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# Determination of the thrombin inhibitor AZD0837 and its metabolites in human bile using mixed mode solid phase extraction and LC–MS/MS

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ABSTRACT

A method for the determination of AZD0837 and its two metabolites AR-H069927 and AR-H067637 in human bile was developed and validated. All three analytes and their stable isotope-labeled internal standards were isolated from bile using solid phase extraction on a mixed mode reversed phase/anion exchange column. Elution was done at high ionic strength with 0.125 M ammoniumacetate in 50% methanol. The extraction recoveries were >75%. Due to the high concentration of AR-H067637 a portion of the extract was diluted before injection on to the LC column, while undiluted extract was directly injected for the analysis of AZD0837 and AR-H069927. Chromatographic separation of all three analytes was achieved in a single system utilizing a C18 column based on fused core particle technology at high flow rate. The two metabolites were eluted when a gradient from 30 to 57% methanol was applied while the more hydrophobic pro-drug, AZD0837, eluted during a steeper second gradient from 57 to 80% methanol with the ammonium acetate concentration and acetic acid concentration kept constant at 3.8 mmol/L and 0.1%, respectively. The total cycle time was 3.2 min. Detection was performed using positive electrospray ionization tandem mass spectrometry. The linearity range was 0.02–20 μmol/L for AZD0837 and AR-H069927, and 1–1000 μmol/L for AR-H067637. The repeatability and the overall precision were less than 15% (RSD) and the accuracy was within the interval 93–100%.



## 1. Introduction

AZD0837 is a novel orally available direct thrombin inhibitor developed by AstraZeneca with intended use as anticoagulant therapy for the prevention and treatment of thromboembolic diseases [1–3].

AZD0837 is a pro-drug that via the intermediate AR-H069927 is bioconverted to the active direct thrombin inhibitor AR-H067637.

The biliary excretion of AZD0837 and its metabolites has been studied in two clinical trials. For this purpose a validated method for the determination of AZD0837 and its two metabolites in human bile was needed.

Validated methods have earlier been developed for the determination of AZD0837 and its two metabolites in human and animal plasma. In addition, a preliminary, partially validated method for pig bile has also been developed. This article describes a fully validated, automated method for the determination of the analytes in human bile that within a short cycle time gives complete separation of all three analytes.

## 2. Experimental

### 2.1. Materials

AZD0837, AR-H069927 and AR-H067637 and their respective deuterium (D) labeled internal standards (Fig. 1) were supplied by AstraZeneca R&D, Mölndal, Sweden. Acetonitrile and methanol were of HPLC grade from Rathburn (Walkerburn, UK). Acetic acid was of analytical grade from Merck (Darmstadt, Germany) and ammonium acetate was of puriss p.a. quality (>98.0%) from Fluka. The solid-phase extraction tubes were Isolute Array HCX, Isolute Array HCX-3 and Isolute HCX-5, 25 mg/1 mL (Biotage AB, Uppsala, Sweden).

### 2.2. Sample collection

Blank bile was collected from drug free volunteers. Study samples were obtained from two clinical studies, involving 8 and 17 healthy male volunteers respectively, and aiming at investigating the involvement of biliary excretion of AZD0837 and its metabolites in the interaction with ketoconazole. Bile was then continuously sampled in 20-min fractions from the duodenum during 3 h after the dosing of AZD0837. To aspirate bile from the intestine, the Loc-I-Gut® perfusion tube (Synectics Medical, Stockholm, Sweden)

