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Determination of unbound ticagrelor and its active metabolite (AR-C124910XX) in human plasma by equilibrium dialysis and LC–MS/MS

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ABSTRACT

Ticagrelor is the first direct acting reversibly binding oral platelet $P2Y_{12}$ receptor antagonist. The parent molecule and the main metabolite (AR-C124910XX) are both able to block adenosine diphosphate-induced receptor signaling with similar potency. Drug binding to plasma proteins reduces free drug available for pharmacologic activity. Therefore, assessing unbound drug is important for interpretation of pharmacokinetic/pharmacodynamic findings. This paper describes the development and validation of an equilibrium dialysis/LC–MS/MS method for measuring unbound ticagrelor and AR-C124910XX in human plasma. Plasma samples (200 μl) were dialysed against phosphate buffered saline (37 °C, 24 h) in 96-well dialysis plates to separate unbound analytes. Drug–protein binding alterations during dialysis were minimized by maintaining physiologic conditions (pH 7.4, 37 °C). Ticagrelor and AR-C124910XX were quantified in dialysates (unbound fraction), retentates and plasma (total concentration) using liquid chromatography–mass spectrometry/mass spectrometry (LC–MS/MS) methods. Calibration curves were established for the retentate and plasma (total concentration) in the ranges 5–5000 ng/ml (ticagrelor) and 2.5–2500 ng/ml (AR-C124910XX), and for the dialysate in the range 0.25–100 ng/ml (both analytes). Both ticagrelor and AR-C124910XX were highly protein bound (>99.8%), i.e. unbound fraction <0.2%. Yet, the methodology was successfully applied to determine unbound concentrations of ticagrelor and AR-C124910XX in clinical samples.

1. Introduction

Ticagrelor is a cyclopentyl-triazolo-pyrimidine, a novel chemical class of antiplatelet agents [1]. Ticagrelor is the first direct acting reversibly binding oral $P2Y_{12}$ receptor antagonist blocking adenine diphosphate (ADP)-induced platelet aggregation [2]. Through its direct and reversible mode of action, ticagrelor exhibits rapid onset and offset of effect, which closely follow drug exposure levels [3]. Unlike the thienopyridines (ticlopedine, clopidogrel and prasugrel), ticagrelor does not require metabolic activation [2,3]. However, its main metabolite, AR-C124910XX, circulates in blood at concentrations approximately one third those of ticagrelor [4,5], and is approximately equipotent in inhibiting the $P2Y_{12}$ receptor [AstraZeneca, data on file]. Hence, the pharmacodynamic response is dependent upon combined exposure to both compounds.

Ticagrelor is approved in the European Union for the prevention of thrombotic events in patients with acute coronary syndromes [6]. The two DISPERSE phase IIb trials demonstrated that, compared with clopidogrel, ticagrelor more rapidly inhibited ADP-induced platelet aggregation to a greater and more consistent extent [7], without increasing minor and major bleeding events [7,8]. The efficacy and safety of ticagrelor versus clopidogrel has been evaluated in PLATO (PLATelet inhibition and patient Outcomes; NCT00391872), a phase III trial with approximately 18,000 patients [9,10]. In this trial, ticagrelor significantly reduced the rate of the primary composite of myocardial infarction, stroke and death from vascular causes compared with clopidogrel in the presence of aspirin [10].

A multiple-dose pharmacokinetic study demonstrated that maximum plasma levels of total ticagrelor occurred 1.5–3.0 h after dosing, and the half-life ranged from 6.2 to 13.1 h [4]. Drug binding to plasma proteins differs between drugs, and only unbound drug is available for distribution, elimination and producing pharmacologic activity. Other antiplatelet therapies have shown a high degree of protein binding, e.g. clopidogrel and its main active metabolite are both highly protein bound (>94% [11]). Certain conditions can affect plasma protein concentrations (e.g. age,

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pregnancy, liver disease, renal failure). Furthermore, drug displacement may occur under certain pathologic conditions (e.g. diabetes), and in the presence of other drugs/factors competing for protein binding sites. Both phenomena may influence the unbound concentration (free fraction) [12,13]. Therefore, assessing unbound drug concentration is important for pharmacokinetic and pharmacodynamic interpretation [12,14].

Measurement of unbound drug fractions typically requires a separation technique combined with a sensitive assay. Several separation methods have been proposed, including, ultrafiltration [15], ultracentrifugation [16], equilibrium dialysis [17], protein precipitation [18], high performance frontal analysis (HPFA [19]), and high-performance-capillary electrophoresis with HPFA [20]. Each technique has advantages and limitations [12]. The most common methods used routinely are ultrafiltration and equilibrium dialysis.

Ultrafiltration is the simplest and quickest method providing a direct measure of unbound drug concentrations without a dilutional effect. However, drug adsorption to the device and membrane can be a significant problem. If drug adsorption occurs (e.g. as with basic, lipophillic compounds), this technique will always underestimate free drug fraction [21]. Equilibrium dialysis is commonly used due to its relative simplicity and applicability to high throughput assays, and has long been considered a reference method for assessing free drug fraction [12]. Drug adsorption is less of an issue with this technique (except for extremely hydrophobic drugs and drugs with concentration-dependent binding) provided that sufficient time is allowed to attain equilibrium and drug concentrations on both sides of the membrane are measured [12]. However, for the determination of unbound concentration, three sample aliquots, the plasma (total concentration), the dialysate and the retentate, need to be analyzed.

