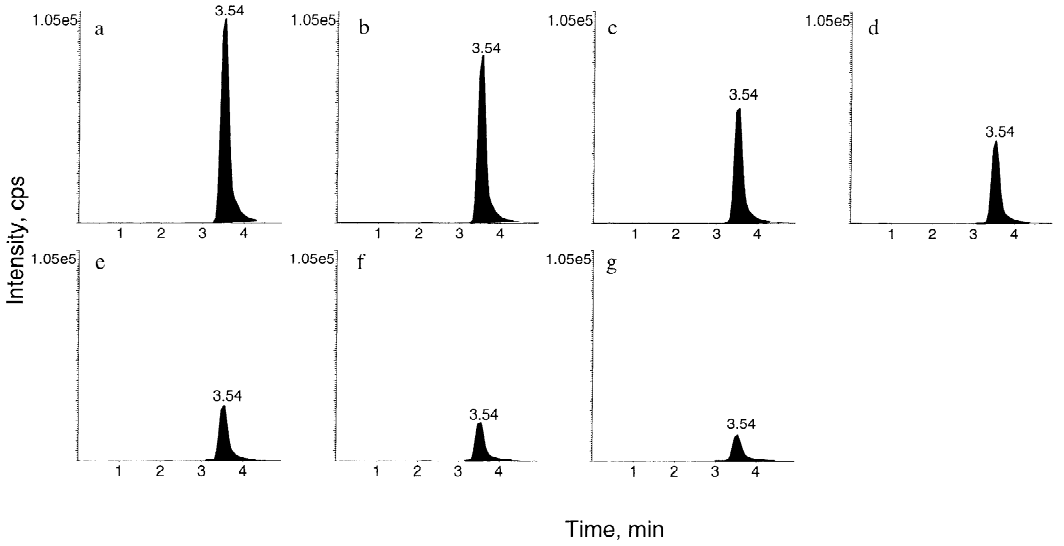

Fig. 2. Direct HPLC–APCI-MS–MS chromatograms of the test compound in rat plasma after (a) 5-min, (b) 29-min, (c) 53-min, (d) 77-min, (e) 125-min, (f) 149-min and (g) 173-min incubation at 37 °C.

test compound, shown in Fig. 3. The signals of the second peak in the mass chromatograms (Fig. 4) were contributed from the test compound (M) due to the M+1 isotope (23% relative abundance at $m/z=$ 521) showing the need for chromatographic separation between the test compound and its M+1 metabolite. The PCMF column was not expected to offer large plate numbers for analyte separation, however, it provides sufficient chromatographic efficiency for high speed HPLC–MS–MS analysis. As shown in Fig. 4, baseline separation of the parent compound and its M+1 metabolite was achieved with the PCMF column. Also, no interference peak was observed in the mass chromatograms of the two analytes obtained from the blank rat plasma (data not shown). The retention times and peak shape for all analytes (as indicated in Figs. 2 and 4) were found to be reproducible throughout the experiment. The proposed method sequentially provides the results of drug stability in plasma from four different species in one simple experiment. The changes in the peak areas of both analytes in the rat plasma are plotted against incubation times in Fig. 5. The degradation rates of the test compound in the rat plasma substantially decrease after the 2-h incubation at 37 °C while the growth rates of the M+1 metabolite appear to be reaching chemical equilibrium. The degradation reaction observed in this study was possibly catalyzed by an amidase in the rat plasma [16].

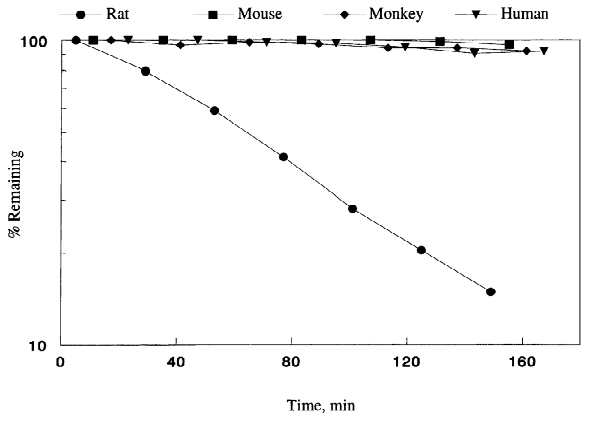

Fig. 3. Stability of the test compound in rat, mouse, monkey and human plasma as a function of incubation time at 37 °C.

