Journal of Chromatography B, 878 (2010) 2299–2306

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Determination of ticagrelor and two metabolites in plasma samples by liquid chromatography and mass spectrometry

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ARTICLE INFO

Article history:
Received 15 April 2010
Accepted 15 June 2010
Available online 25 June 2010

Keywords:
Ticagrelor
AZD6140
$P2Y_{12}$ receptor inhibitor
Platelets
Incurred sample reproducibility
Incurred sample stability

ABSTRACT

Rapid and sensitive analytical methods using liquid chromatography with tandem mass spectrometry (LC/MS/MS) were developed for the determination of ticagrelor, the first reversible oral platelet $P2Y_{12}$ receptor inhibitor, and its metabolites AR-C124910XX and AR-C133913XX in human plasma. Ticagrelor and its metabolites were extracted using protein precipitation with acetonitrile. Chromatographic separations were performed on reversed phase columns and detection using atmospheric pressure chemical ionization (APCI). Ticagrelor and AR-C124910XX were analyzed in the same assay, with the internal standard, d7-ZD6140, on a C18 column using negative ionization; AR-C133913XX analyzed separately on a phenyl column using positive ionization. Full validation of the methods was performed including selectivity, lower limit of quantification, accuracy, precision stability and incurred sample reproducibility and incurred sample stability. Total analytical run time was short (2 min). Calibration curves were established in the range 5–5000 ng/mL for ticagrelor, 2.5–2500 ng/mL for AR-C124910XX and 2–1000 ng/mL for AR-C133913XX. Lower limits of quantification for ticagrelor, AR-C124910XX and AR-C133913XX were determined to be 5, 2.5 and 2.0 ng/mL, respectively from 100 μL of human plasma. For ticagrelor, AR-C124910XX and AR-C133913XX, mean intra-batch accuracy was 91.9–109.0%, 86.8–109.2% and 100.5–112.0%, respectively; intra-batch precision was 4.0–8.4%, 5.2–16.9% and 3.9–12.3%, respectively. The methods were also applied to quantification of ticagrelor, AR-C124910XX and AR-C133913XX in rabbit, rat, mouse and marmoset, using 25 μL of animal plasma. A modified methodology was developed to quantify ticagrelor and AR-C124910XX in plasma from dog and cynomolgus monkey. Human incurred samples were found to generate consistent reproducibility and stability results. This method was successfully applied to determine plasma concentrations following administration of ticagrelor in human volunteers and patients, and animal safety evaluation studies. This validated methods has the advantages of being straightforward, robust and allows a fast throughput of samples.

1. Introduction

Ticagrelor (AZD6140) is the first reversibly binding oral platelet $P2Y_{12}$ receptor antagonist [1], which has shown potential benefits over current thienopyridine therapy [2]. In preclinical models, ticagrelor demonstrated a rapid and reversible concentration-dependent binding to the $P2Y_{12}$ receptor [3], which blocked adenosine phosphate (ADP)-induced platelet aggregation [4]. These characteristics are associated with a wider separation between antithrombotic and bleeding effects than seen with current agents [2,5]. Consistent with these data, phase IIb results showed that ticagrelor has a rapid onset of action with greater and more consistent inhibition of ADP-induced platelet aggregation than clopidogrel without increasing major plus minor bleeding [6]. Ticagrelor offers potential advantages in managing patients at risk for thrombotic events because of rapid onset and offset of antiplatelet effect. The safety and efficacy of ticagrelor has been evaluated in approximately 18,000 patients with acute coronary syndromes, in the phase III trial, PLATO (NCT00391872) [7,8].

Ticagrelor does not require metabolic activation for its antiplatelet activity, but it does have one equipotent metabolite, AR-C124910XX, which has blood concentration of approximately one third that of ticagrelor [2]. In addition, one metabolite, AR-C133913XX, with a relative high circulating concentration has also been monitored [9].

A sensitive, reliable and validated analytical method to quantify drug concentrations in plasma has an essential role in toxicology and clinical pharmacology. Our objective was therefore to develop a straightforward yet reliable assay for the measurement of the novel drug, ticagrelor, and its metabolites in human and

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doi:10.1016/j.jchromb.2010.06.018

animal plasma. Liquid chromatography with mass spectrometry is a well-established technique, and has been successfully used in the determination of antiplatelet agents and their metabolites in plasma [8,10,11,12]. This paper describes the development and validation of fast, high-throughput methods for liquid chromatography with tandem mass spectrometry (LC/MS/MS) for determination of ticagrelor and its metabolites AR-C124910XX and AR-C133913XX in plasma. The applicability of these methods in assessing plasma ticagrelor following administration in human healthy volunteers and patients is also described.

2. Experimental

2.1. Background

LC/MS/MS was used to develop an uncomplicated, fast-turnaround and reliable methodology for the determination of ticagrelor and its active metabolite, AR-C124910XX. As ticagrelor and AR-C124910XX co-eluted, the analysis could be simplified by use of only one internal standard. Given the simplicity and performance of this method, when quantification of the metabolite AR-C133913XX was also required, we modified the methodology rather than developed a new method to measure the three analytes together.

Because of a difference in hydrophobicity and ionization polarity between the analytes (ticagrelor and AR-C124910XX, negative; AR-C133913XX, positive) it was not preferred to use the same chromatographic system. As a result, following protein precipitation and production of supernatant, the methodology incorporated a separate chromatographic analysis of AR-C133913XX. However, since the same extraction procedure was used this additional analysis of AR-C133913XX did not require any additional sample.