Ultrafiltration was evaluated and found to be unsuitable for determination of unbound fractions of ticagrelor and AR-C124910XX due to high non-specific binding; therefore equilibrium dialysis was selected as the most suitable method. This paper describes the validation of an equilibrium dialysis method with liquid chromatography–mass spectrometry/mass spectrometry (LC–MS/MS) to evaluate the unbound fraction of ticagrelor and AR-C124910XX in human plasma samples. Due to high protein binding of both analytes, enhanced assay sensitivity was required. Thus, the previously reported LC–MS/MS plasma method [22] was adapted and validated using phosphate buffered saline as the matrix for the dialysate analysis. Furthermore, a topic under discussion is whether the anticoagulant heparin affects the unbound fraction of protein-bound drugs [23], thus, the validation of the LC–MS/MS method in human potassium ethylenediaminetetraacetic acid (K_2-EDTA) plasma samples was also conducted for the analysis of retentate and total concentrations.

2. Experimental

2.1. Background

Initially, ultrafiltration was evaluated to separate unbound ticagrelor and AR-C124910XX. A high degree (>90%) of non-specific binding of both analytes occurred with the following membranes: Amicon Ultrafree®-MC Centrifugal Filter Units molecular weight cut-off 5000 Da; Millipore hydrophilic polytetrafluoroethylene membrane (MF) 0.45 μm; Millipore Biomax Polyethersulfone membranes (5000 and 10,000 Da cut-off); regenerated cellulose (Millipore, 10,000 and 30,000 Da cut-off) (Millipore, Consett, County Durham, UK) and cellulose triacetate (VivaScience, Epsom, Surrey, UK) 5000 and 10,000 Da cut-off. As both compounds are non-polar, binding to the vessel and ultrafiltration membrane was not unexpected in aqueous conditions. Consequently, equilibrium dialysis was considered as an alternative analytical technique.

2.2. Chemicals and reagents

Ticagrelor (99.2% pure), AR-C124910XX (93.0%) and internal standard (D_7-ZD6140; 99.7%) were supplied by AstraZeneca R&D (MöIndal, Sweden). Methanol (HPLC grade) and ammonium acetate (analytical grade) were obtained from Fisher (Loughborough, UK). Acetonitrile (HPLC grade) and dimethyl sulphoxide (DMSO, analytical grade) were purchased from Sigma–Aldrich (Dorset, UK). Phosphate buffered saline (PBS, analytical grade) was obtained as pre-weighed packets from Sigma–Aldrich. Purified water (NANOpure infinity or HPLC grade) was used.

2.3. Control matrix and test samples

Since clinical samples to be analyzed for unbound ticagrelor and AR-C124910XX need to contain the anticoagulant K_2-EDTA, the methods were validated using K_2-EDTA human plasma (Bioreclamation, New York, NY, USA). For test samples, venous blood was collected from subjects into K_2-EDTA tubes, mixed gently by inversion and placed on ice. Within 1 h of collection, samples were centrifuged (1500 × *g*; 10 min, 4 °C) and the plasma immediately frozen (−80 °C) in polypropylene tubes.

2.4. Preparation of calibration standards, quality control samples and internal standard

Duplicate stock solutions (2000 μg/ml, corrected for purity) of ticagrelor, and AR-C124910XX, were prepared separately in DMSO. Working solutions were made from these stocks by further dilution with DMSO.

For plasma (total concentration) and retentate analyses, calibration standards were freshly prepared by diluting standard working solutions (ticagrelor: 0.2–200 μg/ml; AR-C124910XX: 0.1–100 μg/ml) in control human K_2-EDTA plasma to produce standards for ticagrelor (5, 10, 50, 250, 1000, 2500, 4000 and 5000 ng/ml), and AR-C124940XX (2.5, 5, 25, 125, 500, 1250, 2000 and 2500 ng/ml). For dialysate analysis, standard working solutions (6.25–250 μg/ml) were diluted in PBS (pH 7.4):acetonitrile (50:50, v/v), containing internal standard (total concentration 25 ng/ml). The final calibration concentrations for dialysate analysis were 0.25, 1, 2, 10, 20, 25, 50, 90 and 100 ng/ml for both analytes.

Quality control (QC) samples for plasma analysis were prepared by diluting working solutions (ticagrelor: 0.6–1000 μg/ml; AR-C124910XX: 0.3–500 μg/ml) in control human plasma. The final concentrations were: 5, 15, 800, 3500, 5000 and 8000 (dilution QC) ng/ml (ticagrelor); 2.5, 7.5, 400, 1750, 2500 and 4000 (dilution QC) ng/ml (AR-C124910XX). Sub-aliquots were stored frozen (−80 °C) for up to 101 days. QC samples for dialysate analysis were freshly prepared as described for calibration standards (dialysate analysis) to produce final QC concentrations for both analytes of 0.25, 1, 3, 10, 80, 100 and 1000 (dilution QC) ng/ml.

A stable isotope labeled internal standard (D_7-ZD6140) was used to quantify ticagrelor (formerly AZD6140) and AR-C124940XX. A stock solution (1000 μg/ml in DMSO) was diluted in methanol:ammonium acetate (10 mM, pH native, 50:50, v/v) for plasma analysis (1000 ng/ml; stored at 4 °C). For dialysate analysis, the internal standard stock solution was freshly diluted in acetonitrile (50 ng/ml; for samples from equilibrium dialysis plates) or PBS (pH 7.4): acetonitrile (50:50, v/v) (25 ng/ml; for dialysate calibration standards and QC samples).