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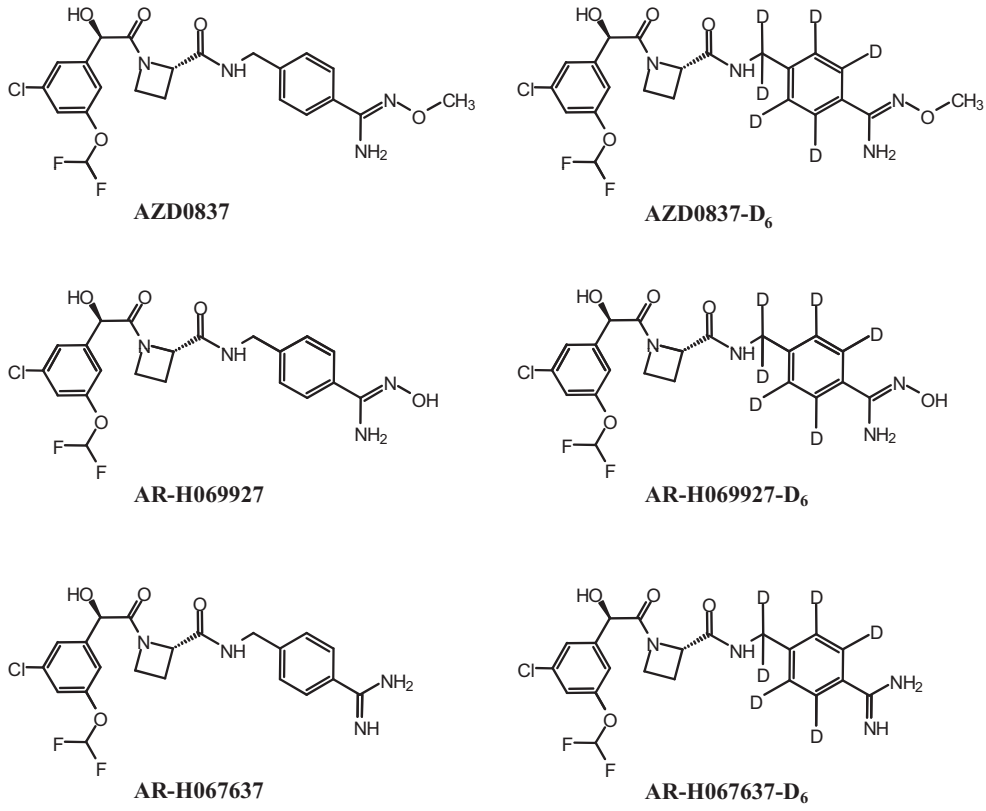


**Fig. 1.** Chemical structures of AZD0837, AR-H069927 and AR-H067637 and their respective deuterium labeled internal standards.

was utilized; this method has previously been described in detail [4,5]. The bile was then collected on ice in polypropylene tubes and immediately frozen at −70 °C.

### 2.3. Pipetting robots

The SPE system consisted of a robotic sample processor, Genesis RSP 150 (Tecan, Hombrechticon, Switzerland), a manifold (Tomtec Inc., Hamden, MA, USA) and an on-site assembled ejector pump for underpressure control. The 96-channel robot was a BRAVO 96 LT liquid handling robot (Agilent technologies, CA, USA).

### 2.4. Calibration standards and quality control samples

Stock solutions of each analyte were prepared by first dissolving the analyte in methanol (20% of total volume) and then diluting with ammonium acetate (0.05 mol/L) to obtain a final concentration of 1000 μmol/L (AZD0837 and AR-H069927) or 100 000 μmol/L (AR-H067637). Two sets of working solutions were then prepared in 20% methanol in 0.05 mol/L ammonium acetate. One set contained a mixture of AZD0837 and AR-H069927 at six concentrations levels in the range 0.4–400 μmol/L and one set contained AR-H067637 at six levels in the range 20–20 000 μmol/L.

Calibrations standards containing a mixture of all three analytes were prepared by a 20-fold dilution of each working solution in human blank bile (e.g. at each concentration level, 175 μL of each of the two working solutions was added to 3150 μL human bile). This resulted in the calibration range 0.02–20.0 μmol/L for AZD0837 and AR-H069927 and 1–1000 μmol/L for AR-H067637. If not used fresh, the calibration standards were stored at −70 °C.

Quality control samples were prepared in bulk from the working standard solutions at the levels "High" (16.0 μmol/L for AZD0837 and AR-H069927 and 780 μmol/L for AR-H067637), "Middle" (0.600 μmol/L for AZD0837 and AR-H0699277XX and 30.0 μmol/L for AR-H067637) and "Low" (0.061 μmol/L for AZD0837 and AR-H069927 and 3.00 μmol/L for AR-H067637). A maximum of 5% dilution of the bile matrix was allowed. The quality control samples were stored in aliquots at −70 °C.

A working solution containing the three internal standards were prepared in 4% methanol in 0.05 mol/L ammonium acetate at the concentrations 3.9 μmol/L (AZD0837-$D_6$), 2.0 μmol/L (AR-H069927-$D_6$) and 530 μmol/L (AR-H067637-$D_6$).

### 2.5. Sample extraction

The bile samples were thawed at room temperature, homogenized by vortex-mixing and centrifuged at 2100 × *g* for 5 min at room temperature.