2.2. Materials and reagents

Ticagrelor, AR-C124910XX, AR-C133913XX and internal standard (d7-ZD6140) (chemical structures shown in Fig. 1) were supplied by AstraZeneca R&D, Charnwood, UK, or AstraZeneca R&D, Södertälje, Sweden. Methanol (HPLC grade) and ammonium acetate (analytical grade) were purchased from Fisher (Loughborough, UK). Acetonitrile (HPLC grade) and dimethyl sulfoxide (DMSO) (analytical grade) were purchased from Sigma–Aldrich (Dorset, UK). High purity water (NANOpure Infinity or HPLC grade) was used.

2.3. Preparation of calibration standards and quality control samples

Calibration standards and quality control (QC) samples were prepared from independently weighed stocks. Standard stock solutions of the three analytes were prepared in DMSO to a concentration of 2000 μg/mL. For quantification of ticagrelor and AR-C124910XX, the internal standard d7-ZD6140 (1000 μg/mL in DMSO) was used. Internal standard was not used for quantification of AR-C133913XX.

Ticagrelor calibration standards in control plasma were prepared using ticagrelor standard working solutions (200 ng/mL to 200 μg/mL in DMSO) resulting in calibration standards of 5, 10, 50, 250, 1000, 2500, 4000 and 5000 ng/mL. AR-C124910XX calibration standards in control plasma were prepared using AR-C124910XX standard working solutions (100 ng/mL to 100 μg/mL in DMSO) resulting in calibration standards of 2.5, 5, 25, 125, 500, 1250, 2000 and 2500 ng/mL. AR-C133913XX calibration standards in control plasma were prepared using AR-C133913XX standard working solutions (80 ng/mL to 40 μg/mL in DMSO) resulting in calibration standards of 2, 4, 20, 100, 400, 500, 800 and 1000 ng/mL.

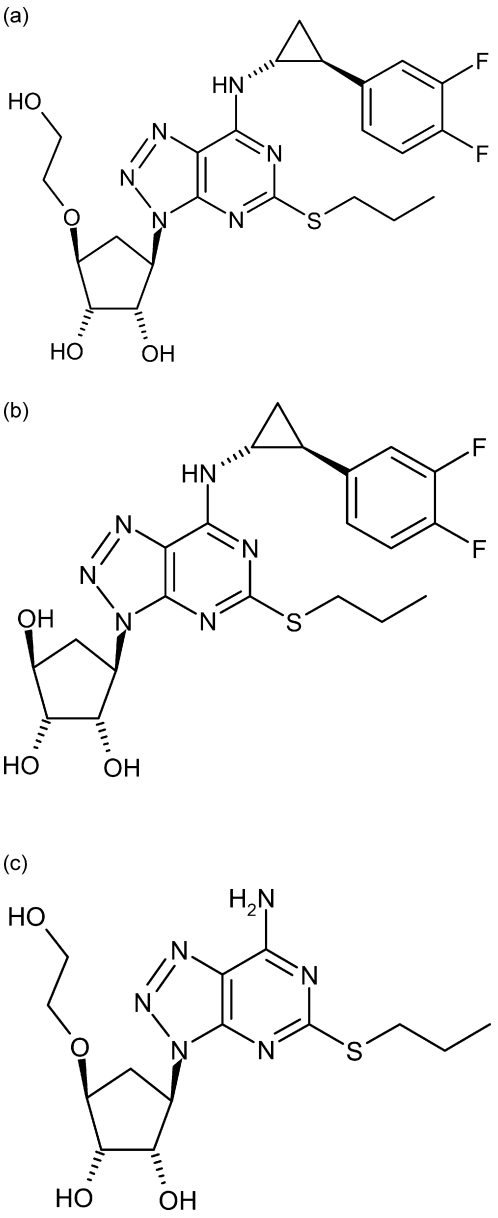

Fig. 1. Chemical structure of (a) ticagrelor, (b) AR-C124910XX, and (c) AR-C133913XX.

Internal standard was diluted to a concentration of 1000 ng/mL (in methanol:10 mM ammonium acetate [50:50 (v/v)]).

QC solutions were similarly prepared resulting in QC samples of 15, 800, 3500, 8000 and 25,000 ng/mL for ticagrelor, 7.5, 400, 1750, 4000 and 12,500 ng/mL for AR-C124910XX and 6, 45, 800, 1500 and 5000 ng/mL for AR-C133913XX.

2.4. Collection and storage of plasma samples

Venous blood samples were collected from humans and several animal species (rabbit, rat, mouse, marmoset, cynomolgus monkey and dog) into lithium heparinized tubes. Tubes were gently inverted and placed in an ice bath until centrifugation. After centrifugal separation (1500 × *g*; 10 min, 4 °C), plasma was transferred to polypropylene tubes and immediately frozen (−20 °C) until analysis. All samples were processed within an hour. Collection of blood samples into tubes with ethylenediaminetetraacetic acid (EDTA) as the anticoagulant was also validated (data not shown).

2.5. Sample preparation

Samples were thawed (<30 °C), vortex mixed, centrifuged (1800 × *g*, 5 min, 20 °C) and aliquotted (100 μL for human plasma samples, 25 μL for animal plasma samples) into 96-well multiplates. Internal standard (1000 ng/mL; 25 μL) was added to all samples (except blanks [25 μL water]), and vortex mixed (10 min). Samples were extracted by acetonitrile protein precipitation. Acetonitrile (200 μL) was added and samples vortex mixed (10 min), followed by centrifugation (1800 × *g*, 5 min, 20 °C).

For combined analysis of ticagrelor and AR-C124910XX, 150 μL of each supernatant was added to a well of a 96-well multiplate containing 150 μL water. The plate was vortex mixed (5 min) and submitted for LC/MS/MS. For the separate analysis of AR-C139913XX, 100 μL of each supernatant was added to a well of a 96-well multiplate and evaporated to dryness under nitrogen. Samples were then reconstituted in 80 μL mobile phase (acetonitrile:10 mM [pH native] ammonium acetate [25:75 (v/v)]); the plate was vortex mixed (5 min), centrifuged (1800 × *g*, 5 min, 20 °C) and then submitted for LC/MS/MS.