2.5. Analytical procedures

2.5.1. Sample preparation

Sample processing from frozen plasma to LC–MS/MS analysis is summarized in Fig. 1. Frozen plasma samples were thawed at room temperature (<30 °C), vortexed for 5 min then centrifuged (1800 × *g*, 5 min, 20 °C). Prior to equilibrium dialysis, batch-wise pH adjustment (to pH 7.4) of calibration standards, QC samples, and plasma samples was conducted in a CO_2 incubator at 37 °C using a slight modification of the method of Nilsson and Schmidt [24] 500 μl aliquots were used, the 96-well plates were shaken at 1000 rpm, and the pH of plasma in control wells was tested using pH strips (Fisherbrand pH-Fix 6.0–7.7 (0.1 pH unit accuracy), EU code: FB33013).

2.5.2. Equilibrium dialysis

96-well format equilibrium dialysis plates, 5000 Da molecular weight cut-off, were obtained from Harvard Apparatus Ltd (Edenbridge, Kent, UK). On the buffer side, 200 μl PBS was added to each well and the filled wells were gently sealed with cap strips. Following inversion, 200 μl aliquots of plasma samples were added to the plasma side and the wells were sealed. Plates were rotated on a plate rotator (IKA Vibrax-VXR) in a CO_2 incubator at 37 °C for approximately 24 h.

For retentate and plasma (total concentration) analyses by LC–MS/MS, the sample preparation was the same as that previously described [22] and is summarized in Fig. 1. This figure also shows the sample preparation for dialysate samples. To reduce the non-specific binding of analytes in an aqueous environment, dialysate was removed from the dialysis plates and added to acetonitrile as soon as possible following equilibrium.

2.5.3. Instrumentation and LC/MS conditions

Prepared plasma and retentate samples were analyzed for ticagrelor and AR-C124910XX by LC–MS/MS as previously described [22]. As analyte concentrations in the dialysate were very low, a lower limit of quantification was required for dialysate analysis. The same conditions as previously published [22] were used for dialysate with the exception of using a more sensitive mass spectrometer API5000 (PE Sciex).

The LC 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 × 4.6 mm; Jones Chromatography, Glamorgan, UK) at ambient temperature in an air-conditioned laboratory, using a degassed mobile phase (Agilent 1100 Series Degasser) of acetonitrile (10 mM; pH native) and 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 (plasma) or an API5000 mass spectrometer using atmospheric pressure chemical ionization 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 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 521.2 → 361.1 for ticagrelor, m/z 477.2 → 361.1 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 was performed using Analyst software (initially version 1.2 and later upgraded to version 1.4.2 Applied Biosystems – PE Sciex). Calibration curves plotted nominal concentrations of calibration standards versus the analyte to internal standard peak area ratio. Sample concentrations were calculated by quadratic (plasma/retenate; $y = ax^2 + bx + c$) or linear (dialysate; $y = ax + c$) regression analysis, using the reciprocal of concentration ($1/x$) as weighting.

2.6. Validation

The methods were validated to meet the FDA requirements for bioanalytical method validation [25]; not all data are shown. However, stability, selectivity, matrix effect, carry over and recovery results were within established criteria. Validation of the LC–MS/MS method for total ticagrelor and total AR-C124910XX analyses in plasma have been previously reported [22].

2.6.1. Precision, accuracy and lower limit of quantification [LLOQ] of LC/MS for plasma and dialysate samples

Intra-batch assay precision (percentage of coefficient of variation [% CV]) was determined at each QC level, of both ticagrelor and AR-C124910XX, for 4 runs for dialysate and 1 run for total plasma analysis. Inter-run precision (dialysate analysis only) was expressed as the % CV for all QC replicates at a given concentration. For acceptable assay precision, the CV was required to be ≤15%, and ≤20% at the LLOQ.

Assay accuracy was expressed as the relative percentage of errors. Intra-run accuracy (dialysate and total plasma) was determined by expressing the mean concentration of all QC samples analyzed in each run as a percentage of their nominal concentration. Mean intra-run accuracy was determined from accuracy values obtained from six replicates of QC samples at each concentration level in each run. Mean inter-run accuracy (dialysate only) was determined by expressing the mean concentration of all QC samples analyzed in the runs. For acceptable assay accuracy, the mean accuracy values were required to be 85–115% of the nominal concentrations at all levels, except for the LLOQ (80–120%).

The assay LLOQ was the lowest concentration that yielded acceptable precision (≤20%) and accuracy (80–120%) using samples other than calibration standards.

2.6.2. Time to equilibrium and non-specific binding/adsorption

Control human K_2–EDTA plasma samples were spiked with low (15 ng/ml; 7.5 ng/ml), medium (800 ng/ml; 400 ng/ml) or high (3500 ng/ml; 1750 ng/ml) QC levels of ticagrelor or AR-C124910XX and subjected to equilibrium dialysis. Plasma retentate and dialysate samples were collected at 0, 0.5, 1, 2, 4, 6 and 24 h and analyzed. The optimal time for dialysis equilibration was considered to be when there was the smallest change in response per time unit between time points.

2.6.3. Precision of unbound concentration determinations

Six replicates of the high plasma QC (3500 ng/ml ticagrelor; 1750 ng/ml AR-C124910XX) samples, and six replicates of a plasma sample spiked with both ticagrelor (300 ng/ml) and AR-C124910XX (300 ng/ml) were subjected to equilibrium dialysis and LC–MS/MS analysis. Precision was expressed as % CV of the mean observed ratio between unbound and total concentrations of ticagrelor and AR-C124910XX in the plasma.

