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An easy and fast adenosine 5'-diphosphate quantification procedure based on hydrophilic interaction liquid chromatography-high resolution tandem mass spectrometry for determination of the in vitro adenosine 5'-triphosphatase activity of the human breast cancer resistance protein ABCG2

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Abstract

Interactions with the human breast cancer resistance protein (hBCRP) significantly influence the pharmacokinetic properties of a drug and can even lead to drug-drug interactions. As efflux pump from the ABC superfamily, hBCRP utilized energy gained by adenosine 5'-triphosphate (ATP) hydrolysis for the transmembrane movement of its substrates, while adenosine 5'-diphosphate (ADP) and inorganic phosphate were released. The ADP liberation can be used to detect interactions with the hBCRP ATPase. An ADP quantification method based on hydrophilic interaction liquid chromatography (HILIC) coupled to high resolution tandem mass spectrometry (HR-MS/MS) was developed and successfully validated in accordance to the criteria of the guideline on bioanalytical method validation by the European Medicines Agency. ATP and adenosine 5'-monophosphate were qualitatively included to prevent interferences. Furthermore, a setup consisting of six sample sets was evolved that allowed detection of hBCRP substrate or inhibitor properties of the test compound. The hBCRP substrate sulfasalazine and the hBCRP inhibitor orthovanadate were used as controls. To prove the applicability of the procedure, the effect of amprenavir, indinavir, nelfinavir, ritonavir, and saquinavir on the hBCRP ATPase activity was tested. Nelfinavir, ritonavir, and saquinavir were identified as hBCRP ATPase inhibitors and none of the five HIV protease inhibitors turned out to be an hBCRP substrate. These findings were in line with a pervious publication.

Keywords ADP quantification, HILIC-HR-MS/MS, hBCRP ATPase, hBCRP substrate, hBCRP inhibitor, HIV protease inhibitors

1. Introduction

Membrane transporters such as the human breast cancer resistance protein (hBCRP, also known as ABCG2 or MXR) are gaining more and more attention not only during development, but also for better understanding of pharmacokinetics and drug interactions [1]. In 2010, the International Transporter Consortium (ITC) highlighted the importance of seven key membrane transporters in drug development because of their major influence on the pharmacokinetic, safety, and efficacy profiles of drugs [1]. In 2012, the European Medicines Agency (EMA) and Food and Drug Administration (FDA) included these transport proteins in their guidelines on the investigation of drug interactions [2, 3].

One of the transporters highlighted by the ITC is hBCRP, an adenosine 5'-triphosphate (ATP)-dependent efflux pump from the ABC superfamily, closely related to P-glycoprotein [1]. hBCRP is not only highly expressed in several cancer cells, where it was initially discovered, but also in normal human tissues including the small intestine, liver, brain endothelium, and placenta. It plays therefore an important role in the absorption, elimination, and tissue distribution of drugs and other xenobiotics [4]. For the transmembrane movement of its substrates, hBCRP utilized energy gained by ATP hydrolysis, while adenosine 5'-diphosphate (ADP) and inorganic phosphate are released [5].

Besides more complicated models such as cell-based assays, intact organs, or transporter-deficient animals, membrane-based systems were often used to identify hBCRP substrates or inhibitors. Substrate-dependent ATP hydrolysis has been measured to evaluate the interactions with some ABC transporters usually by colorimetric analysis of the inorganic phosphate release [1]. Unfortunately, this reaction can be disturbed by colored samples [6]. Another approach, the analysis of not consumed ATP, was measured by a bioluminescence reaction using luciferase [7]. This method is also known to be interference-prone, particularly due to substrate instability [8]. Furthermore, the linear range of the reaction is below the concentrations expected in the reaction mixtures and all incubated samples had to be diluted prior to analysis [7]. So far, only a few studies were published using LC-MS for quantification of ADP [9-12] but none of them were applicable for the direct measurement of ADP in in vitro hBCRP ATPase activity studies.

Therefore, the aim of the present study was the development of such a method using hydrophilic-interaction liquid chromatography (HILIC) coupled to high resolution

tandem mass spectrometry (HR-MS/MS) for ADP quantification and detection of ATP and adenosine 5'-monophosphate (AMP). The workup and analysis should be validated in accordance to international guidelines for bioanalytical procedures [13]. Furthermore, the applicability of the developed setup should be demonstrated by determining the influence of five HIV protease inhibitors on the in vitro hBCRP ATPase activity, from which were already data available for comparison [14].

2. Materials and methods

2.1. Chemicals and enzymes

The baculovirus-infected insect cell microsomes (Supersomes) containing human complementary DNA-expressed BCRP (Arg482, 5 mg protein/mL) and wild-type Supersomes without hBCRP (control membrane, 5 mg protein/mL) used as negative control were obtained from Corning (Amsterdam, The Netherlands). After delivery, Supersomes were thawed at 37 °C, aliquoted, snap-frozen in liquid nitrogen, and stored at -80 °C until use.