2.6. Instrumentation and LC/MS/MS conditions

2.6.1. Quantification of ticagrelor and AR-C124910XX

The liquid chromatographic system consisted of a PAL CTC Autosampler and an LC-10ADVP pump (Shimadzu, Milton Keynes, UK). Chromatographic separation was achieved on an Genesis C18 analytical column (particle size 3 μm, 50 mm × 4.6 mm; Jones Chromatography, Glamorgan, UK) at ambient temperature in air conditioned laboratory, using a degassed mobile phase (Agilent 1100 Series Degasser) of acetonitrile:10 mM (pH native) ammonium acetate (60:40 (v/v)), at a flow rate of 1.0 mL/min. Sample injection volume was 10–30 μL and the analytical run time was 2 min. Under these conditions, the analytes co-eluted with the internal standard.

The eluent from the HPLC column was coupled to an API 3000 mass spectrometer (MDS Sciex) using atmospheric pressure chemical ionization (APCI) set at a temperature of 550 °C. The mass spectrometer was operated in negative mode at a unit resolution for both Q1 and Q3 using multiple reaction monitoring (MRM) with a dwell time of 125 ms for ticagrelor and AR-C124910XX and 75 ms for internal standard. The transition of precursor to product ion was monitored at m/z 521.4 → 361.1 for ticagrelor, m/z 477.2 → 361.2 for AR-C124910XX, m/z 528.3 → 368.1 for internal standard. The collision energy was set at −30, −28 and −30 eV for ticagrelor, AR-C124910XX and internal standard, respectively.

Data acquisition and processing were performed using Analyst software (initially version 1.2 and later upgraded to version 1.4.2, Applied Biosystems; MDS Sciex). The calibration curves were generated by plotting the nominal concentrations of ticagrelor or AR-C124910XX calibration standards against the peak area ratio of ticagrelor or AR-C124910XX to internal standard, and by quadratic regression analysis using the reciprocal of concentration (1/×) as weighting.

2.6.2. Quantification of AR-C133913XX

The LC/MS/MS system was the same as previously mentioned. Chromatographic separation was achieved on a BDS Hypersil Phenyl analytical column (particle size 3 μm, 50 mm × 4.6 mm; Jones Chromatography, Glamorgan, UK) with the column at room temperature using a degassed mobile phase of 50 mM (pH native) ammonium acetate:acetonitrile (75:25 (v/v)), at a flow rate of 1.0 mL/min. Sample injection volume was 30 μL and the analytical run time was 2 min. Twenty percent of the eluent from the column was introduced to the mass spectrometer; the remaining 80% was discarded. The mass spectrometer was operated in positive mode at a unit resolution for both Q1 and Q3 using MRM with a dwell time of 250 ms for AR-C133913XX. The transition of precursor to product ion was monitored at m/z 370.9 → 182.9. The collision energy was set at 30 eV.

Data acquisition and processing were performed as earlier described using Analyst software (version 1.2 or version 1.4.2). The calibration curve was generated measuring the area by linear regression analysis, using the reciprocal of concentration (1/×) as weighting.

2.7. Performance of method and validation

Full validation of the analytical methods described in this report was performed in line with US Food and Drug Administration guidelines for validation of bioanalytical methods [13], including tests for selectivity; calibration curve and lower limit of quantification (LLOQ); accuracy and precision; and stability. Only key validation data are reported for the methodology used to determine ticagrelor and two metabolites in human samples. Full validation was also performed for the determination of these analytes in the animal species.

2.7.1. Selectivity

Selectivity was verified by examining any interference with the peaks for ticagrelor, AR-C124910XX, AR-C133913XX and internal standard in the MRM chromatograms from using six extracted individual blank human plasma samples compared to six replicates of plasma spiked to contain analytes at the LLOQ prior to extraction.

2.7.2. Calibration curve and LLOQ

Eight calibration standards and zero samples with and without internal standard were extracted. Zero samples were not included in the curve fit. One replicate of each standard was extracted at each concentration level per analytical run with the exception of the limits of quantification where duplicate standards were extracted. For calibration curves to be deemed acceptable, >75% of the back-calculated standards were required to be within 85–115% of nominal concentration (within 80–120% at the LLOQ).

2.7.3. Precision and accuracy

The precision of the assay was expressed as the percentage of coefficient of variation of each concentration level (CV %). Accuracy was expressed as a percentage of their theoretical concentration. Intra-batch and inter-batch precision and accuracy were determined in three runs on different days using validation samples at the concentration ranges given in Table 1. The validation samples with concentrations outside the calibration ranges were diluted 10 times in blank control plasma (lithium heparin) before extraction.

The intra-run precision was determined from the observed concentrations of six replicates at each of the validation sample levels for each of the three runs. The inter-run precision was determined from the observed concentration of all validation sample replicates (n = 18) at a particular level analyzed in the three runs. To be acceptable, these were required to be ≤15% at all levels (except for ≤20% at the LLOQ).

The intra-run accuracy was determined from the observed concentration of six replicates at each of the validation sample levels for each of the three runs. The inter-run accuracy was determined from the observed concentration of all validation samples replicates (n = 18) at a particular level analyzed in the three runs. To be acceptable, these were required to be 85–115% at all levels (except for 80–120% at the LLOQ).

2.7.4. Stability

Stability of ticagrelor, AR-C124910XX and AR-C133913XX was examined under different conditions described below, each using

Table 1
Intra-batch precision and accuracy of QC data for ticagrelor, AR-C124910XX and AR-C133913XX.