2.6.4. Stability of unbound concentration in human plasma

In addition to stability of ticagrelor and AR-C124910XX in plasma (total concentration; short- and long-term stability, repeated freeze–thaw), the stability of the partitioning between bound and unbound fractions was assessed to verify that the

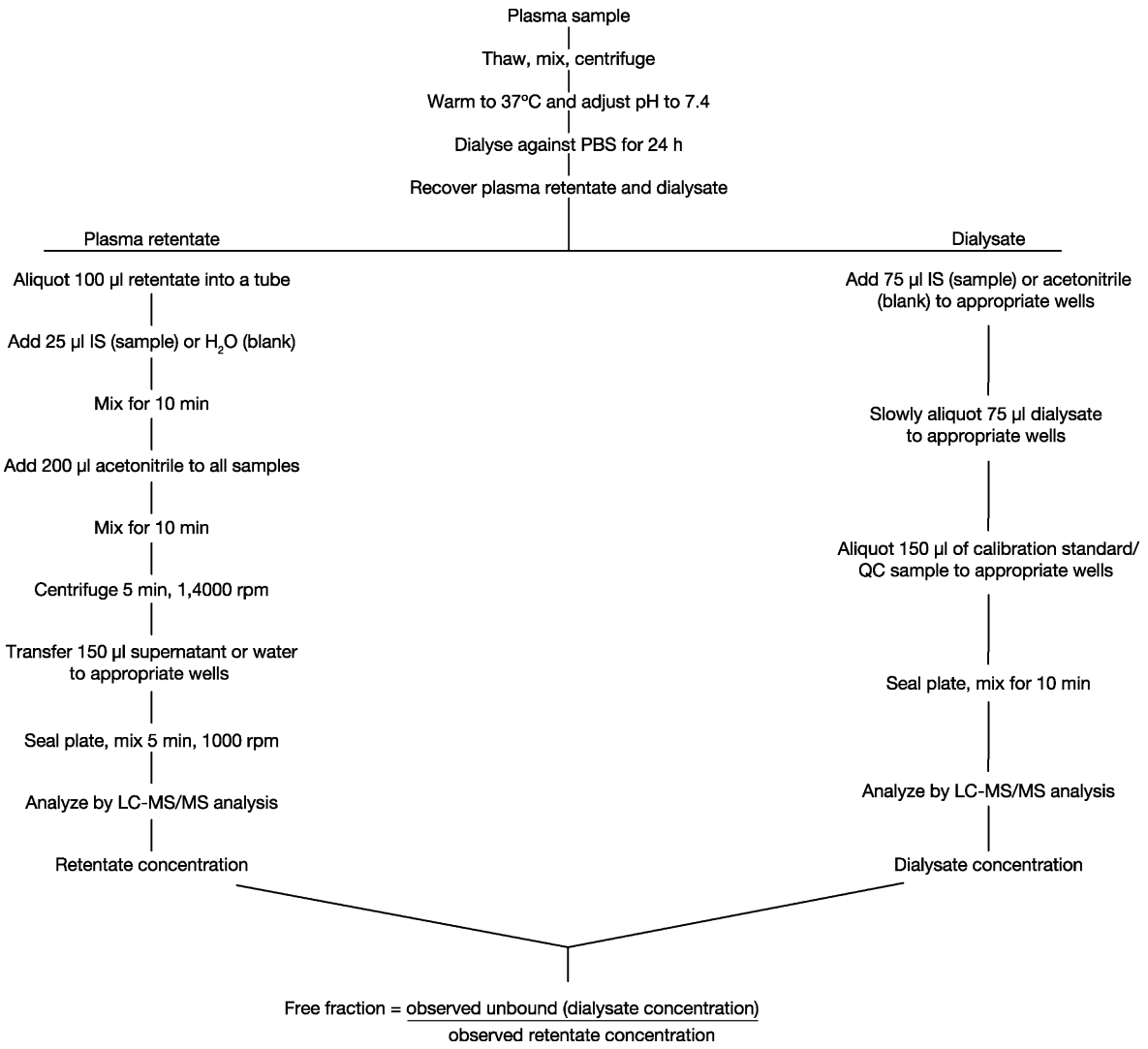

Fig. 1. A flow diagram summarizing the processing of samples for free and total ticagrelor and AR-C124910XX analysis by equilibrium dialysis-LC/MS.

method could be used to support clinical studies. Therefore, equilibrium dialysis was performed on plasma samples stored for 24 h at room temperature and 101 days stored at −80 °C. Equilibrium dialysis was also performed on samples exposed to three freeze–thaw cycles. Three replicates of the high plasma QC (3500 ng/ml ticagrelor; 1750 ng/ml AR-C124910XX) were used in each of the assessments.

2.7. Calculation of unbound fraction and unbound concentration

The unbound fraction was calculated using: observed unbound (dialysate) concentration/observed retentate concentration. The unbound concentration was calculated by: unbound fraction × total concentration.

2.8. Application to clinical data

The method was applied to the analysis of concentrations of unbound ticagrelor and AR-C124910XX in plasma samples from clinical studies following administration of single doses of ticagrelor (all subjects provided informed consent and local ethical board approval was obtained).

3. Results

3.1. Precision, accuracy and LLOQ of LC/MS for plasma and dialysate samples

Overall assay performance (precision and accuracy) for both ticagrelor and AR-C124910XX in K2-EDTA plasma were within acceptable limits with a mean intra run precision between 2.0–12.9% and 2.5–11.7%, a mean intra run accuracy between 97.1–109.0% and 98.9–104.1% for ticagrelor and AR-C124910XX, respectively.

