Stereoselective Metabolism of Prasugrel in Humans Using a Novel Chiral Liquid Chromatography-Tandem Mass Spectrometry Method

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ABSTRACT:
A liquid chromatography-tandem mass spectrometry method was developed to chromatographically separate the four stereoisomers of the active metabolite of prasugrel, R-138727, in human plasma after derivatization with bromomethoxyacetophenone to stabilize the molecule. This technique was designed to determine the relative contribution of each stereoisomer, based on statistical analyses of each stereoisomer’s chromatographic peak areas. The methodology was validated and used for the analysis of clinical samples in which R-138727 had been derivatized at the time of blood collection. This technique can be useful to determine the ratios of stereoisomers in biological samples (e.g., plasma) especially in situations in which authentic standards of each individual stereoisomer are scarce or unavailable. In humans, the metabolic formation of R-138727 from prasugrel was found to be stereoselective, where 84% of R-138727 was present as RS and RR, the two most pharmacologically potent isomers, whereas the SR and SS enantiomers accounted for 16%. The ratios of the R-138727 stereoisomers were consistent among subjects, regardless of the dose or time of sample collection or whether the blood was sampled after the first dose or after 4 weeks of therapy.

Prasugrel, a novel thienopyridine antiplatelet compound, is a prodrug that is rapidly hydrolyzed in vivo to the pharmacologically inactive thiolactone R-95913, which is then metabolized by various cytochrome P450 enzymes to the pharmacologically active R-138727 (Fig. 1) (Rehmel et al., 2006; Farid et al., 2007). The pharmacological action of prasugrel is the result of R-138727 binding irreversibly to the P2Y12 platelet adenosine diphosphate receptor, thus inhibiting platelet activation and aggregation (Jakubowski et al., 2006). R-138727 possesses two chiral centers, and it has been reported that the four stereoisomers possess differing antiplatelet activity, with the two stereoisomers with the R-configuration at the thiol group (R-125689 and R-125690) being the most potent (Hasegawa et al., 2005).

Xenobiotics that contain multiple chiral centers (stereocenters) pose a challenging dimension to the overall drug development process. The chemistry and pharmacological significance of multiple stereocenters have been reviewed (Testa et al., 1993; Tomaszewski and Rumore, 1994; Reist et al., 1995; Van Miert, 2003). A compound with two chiral centers can exist in four stereoisomeric forms: RR, SS, RS, and SR. Enantiomers are stereoisomers that are mirror images (RR and SS are enantiomers as are RS and SR). Diastereomers are stereoisomers but are not mirror images, e.g., RR is a diastereomer of both RS and SR; likewise, SS is a diastereomer of both RS and SR. Such molecules pose a significant challenge for the bioanalytical chemist when analyzing plasma samples for pharmacokinetic analyses (Marzo and Heftman, 2002). Typically, “pure” standards of these stereoisomers are not available; therefore, the molecule is quantified as a single peak (achiral assay) or as doublets (RR/SS and RS/SR) using reverse-phase chromatography.

An achiral assay that determines the concentration of R-138727 in human plasma samples (after derivatization to stabilize the molecule with 2-bromo-3’-methoxyacetophenone) has been validated and used to support clinical studies (Farid et al., 2007). However, the extent of formation of each of the R-138727 stereoisomers in animals or humans has not been investigated previously. The objective of this research was to develop an assay to separate and determine the relative contribution of each of the four stereoisomers to the total R-138727 present in human plasma after administration of varying single and multiple doses of prasugrel. The chiral assay was also designed to statistically identify divergence in the individual peak area ratios, if any, compared with the “standard” comparators [quality control (QC) samples]. The method used a stable isotope-labeled internal standard, and the sample preparation was similar to that of the achiral method. Theoretically, the ratio of the four peaks comprising R-138727 in the standards used in the assay should be equimolar (1:1:1:1); therefore, the calculated ratio is considered equivalent to 1:1:1:1.