Nominal ticagrelor concentration (ng/mL)						
	5	15	800	3500	5000	25,000[*,**]
Observed ticagrelor concentration (ng/mL)						
Intra-batch mean ± SD	5.45 ± 0.304	14.1 ± 1.18	735 ± 49.2	3540 ± 230	4830 ± 380	24,100 ± 965
Intra-batch precision (CV %)	5.6	8.4	6.7	6.5	7.9	4.0
Intra-batch accuracy (%)	109.0	94.0	91.9	101.1	96.6	96.4
Nominal AR-C124910XX concentration (ng/mL)						
	2.5	7.5	400	1750	2500	12,500[*,**]
Observed AR-C124910XX concentration (ng/mL)						
Intra-batch mean ± SD	2.73 ± 0.462	7.15 ± 0.762	353 ± 23.2	1660 ± 86.1	2170 ± 155	11,300 ± 799
Intra-batch precision (CV %)	16.9	10.7	6.6	5.2	7.1	7.1
Intra-batch accuracy (%)	109.2	95.3	88.3	94.9	86.8	90.4
Nominal AR-C133913XX concentration (ng/mL)						
	2.0	6.0	45	800	1000	5000[**]
Observed AR-C133913XX concentration (ng/mL)						
Intra-batch mean ± SD	2.01 ± 0.248	6.05 ± 0.513	46.6 ± 2.53	859 ± 33.2	1120 ± 76.3	5570 ± 253
Intra-batch precision (CV %)	12.3	8.5	5.4	3.9	6.8	4.5
Intra-batch accuracy (%)	100.5	100.8	103.6	107.4	112.0	111.4

$N = 6$.

[*] Except for highest concentration of ticagrelor and AR-C124910XX where $n = 5$ as data exclude statistical outlier (Dixon's Q-test, $P \leq 0.05$). When included, ticagrelor precision = 23.1% and accuracy = 88.4%. AR-C124910XX, precision = 22.9% and accuracy = 82.4%.

[**] Highest concentration was diluted 10-fold with control plasma.

six replicates of low, medium and high concentration QC samples (ticagrelor: 15, 800 and 3500 ng/mL; AR-C124910XX: 7.5, 400 and 1750 ng/mL, AR-C133913XX: 6 and 800 ng/mL [no medium QC sample]), unless stated otherwise. Acceptable stability was considered to be within 85–115% of the relevant theoretical concentration.

Short-term stability of ticagrelor and its metabolites was examined (a) in human plasma (by comparing the concentration of QC samples stored at room temperature for 24 h after spiking and mixing, before being frozen [nominal −20 °C], prior to analysis) and (b) in human whole blood (by comparing samples stored at room temperature for 1 and 4 h [AR-C133913XX only] with samples analyzed immediately after preparation).

Long-term stability in spiked samples for ticagrelor and AR-C124910XX were determined by comparing the concentration of samples stored at −20 °C with their theoretical concentration after approximately 30 months. Concentrations for ticagrelor were 3, 50, 400 ng/mL and for AR-C124910XX 7.5, 50, 400 ng/mL. Similarly, the long-term stability in spiked samples for AR-C133913XX was determined by comparing the concentration of samples stored at −20 °C with their theoretical concentration after approximately 12 months.

Long-term stability of ticagrelor and AR-C124910XX in incurred samples (pooled to give concentrations for ticagrelor of approximately 15, 800 and 3500 ng/mL) stored at −20 °C was determined by comparing the concentrations of samples analyzed after 1–3 years with baseline values determined immediately after preparation. In addition, the long-term stability of AR-C133913XX in incurred samples ($n = 20$, 5–800 ng/mL) was determined by comparing the concentration of samples analyzed after up to approximately 12 months to initially determined concentrations.

Stability was also assessed in spiked sample extracts (data not shown) and incurred sample extracts ($n = 20$, 100–5000 ng/mL for ticagrelor, 10–2000 ng/mL for AR-C124910XX and $n = 20$, 6–700 ng/mL for AR-C133913XX) stored at room temperature. This was determined by comparing the concentrations of samples analyzed after 9 days (ticagrelor and AR-C124910XX) and 1 day (AR-C133913XX) with baseline values determined immediately after preparation.

Freeze–thaw stability was also assessed by comparing the concentration of QC samples after four freeze–thaw cycles with those which had only undergone one freeze–thaw cycle.

2.8. Application to measurement in other species

The suitability of the present method was assessed for allowing the determination of ticagrelor, AR-C124910XX and AR-C133913XX in other species (mouse, rat, rabbit and marmoset). The possibility of extending the range of quantification to an upper limit of 200 μg/mL was examined by diluting with blank plasma.

These methods were then fully validated, as for human samples (including selectivity; calibration curve and LLOQ; accuracy and precision; and stability). Stability of ticagrelor, AR-C124910XX and AR-C133913XX following long-term storage was determined by comparing the concentrations of samples stored at −20 °C over a 6-month period with the concentration of samples analyzed immediately after preparation. Freeze–thaw stability was also examined as described for human samples.

In cynomolgus monkey and dog, only methods to quantify ticagrelor and AR-C124910XX were developed.

2.9. Application to clinical studies

The method was applied to the analysis of more than 30,000 human plasma samples obtained from over 7000 participants in approximately 50 clinical studies following administration of single and multiple doses of ticagrelor (all patients provided informed consent and local ethical review board approval was obtained). Participants were diverse and included healthy Caucasian, Chinese, male and female subjects, as well as patients with either liver or renal impairment, patients with acute coronary syndrome and patients with stable coronary artery disease. Blood sampling, plasma isolation and storage were as described above.

Each analytical run had a maximum of 96 samples (including eight calibration standards at different levels covering the range and six QC samples at three different levels). Six of the calibration standards should have a back-calculated concentration within ±15% of the nominal value (±20% for the LLOQ). At least four out of six, and one at each level, QC samples had to fall within ±15% of the nominal concentration.

