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# Simultaneous determination of acrylamide, its metabolite glycidamide and antipyrine in human placental perfusion fluid and placental tissue by liquid chromatography–electrospray tandem mass spectrometry

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ABSTRACT

A rapid and sensitive method using liquid chromatography–tandem mass spectrometry (LC–MS/MS) was developed for the simultaneous determination of acrylamide (AA) and its genotoxic metabolite glycidamide (GA) with a test marker antipyrine (AP) in placental tissue and perfusion medium used in human placental perfusion studies. An internal standard ($^{13}$C-acrylamide) was added to the samples which were then deproteinized with acetonitrile. Chromatographic separation was performed on a reversed phase column with a gradient elution of acetonitrile and 0.01% formic acid at a flow rate of 0.3 mL/min. Detection and quantification of the analytes were carried out with a triple quadrupole mass spectrometer using positive electrospray ionization (ESI) and multiple reaction monitoring (MRM). The method was validated and linear over a concentration range of 0.5–20 μg/mL for acrylamide and glycidamide and 5–200 μg/mL for antipyrine. The lower limit of quantification for acrylamide and glycidamide was 0.5 μg/mL and for antipyrine 5 μg/mL. The method was selective, and good accuracy, precision, recovery, and stability were obtained for concentrations within the standard curve. The method was successfully used to analyze the placental perfusion medium and tissue samples in a toxicokinetic study for transplacental transfer of acrylamide and glycidamide. This is the first time that acrylamide, glycidamide and antipyrine are measured simultaneously.



## 1. Introduction

The neurotoxic, carcinogenic and mutagenic acrylamide (AA) is formed by Maillard reaction in heated food containing asparagine and reducing sugars [1,2]. AA is metabolized into a more mutagenic and genotoxic epoxide metabolite, glycidamide (GA) via CYP2E1 and forms haemoglobin and DNA adducts in vivo [3]. GA is the most reactive and biologically most important metabolite of AA [4]. GA has been found in human urine after the exposure to AA in food [4]. Because there are concerns of AA and especially GA as possible transplacental carcinogens, data of AA and GA transfer in human placenta is needed. For such studies human placental perfusion is potentially the best method because placenta is an organ which differs the most between species. In perfusion human placenta can be kept physiologically functional if the perfusion is initiated right after delivery. Placental perfusion has been mainly used to study placental transfer of drugs but it is also applicable in studies of toxic environmental chemicals [5]. In placental perfusion studies methods for the analysis of the studied compounds in perfusion medium and placental tissue are the prerequisite for experiments.

AA in drinking water [6] and in various food products [7–10] has been analyzed by many techniques. For the analysis of AA, several different sample preparation procedures have been used, such as liquid–liquid extraction (LLE) and solid phase extraction (SPE) based either on non-retentive SPE, or double SPE with a combination of a hydrophilic/lipophilic stationary phase column and mixed mode sorbent column [9,11,12]. AA and GA are highly water soluble compounds and they have minute retention on reversed phase columns, which makes the development of a specific and selective HPLC-UV method challenging. Therefore, in addition to reversed phase a large number of chromatographic techniques have been used including normal phase, ion exchange, gel permeation

*Abbreviations:* AA, acrylamide; GA, glycidamide; AP, antipyrine; LC–MS/MS, liquid chromatography with tandem mass spectrometric detection; HPLC-UV, high performance liquid chromatography with ultraviolet detection; FD, fluorescence detector; FID, flame ionization detector; ECD, electron capture detector; IS, internal standard; QC, quality control; R.S.D., relative standard deviation; LLOQ, lower limit of quantification; SPE, solid phase extraction; LLE, liquid–liquid extraction.

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chromatography or various combinations of the above techniques [13,14]. Ultraviolet (UV), fluorescence (FD) and mass spectrometric detection (MS or MS/MS) have been used with liquid chromatography [11,15]. AA has also been analyzed by gas chromatography as brominated or silylated derivatives with flame ionization (FID), electron capture (ECD) and mass spectrometric detection (MS and MS/MS) [11]. These methods usually require tedious multi-step sample treatment and heating, which can lead to polymerization of AA. Above mentioned instrumental techniques has been also exploited, for analyzing AA and GA from biological samples, such as human urine [4], mouse serum [16] and rat plasma [17].