ABBREVIATIONS: R-95913, 2-[2-oxo-6,7-dihydrothieno[3,2-c]pyridin-5(4H)-yl]-1-cyclopropyl-2-(2-fluorophenyl)ethanone; R-138727, 2-[1-2-cyclopropyl-1-(2-fluorophenyl)-2-oxoethyl]-4-mercaptop-3-piperidinylidene acetic acid; QC, quality control; HPLC, high performance liquid chromatography; LC/MS/MS, liquid chromatography/tandem mass spectrometry; SPE, solid phase extraction.
Materials and Methods

Reagents and Materials. All chemicals were of analytical grade and used without further purification. Methanol and acetonitrile were purchased from Honeywell Burdick & Jackson (Muskegon, MI), formic acid (96%), ammonium acetate (97%), ammonium hydroxide (28–30%), ammonium chloride, and 2-bromo-3'-methoxy-acetophenone (98%) from Sigma-Aldrich (St. Louis, MO), and dimethyl sulfoxide (99.9%) from Mallinckrodt (Hazelwood, MO). A Waters MilliQ system (Waters, Billerica, MA) was used to obtain purified water. ScreenMates 96-well plate vials (1.4 ml) and ScreenMates 96 Cap strips were purchased from Matrix Technologies (Cheshire, UK). Human EDTA plasma was purchased from Biochemical Pharmacologicals (Winchester, VA) and was used to prepare all control, standard curve, QC, and validation samples.

Analytical Standards. R-138727 and [2H₄]R-138727 were obtained from Sankyo Co., Ltd. (Tokyo, Japan) and stored at −20°C (Fig. 2). Additionally, small amounts of each of the R-138727 stereoisomers (R-125687, R-125688, R-125689, and R-125690) were also provided by Sankyo Co. for positive identification after chromatographic separation.

LC-MS/MS. A PE Sciex API 4000 mass spectrometer (Applied Biosystems, Foster City, CA) was used with a TurboIonSpray interface operated in positive ion mode. Nitrogen for use as the curtain and collision gases was from an Air Liquide in-house system. The high-performance liquid chromatography (HPLC) system comprised two Shimadzu LC-10ADvp pumps, a Shimadzu DGU-14A solvent degasser, a Shimadzu SCL-10Avp system controller (Shimadzu, Kyoto, Japan), a Jones Chromatography column heater (Jones Chromatography, Barnstaple, UK), and a Gilson 215 autosampler (Gilson, Inc., Middleton, WI). Samples (10 μl) were injected onto a 10 × 2 mm BetaBasic C4 Javelin Guard (Thermo Electron Corporation, Waltham, MA) connected to two (150 × 2 mm and 250 × 2 mm) ProntoSIL Chiral AXQN columns in series (MAC-MOD Analytical, Chadds Ford, PA) (Fig. 3). An isotropic mobile phase consisting of 200:1:2:0:6:0.0025 methanol/acetic acid/water/0.1 M ammonium acetate was used at a flow rate of 170 μl/min. The column temperature was maintained at 30°C. Methanol was used as the autosampler wash solution. The total cycle time was 20–25 min/injection.

R-138727 was monitored as its derivatized form by following the selected reaction monitoring transition from m/z 498.2 to m/z 348.3 using a dwell time of 1000 ms (Fig. 4). The deuterated internal standard ([2H₄]R-138727) was similarly monitored using the selected reaction monitoring transition from m/z 502.2 to m/z 352.3 using a dwell time of 200 ms. The following mass spectrometer settings were used: source temperature of 500°C, TurbolonSpray voltage of 5500 V, gas 1 setting at 50, gas 2 setting at 50, curtain gas setting at 10, collision gas setting at 4, declustering potential at 75 V, entrance potential at 10 V, and collision energy at 29 V.

Derivatization of R-138727 and the Internal Standard for Analysis. A 50 μg/ml stock solution of R-138727 or [2H₄]R-138727 in the derivatized form was prepared by the addition of 4.0 ml of 0.1 M ammonium hydroxide-0.1 M ammonium chloride (1:4, v/v) to 1.25 mg of R-138727, followed by 0.5 ml of 0.1 M 2-bromo-3'-methoxyacetophenone (the derivatizing reagent) for at least 10 min and dilution to 25 ml with acetonitrile as described previously (Farid et al., 2007). The internal standard was further diluted with acetonitrile to yield a 100 ng/ml working solution. All solutions were stored at −20°C in polypropylene vials.