The extraction was performed using a Tecan pipetting robot for the transfer and addition of samples and liquids. Activation and conditioning of the SPE plates (Isolute Array 96-well HCX-5, 25 mg/1 mL) were performed by first adding 450 μL of methanol to each column and then, after adjusting the pressure level in the manifold to 6–9 mbar below ambient pressure, 250 μL of water. Prior to the loading step, 50 μL of the sample, 25 μL of a mixture of the internal standards and 50 μL of a dilution buffer (5% methanol in 0.05 mol/L ammonium acetate) were premixed in a 96-well plate. Mixing was accomplished by repeated aspiration and redispension into the well. The content of the well was then transferred to the SPE plates. Acid interferences were removed by passing 450 μL acetic acid, 1 mol/L, through the SPE plates and then 450 μL methanol was added to remove neutral interferences. The underpressure was released to ambient, the waste plate removed and a 96-well collection plate inserted in the manifold and the pressure was again adjusted to 6–9 mbar below ambient pressure. The analytes were eluted at high ionic strength with two portions (2 × 250 μL) of a mixture of 50% methanol and 50% 0.125 mol/L

**Table 1**
Selected reaction monitoring (SRM) transitions for quantification and mass spectrometric settings.

| Compound | Precursor ion ($m/z$) ($M+H^+$) | Product ion ($m/z$) | Declustering potential (V) | Collision energy (eV) | Dwell times (ms) |
|---|---|---|---|---|---|
| AZD0837 | 497.1 | 163.1 | 96 | 35 | 50 |
| AZD0837-$D_6$ | 503.2 | 169.1 | 96 | 35 | 50 |
| AR-H069927 | 483.1 | 149.1 | 76 | 31 | 50 |
| AR-H069927-$D_6$ | 489.2 | 155.1 | 76 | 31 | 50 |
| AR-H067637 | 467.1 | 106.1 | 81 | 77 | 100 |
| AR-H067637-$D_6$ | 473.2 | 112.1 | 81 | 77 | 100 |

ammonium acetate. After sealing and vortex-mixing, the collection plate was centrifuged at 1500 × *g* for 1 min.

The plate was moved to a 96-channel robot in order to transfer a portion (25 μL) of each extract simultaneously from each well into a new collection plate. A dilution liquid (700 μL) consisting of 50% methanol and 50% 0.125 mol/L ammonium acetate had earlier been added to each well of this plate by the 96-channel robot. Also this plate was sealed, vortex-mixed and centrifuged at 1500 × *g* for 1 min.

The first collection plate, with the undiluted extracts, was used for the analysis of AZD0837 and AR-H069927 and the second plate, with the diluted extracts, for the analysis of AR-H067637.

After the completion of the extraction step, the extracts, 5 μL of the undiluted extracts and 3 μL of the diluted extracts, were injected onto two separate but identical chromatographic systems.

### 2.6. Liquid-chromatography–mass spectrometry

Two identical LC–MS/MS systems were used and diluted and undiluted extracts were analyzed in parallel. The liquid chromatography system consisted of two LC-10AD VP pumps and a SIL-HTc autosampler from Shimadzu (Kyoto, Japan). The mass spectrometer was an API 4000 triple quadrupole with electrospray (turbo-ion spray) interface (Sciex, Concord, Canada). Data were processed and evaluated on Analyst 1.4 software from Applied Biosystem MDS Sciex (Foster City, USA). The analytical column used was a reversed-phase Ascentis Express with fused core C18, 2.7 μm particles (50 mm × 3.0 mm id) column from Supelco (Bellefonte, PA, UK).

The mobile phases consisted of 30% methanol (phase A) or 80% methanol (phase B) in 3.8 mmol/L ammonium acetate and 0.1% acetic acid. A linear gradient from 0 to 55% of mobile phase B was applied during 1.6 min followed by a steeper gradient from 55 to 100% of mobile phase B during 0.4 min. The flow rate was 1.2 mL/min. After the elution of the analytes, the column was washed during 0.8 min with 100% mobile phase B and then reequilibrated with 100% A for 0.3 min. The cycle time was 3.2 min. This applied to the mobile phases for both systems.