Antipyrine (AP) is a commonly used marker for transfer by passive diffusion. Antipyrine has in addition to many human placental perfusion studies also been used in other experimental models for toxicokinetic studies [18,19]. In placental perfusion, AP is used to determine the overlap between maternal and fetal circulation and to normalize variation between perfusions [20]. AP has been analyzed in perfusion medium by HPLC-UV [21] or by scintillation counting using $^{14}$C labelled AP [22]. It would be more effective to measure AP simultaneously with the studied compounds. However, AP is more lipid soluble than AA and GA making the combined analysis challenging. Consequently, no methods exist for simultaneous analysis of AA, GA and AP in human tissue and perfusion medium. If applicable to human plasma, such a method could also improve the risk assessment of AA [23].

None of the above mentioned published methods [4,16,17] were developed to analyze simultaneously AA, GA and AP from perfusion medium and placental tissue. This is the first study describing the development and validation of a simple and fast LC–MS/MS method with a single step sample preparation for the simultaneous analysis of AA, GA and AP in placental perfusion medium and perfused placental tissue from human placental perfusion studies. This method was validated in terms of selectivity, linearity, precision, accuracy and stability, and proved to be appropriate for its purpose.

## 2. Experimental

### 2.1. Chemicals and reagents

AA (purity 99.9%) and AP (purity >98%) were purchased from Sigma (St. Louis, MO, USA), GA from Toronto Research Chemicals (Toronto, Canada) and $^{13}$C-acrylamide, 1,2,3-$^{13}C_3$ (isotope purity 99%), 1 mg/mL in methanol from Cambridge Isotope Laboratories (Andover, MA, USA). Acetonitrile was obtained from J.T. Baker (Deventer, Holland) and formic acid from Merck (Darmstad, Germany). Perfusion medium consisted of RPMI 1640 cell culture medium (Cambrex, Verviers, Belgium) with dextran (2 g/l, Sigma), human albumin (2 g/l, The Finnish Red Cross, Finland), heparin (25 IU/mL, Leo Pharma, Malmö, Sweden), sodium puryvate (1 mM, Cambrex, Fluka), non-essential amino acid solution (10 mL/l, Cambrex, Fluka), penicillin–streptomycin (25 U/mL Cambrex, Fluka) and L-glutamine (8 nM, Cambrex, Fluka). De-ionized $H_2O$ was produced using a Milli-Q water purification system from Millipore (Milford, MA, USA). All reagents were of analytical grade, and the solvents were of HPLC grade.

### 2.2. Equipment and chromatographic conditions

The HPLC system comprised of an Agilent 1200 Series Rapid Resolution LC System (Agilent Technologies, Waldbronn, Germany) with a Zorbax SB-Aq column (100 mm × 2.1 mm, 3.5 μm) (Agilent Technologies, Palo Alto, CA, USA). The mass analysis was carried out with an Agilent 6410 Triple Quadrupole mass spectrometer equipped with an electrospray ionization source (Agilent Technologies, Palo Alto, CA, USA). Data acquisition and quantification was conducted using Agilent MassHunter Workstation software B.01.00.

Chromatographic separations were performed using a gradient elution with 0.01% formic acid (A) and acetonitrile (B) as follows: 0–1.0 min, 5% B; 1.0–1.3 min, 5% B → 40% B; 1.3–4.0 min, 40% B; 4.0–4.1 min, 40% B → 5% B; 4.1–7.0 min, 5% B. Flow rate was 0.3 mL/min, column temperature was maintained at 25 °C and autosampler tray temperature was set at 10 °C. Injection volume was 10 μl. The following ionization conditions were used: electrospray ionisation (ESI) positive ion mode, drying gas (nitrogen) temperature 300 °C, drying gas flow rate 10 l/min, nebulizer pressure 50 psi and capillary voltage 4000 V. Detection was performed using multiple reaction monitoring (MRM) with the following transitions: $m/z$ 72 → 55 for AA, $m/z$ 75 → 58 for $^{13}$C-AA, $m/z$ 88 → 44 for GA and 189 → 56 for AP. Fragmentor voltage and collision energy for AA, IS and GA were 60 and 10 V, respectively. For AP the fragmentor voltage and collision energy were 60 and 30 V, respectively. Dwell time was 100 ms and mass resolution (peak width) for MS1 and MS2 quadrupoles were 1.2 FWHM. Divert valve was programmed to allow eluent flow into the mass spectrometer from 0.9 to 5.0 min of each run. Internal standard method was used for the analysis of AA and GA. External standard method was used for the AP.