AMP disodium salt, ADP sodium salt, ATP magnesium salt, guanosine 5'-diphospate (GDP) sodium salt, uridine 5'-phosphate (UDP) sodium salt hydrate, sulfasalazine, sodium orthovanadate, amprenavir, indinavir, nelfinavir, ritonavir, saquinavir mesylate, ammonium acetate, MES hydrate, and Trizma base were obtained from Sigma-Aldrich (Taufkirchen, Germany), formic acid (MS grade) from Fluka (Neu-Ulm, Germany), acetonitrile, methanol (both LC-MS grade), and all other chemicals from VWR (Darmstadt, Germany).

Stock solutions were prepared in bidistilled water for sodium orthovanadate (10 mM), AMP, ADP, ATP, GDP, and UDP (20 mM, respectively) or in methanol for sulfasalazine (0.5 mg/mL), amprenavir, indinavir, nelfinavir, ritonavir, and saquinavir (1 mg/mL, respectively). Stock solutions were aliquoted and stored at -20 °C until use.

2.2. HILIC-HR-MS/MS apparatus

A Thermo Fisher Scientific (TF, Dreieich, Germany) Dionex UltiMate 3000 Rapid Separation (RS) LC system with a quaternary UltiMate 3000 RS pump and an UltiMate 3000 RS autosampler was used and controlled by the TF Chromeleon

software version 6.80. It was coupled to a TF Q-Exactive Plus equipped with a heated electrospray ionization II source (HESI-II). The gradient elution was performed on a Macherey-Nagel (Düren, Germany) HILIC Nucleodur column (125 × 3 mm, 3 µm) using aqueous ammonium acetate (200 mM, eluent A) and acetonitrile containing 0.1% (*v/v*) formic acid (eluent B). The flow rate was set to 700 µL/min and an isocratic elution with a duration of 6 min using 65% eluent B was performed at 40 °C column temperature, maintained by a Dionex UltiMate 3000 RS analytical column heater. The injection volume for all samples was 1 µL. HESI-II conditions were as already described before [15]: sheath gas, 60 arbitrary units (AU); auxiliary gas, 10 AU; spray voltage, 4.00 kV; heater temperature, 320 °C; ion transfer capillary temperature, 320 °C; and S-lens RF level, 60.0. Mass calibration was done prior to analysis according to the manufacturer's recommendations using external mass calibration. For evaluating the chromatographic separation, a full scan experiment was used with the following scan parameters: polarity, negative; in-source collision-induced dissociation (CID), 0 eV; microscan, 1; resolution, 35,000; automatic gain control (AGC) target, 1e6; maximum injection time (IT), 120 ms; and acquisition range, 100-600 *m/z*. The final quantification was performed using a targeted single ion monitoring (t-SIM) and a subsequent data-dependent MS^2 (dd-MS^2) mode with an inclusion list containing the exact masses of negatively charged AMP (*m/z* 346.0558), ADP (*m/z* 426.0221), and ATP (*m/z* 505.9885). The settings for the t-SIM mode were as follows: polarity, negative; in-source CID, 0 eV; microscan, 1; resolution, 35,000; AGC target, 5e4; maximum IT, 100 ms; and isolation window, 4 *m/z*. The cycle time for the t-SIM was 2.3 Hz. The settings for the dd-MS^2 mode were as follows: microscan, 1; resolution, 35,000; AGC target, 2e5; maximum IT, 100 ms; isolation window, 4 *m/z*; and dynamic exclusion, 4 s. Limited by the dynamic exclusion, the cycle time for the dd-MS^2 was set to 0.25 Hz. Quantification was performed using t-SIM, while dd-MS^2 was only used for identification. TF Xcalibur Qual Browser 2.2 software was used for data handling. The settings for automated peak integration were as follows: mass tolerance, 5 ppm; peak detection algorithm, ICIS; area noise factor, 5; and peak noise factor, 300. GraphPad Prism 5.00 (GraphPad Software, San Diego, USA) was used for statistical evaluation.

2.3. Method validation

The ADP quantification method was validated in accordance to the "Guideline on bioanalytical method validation" published by the EMA [13]. Briefly, the method was tested for selectivity (using ten blank samples containing 0.2 mg/mL control membrane with and without ATP, respectively), carry-over (using a blank sample without ATP following the high quality control, QC), lower limit of quantification (LLOQ, equal to the lowest calibration standard), within-run accuracy and precision (analyzed in a single run six samples per level at four concentration levels: LLOQ QC, low QC, medium QC, and high QC), between-run accuracy and precision (analyzed in three different runs on three different days six samples per level at four concentration levels: LLOQ QC, low QC, medium QC, and high QC), dilution integrity (analyzed five samples spiked above the calibration range and diluted by factor five with blank matrix), matrix effect (using six samples with matrix and six samples without matrix at two concentration levels: low QC and high QC), and stability of processed samples in the autosampler (analyzed immediately after preparation and again after 24 h in the autosampler, three samples per level at two concentration levels: low QC and high QC). The calibration consisted of six concentration points (given in Table 1) equally distributed over the entire range. The concentrations of LLOQ QC, low QC, medium QC, and high QC were as follows: 50, 125, 250, and 375 µM. Calibration standards and QCs were prepared from different stock solutions that were serially diluted with bidistilled water to obtain the final concentrations. Control membrane, diluted to a final concentration of 0.2 mg/mL with 50 mM Tris-MES buffer (pH 6.8), was used for sample preparation. Unless otherwise stated, 4 mM ATP was also present in the samples, which were not incubated. The final volume was 30 µL. Finally, the samples were diluted with the same volume of acetonitrile, centrifuged for 2 min at 10,000×*g*, the supernatant was transferred to an autosampler vial, and analyzed by HILIC-HR-MS/MS. For quantification, the mean ADP area was used calculated after running each sample twice.