It is well-known that the temperature plays an important role in the reaction rates of enzymatic or non-enzymatic reactions. As illustrated in Fig. 6, the drug plasma stability improved as the incubation temperature decreased. Furthermore, the test compound is found to be stable in rat plasma for the 2-h incubation time at 4 and 20 °C following the addition of sodium thiosulfate to the sample. One concern

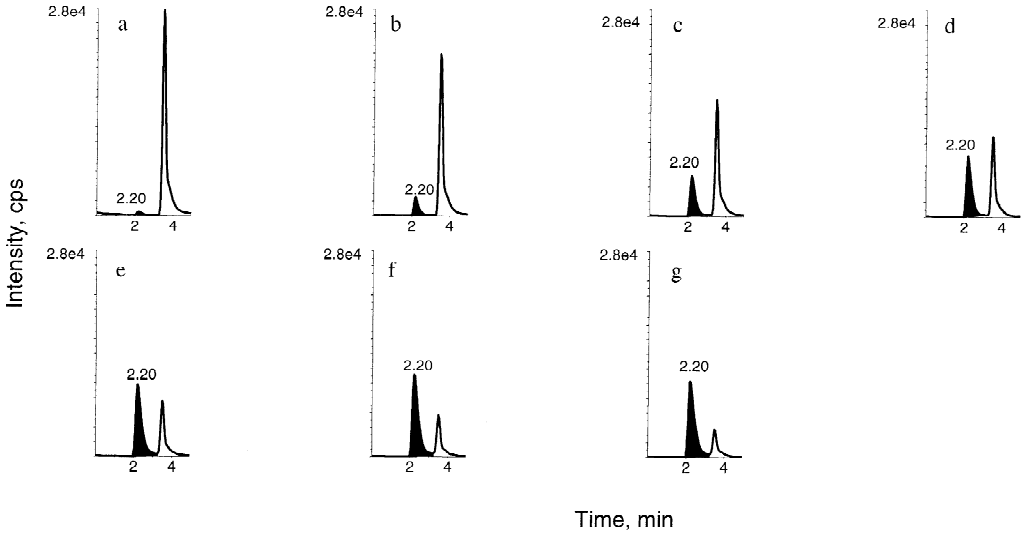

Fig. 4. Direct HPLC–APCI-MS–MS chromatograms of the carboxylic acid metabolite in rat plasma after (a) 5-min, (b) 29-min, (c) 53-min, (d) 77-min, (e) 125-min, (f) 149-min and (g) 173-min incubation at 37 °C.

when placing plasma samples in a 96-well polypropylene plate was heat transfer. However, as demonstrated in Fig. 6, the results of rat plasma stability of the test compound obtained by the proposed method are in good agreement with those obtained by the traditional manual incubation method.

Comparison of the peak area responses of the test compounds in spiked plasma with those from the spiked supernatant solution using the protein precipitation technique provided an indication of recovery for each drug candidate for this on-line column extraction procedure as described previously [11–14]. The loss of signal was due to the sum of losses from the extraction efficiency of the analytes through the PMCF column and the other possible negative effects such as matrix ion suppression. The apparent on-column recovery of the test compound was studied with four different plasma samples spiked at the 250 ng/ml concentration level. The apparent

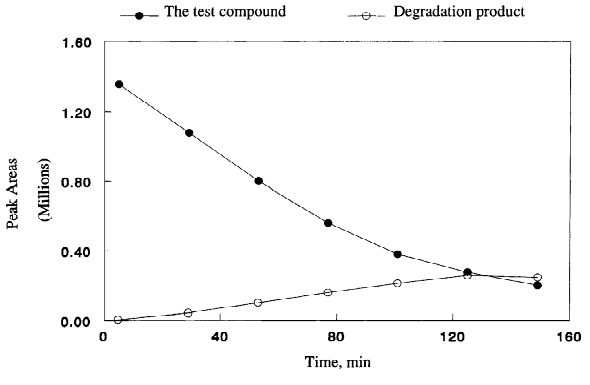

Fig. 5. The disappearance of the test compound in rat plasma is correspondent to the growth of its M+1 metabolite.

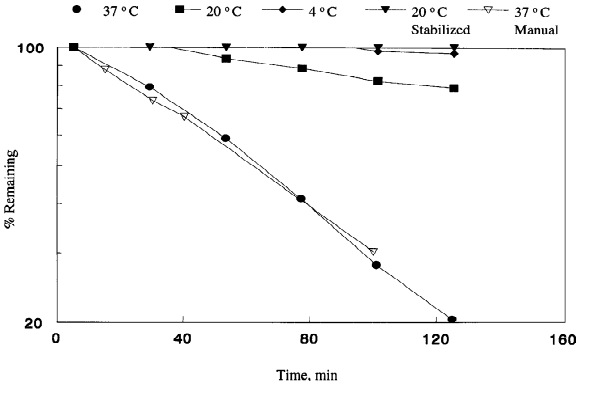

Fig. 6. Stability of the test compound in raw and stabilized rat plasma obtained by the conventional manual method and the proposed automated direct injection method as a function of incubation temperature.

on-column recovery values for the test compounds (five sample injections) using the direct injection method were determined to be 98.8% (%CV=5.7), 95.8% (%CV=4.1), 91.8% (%CV=3.4) and 90.3% (%CV=6.4) in rat, mouse, monkey and human plasma, respectively. So far, we have tested over 25 drug candidates from different discovery programs based upon this simple procedure which has provided plasma stability data for rat, mouse, monkey and human plasma for these compounds.

4. Conclusions

A simple procedure which uses an autosampler as an incubator combined with a single mixed-function column LC–APCI-MS–MS system has been shown to provide plasma stability results for drug candidates. The proposed method should prove to be reliable as a screen-type assay for evaluation of drug plasma stability.

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