Mean intra-run precision for ticagrelor in dialysate ranged from 7.0 to 12.4%, and mean inter-run precision was between 8.0% and 14.5% (Table 1). Assay accuracy for ticagrelor in dialysate was also within pre-defined limits. Mean intra-run accuracy was between 92.5% and 110.0%, and mean inter-run accuracy ranged from 92.7–119.6% (Table 1).

For AR-C124910XX dialysate analysis, mean intra-run (9.1–11.5%), and inter-run (9.8–14.0%) precision were acceptable for 3–100 ng/ml. At 1 ng/ml, the precision values were slightly higher than the limit (15.9% intra-run and 18.6% inter-run). Mean intra- and inter-run precision for the LLOQ (0.25 ng/ml) were acceptable at 19.9% for both (Table 1). Mean intra-run and inter-run accuracy for AR-C124910XX in dialysate

Table 1
Precision and accuracy of quality control data for ticagrelor and AR-C124910XX – dialysate analysis.

Nominal ticagrelor concentration (ng/ml)	0.25	1	3	10	80	100
Observed ticagrelor concentration (ng/ml)						
Mean intra-run precision (% CV, $n=4$)[b]	7.0[a]	12.2	12.4	12.3	10.1	10.7
Mean intra-run mean accuracy (%, $n=4$)[c]	110.0[a]	97.5	92.5	96.8	95.8	94.8
Inter-run mean ± SD	0.274 ± 0.022	0.977 ± 0.122	2.78 ± 0.347	9.63 ± 1.40	76.8 ± 9.81	94.7 ± 11.0
	$n=11$	$n=23$	$n=23$	$n=21$	$n=23$	$n=23$
Inter-run precision (CV %)[d]	8.0	12.5	12.5	14.5	12.8	11.6
Inter-run accuracy (%)[e]	109.6	97.7	92.7	96.3	96.0	94.7
Nominal AR-C124910XX concentration (ng/ml)	0.25	1	3	10	80	100
Observed AR-C124910XX concentration (ng/ml)						
Mean intra-run precision (% CV, $n=4$)[b]	19.9[a]	15.9	11.5	9.9	9.1	9.5
Mean intra-run mean accuracy (%,$n=4$)[c]	105.6[a]	103.2	98.9	97.9	93.8	93.2
Inter-run mean ± SD	0.266 ± 0.053	1.03 ± 0.192	2.95 ± 0.414	9.89 ± 0.972	74.9 ± 8.45	93.2 ± 10.2
	$n=11$	$n=23$	$n=22$	$n=18$	$n=21$	$n=21$
Inter-run precision (CV %)[d]	19.9	18.6	14.0	9.8	11.3	10.9
Inter-run accuracy (%)[e]	106.4	103.0	98.3	98.9	93.6	93.2

[a] $n=2$ runs.
[b] Mean intra-run precision determined from the values obtained from each run.
[c] Mean intra-run accuracy determined from accuracy values from 6 replicates at each concentration level in each run.
[d] Inter-run precision for all QC replicates at a particular level within the runs.
[e] Inter-run accuracy from the mean concentration of all QCs analyzed in the runs. Any sample not included in the statistics was due to mis-injection or mis-extraction.

ranged from 93.2 to 105.6% and 93.2 to106.4%, respectively (Table 1).

The LLOQs were 5 ng/ml (ticagrelor) and 2.5 ng/ml (AR-C124910XX) for plasma analysis, and 0.25 ng/ml for both analytes in dialysate. At the LLOQ, the signal-to-noise ratio was greater than five for ticagrelor and AR-C124910XX for both dialysate (Fig. 2) and plasma analyses (data not shown).

3.2. Time to equilibrium and non-specific binding to equilibrium dialysis plate

The ratio of both ticagrelor (Fig. 3a) and AR-C124910XX (Fig. 3b) in dialysate versus retentate increased with time. Both analytes were first detectable in dialysate at 1 h (high QC samples) and 2 h (medium QC samples). For low QC samples, AR-C124910XX (7.5 ng/ml) was not detectable in dialysate at any time point, whereas ticagrelor (15 ng/ml) was present from 6 h onwards. At 24 h, the % CVs were low (<12%, $n=3$ per concentration) for both analytes in dialysate following equilibrium dialysis of the medium and high QC samples. Although it could not be confirmed by an additional time point that equilibrium had been achieved after 24 h, the change in concentration between 6 and 24 h was in the range of a factor of 2 and was considered small based on the 18 h time period. Based on these results, the time selected for dialysis equilibration was 24 h at 37 °C.

Even though non-specific binding to the equilibrium dialysis plate occurs in the buffer, the degree of such binding could not be determined due to the observation that the overall decrease in the concentration of analytes in the retenate were not quantifiable.