After completed validation, all analytical runs consisted of two blank samples, the calibration standards in duplicate, three levels of QC samples (low, medium, and high) in duplicate, and the study samples. All samples were analyzed twice and the mean ADP area minus mean ADP area in blank samples was used for quantification. All calculations were done using GraphPad Prism 5.00 software.

2.4. Incubation conditions for detection of hBCRP ATPase activity

Reaction conditions were adapted from Sarkadi et al. [16] with the following modifications. All reactions were carried out in 500 µL reaction tubes. Sulfasalazine, a known hBCRP substrate [17], was diluted with bidistilled water and used at a final concentration of 10 µM to ascertain appropriate incubation conditions. To check the protein dependency of the ATPase activity, the content of hBCRP membrane was varied between 0.1 and 0.8 mg/mL. To check the time dependency of the ATPase activity, incubation duration was varied between 5 and 60 min. To check the ATP dependency of the ATPase activity, ATP content was varied between 0.25 and 4 mM. All incubations were conducted in duplicate.

Final incubation mixtures contained 0.2 mg/mL hBCRP membrane and 4 mM ATP, as well as an hBCRP substrate or a mixture of an hBCRP substrate and an hBCRP inhibitor. ATP and substrate/inhibitor were diluted with bidistilled water and hBCRP membrane with Tris-MES buffer prior to incubations. The reaction was started by addition of ATP and stopped after 10 min of incubation at 37 °C by addition of 30 µL of ice-cold acetonitrile. The mixture was centrifuged for 2 min at 10,000×*g*, the supernatant transferred to an autosampler vial, and analyzed by HILIC-HR-MS/MS.

Incubations with sulfasalazine and 400 µM sodium orthovanadate, an inhibitor of ABC efflux pumps such as hBCRP [17], were also conducted.

2.5. Application for determination of interactions with HIV protease inhibitors

To test the influence of amprenavir, indinavir, nelfinavir, ritonavir, and saquinavir on hBCRP ATPase activity, six different sample sets consisting of three samples each were used as shown in Fig. 1. Incubation conditions were the same as described above. Sample set one contained one of the test compounds, set two one of the test compounds and sulfasalazine, sets three and five only sulfasalazine, set four sulfasalazine and orthovanadate, and set six none of these substances. All reactions were started by addition of ATP and sample sets one to five contained hBCRP membrane, while sample set six contained control membrane. Reactions of sample sets one to four and six were stopped by addition of pure acetonitrile, while acetonitrile used for set five contained the test compounds in addition. The HIV protease inhibitors were diluted with bidistilled water prior to incubations and had a final concentration of 50 µM. The ADP formation in sets one to five minus ADP formation in set six was then compared to each other. For statistical analysis of data,

a one-way ANOVA followed by Dunnett's multiple comparison test with set three as reference group (significance level, $P < 0.05$, 95% confidence intervals) was used.

3. Results and discussion

3.1. Development of the method

For ABC transporters such as hBCRP, the transport process is associated with ATP binding and hydrolysis to provide energy for substrate translocation [4]. In the presence of ATP and a substrate, the hBCRP ATPase is activated and ATP consumed, while ADP and inorganic phosphate are released. If membrane fragments expressing the investigated transporter are used measurement of the substrate translocation is not possible, but the ATPase activity can be used as marker for interactions with hBCRP. Colorimetric analysis of the inorganic phosphate may provide a simple and practical approach [1]. As ATP has to be present in excess to be not the limiting factor of the reaction, the quantification of remaining ATP after termination is another possibility [7]. However, as already mentioned, both methods have several limitations such as disturbance by colored samples and substrate instability [6, 8]. Thus, the current method used hBCRP membranes and targeted the quantification of ADP that was not used as marker for determination of ATPase activity before. Furthermore, none of the described methods used the high flexibility and sensitivity of HR-MS/MS. HILIC was shown to provide sufficient retention and separation of small and polar compounds [18, 19], but also of the highly polar adenosine nucleotides [12, 20]. However, the method by Dowood et al. was developed to quantify 3'-phosphoadenosine-5'-phosphosulfate, while ADP and ATP were only qualitatively included to prevent interferences [20]. Li et al. quantified ADP, ATP, and four other cofactors in *E. coli* cells [12], but the linear range for ADP was below the concentrations that were expected in incubations with hBCRP membranes. Furthermore, the procedure was not validated in accordance to international guidelines and solid phase extraction followed by an analytical run time of 37 min would be far too time-consuming to screen a high number of samples for interactions with the hBCRP ATPase activity [12].