Standard Curve and QC Samples. Standard curve and QC samples were prepared in plasma at R-138727 concentrations from 4 to 256 ng/ml. Each standard curve point was prepared in duplicate for the validation. Stability and QC samples were prepared at 4, 16, 64, and 256 ng/ml. Each concentration of QC samples was prepared in replicates of six for validation experiments and in triplicate for routine analyses.

Stability Experiments. Room temperature, freeze/thaw, and frozen-storage stability experiments were conducted using triplicates of each QC concentration. Long-term frozen stability was tested at −20°C. The objective of these stability experiments was to establish that there were no changes in the ratio of the four stereoisomers upon storage.

Ninety-Six-Well Extraction Procedure. All samples were vortex-mixed and centrifuged (3500 rpm for 10 min at 15°C) to remove any particulate matter, after which 200-μl aliquots of each sample [blanks, standard curve samples, QC samples (also used as validation samples), and study samples] were pipetted into designated 96-well polypropylene tubes. The internal standard (20 μl of the 100 ng/ml solution) was added to all vials except the plasma blanks, to which 20 μl of 1:1 methanol-water (v/v) was added. To all samples, 300 μl of 1% formic acid was added, and the vials were capped, vortex-mixed, and centrifuged (3000 rpm for 15 min at room temperature). Empore C8-SD 96-well solid-phase extraction (SPE) plates (3M, St. Paul, MN) were used for SPE, which was performed on a Tomtec Quadra 96 liquid-handling station (Tomtec, Hamden, CT). The SPE plate was conditioned with 100 μl of methanol followed by 200 μl of water. The entire sample supernatant was loaded onto the SPE plate using the vacuum manifold, washed with 500 μl of 1% formic acid, 500 μl of 50 mM ammonium acetate, and 500 μl of 30% methanol-water, respectively. The dry SPE plate was placed on a 96-well polypropylene collection plate, and the sample was slowly eluted with 150 μl of methanol. The plate was capped and centrifuged, and the supernatant was transferred to a 96-well plate for chiral electrospray LC-MS/MS analysis.

Plasma Samples. R-138727 was derivatized with 2-bromo-3'-methoxyacetophenone at the time of blood collection to ensure stabilization of the thiol moiety of the compound (Farid et al., 2007).

Method Validation. Validation of this methodology consisted of analyzing...
considered equimolar. The low and high limits (stereoisomer was then used to define low and high limits that would be
IS is internal standard. The overall mean of the peak area ratio for each
multiplicity was also applied. Thus, an
(25% of each), a Bonferroni correction (Milliken and Johnson, 1992) for
from the two independent chiral centers yielding equimolar concentrations. Although the theoretical value for each stereoisomer is 25%
(100%), the absolute peak areas generated could be different. The
techique were calculated using the total peak area at each of the four QC
samples (stability samples, clinical samples, etc.) with individual peak area
there was no intent to generate actual concentrations in the absence of indi-
vidual standards for each stereoisomer. All plasma samples were positioned
within each batch in a manner that they were bracketed by the QC samples.
Acceptance of the batch was based on at least five of the six replicates at each
validation QC concentration falling within the above-defined low and high
limits and the overall precision of the individual peak-area ratio percentages
being <15% relative standard deviation.

Results
Characteristics of the New Chiral LC/MS/MS Methodology. The
identity of each stereoisomer of R-138727 was determined by
individually analyzing a small authentic sample of each stereoisomer
(following the same derivatization procedure). The four stereoisomers eluted in the following sequence: SR, SS, RS, and RR. Chromato-
grams representing the lowest standard and a blank plasma sample
along with the identification of the individual stereoisomer peaks are
presented in Fig. 5.

Typical retention times for the four peaks were ~11.0 (R-125688),
~12.0 (R-125687), ~15.0 (R-125690), and ~17.0 (R-125689) min.
The assay lower and upper limits of quantification for R-138727 were
4 ng/ml and 256 ng/ml, respectively. The intrabatch accuracy (relative
error) for R-138727 ranged from ~8.8 to 7.3%, and the intrabatch
precision ranged from 1.2 to 6.5% at the concentrations tested (Table
1), thus demonstrating validation of the methodology. The correlation
coefficient ($r^2$) for derivatized R-138727 (quantified as the total of all
four stereoisomers) was $\approx 0.9986$ during validation.