### 2.3. Preparation of standards

The stock solutions of AA, GA and AP were prepared by dissolving the compounds in $H_2O$ to give a final concentrations of 10 mg/mL and 1 mg/mL for $^{13}$C-AA which was used as an IS for AA and GA. The stock solutions were stored at −20 °C. The solutions of AA, GA and AP were daily diluted in $H_2O$ to obtain standard working solution at a concentration of 100 μg/mL for AA and GA and 1000 μg/mL for AP. The calibration standards were prepared by adding the analytes in perfusion medium or in supernatant of centrifuged control tissue homogenate to give concentrations of 0.5, 1.0, 2.0, 4.0, 8.0, 12, 15 and 20 μg/mL of AA and GA and 5, 10, 20, 40, 80, 120, 150 and 200 μg/mL of AP. Internal standard (1.0 μg/mL of $^{13}$C-AA) was added into each sample. Quality control (QC) samples containing AA (1.0, 4.0, 15 μg/mL), GA (1.0, 4.0, 15 μg/mL) and AP (10, 40, 150 μg/mL) were prepared from stock solutions by adding the analytes into blank perfusion medium or placental tissue homogenate.

### 2.4. Sample preparation

During the 4 h perfusions perfusion medium samples from maternal and fetal circulation were collected every half an hour for the first 2 h and once per hour thereafter. To remove the red blood cells, samples were centrifuged at 12,000 × $g$ for 15 min and the supernatant was stored in −20 °C. For measuring AA, GA and AP in perfused tissue, a 0.5 g piece of tissue was homogenized with 1 mL of $H_2O$ and centrifuged at 12,000 × $g$ for 15 min before the sample preparation and analysis. Before LC–MS/MS analysis the proteins in all samples were precipitated with acetonitrile. Acetonitrile (300 μL) was added to 100 μL of perfusion medium samples or the supernatant of 0.5 g of tissue homogenate sample. After vortexing and centrifugation at 12,000 × $g$ for 15 min, 100 μl of clear supernatant was diluted with 900 μl of $H_2O$ and injected into the instrument.

### 2.5. Validation

Validation of our method was based on the FDA guidelines for bioanalytical methods [24]. The selectivity was assessed by analyzing reference standards with and without background. The matrix effect was studied by a post-column infusion experiment [25]. The

linearity of the assay for each of the analytes, in both buffer and tissue homogenate were assessed by analyzing the calibration curves from eight concentrations of dilution series in duplicate covering the range of 0.5–20 for AA or GA and 5–200 μg/mL for AP, respectively. The calibration curve included samples of perfusion matrix without analytes including IS (a blank sample) and excluding IS (a zero sample). The lower limit of quantification (LLOQ) was determined by calculating precision and accuracy for five LLOQ samples that were independent of the calibration curve. The intra-day precision of the assay was assessed by calculating the relative standard deviation (R.S.D.) for the analysis of QC samples in six replicates, and inter-day precision was determined by the analysis of QC samples on 3 days. Accuracy was assessed by calculating the deviation of the measured value from the nominal value, which compared the calculated and known concentrations. Recovery of the analytes was expressed as a percentage area of the QC sample relative to that of the corresponding standard sample in six replicates. The stability of the analytes was investigated in three replicates of QC1 and QC3 before sample preparation, both in perfusion medium (−20 °C) and in tissue homogenate (−80 °C) and by comparing the concentrations to those of freshly prepared standards. The freeze and thaw stability was determined after three freeze–thaw cycles. The short-term temperature stability was investigated by keeping the samples for 4 h at room temperature before sample preparation. The long-term stability was evaluated by analyzing the samples stored up to 30 days in freezer. The stock solution stability was investigated by comparing freshly prepared standards to standards prepared from a stock which had been frozen for 30 days and kept at room temperature for 6 h after thawing. The post-preparative stability was assessed by keeping the samples in autosampler at 10 °C for 24 h.