Chemical structures and HR-MS/MS spectra of AMP, ADP, and ATP are given in Fig. 2. Except for AMP, the mass of the precursor ion could not be detected in the

HR-MS/MS spectrum but the substances could be differentiated thanks to specific fragments anyway. To ensure chromatographic separation, a mixture of the pure substance solutions was used containing AMP, ADP, and ATP in water:acetonitrile 1:1 (*v/v*) at a concentration of 2 mM, each. After successful separation, two peaks appeared in the t-SIM chromatogram of ADP as shown in Fig. 3A and both were most likely identified to be ADP based on the dd-MS^2 spectrum. If ADP was injected alone, only one peak @ 3.5 min was detected. As the second ADP peak @ 4.0 min appeared only in solutions containing also ATP or exclusively ATP, this was most probably due to in-source fragmentation of ATP to ADP. However, as both peaks were chromatographically separated, it was possible to only integrate the prior one and use its area for ADP quantification in all samples.

To correct experimental variability, an internal standard structurally similar to ADP such as GDP should be added. Unfortunately, even changes in the ratio of the eluents and an increased run time did not lead to complete separation of the analytes and addition of GDP caused tailing of the ADP signal, probably as result of column saturation during co-elution. Therefore, UDP was tested as internal standard. Surprisingly, the UDP signal increased with an increasing amount of ADP in the sample for unclear reasons. As these two compounds with structural similarity to ADP did not provide any benefit, no internal standard was used instead and results were still sufficient but to correct fluctuations during analysis, all samples were run twice and the mean ADP area was used.

3.2. Method validation

The analytical procedure based on HILIC-HR-MS/MS in t-SIM mode with a subsequent dd-MS^2 mode allowed detection and identification of AMP, ADP, and ATP. While AMP and ATP were only qualitatively included, the quantification of ADP was successfully validated in accordance to the criteria of an international guideline [13]. To avoid imprecision in ADP quantification by permanent MS^2 recording, dynamic exclusion of 4 s was used, what allowed repeated MS^2 recording of the same precursor ion only after 4 s had passed. Experimental variability during analysis was corrected by duplicate analysis of each sample and calculation of the mean ADP area. Mean coefficient of determination for calibration curves are given in Table 1. Curve was fitted using linear regression without weighting.

The LLOQ was set equal to the lowest calibration standard as the practically relevant concentration range were way above the real LOQ. The method was selective at LLOQ levels if no ATP was contained in the analyzed samples as shown in Fig. 3B. In presence of ATP, ADP was detectable (Fig. 3C). ADP was already contained in the ATP pure substance solution as well. Therefore, it could either be an impurity in the ATP pure substance, that is isolated from a microbial source by the manufacturer, or formed during ATP dissolving. ATP is known to be stable for months in aqueous solution stored at -15 °C and only for approximately one week at 0 °C [21]. Therefore, it was necessary to use always a freshly thawed ATP aliquot and to prepare two blank samples with ATP and to subtract the ADP area detected in these samples from the ADP area detected in all following samples. No carry-over was observed. The LLOQ for ADP was defined as 50 µM, which is the lowest ADP concentration that can be quantified reliably. The mean within-run and between-run accuracies ranged from 3 to 14% and were within 20% of the nominal values for the LLOQ QC and within 15% for the low, medium, and high QC samples. The mean within-run and between-run precisions ranged from 3 to 7%. Precisions were within 20% for the LLOQ QC and within 15% for the low, medium, and high QC samples. Accuracy and precision data are summarized in Table 2. To investigate the matrix effect, the ratio of the peak area in presence of matrix to the peak area in absence of matrix was used. Those matrix factors were 1.5 and 1.3 for low and high QC levels with coefficients of variation of 9% and 6%, respectively, and thus not greater than 15%. Chromatograms of a LLOQ QC and a high QC can be found in Fig. 3D and 3E, respectively. Fig. 3C-E show also, that AMP was detectable in samples containing ADP and/or ATP, even if they were not fortified with AMP. This was most likely due to an impurity in the pure substances of ADP and/or ATP. Processed samples provided stability in the autosampler for at least 24 h, corresponding to the maximum duration of the analytical runs, as mean concentrations of low and high QC levels were within ±15% of the nominal values.

3.3. Detection of hBCRP ATPase activity

The hBCRP substrate sulfasalazine was used to demonstrate the detectability of ADP formed in in vitro incubations by hBCRP ATPase activity. Incubation time and enzyme concentration were varied and final conditions set in the linear range of ADP formation. Further incubations were therefore conducted with 0.2 mg/mL hBCRP

membrane for 10 min. To avoid non-specific protein binding, the protein concentrations were chosen as low as analytically possible as recommended by Baranczewski et al. [22]. The dependency of the ATP concentration was also tested because ATP should not be the limiting factor of the reaction. The highest amount of ADP was formed with 4 mM ATP. The final incubation conditions were similar to the hBCRP membrane manufacturer's recommendations, but the protein concentration could be chosen lower than suggested, thanks to the high sensitivity of HR-MS/MS reducing the risk of non-specific protein binding, as well as material costs.