To examine incurred sample reproducibility of ticagrelor and AR-C124910XX in human plasma, 266 samples (covering all participant groups mentioned above) were analyzed twice in separate runs. To evaluate the reproducibility, the two results were compared and the number of repeated ticagrelor and AR-C124910XX samples within ±20% of original analysis value was calculated.

3. Result and discussion

3.1. Chromatographic performance

3.1.1. Quantification of ticagrelor and AR-C124910XX

Ticagrelor, AR-C124910XX and the internal standard were retained on the column with retention times for all three analytes of approximately 1.3 min. Ticagrelor, AR-C124910XX and the internal standard were detected in the negative MRM mode with acceptable sensitivity; product ion spectra of these analytes are shown in Fig. 2a, b and d.

Selectivity in spiked samples was within the acceptable criteria and no endogenous peaks interfering with ticagrelor or its metabolite were observed in the MRM chromatograms of blank human plasma samples. Typical MRM chromatograms of blank human plasma samples alone and with the addition of ticagrelor, AR-C124910XX and internal standard, respectively (Fig. 3a–f) are shown.

3.1.2. Quantification of AR-C133913XX

In the separate analysis of AR-C133913XX, this metabolite was retained on the column with a retention time of approximately 1.2 min. AR-C133913XX was detected in the positive MRM mode with high sensitivity; product ion spectrum of the analyte is shown in Fig. 2c.

Selectivity in spiked samples was within the acceptable criteria and no endogenous peaks that interfered with detection of AR-C133913XX were observed in the MRM chromatograms of blank human plasma samples. A typical MRM chromatogram of a blank human plasma sample alone (Fig. 3g) and with AR-C133913XX (2.0 ng/mL) added (Fig. 3h) are shown.

3.2. Performance of method and validation

3.2.1. Calibration curve and LLOQ

Calibration curves were established in the range of 5–5000 ng/mL for ticagrelor, 2.5–2500 ng/mL for AR-C124910XX and 2–1000 ng/mL for AR-C133913XX, and complied with the predefined acceptance criteria. Quadratic equation curves ($n = 3$, separate days) exhibited an excellent relationship with a mean ± SD correlation coefficient (r^2) of 0.9995 ± 0.0001 for ticagrelor and 0.9994 ± 0.0002 for AR-C124910XX. The calibration curve for AR-C133913XX exhibited good linearity with mean ± SD correlation coefficient (r^2) of 0.9982 ± 0.0016. The LLOQ for ticagrelor, AR-C124910XX and AR-C133913XX were determined to be 5, 2.5 and 2.0 ng/mL, respectively. Signal-to-noise ratios at the LLOQ were greater than five for each analyte.

To better reflect the sample concentrations in a few studies, the method was also validated using a calibration range of 1–2000 ng/mL for ticagrelor and 2.5–1000 ng/mL for AR-C124910XX, with a 100 μL sample volume. In this methodology, only ticagrelor and AR-C124910XX were determined (data not presented).

3.2.2. Precision and accuracy

Table 1 shows the intra-batch precision and accuracy of the analytes. Intra-batch precision for ticagrelor, AR-C124910XX and AR-C133913XX was acceptable, with CV no more than 8.4%, 16.9% (at LLOQ) and 12.3% (at LLOQ), respectively. Intra-batch accuracy ranged between 91.9% and 109.0% for ticagrelor between 86.8% and 109.2% for AR-C124910XX and between 100.5% and 112.0% for AR-C133913XX (Table 1). It should be noted that precision and accuracy at the highest concentration of ticagrelor and AR-C124910XX were calculated (using $n = 5$) after excluding one statistical outlier (Dixon's Q-test, $P \leq 0.05$) (Table 1).