3.3. Precision of equilibrium dialysis

The precision of the mean unbound fraction of ticagrelor and AR-C124910XX was 17.7% (300 ng/ml, $n=6$) and 14.8% (3500 ng/ml, $n=6$), and 25.0% (300 ng/ml, $n=6$) and 11.0% (1750 ng/ml, $n=6$), respectively. Although the precision for ticagrelor and AR-C124910XX at 300 ng/ml was outside the generally accepted 15%, this result was considered acceptable considering the dialysate and retentate are analyzed independently, and that the concentration of analytes in the dialysate aliquot were very close to LLOQ of the assay. For ticagrelor intra-batch precision for dialysate concentrations close to the LLOQ were between 10 and 15%, and for AR-C124910XX were up to 20%. Intra-batch precision in plasma for both analytes, at 300 ng/ml, was typically less than 10%. Thus, the estimated precision for the unbound fraction is approximately 18% (SQR[15^2+10^2]) for ticagrelor and 22% (SQR[20^2+10^2]) for AR-C124910XX. In addition to the separate LC/MS assays used, equilibrium dialysis will also impact on the precision of the overall method. The degree of protein binding for both ticagrelor and AR-C124910XX was very high (>99.8%) and a CV of 17.7% corresponds to a standard deviation of approximately 0.035% for the unbound fraction of ticagrelor.

3.4. Stability of analytes in human plasma

Total ticagrelor and total AR-C124910XX were stable in K_2-EDTA human plasma subjected to three repeated freeze–thaw cycles, at room temperature for 24 h, and frozen at −80 °C for 6 months (data not shown). Furthermore, under equilibrium dialysis conditions (37 °C for 26 h), total ticagrelor and AR-C124910XX were stable. The percent differences in concentration from baseline were −8.0% and 5.9% (ticagrelor) and −7.4% and −3.5% (AR-C124910XX) for the low and high QC samples, respectively.

Evaluation of the unbound fraction of ticagrelor and AR-C124910XX in K_2-EDTA human high QC samples subjected to three repeated freeze–thaw cycles, stored at room temperature for 24 h, and stored frozen at −80 °C indicated stability under these storage conditions. The range of the relative percent differences from baseline for unbound fraction of ticagrelor was −23.2 to −1.0%, and for unbound AR-C124910XX −10.3 to +9.2% in high QC samples (Table 2). The largest difference in concentration was –23.2%, equivalent to an overall apparent change in unbound fraction of ticagrelor of −0.068%, and was, therefore, considered acceptable. Based on these results, the unbound fraction of ticagrelor and AR-C124910XX was deemed stable at −80 °C for at least 101 days, 3 repeated freeze/thaw cycles and 24 h at room temperature.

3.5. Application to clinical data

The validated method was successfully applied to determine the unbound fraction of ticagrelor and AR-C124910XX in clinical studies after a single oral dose of ticagrelor [26]. Analyzing samples from several subjects showed protein binding >99.8% for both analytes, with a relative standard deviation of unbound fraction of 15.9% ($n=88$) and 20.7% ($n=13$, due to many samples below LLOQ) in samples with total analyte concentrations of 10.1–2250 ng/ml and 10.3–556 ng/ml, for ticagrelor and AR-C124910XX, respectively (Fig. 4). The within-subject relative standard deviations ranged

Table 2
Stability of unbound fraction of ticagrelor and AR-C124910XX in high QC samples.

Storage conditions	Ticagrelor		AR-C124910XX	
	Relative change[a] (%)	Absolute change[b] (%)	Relative change[a] (%)	Absolute change[b] (%)
24 h at room temperature	−3.4	−0.005	3.6	0.005
3× freeze–thaw cycles	−1.0	−0.001	9.2	0.013
3 months at −80 °C	−23.2	−0.068	−10.3	−0.026

[a] Relative percentage change compared to baseline value.
[b] Absolute change in unbound fraction of analyte.

from 3.5% to 22.2% (n = 2–6 samples/subject) for ticagrelor. Due to very high protein binding, many samples were below the LLOQ in the dialysate for AR-C124910XX and no within subject relative standard deviation could be calculated. No major differences in the unbound fraction were seen at different concentrations of the analytes (Fig. 4) or between healthy volunteers and subjects with mild hepatic impairment [26]. Based on several limitations, i.e., both compounds are highly protein bound (>99.8%), the analyses were conducted in several batches, and that the determination required equilibrium dialysis followed by two further analyses, these results were considered to be acceptable.

4. Discussion

A method to determine the unbound fraction of ticagrelor and AR-C124910XX in human plasma samples using equilibrium dialysis was successfully developed. A slight adaptation of the established LC–MS/MS method for total ticagrelor and AR-C124910XX [22] increased the assay sensitivity sufficiently for the quantification of unbound analytes in dialysate. The overall method was validated over the concentration ranges 5–5000 ng/ml (ticagrelor) and 2.5–2500 ng/ml (AR-C124910XX) in plasma (total concentration) and retentate, using a 100 μl sample aliquot; and 0.25–100 ng/ml (ticagrelor and AR-C124910XX) in dialysate, using a 75 μl sample aliquot. Moreover, the LC–MS/MS method for total ticagrelor and AR-C124910XX was validated for human K_2-EDTA plasma samples.

Initial evaluations with ultrafiltration to separate unbound ticagrelor and AR-C12490XX were unsuccessful due to the very high (>90%) non-specific binding of these non-polar compounds to the device and membrane in aqueous conditions. Thus, equilibrium dialysis became the preferred analytical method. This well-established method is robust, providing accurate information on unbound drug fraction. Furthermore, the availability of single-use 96-well dialysis plates means that equilibrium dialysis can be easily applied, inexpensively, to large sample numbers. Another advantage is that only small sample volumes (e.g. 50–200 μl) are required.

Equilibrium dialysis was used successfully to achieve the unbound fractions of ticagrelor and AR-C12490XX from 200 μl aliquots of K_2-EDTA plasma. However, this separation technique is associated with several limitations that require adequate control as discussed below.