As ADP was also detected in incubations without sulfasalazine, the basal ATPase activity was determined. Therefore, hBCRP membranes were incubated with the hBCRP inhibitor orthovanadate in presence of sulfasalazine. The amount of formed ADP was comparable to that formed in incubations with control membrane. Control membranes provided constant, reproducible ATP consumption that was independent of the presence of other substances, such as sulfasalazine or orthovanadate. Therefore, the amount of ADP formed in incubations with control membrane could be used as blank samples and subtracted from that formed in incubations with hBCRP membrane.

3.4. Effect of HIV protease inhibitors on hBCRP ATPase activity

The experimental setup with six different sample sets (Fig. 1) allowed identification of hBCRP ATPase activity activators as well as inhibitors. Therefore, set one was used as activator test set and set two as inhibitor test set. Set three provided the activator positive control, using a known hBCRP substrate leading to activation of the hBCRP ATPase activity, and set four the inhibitor positive control. The ADP formation in set three was set to 100% hBCRP ATPase activity and the ADP formation in set four was below 10%, suggesting almost complete hBCRP ATPase activity inhibition. Set five allowed exclusion of mass spectral ion suppression or enhancement effects on the ADP detection caused by the test compounds [23]. Those interfering samples were mandatory, as only the adenosine nucleotides were monitored by the analytical method and co-eluting analytes could lead to false positive (in case of ion suppression) or false negative (in case of ion enhancement) results. For the five HIV protease inhibitors, no analytical interferences could be detected. A one-way ANOVA followed by Dunnett's multiple comparison test was used to decide whether ADP formation in sets one, two, four, or five was

statistically significantly different from ADP formation in set three. Similar initial screening strategies were published by Dinger et al. and Wagmann et al. to identify CYP or MAO inhibitors, respectively [19, 24]. ATPase activity in sample sets one and two are given in Fig. 5. Out of five test compounds, none could activate the hBCRP ATPase in a way comparable to sulfasalazine. Furthermore, amprenavir and indinavir were shown to have no hBCRP ATPase activity inhibition potential, while nelfinavir, ritonavir, and saquinavir were identified as hBCRP ATPase activity inhibitors. These results are in line with findings of Gupta et al. who studied hBCRP substrate or inhibitor properties of those five HIV protease inhibitors with human embryonic kidney cells stably expressing hBCRP by measuring intracellular mitoxantrone fluorescence using flow cytometry [14].

4. Conclusion

The presented method was the first using ADP quantification by HILIC-HR-MS/MS to detect in vitro hBCRP ATPase activity. The workup and analysis were validated according to international guidelines. Due to its high sensitivity, only small amounts of hBCRP membrane were needed, thus, reducing the risk of non-specific protein binding as well as material costs. Sample preparation by protein precipitation was simple and fast and the analysis time of 6 min for one analytical run allowed high throughput. Nevertheless, some shortcomings should be considered. The used orbitrap-based mass spectrometer is rather expensive and therefore not available for everyone but the use of a triple quadrupole mass spectrometer might be an alternative. Furthermore, no internal standard could be recommended as all tested compounds turned out to be inappropriate but ADP quantification could still be successfully performed.

The approach was successfully applied to study interactions between hBCRP and five HIV protease inhibitors. Nelfinavir, ritonavir, and saquinavir were identified as hBCRP ATPase activity inhibitors, while amprenavir and indinavir did not inhibit hBCRP ATPase activity. None of the five HIV protease inhibitors turned out to be an hBCRP substrate. These findings were in line with published data [14]. Therefore, this approach should be able to predict possible interactions between the hBCRP ATPase and compounds of interest.

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Table 1 Calibrator concentrations for ADP quantification and mean coefficient of determination (R^2, ± coefficient of variation, CV)

	Calibrator						mean R^2 (±CV)
	1	2	3	4	5	6	
ADP conc., µM	50	100	200	300	400	500	0.9849 (±0.5)

Table 2 Validation results for ADP quantification method: within-run and between-run accuracy and precision

	LLOQ QC	low QC	med QC	high QC
Within-run accuracy, %	9	6	3	14
Between-run accuracy, %	7	8	10	10
Within-run precision, %	4	3	3	3
Between-run precision, %	4	7	4	3

Legends to the figures

Fig. 1 Incubation scheme for detection of human breast cancer resistance protein (hBCRP) ATPase activity using six sample sets (ATP: adenosine 5'-triphosphate, ACN: acetonitrile).

Fig. 2 Chemical structures and HR-MS/MS spectra of adenosine 5'-monophosphate (AMP), adenosine 5'-diphosphate (ADP), and adenosine 5'-triphosphate (ATP).

Fig. 3 Targeted-SIM chromatograms of adenosine 5'-monophosphate (AMP), adenosine 5'-diphosphate (ADP), and adenosine 5'-triphosphate (ATP) in a mixture of pure sample solutions (2 mM, each; A), a blank sample (B), a blank sample spiked with 4 mM ATP (C), a LLOQ QC (50 µM ADP and 4 mM ATP; D), and a high QC (375 µM ADP and 4 mM ATP; E).

Fig. 4 Overlaid targeted-SIM chromatograms of adenosine 5'-diphosphate (ADP) in incubated samples containing adenosine 5'-triphosphate (ATP, 4 mM), hBCRP membrane (0.2 mg/mL), and an hBCRP substrate (10 µM sulfasalazine; sample A), additionally an hBCRP inhibitor (400 µM orthovanadate; sample B) or ATP (4 mM) and control membrane (0.2 mg/mL; sample C). The peaks @ 3.56 min were used for ADP quantification, while peaks @ 4.03 min were most likely caused by in-source fragmentation of ATP.