### 2.6. Application—human placental perfusion study

The method described in this paper is primarily meant to be used for analysis of samples from ex vivo perfused fresh human placentas, to study transplacental transfer and tissue accumulation of AA, GA and AP. To get a human placenta after birth, approval from the official Research Ethics Committee of the University Hospital District of Kuopio region was gained (11th of May 2005). Details of the human placental perfusion method are described by Myllynen et al. [21]. In pilot experiments, one perfusion with AA (4 μg/mL) and one with GA (5 μg/mL) were carried out. The amount of analytes in perfusions was about 1 mg which is comparable to the estimated average daily intake of AA for a 70 kg adult, 0.35 mg/day [26].

## 3. Results and discussion

### 3.1. Development of the method

During the method development several different reversed phase (RP) and hydrophilic interaction chromatography (HILIC) columns were tested with HPLC-UV instrumentation. As AA and GA are both small (MW 71.08 and 87.08, respectively), polar and hydrophilic compounds, the reversed phase technique with HPLC-UV provided poor retention to the columns tested even with columns designed for polar compounds and aqueous mobile phases such as alkylamide and “Aqua type” columns. Because all tested columns provided only minute retention, attention was focused to sample preparation to separate AA and GA from various inorganic salts eluting at the solvent front and the endogenous compounds present in perfusion samples. For this purpose, together with protein precipitation various solid phase extraction columns, including polymer-based stationary phase (OASIS HLB, Waters, MA, USA), mixed-mode (Strata XC, Phenomenex Inc., USA) and ion chromatography (SAX and SCX, Varian Inc., USA), and liquid–liquid extraction with ethyl acetate were tested to provide a cleaner extract for the injection. Although with protein precipitation the recovery was high, the method was not selective enough to separate AA and GA from the background. Furthermore, although SPE columns have shown to be accurate and precise for AA analysis in food [27,28], in our study all of the tested columns were inadequate to provide a method with acceptable precision. With the tried method it varied from 6 to 24%, especially the precision of GA at QC1 which was even more than 180%. Moreover, the recovery of SPE columns was only around 40%. Because the highest precision from replicate measurements was obtained with protein precipitation during the preliminary method development, it was selected as the sample preparation method with LC–MS/MS.

As a conclusion from the preliminary method development, the more selective mass spectrometric detection was chosen for instrumental technique. Although AA and GA were poorly retained in the reversed phase HPLC column, we found a triple quadrupole mass spectrometer with highly selective MRM together with the reversed phase technique adequate for separating the analytes from the disturbance caused by matrix and inorganic salts. With LC–MS/MS, the best combination of peak shape and capacity factor ($k$) were achieved with a narrow bore C18 column optimized for acidic aqueous solutions (Zorbax SB-Aq, Agilent) with a mobile phase of water–acetonitrile–formic acid (95:5:0.01, v/v/v). It should be noted that the AA peak showed some tailing with all columns and mobile phase compositions tested. To be able to include antipyrine (AP) in the analysis, it was necessary to add a steep ACN gradient at the end of the run to elute this relatively lipophilic compound. The HPLC instrument was optimized for low gradient volumes to allow fast response to the applied change in the mobile phase composition.

Mass spectrometric detection was performed using a highly selective MRM mode. The amount of formic acid in mobile phase was not critical for the chromatography and 0.01% was found to produce the most intense ESI ionization for AA and GA. The use of a volatile ion-pairing additive TFA was not feasible as it created a substantial amount of ion suppression. Using full-scan MS experiments, the molecular ions for AA, IS ($^{13}$C-AA), GA and AP were found to be $m/z$ 72, $m/z$ 75, $m/z$ 88 and $m/z$ 189, respectively. By monitoring transitions to the most intensive product ions (Fig. 1), a specific and sensitive assay was developed. Following transitions were used: $m/z$ 72 → 55 for AA, $m/z$ 75 → 58 for $^{13}$C-AA, $m/z$ 88 → 44 for GA and 189 → 56 for AP. Similar transition for AA was selected as in other published methods based on the abundance of production ion [29–31]. A stable isotope-labelled analogue of AA was selected as an internal standard to ensure uniform behaviour of the AA and IS in the entire analytical instrumentation as well as during the sample preparation. Despite being an isotope analogue of AA, this IS was also found to be well suited for the analysis of GA. No problems with the isotopic integrity of the label were observed. AP was analyzed by using an external standard method.