Typical instrument settings of the mass spectrometers were 76–96 V for the declustering potential, 31–77 eV for the collision energy and 550 °C for the turbo heater temperature (Table 1). Other settings, including gas flow, lens and quadrupole voltages and parameters for mass resolution of the separating quadrupole were used as obtained during routine optimization of the instrument.

Mass spectral peak width ($w_{1/2}$) was measured in unit mass resolution mode. The SRM transitions of the precursor ions ($M+H^+$), the corresponding product ions and the dwell times are shown in Table 1.

### 2.7. Calibration

In each analytical run, calibration was made from human bile standards at 6 concentration levels covering the range 0.02–20.0 μmol/L for AZD0837 and AR-H069927 and the range 1–1000 μmol/L for AR-H067637. Linear regression with $1/x^2$ weighting was used.

## 3. Results and discussions

### 3.1. Extraction

Methods for AZD0837 and its two metabolites have earlier been developed for the determination of these analytes in human [3] and animal [6] plasma and pig bile [6].

The extraction of the analytes from plasma was based on solid phase extraction (SPE) on hexyl silica. When the plasma methods were tested on pig bile, interfering peaks from the bile matrix as well as ion suppression effects were seen (data not shown). The pro-drug and its two metabolites are very different regarding their degree of hydrophilicity. Therefore, the conditions for washing off potentially interfering components were quite mild to avoid loss of the more hydrophilic analytes, while the conditions for elution were rather harsh to recover the most hydrophobic analyte. This may explain the failure to produce bile extracts free of interferences. Due to this, no attempts were made to apply these methods on human bile samples.

The method for the determination of AZD0837 and its two metabolites in pig bile was based on a simple dilution of the bile sample followed by a chromatographic method optimized to separate the analytes from the interfering bile components on the LC column [6]. When this method was tested on human bile samples, the chromatogram obtained from a blank bile samples seemed to be free from interfering substances at the retention times of the three analytes. However, this method did not seem to be robust enough in terms of the precision at the LLOQ level.

A method based on mixed mode solid phase extraction was earlier developed for the determination of the oral thrombin inhibitor Exanta and its three metabolites in plasma [7]. This technique was then found especially suitable for isolation of analytes, which differ in hydrophobicity. As also AZD0837 and its metabolites carry charged groups and differ in hydrophobicity, extraction using mixed mode SPE was investigated.

Three different SPE materials (Isolute HCX5 (C4), Isolute HCX (C8) and Isolute HCX3 (C18)) were tested. The sample load was followed by two washing steps, using acetic acid and methanol respectively. The elution liquid was optimized regarding the content of organic modifier and ionic strength. Isolute HCX5, giving the highest recovery, was chosen. The recoveries found were within the interval 76.8–93.4% (Table 2). The recoveries of the internal standards were of the same magnitude.

One of the challenges of the method development was the large difference in concentration between the analytes, with AR-H067637 having up to a 50 000-fold higher concentration compared to the analyte with the lowest concentration. Therefore a test was performed to investigate if the high concentration of AR-H067637 had any effect on the recoveries of AZD0837 and AR-H069927. Then a bile sample spiked with AR-H067637 to the ULOQ level and with AR-H069927 and AZD0837 to the LLOQ level was used. The recoveries in this mixture were found to be

**Table 2**
Extraction recovery.

| Analyte | Concentration (μmol/L) | Recovery (%) ($n = 3$) | CV (%) |
|---|---|---|---|
| AZD0837 | 0.0623 | 87.4 | 0.4 |
| | 0.623 | 93.4 | 6.9 |
| | 19.5 | 86.9 | 0.9 |
| AR-H069927 | 0.0628 | 81.5 | 2.4 |
| | 0.628 | 91.8 | 3.8 |
| | 19.6 | 76.8 | 0.7 |
| AR-H067637 | 3.03 | 92.2 | 2.7 |
| | 30.3 | 92.9 | 3.0 |
| | 1000 | 83.7 | 1.5 |

approximately 10% lower compared to the recoveries obtained using bile samples spiked with only one analyte, but these differences were fully compensated for by the stable isotope labeled internal standards.

### 3.2. Chromatographic separation

The liquid chromatographic method for the determination of the analytes in pig bile is based on a HyPurity C18 column (5 μm, 50 mm × 2.1 mm) using a flow rate of 0.2 mL/min. A linear gradient from 30% methanol to 80% methanol in 3.8 mmol/L ammonium acetate and 0.1% acetic acid, followed by a washing step and a reequilibration step resulted in a total cycle time of 8 min. This method was further developed for the human bile samples with the aim to reduce the cycle time.