Analysis of a plasma blank injected after each injection of the
highest standard resulted in no quantifiable peaks, indicating no
detectable system carryover and enabled the use of this methodology
for routine analyses without additional method development to
counter carryover. The individual stereoisomers peak area ratios of
the validation QC samples were within the low and high limits defined on
the basis of the standard curve samples, indicating that the ratios of the
stereoisomers were similar between the standards and QC samples.

Data Analysis and Statistical Methodology. Each individual peak was
reported as a percentage of the total peak area using eq. 1:

$$\frac{\text{Individual peak area}}{\text{Total IS peak area}} \times 100$$

IS is internal standard. The overall mean of the peak area ratio for each
stereoisomer was then used to define low and high limits that would be
considered equimolar. The low and high limits ($\alpha = 0.05/2$ to account for a
two-tailed test) were defined using eq. 2 based on the Student’s $t$ distribution
(Milliken and Johnson, 1992):

$$\text{Mean} \pm t_{\alpha} \times \text{SD} \times (1 + 1/n)^{1/2}$$

Because there are four mutually exclusive outcomes (stereoisomers) resulting
from the two independent chiral centers yielding equimolar concentrations
(25% of each), a Bonferroni correction (Milliken and Johnson, 1992) for
multiplicity was also applied. Thus, an $\alpha$ value of 0.05/4 was used. Unknown
samples (stability samples, clinical samples, etc.) with individual peak area
ratio percentages that fell outside the low and high limits determined for each
batch (the acceptance criteria based on the QC samples) for at least two
stereoisomers (peaks) were considered to be nonconforming with the theoretical
ratio of 1:1:1:1.

Analysis of Plasma Samples after Prasugrel Administration. Plasma
samples were analyzed to determine the ratio of R-138727 stereoisomer after
single oral doses of prasugrel of 40 or 60 mg and multiple doses ranging from
5 to 15 mg for 1 month. Triplicate samples of each QC concentration were
simultaneously analyzed. Standard curve samples were not included because
there was no intent to generate actual concentrations in the absence of indi-
vidual standards for each stereoisomer. All plasma samples were positioned
within each batch in a manner that they were bracketed by the QC samples.
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limits and the overall precision of the individual peak-area ratio percentages
being <15% relative standard deviation.
The results of the stability experiments demonstrated that the ratio of the four stereoisomers of R-138727 remained constant in human plasma when they were exposed to room temperature for 24 h, subjected to four freeze/thaw cycles, or held during long-term frozen storage for 31 days at 20°C.

Analysis of Human Plasma Samples after Prasugrel Administration. Ninety samples obtained from multiple subjects from different prasugrel treatment groups (doses) were analyzed to determine the R-138727 stereoisomer ratios. The percentage peak area limits for each stereoisomer corresponding to a 1:1:1:1 ratio were calculated using the QC samples and the data are shown in Table 2. A summary of the results is shown in Table 3, and a representative chromatogram depicting a subject plasma concentration is shown in Fig. 6.

The stereoisomer ratios of R-138727 in plasma samples of subjects given prasugrel diverged from the 1:1:1:1 ratio obtained in the QC samples. Overall, the four stereoisomers, R-125688, R-125687, R-125690, and R-125698 exhibited a 1:1:4:4 ratio. On average, the diastereomers RS (R-125690) and RR (R-125689) comprised ~84% of the R-138727 present in plasma, whereas the diastereomers SR (R-125688) and SS (R-125687) accounted for ~16% (Table 3).

Discussion

This is the first report describing the separation of the enantiomers of the active metabolite of prasugrel and its clinical application. The data show the successful development and validation of a novel chiral LC/MS/MS methodology that can be used to identify divergence in diastereomer peaks in the absence of authentic standards. This technique can also be extremely valuable in situations in which selective disposition of diastereomers can be implicated in understanding clinical pharmacology. The use of two HPLC columns in tandem was required to enable the separation of the R-138727 stereoisomers. There was no divergence in peak area ratios observed during conduct of stability studies.