Fig. 5 Effect of 50 µM amprenavir, indinavir, nelfinavir, ritonavir, or saquinavir on hBCRP ATPase activity. Sample set one contained human breast cancer resistance protein (hBCRP) membrane, adenosine 5'-triphosphate (ATP), and one of the HIV protease inhibitors, while sample set two contained sulfasalazine in addition. Percentage of activity represented the percentage of adenosine 5'-diphosphate (ADP) formation in relation to hBCRP ATPase activator positive control incubations only with sulfasalazine (100%). Values are expressed as mean and were tested for significance (n = 3, ***, $P < 0.001$, **, $P < 0.01$, *. $P < 0.1$ for ADP formation in incubations with test compound versus ADP formation in activator positive control incubations).

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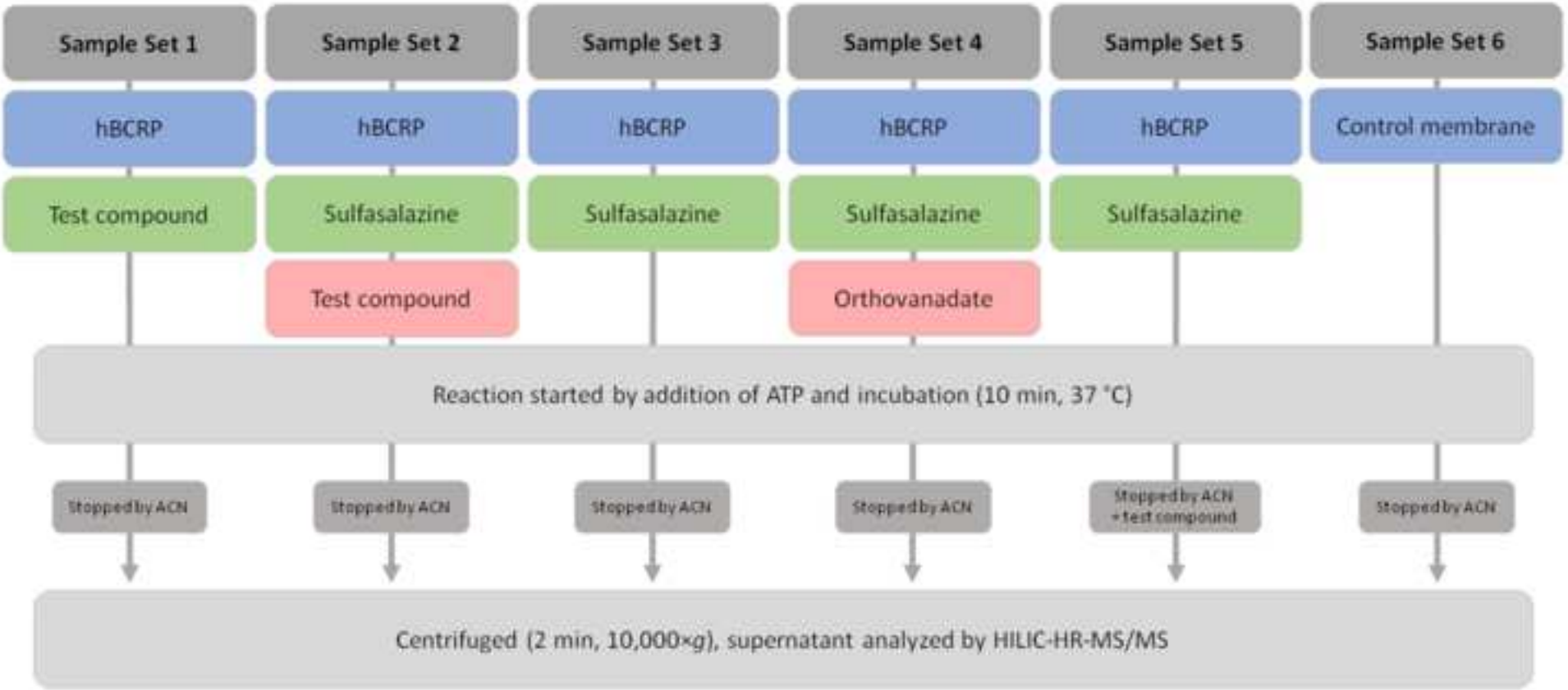