A higher flow rate on the HyPurity column could not be applied without compromising the separation. However, the use of an LC column based on fused–core particle technology (Ascentis Express, 2.7 μm, 50 mm × 3 mm) allowed a flow rate of 1.2 mL/min. The two most hydrophilic analytes, AR-H067637 and AR-H069927, eluted during a first linear gradient from 30 to 57% methanol during 1.6 min giving the retention times 1.1 min and 1.3 min, respectively. The more hydrophobic analyte AZD0837 eluted at a retention time of 1.9 min during a second steeper gradient from 57 to 80% methanol during 0.4 min. A washing step and a reequlibration step were added, which resulted in a cycle time of 3.2 min. The ammonium acetate concentration and acetic acid concentration were kept constant at 3.8 mmol/L and 0.1%, respectively. All three analytes were well separated and with good peak shapes (Fig. 2).

To avoid the risk of affecting the determination of AR-H067637 in diluted extract by contamination from remainder of this analyte in the LC/MS instrument from earlier injected undiluted extracts, separate instruments were used for undiluted and diluted extracts.

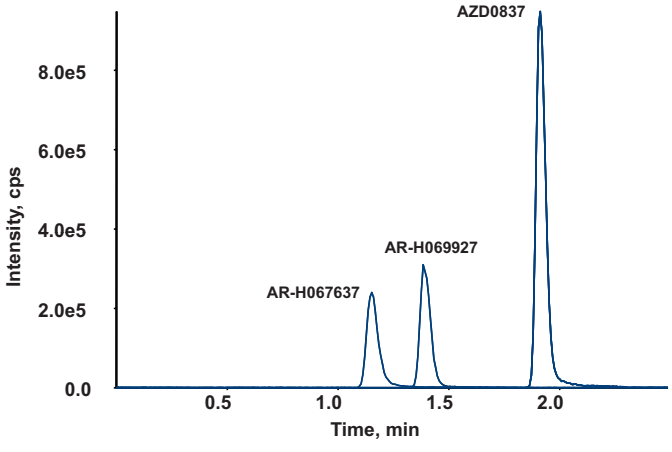


**Fig. 2.** Chromatogram showing the separation of AZD0837, AR-H069927 and AR-H067637.

**Table 3**
Precision and accuracy.

| Analyte | Nominal concentration (μmol/L) | Overall precision (%) ($n = 5$, $p = 3$) | Repeatability (%) ($n = 5$, $p = 3$) | Accuracy (%) |
|---|---|---|---|---|
| AZD0837 | 0.0205 | 8.5 | 7.9 | 94.2 |
| | 0.0630 | 5.6 | 5.3 | 95.3 |
| | 19.9 | 2.0 | 1.2 | 93.3 |
| AR-H069927 | 0.0207 | 13.1 | 8.7 | 100.1 |
| | 0.0630 | 3.8 | 3.6 | 97.2 |
| | 20.1 | 1.9 | 1.9 | 93.5 |
| AR-H067637 | 0.999 | 6.1 | 2.2 | 94.7 |
| | 3.04 | 4.0 | 3.2 | 99.1 |
| | 998 | 1.4 | 1.1 | 97.5 |

In addition, this made it possible to analyze the two different extracts in parallel.

### 3.3. Selectivity

No interfering peaks from endogenous bile components were seen when bile samples from six different human individuals were examined. Typical chromatograms are shown in Fig. 3.

### 3.4. Matrix effects on ionization

The sample matrix effect on ionization was determined by comparing the peak areas for AZD0837 and its metabolites added to extracted drug-free bile samples with those obtained when the compounds were added to a buffer solution. The concentration corresponded to the middle level quality control sample. When six different human bile samples were used, the calculated mean of the matrix factors were 1.13 (AZD0837), 1.04 (AR-H069927) and 1.05 (AR-H067637) with a CV less than 2.5% for all three compounds. Thus the matrix effect on ionization was negligible.

### 3.5. Accuracy, precision and linearity

The within-run precision (repeatability) and the overall precision were determined using spiked human bile samples at three concentrations levels, representing the quality control samples “High”, “Middle” and “Low”. Five replicates at each level were included in three analytical runs performed at different days. The accuracy was determined as the mean accuracy relative the nominal concentration.