In-vitro conditions can artifactually alter drug-protein binding [27]. In the equilibrium method described herein, particular attention was paid to ensure that near physiologic conditions were maintained. As the pH of plasma samples change during storage [24], the pH was adjusted to pH 7.4, to re-establish the ratio of bound and unbound analytes. The dialysis was also conducted at 37 °C to minimize any temperature-dependent alterations in binding. In addition, a 5000 Da molecular weight cut-off was chosen to minimize protein transfer to the dialysate.

As with ultrafiltration, non-specific drug adsorption to the equipment is also a well-recognized limitation of equilibrium dialysis. Although this phenomenon is less of a problem with equilibrium dialysis than ultrafiltration provided that (a) sufficient time is allowed for equilibration, (b) the determinations are performed in a range where the protein binding is independent of concentration, and (c) drug concentrations in both the plasma retentate and dialysate are measured and used to calculate the unbound drug fraction. All these criteria were applied in the current method development. By conducting a time course for a range of QC samples of both analytes, the time-to-equilibration was considered to be established by 24 h at 37 °C. However, given that ticagrelor and AR-C12490XX bind to plastic in aqueous environments, dialysate samples should be analyzed as soon as possible after equilibrium dialysis to reduce the impact of variability on data validity.

Despite the concern regarding non-specific binding and the high protein binding, the precision of the current equilibrium dialysis method showed that the results for the unbound fraction were acceptable. The precision for one ticagrelor (17.7%) and AR-C124910XX concentration (25.0%) was only slightly above what is normally accepted for standard LC–MS/MS methods. Overall, these results are likely to be representative of the method performance due to high levels of non-specific binding and the high protein binding, and are considered acceptable for the intended use of the analytical method.

Due to the high protein binding of ticagrelor and AR-C124910XX, the LC–MS/MS method developed for total concentrations in lithium–heparin plasma samples [22] were not suitable and did not have sufficient limit of quantification (LOQ) for quantifying the dialysate. In addition, high levels of protein binding make determination of the unbound fraction more difficult, and is likely to reduce the accuracy and precision of determination. Thus, the LC–MS/MS method was slightly modified by using less dilution and upgrading the MS system to improve the sensitivity. The lower LOQ of 0.25 ng/ml for both compounds in dialysate was lower than that for ticagrelor (5 ng/ml) and AR-C124910XX (2.5 ng/ml) in plasma. Moreover, the precision and accuracy results for the LC–MS/MS dialysate analyses were considered acceptable. Another modification required was the use of K_2-EDTA as an anticoagulant instead of lithium heparin, which could potentially affect drug-protein binding [23]. The adapted LC–MS/MS method was suitable for K_2-EDTA plasma samples. Calibration curves were established and the precision and accuracy results for total ticagrelor and AR-C124910XX were within acceptable limits. These results were very similar to those reported for lithium–heparin plasma samples [22].

The presence of K_2-EDTA did not affect the stability of total ticagrelor or AR-C124910XX for the time period evaluated. Importantly, these compounds were also stable in plasma under equilibrium dialysis conditions, i.e. 37 °C for 26 h. The unbound fraction of both ticagrelor and AR-C124910XX was also found to be stable to storage under the various conditions monitored. Although a deviation from the specified stability criteria was seen for unbound ticagrelor, this was considered acceptable and likely due to the variability in method performance given the non-specific binding to plastic under aqueous conditions and the high protein binding.