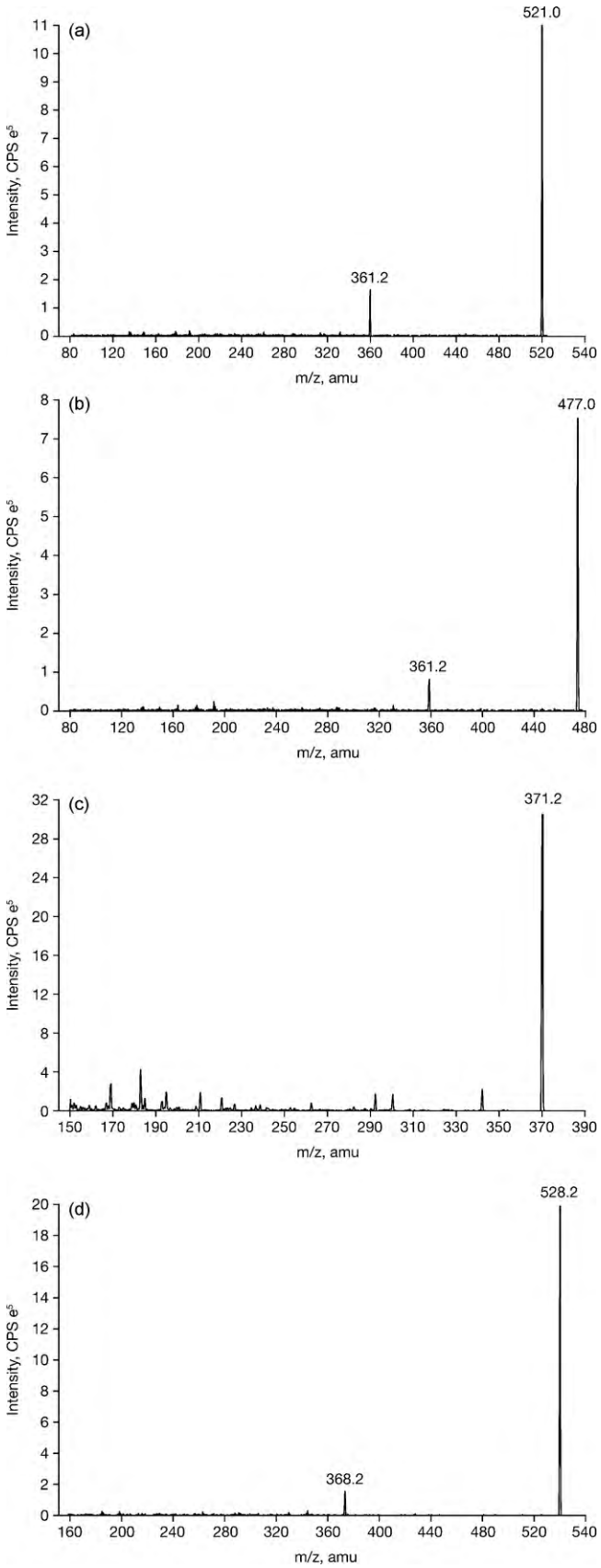

Fig. 2. Product ion spectra of (a) ticagrelor, (b) AR-C124910XX, (c) AR-C133913XX, and (d) internal standard.

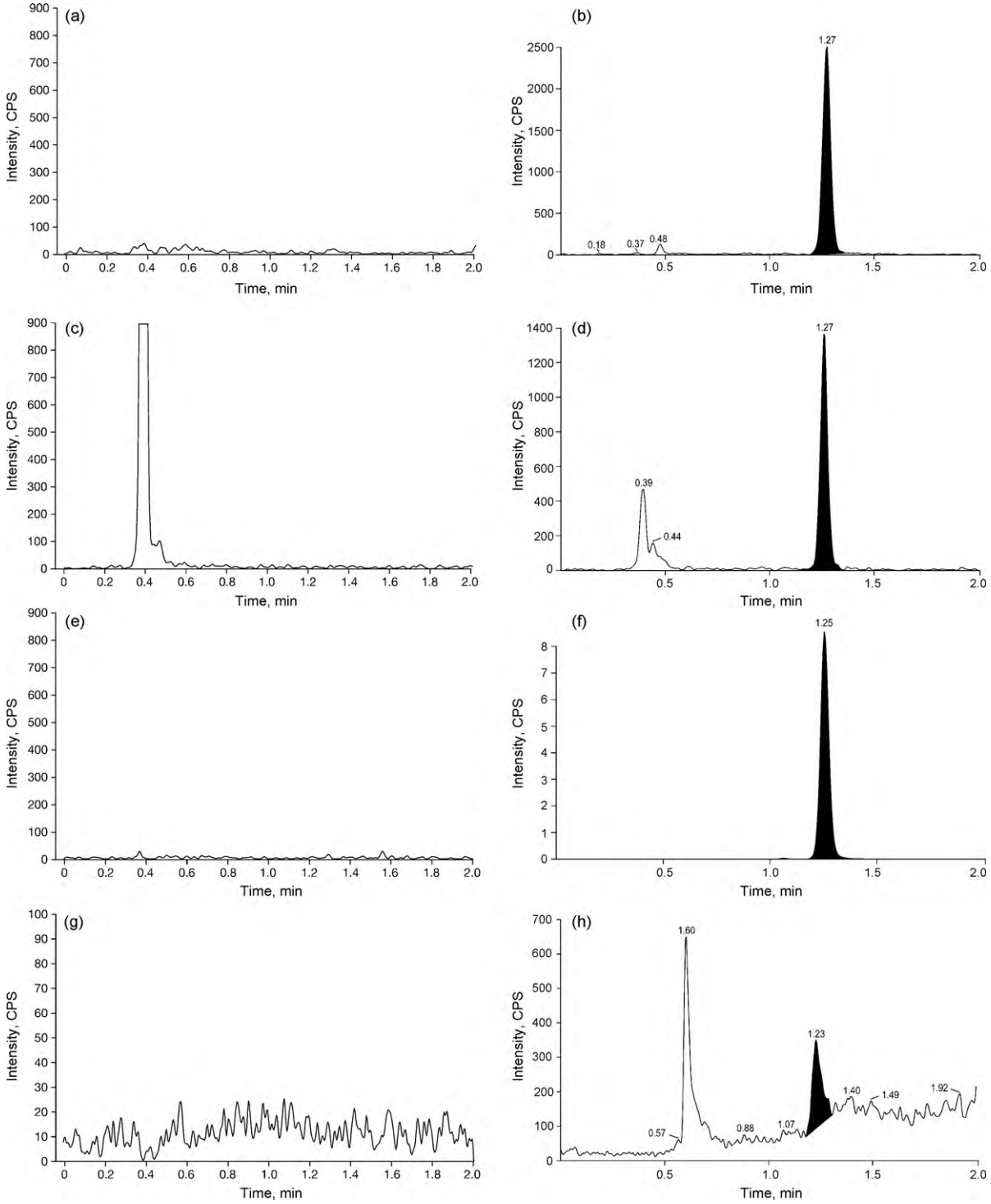

Fig. 3. Typical MRM chromatograms of (a) blank human plasma sample alone and (b) with the addition of ticagrelor (5 ng/mL; LLOQ) obtained at 521.4 → 361.1; (c) blank human plasma sample alone and (d) with the addition of AR-C124910XX (2.5 ng/nL; LLOQ) obtained at 477.2 → 361.2; (e) blank human plasma sample alone and (f) with the addition of internal standard obtained at 528.3 → 368.1; and (g) blank human plasma sample alone and (h) with the addition of AR-C133913XX (2 ng/mL; LLOQ) obtained at 370.9 → 182.9.