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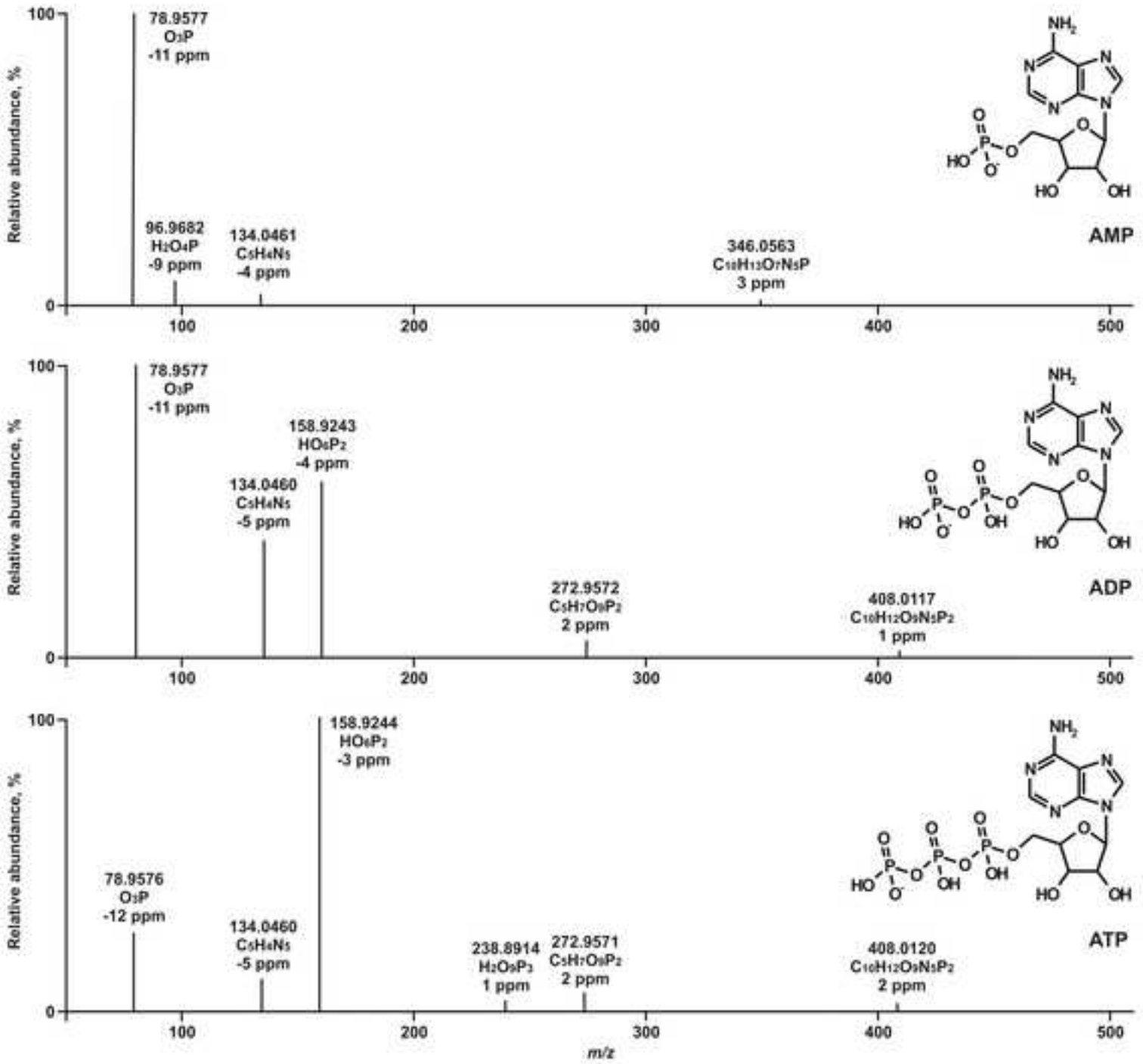