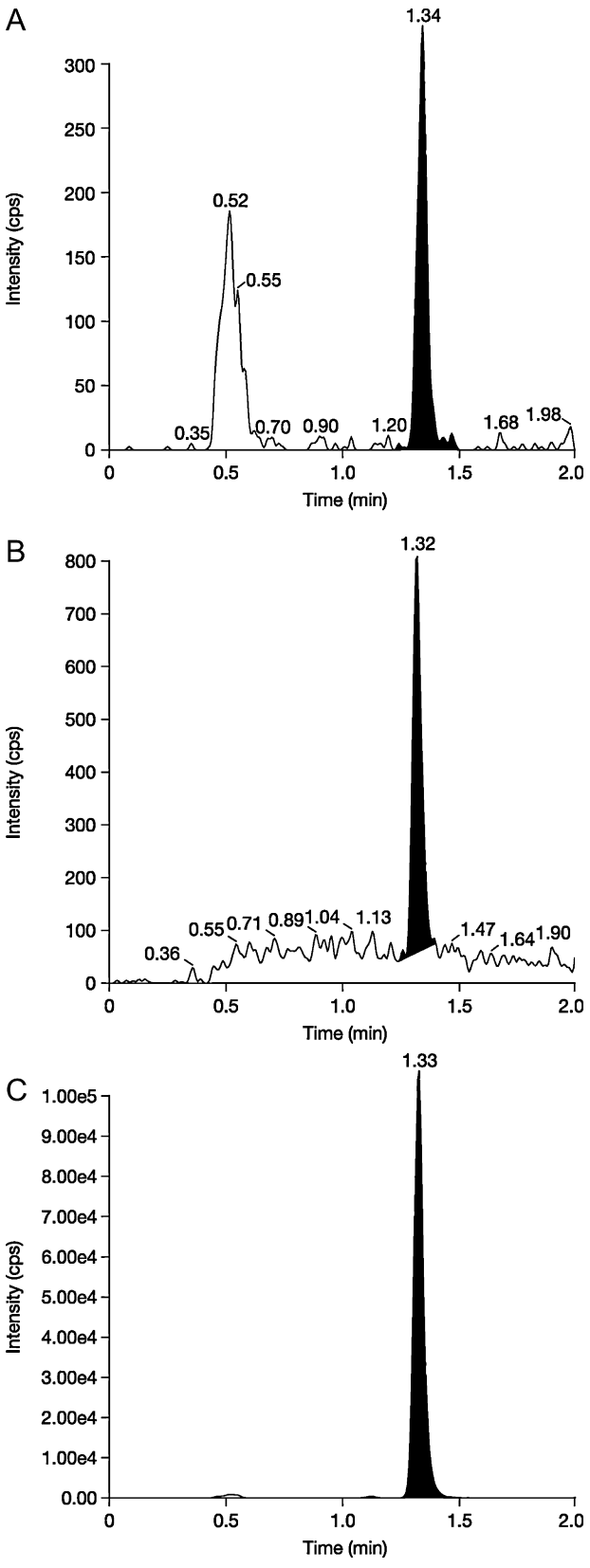

Fig. 2. Typical chromatograms of a dialysate sample with the addition of 0.25 ng/ml ticagrelor (A), 0.25 ng/ml AR-C124910XX (B), and internal standard (C).

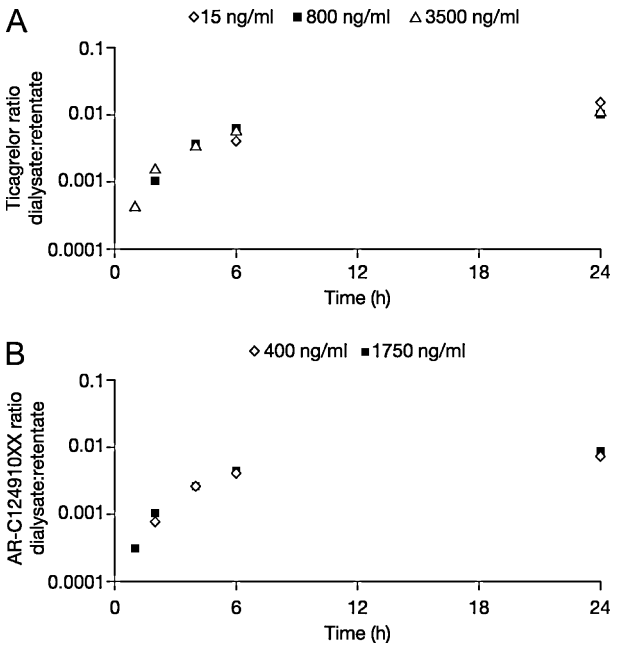

Fig. 3. Time to equilibrium dialysis of a range of QC samples for ticagrelor (A) and AR-C124910XX (B).

When analyzing samples from clinical studies, the unbound fractions of both analytes were similar for all subjects, and were independent of total drug plasma concentrations. Furthermore, samples from test subjects were analyzed in the same batch run as samples from control subjects to allow data comparisons. Therefore, any day-to-day variation was less critical for the purpose of the studies. However, for studies where samples from concurrent control subjects are not available, consideration to monitor any day-to-day variation, e.g. different equilibrium dialysis plates, should be done. One way of doing this is to use spiked QC samples that are processed through equilibrium dialysis and in which the unbound concentration is determined. Even though the true unbound concentration cannot be known, such samples can be used to assess variations in the method performance.

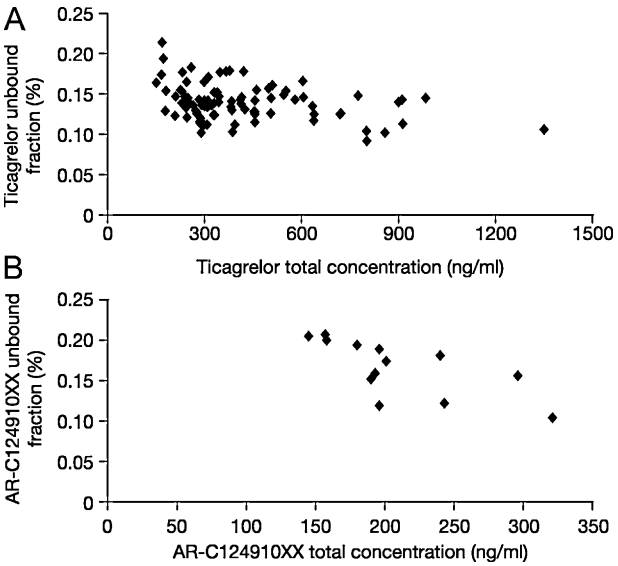

Fig. 4. Fraction of unbound ticagrelor and AR-C124910XX in plasma versus total plasma concentrations in blood samples from 20 human subjects treated with a single oral dose of ticagrelor.

The mean unbound fraction of ticagrelor and AR-C124910XX were relatively small, i.e., <0.2% for all measured concentrations. These results demonstrate that both ticagrelor and AR-C124910XX are highly protein bound in human plasma K_2-EDTA samples.

5. Conclusions

In conclusion, a method for determining the unbound fraction of ticagrelor and its main metabolite AR-C124910XX in K_2-EDTA human plasma was successfully validated over the required concentration ranges. Furthermore, the applicability of the LC–MS/MS method for total ticagrelor and AR-C124910XX in human plasma samples using K_2-EDTA as the anticoagulant was confirmed. Thus, using equilibrium dialysis with LC–MS/MS, a robust, high-throughput, validated analytical method is now available for the quantification of unbound ticagrelor and its active metabolite in clinical samples.

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.

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