### 3.2. Selectivity and matrix effect

To determine the selectivity of the LC–MS/MS method, six reference samples of AA, GA, AP or IS added in non-perfused perfusion medium and prepared with the sample preparation method were analyzed (Fig. 2). The selectivity was ensured with the LLOQ samples from each analyte. The solvents or perfusion medium did not give any interfering peaks or background in any of the samples.

Furthermore, a post-column infusion experiment was performed to evaluate the matrix effect after the injection of the placental perfusion samples. The infusion setup consisted of a

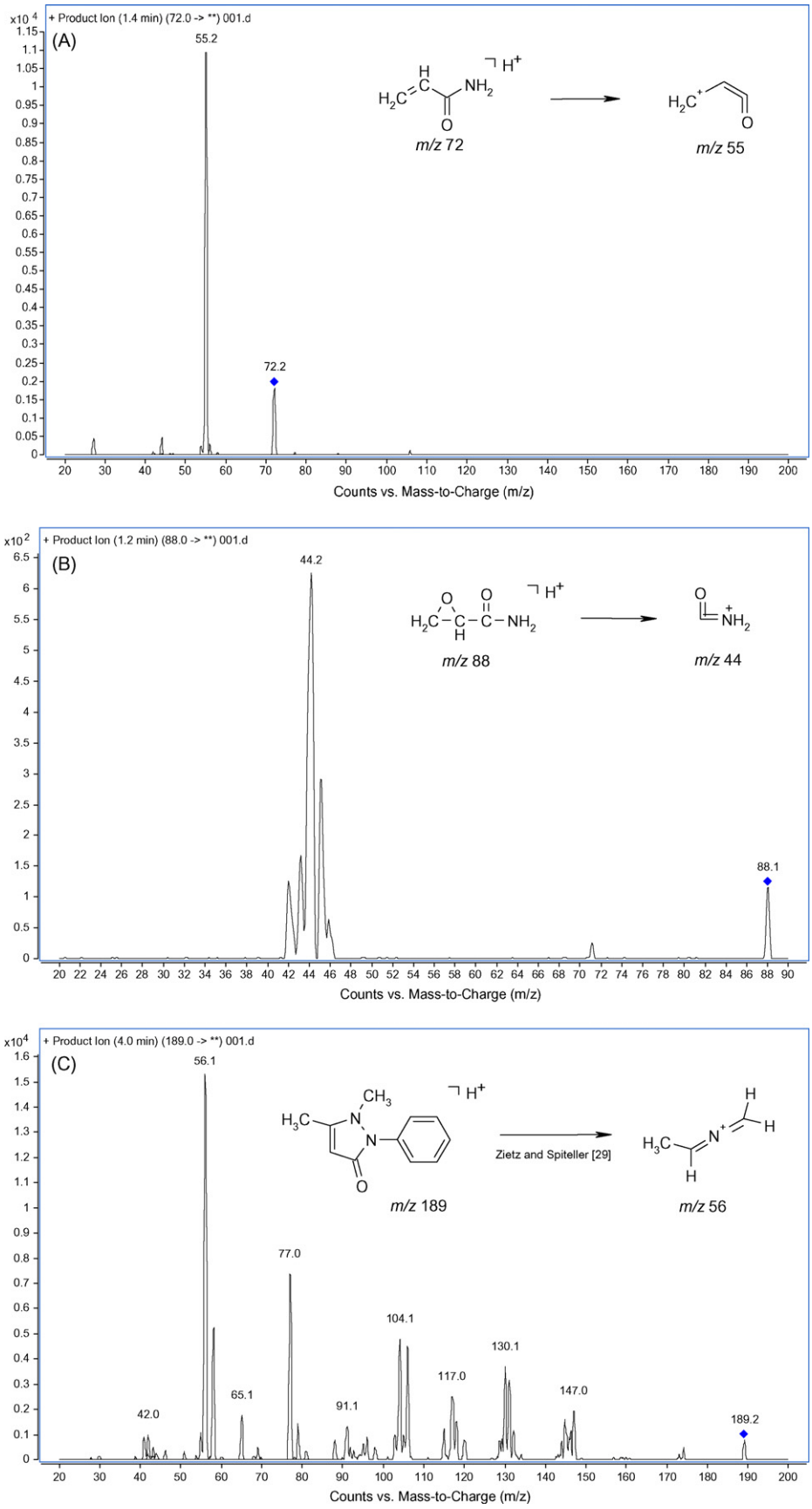


**Fig. 1.** Product ion mass spectra with proposed fragmentation of acrylamide (A), glycidamide (B) and antipyrine (C).

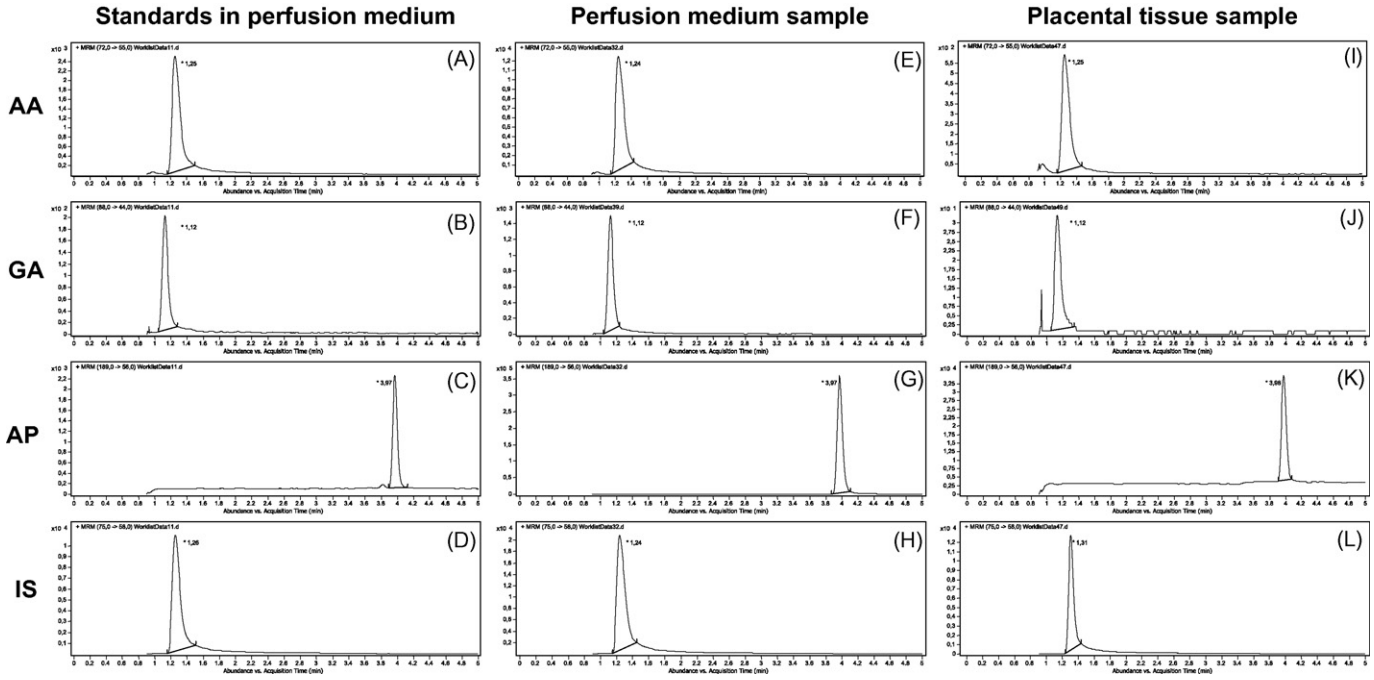


**Fig. 2.** MS chromatograms of (A) standard sample of acrylamide (AA) 4 μg/mL (retention time 1.25 min, MRM $m/z$ 72 → 55), (B) glycidamide (GA) 4 μg/mL (retention time 1.1 min, MRM $m/z$ 88 → 44), (C) antipyrine (AP) 40 μg/mL (retention time 3.97 min, MRM $m/z$ 189 → 56), and (D) internal standard (IS) ($^{13}$C-acrylamide) 1 μg/mL (retention time 1.25 min, MRM $m/z$ 75 → 58) added into placental perfusion medium. (E–H) A sample from maternal circulation in human placental perfusion after 4 h exposure to 4 μg/mL of acrylamide and 100 μg/mL of antipyrine or 5 μg/mL of glycidamide. (I–L) A sample from perfused placental tissue after 4 h exposure to 5 μg/mL acrylamide and antipyrine (I, K, L) or 5 μg/mL of glycidamide (J).