Inter-batch precision for ticagrelor, AR-C124910XX and AR-C133913XX was within predefined criteria, with CV no more than 12.5% (at LLOQ), 13.6% (at LLOQ) and 9.6% (at LLOQ), respectively. Inter-batch accuracy ranged between 93.2% and 102.0% for ticagrelor between 86.8% and 109.2% for AR-C124910XX and between 96.2% and 106.6% for AR-C133913XX.

3.2.3. Stability

Ticagrelor, AR-C124910XX and AR-C133913XX were stable in human plasma when stored for at least 24 h at room temperature, differing from baseline values by no more than 7.1%, 6.2% and 1.5% for ticagrelor, AR-C124910XX and AR-C133913XX, respectively. Removal of one statistical outlier for both ticagrelor and AR-

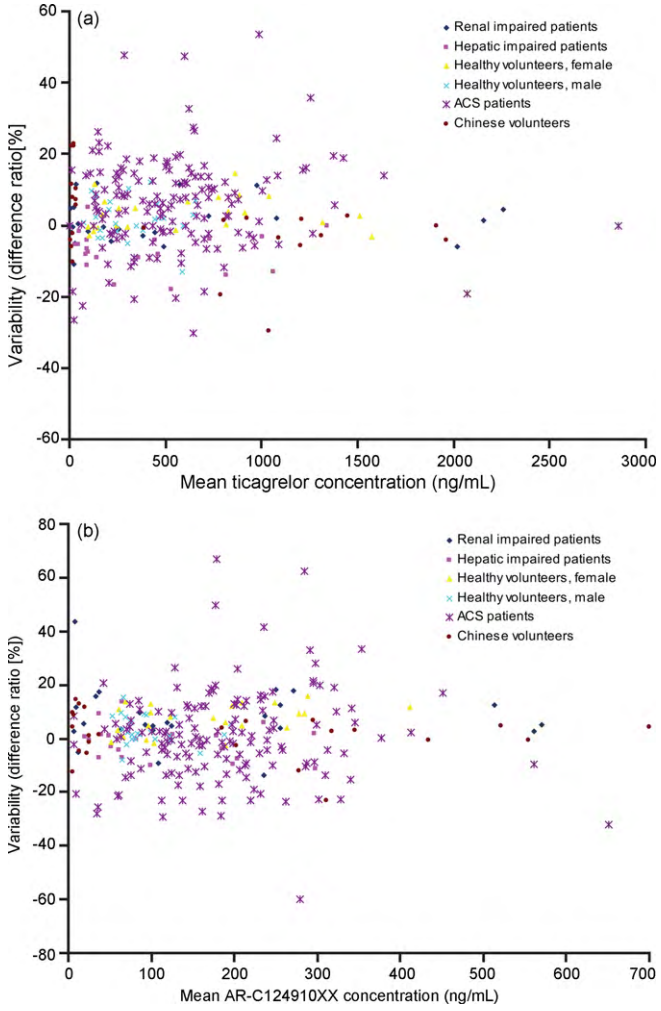

Fig. 4. Bland–Altman plots examining the agreement between duplicate incurred samples of (a) ticagrelor and (b) AR-C124910XX determined in human plasma from several populations. Variability data expressed as ([Repeat] − [Original])/Mean × 100. *Note:* one AR-C124910XX outlier (1145 ng/mL, 7.36% variability) not shown. ACS = acute coronary syndrome.

C124910XX (Dixon's *Q*-test, $P \leq 0.05$) reduced the difference from baseline values to 3.1% for ticagrelor and 3.2% for AR-C124910XX. Stability in whole human blood stored at room temperature for 1 and 4 h (AR-C133913XX) respectively differed from baseline values by 6.7% for ticagrelor, 7.5% for AR-C124910XX and 8.8% for AR-C133913XX, respectively.

Long-term stability in spiked human samples stored at −20 °C up to approximately 30 months showed excellent stability differing from baseline values by at most 4.6% and 12.8% for ticagrelor and AR-C124910XX, respectively. Similarly for AR-C133913XX, spiked human samples stored at −20 °C up to approximately 12 months showed excellent stability differing from baseline values by at most 5.6%.

Long-term stability of incurred samples in human plasma stored at −20 °C up to 3 years (ticagrelor and AR-C124910XX) and 1 year (AR-C133913XX) showed excellent stability, differing in mean observed concentration from baseline values equal to or less than 8.0% (CV 8.2%), 9.8% (CV 11.2%) and 2.7% (CV 14.1%) for ticagrelor, AR-C124910XX and AR-C133913XX, respectively. Stability of incurred sample extracts following 9 days (1 day for AR-C133913XX) storage at room temperature differed in mean concentration from baseline values by 7.1% (CV 6.7%), 3.0% (CV 8.6%) and 8.5% (CV 17.5%) for ticagrelor, AR-C124910XX and AR-C133913XX, respectively.

Ticagrelor and its metabolites were stable in human plasma through multiple freeze–thawing; prepared samples analyzed after four freeze–thaw cycles differed from baseline values by no more than 4.0%.

3.3. Application to measurement in other species

In rabbit, rat, mouse and marmoset, it was possible to successfully quantify ticagrelor, AR-C124910XX and AR-C133913XX over the same range of concentrations as described in humans. In contrast to the above-described methods in humans, where a 100 μL sample volume of human plasma was used, in these animal species, analytes were determined in 25 μL plasma samples. In these species, the LLOQ was 5 ng/mL for ticagrelor, 2.5 ng/mL for AR-C124910XX and 2.0 ng/mL for AR-C133913XX. The upper limit of quantification was 5000 ng/mL for ticagrelor, 2500 ng/mL AR-C124910XX, and 2000 ng/mL for AR-C133913XX. Samples with higher concentrations could be analyzed by dilution with plasma. Using this method it was possible to extend the range such that the upper limit of quantification for ticagrelor was 200 μg/mL.