The overall precision and of the repeatability were <14% and <9%, respectively (expressed as RSD). The accuracy was within the interval 93.3–100.1%. As the overall precision was the same or only marginally larger than the repeatability, it can be concluded that the inter-day variation was small compared to the intra-day variation (Table 3).

Spiked human bile samples at concentrations above ULOQ were, after dilution with drug-free human bile, included in one analytical run. In addition, spiked samples at concentration 2–5 times the

**Table 4**
Precision and accuracy-concentrations >ULOQ.

| Analyte | Nominal concentration (μmol/L) | Repeatability (%) ($n = 5$) | Accuracy (%) |
|---|---|---|---|
| **Dilution** | | | |
| AZD0837 | 1260 | 2.6 | 91.0 |
| AR-H069927 | 152 | 2.0 | 89.2 |
| **Injection volume 1 μL** | | | |
| AZD0837 | 94.8 | 2.6 | 93.2 |
| AR-H069927 | 35.1 | 2.0 | 93.1 |
| AR-H067637 | 1480 | 1.9 | 98.2 |

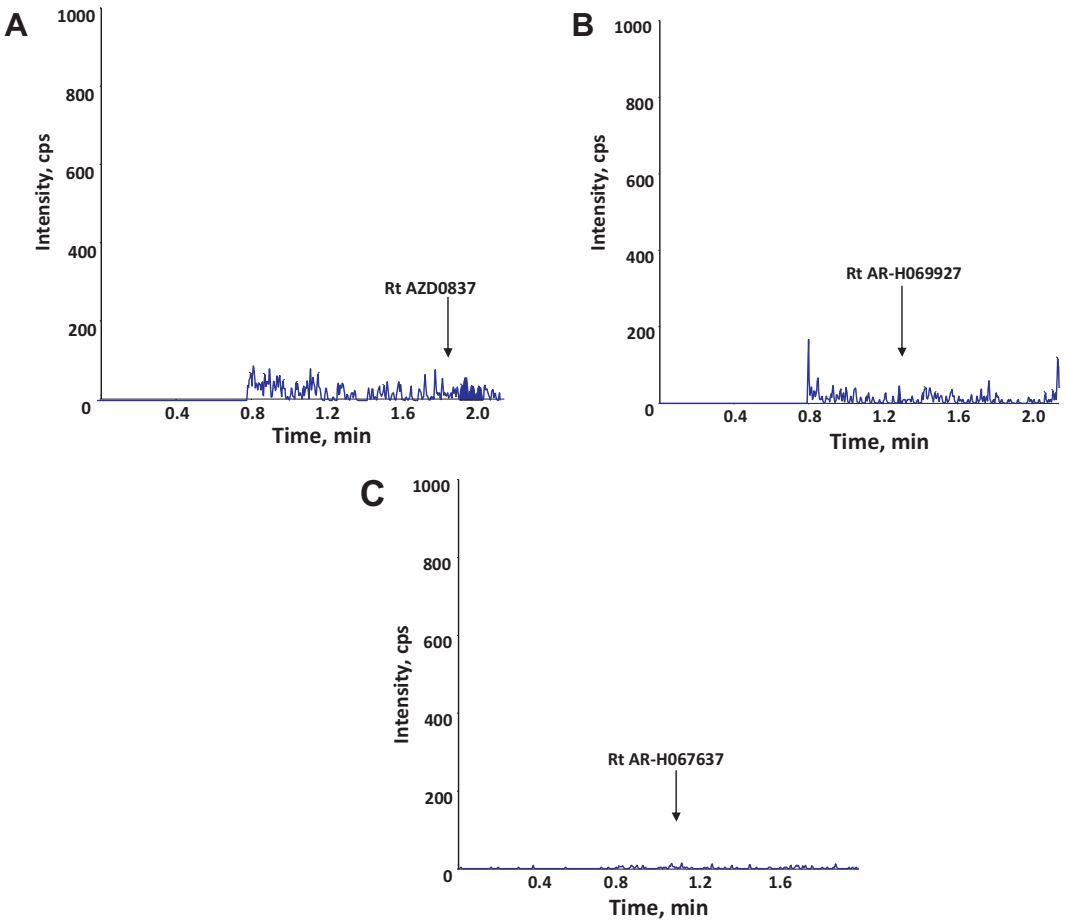


**Fig. 3.** Chromatograms for AZD0837 (A), AR-H069927 (B) and AR-H067637 (C) from a drug free human bile sample.

ULOQ were analyzed in one analytical run using a reduced injection volume of 1 μL for the samples, while keeping the injection volume for the standards according to method.