Plasma samples obtained from multiple subjects from different prasugrel treatment groups (doses) were selected so as to include various time points after the first (loading dose) and/or 1 month of daily maintenance doses of prasugrel. The data showed that regardless of the prasugrel dose administered, the time of blood sampling after the dose (15 min to 12 h), or the duration of treatment, the peak area ratios ranged from 3.1 to 14.5% for stereoisomers R-125688 and R-125687 and from 33.6 to 47.6% for stereoisomers R-125690 and R-125698 (Table 3). These data represent the first report that metabolism of thienopyridines to form the active metabolite is a stereoselective process.

Prasugrel is administered as a racemate. Hydrolysis of prasugrel by esterases produces a 2-hydroxythiophene moiety, which can be tran-

### TABLE 1

<table>
<thead>
<tr>
<th>Inter- and intrabatch accuracy and precision for R-138727 quantified as the summation of all four peaks (RS, RR, SR, and SS)</th>
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</thead>
<tbody>
<tr>
<td>R-138727 Concentration</td>
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<tr>
<td>4 ng/ml</td>
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<tr>
<td>Intrabatch assay</td>
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<tr>
<td>Validation batch 1</td>
</tr>
<tr>
<td>Mean (n = 6)</td>
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<tr>
<td>Accuracy (%RE)</td>
</tr>
<tr>
<td>Precision (%RSD)</td>
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<tr>
<td>Validation batch 2</td>
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<tr>
<td>Mean (n = 6)</td>
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<tr>
<td>Accuracy (%RE)</td>
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<tr>
<td>Precision (%RSD)</td>
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<td>Validation batch 3</td>
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<tr>
<td>Mean (n = 6)</td>
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<tr>
<td>Accuracy (%RE)</td>
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<tr>
<td>Precision (%RSD)</td>
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<tr>
<td>Interassay mean (n = 18)</td>
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<tr>
<td>Low limit</td>
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<tr>
<td>Mean</td>
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<td>High limit</td>
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### TABLE 2

<table>
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<tr>
<th>Calculation of low and high limits for percent peak area ratios, for individual R-138727 stereoisomers, using QC samples from validation</th>
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<tbody>
<tr>
<td>QC Sample Concentration of R-138727</td>
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<tr>
<td></td>
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<tr>
<td>4 ng/ml</td>
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<tr>
<td>16 ng/ml</td>
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<td>64 ng/ml</td>
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<tr>
<td>256 ng/ml</td>
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<tr>
<td>Mean</td>
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<tr>
<td>S.D.</td>
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<tr>
<td>% RSD</td>
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<tr>
<td>Limits (z value)</td>
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<tr>
<td>Low limit</td>
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<td>High limit</td>
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### TABLE 3

<table>
<thead>
<tr>
<th>Ratios of R-138727 stereoisomers in plasma samples from subjects given prasugrel</th>
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<tbody>
<tr>
<td>SR (R-125688)</td>
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<tr>
<td>Percent peak area (mean ± S.D., n = 90)</td>
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<tr>
<td>Percent peak area range</td>
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<tr>
<td>Mean percent peak area of QC samples</td>
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</table>
siently tautomerized to a 1,4-enone, which in turn rearranges to the more thermodynamically stable R-95913. Formation of R-95913 introduces a second chiral center in the molecule, and, considering the postulated route of its formation, all R-95913 diastereomers would be formed in equal proportion. The observed stereoselectivity in the active metabolite formation in humans from R-95913 suggests that either the opening of the thiolactone ring in R-95913, an enzyme-mediated process (Rehmel et al., 2006), is stereoselective and the chirality is preserved up to the step leading to R-138727 formation or the ultimate step leading to R-138727 formation (e.g., reduction of a thioketone) is stereoselective. The results also showed that the two most pharmacologically potent antiplatelet stereoisomers R-125690 and R-125689 represent 84% of the R-138727 circulating in human plasma. The data showed that the ratios of the R-138727 stereoisomers are consistent among subjects, regardless of the dose, time of sample collection, or sampling after the first dose of the drug or after a few weeks of therapy.

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References


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