In cynomolgus monkey and dog, methods to quantify ticagrelor and AR-C124910XX were developed, with successful quantification performed over the range 5–500 ng/mL for both ticagrelor and AR-C124910XX.

3.3.1. Validation

Precision and accuracy data were acceptable at all QC levels in each of the methodologies for quantification in animal species. In rabbit, rat, mouse and marmoset, dilution of samples to extend the upper limit of quantification was also validated.

The methods were also shown to allow selective measurement of the analytes. Ticagrelor, AR-C124910XX and AR-C133913XX were observed to be stable in each of the animal plasma samples stored for up to 3–6 months (in cynomolgus monkeys and dogs only ticagrelor and AR-C124910XX were examined) and following four freeze–thaw cycles.

3.4. Application to clinical study

The validated method was successfully applied to determine the plasma concentrations of ticagrelor and its metabolites after single and multiple oral doses. No significant suppression or build-up of matrix components on the column was seen during analysis of samples from the clinical studies.

In the phase III PLATO trial [7] more than 12,600 samples where analyzed for ticagrelor and AR-C124910XX in a total of 201 analytical runs. Of these, only one run failed to meet the acceptance criteria (and only for one analyte). For ticagrelor, mean inter-run accuracy of the QC sample data was 102.0% (CV 13.7%, $n = 399$) at 15 ng/mL, 100.6% (CV 4.9%, $n = 396$) at 800 ng/mL and 100.9% (CV 5.9%, $n = 399$) at 3500 ng/mL including outliers but not mis-extractions. For AR-C124910XX, mean inter-run accuracy of the QC sample data was 102.1% (CV 11.4%, $n = 395$) at 7.5 ng/mL, 101.3% (CV 6.6%, $n = 392$) at 400 ng/mL and 100.6% (CV 46.3%, $n = 395$) at 1750 ng/mL including outliers but not mis-extractions.

The method proved to be very reproducible and incurred samples ($n = 266$) were found to generate consistent results in all evaluated populations and patient groups. For ticagrelor 91.7% of the incurred samples had a variability of less then 20% and for AR-C124910XX 88.0% had a variability of less then 20% (Fig. 4). No trend in the variability was seen.

4. Summary and conclusion

Rapid and sensitive LC/MS/MS assay methods were developed for the determination of ticagrelor and its metabolites in human

and animal plasma samples. Using these methods, the LC/MS/MS assays have been fully validated and have been used to analyze numerous samples from several clinical trials. The present methodologies have several benefits, including a very fast throughput, a small sample size requirement, ease of sample preparation, and high sensitivity and selectivity.

Each of the steps of the methods is uncomplicated allowing analysis of large numbers of clinical samples. Plasma sample preparation only involves a single-step extraction with acetonitrile, followed by centrifugation. Using the selected isocratic conditions, ticagrelor and AR-C124910XX co-elute, allowing the analysis to be further simplified by the use of only one internal standard. The precision and accuracy data show that the internal standard suitably tracked both ticagrelor and AR-C124910XX, compensating for any changes in detector sensitivity. Indeed, these LC/MS/MS methods demonstrated high accuracy and precision for analysis of all three analytes in human plasma. In addition, the short LC/MS/MS analysis run time of approximately 2 min per sample allows for injection of approximately 500 samples per day. Consequently, the described methods are fast, allowing a high-throughput of samples.

The current methodology requires separate chromatographic analysis of the metabolite, AR-C133913XX. However, this was done part way through extraction and hence only one aliquot of plasma was required. Therefore, given the simplicity and performance of the parallel methods, the development of a single tandem mass spectrometry analysis was considered unnecessary. Additionally, since the chromatographic analysis of AR-C133913XX used the same extraction procedure and was only separated following protein precipitation and production of supernatant, the method did not require any additional sample volume, retaining the small sample size requirement. AR-C133913XX was only determined in a few studies. Therefore the investment in a labeled internal standard was not done. d7-ZD6140 was evaluated, but due to different properties, it was not a realistic choice.

Assay calibration curves were established in the range of 5–5000 ng/mL for ticagrelor, 2.5–2500 ng/mL for AR-C124910XX and 2–1000 ng/mL for AR-C133913XX with LLOQ of 5, 2.5 and 2.0 ng/mL, respectively. The analytical methods also had excellent intra-and inter-batch accuracy and precision. The quantifiable concentrations of ticagrelor by the assay are in the same range as mean plasma concentrations measured in plasma in volunteers treated with ticagrelor 50–200 mg bid or 400 mg qd [14]. Indeed, the validated method was successfully applied to the analysis of thousands of samples in human pharmacokinetic and clinical studies following oral administration of ticagrelor (single and multiple doses).

The analytical methods were also shown to be applicable to measurement of ticagrelor and its metabolites in the plasma from various animal species, and were adaptable to increasing the upper limit of quantification. Furthermore, these methods have been successfully used to determine plasma concentrations of ticagrelor and its metabolites in several animal toxicology studies. This straightforward, robust, fast throughput, validated assay underpins the understanding the clinical pharmacology of ticagrelor.

Potential conflicts of interest

Henrik Sillén and Patty Davis are employed by AstraZeneca. Melanie Cook is employed by York Bioanalytical Solutions, who receive financial support from AstraZeneca.

Acknowledgements

Financial support for the conduct of the research was provided by AstraZeneca. Colleagues at Clinical Pharmacology & DMPK involved in this project are acknowledged for their dedicated work. Colleagues at York Bioanalytical Solutions are acknowledged for their hard work within this project. The authors would also like to acknowledge Patrick Hoggard, PhD (Gardiner-Caldwell Communications, Macclesfield, UK) for assistance in the preparation of the draft and collating author contributions, funded by AstraZeneca.

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