The possibility to use a reduced injection volume to determine concentrations 2 (AR-H069927), 3 (AR-H067637) or 5 (AZD0837) times ULOQ was demonstrated (Table 4). Higher concentrations, 1300 μmol/L of AZD0837 and 150 μmol/L of AR-H069927, were determined after a 100- or 10-fold dilution with acceptable precision and accuracy (Table 4).

The linearity, shown as the accuracy of the back-calculated concentrations of the calibration standards obtained in the three runs used to determine accuracy and precision is presented in Table 5. The mean accuracy was within the interval 96.1–104.5% and the CV was <9.5%. The slopes, intercepts and the coefficients of correlation for the calibration lines are shown in Table 6. The coefficients of correlation were >0.997 in all cases.

### 3.6. Stability

There was no degradation of the analytes observed after storage of spiked human bile samples for six months at −70 °C or for 6 h at room temperature. Stability during 3 freeze-thaw cycles in human

**Table 5**
Accuracy and precision of back-calculated concentrations of the calibration samples from three analytical batches with two replicates in each batch ($n = 6$).

| Nominal concentration (μmol/L) (AZD0837, AR-H069927, AR-H067637) | AZD0837 | | AR-H069927 | | AR-H067637 | |
|---|---|---|---|---|---|---|
| | Accuracy (%) | CV (%) | Accuracy (%) | CV (%) | Accuracy (%) | CV (%) |
| 0.0202, 0.0201, 0.996 | 99.8 | 9.5 | 100.1 | 2.8 | 99.4 | 3.3 |
| 0.160, 0.159, 7.96 | 101.8 | 2.0 | 99.4 | 3.6 | 104.5 | 1.1 |
| 1.27, 1.26, 63.7 | 99.9 | 1.5 | 98.9 | 1.1 | 102.7 | 1.6 |
| 5.03, 5.03, 252 | 102.8 | 3.0 | 101.7 | 1.8 | 98.3 | 1.5 |
| 11.5, 11.5, 579 | 99.1 | 1.3 | 98.9 | 2.2 | 99.1 | 2.1 |
| 20.0, 20.0, 1010 | 96.6 | 1.9 | 101.0 | 1.5 | 96.1 | 3.6 |

**Table 6**
Slopes, intercepts and the coefficient of correlation for the calibration equations from three analytical batches used for estimation of precision and accuracy.

| Batch no | AZD0837 | | | AR-H069927 | | | AR-H067637 | | |
|---|---|---|---|---|---|---|---|---|---|
| | Slope | Intercept | $r$ | Slope | Intercept | $r$ | Slope | Intercept | $r$ |
| 1 | 0.782 | 0.00012 | 0.9990 | 0.962 | −0.00090 | 0.9997 | 1.28 | 0.000011 | 0.9987 |
| 2 | 1.05 | 0.0012 | 0.9996 | 0.982 | −0.0014 | 0.9996 | 1.37 | 0.000069 | 0.9994 |
| 3 | 1.06 | 0.00087 | 0.9977 | 0.978 | 0.0014 | 0.9994 | 1.36 | −0.00011 | 0.9998 |