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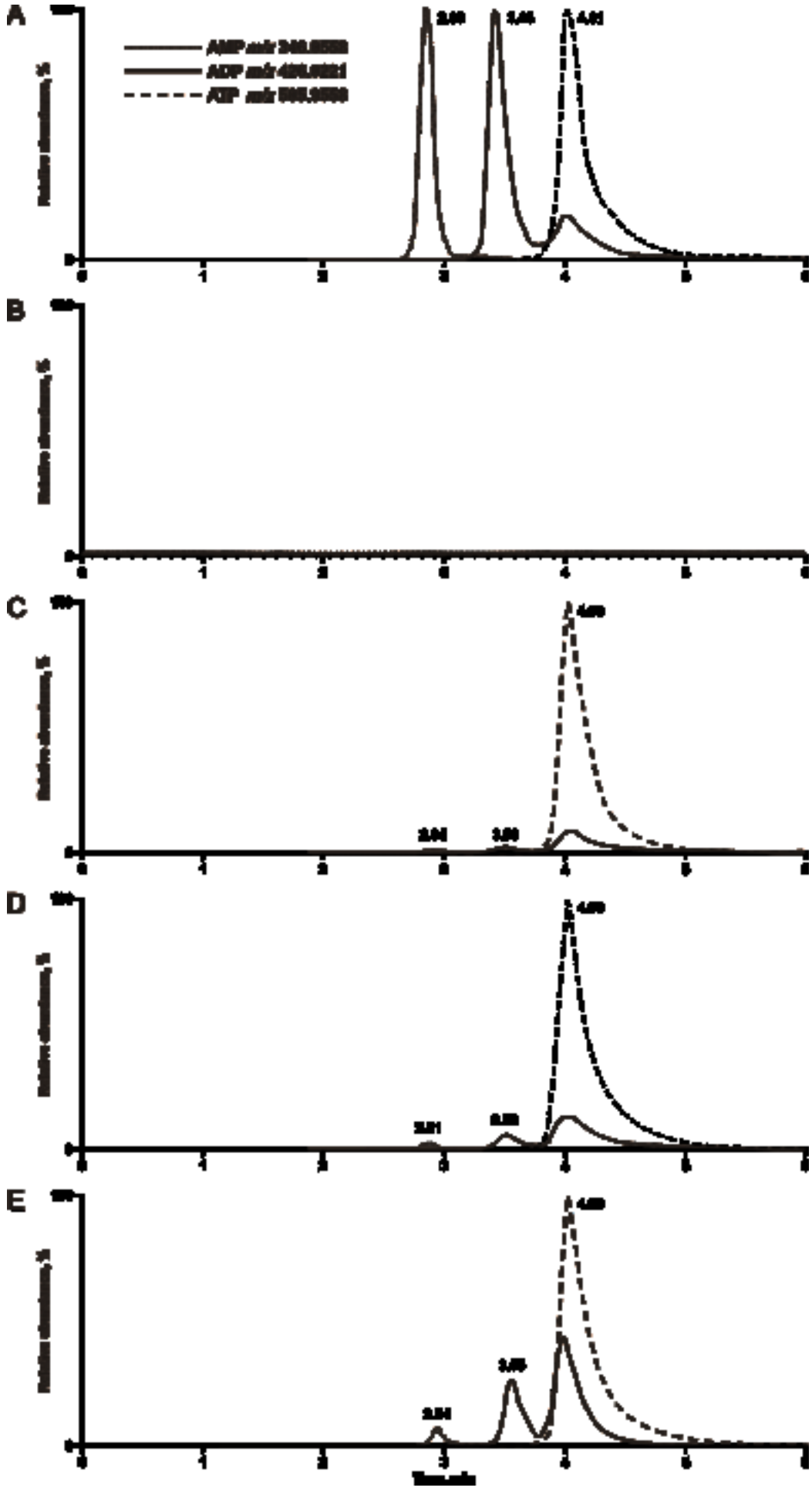

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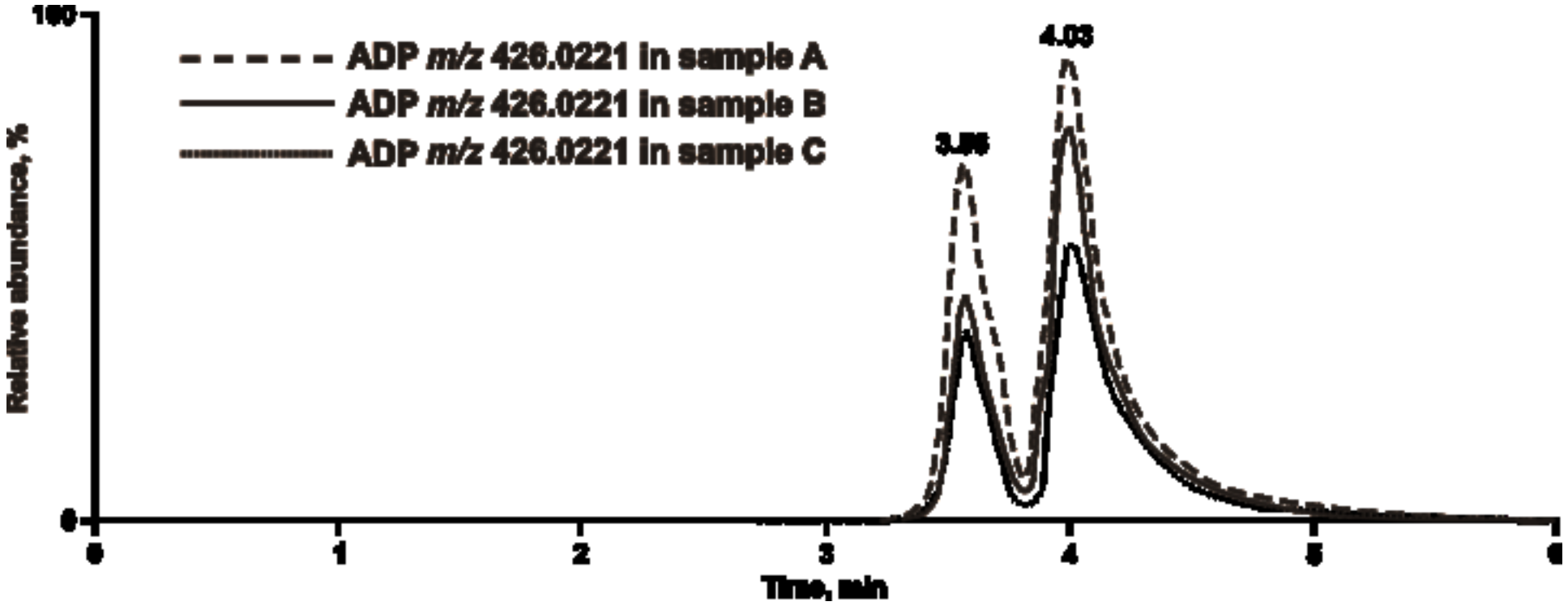

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