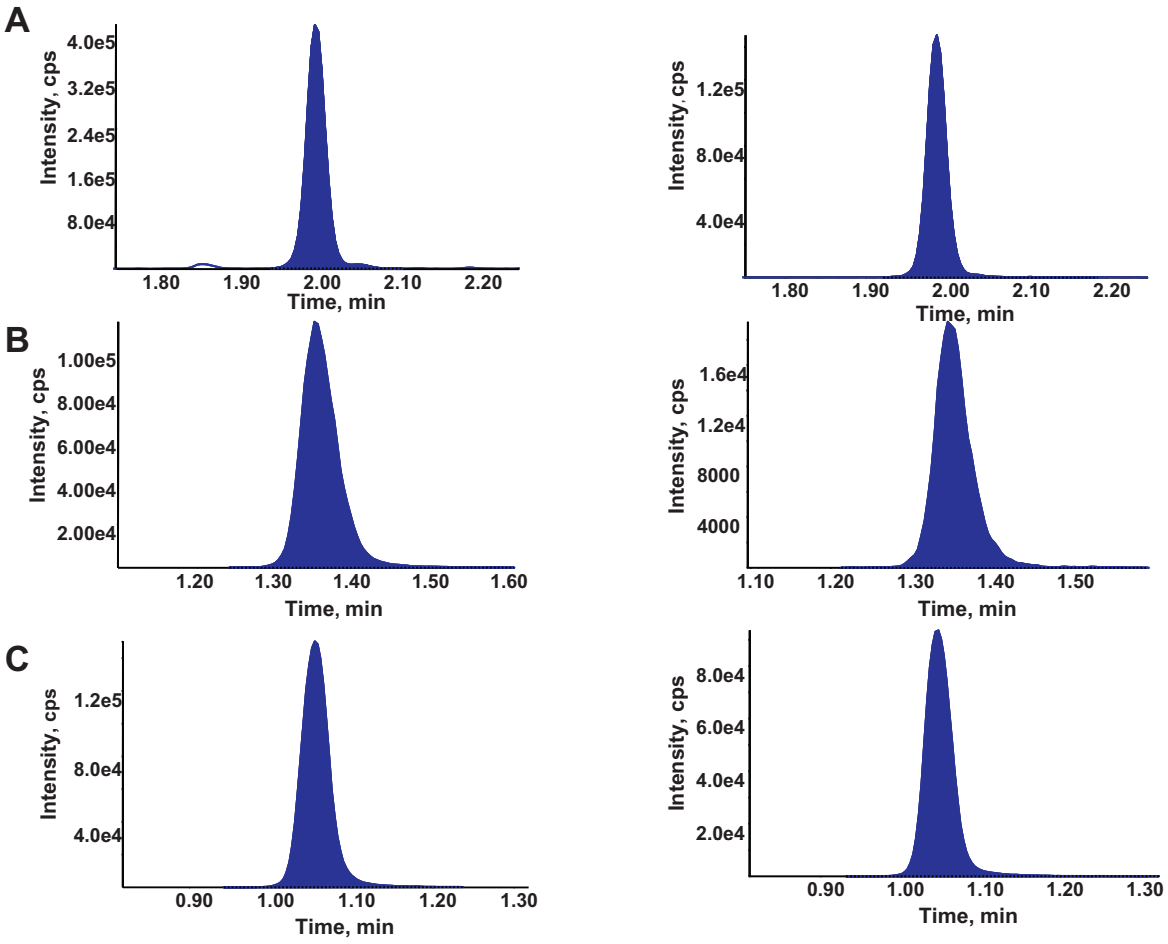


**Fig. 4.** Chromatograms from an authentic human bile sample with added internal standard. The samples is collected 1 h 40 min after an oral dose of AZD0837. Concentration of (A) AZD0837 and its internal standard: 6.67 μmol/L and 1.95 μmol/L, (B) AR-H069926 and its internal standard: 6.63 μmol/L and 1.00 μmol/L, (C) AR-H067637 and its internal standard: 319 μmol/L and 265 μmol/L.

bile was demonstrated. Processed human bile samples could be stored for at least 8 days in a refrigerator or for at least 96 h at room temperature. The accuracy relative the initial concentrations in all stability tests were within 97–102%.

### 3.7. *Incurred sample reproducibility (ISR)*

In one clinical study, a set of 24 randomly selected study samples representative of the study and with concentrations higher than the LLOQ were analyzed for the purpose of demonstrating the reproducibility of the method toward incurred samples (ISR). The difference between the reanalysis result and the original result was less than ±20% for all samples, and less than ±10% for the majority of the samples, for all three analytes. Chromatograms from an authentic human bile sample are shown in Fig. 4.

## 4. Conclusions

A quantitative method for the determination of AZD0837 and the two metabolites AR-H069927 and AR-H067637 in human bile was developed and validated. The procedure for sample clean–up earlier successfully used for plasma samples was changed from reversed-phase SPE to mixed mode SPE. In this way samples extracts with no interfering peaks and no matrix effects on ionization could be produced also for bile samples. A high flow rate on a fused core LC column, in combination with two gradient steps, one for the two hydrophilic analytes and one for the more hydrophobic analyte, resulted in a chromatographic separation of the three analytes within a short cycle time.

The method has successfully been used for the determination of AZD0837 and its two metabolites in human bile samples in two clinical studies.

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