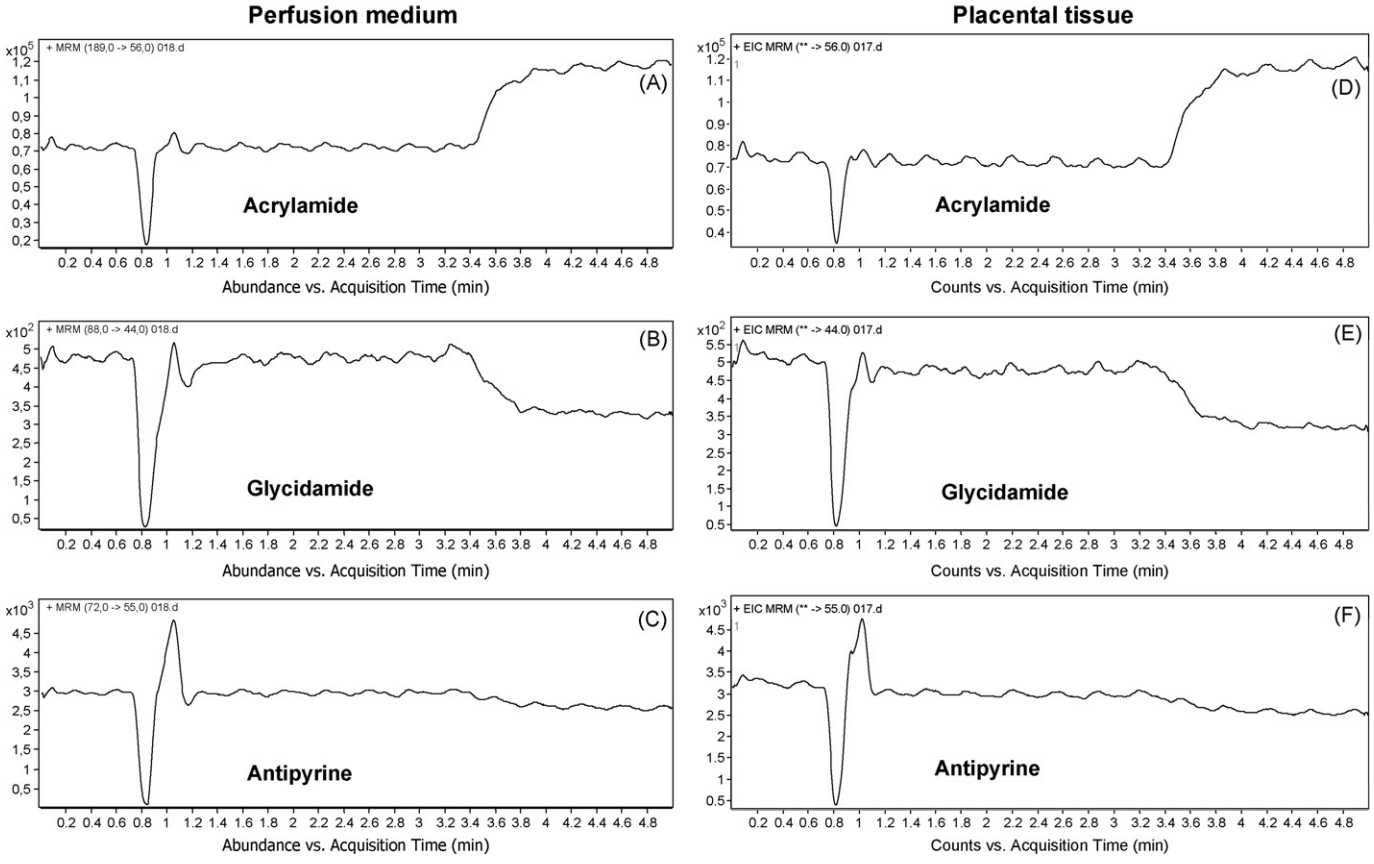


**Fig. 3.** Effect of injecting blank human placental perfusion sample or tissue homogenate sample on column with post-column infusion. Panels A–F are MRM chromatograms for analytes with a protein precipitation for blank perfusion sample (A–C) or tissue sample (D–F) injected on column. Constant flow of standard solution of analytes in 10% ACN was delivered via the T-piece to the mobile phase. Analyte signals were monitored after the injection of a blank sample from placental perfusion experiment.

**Table 1**
The linear range, calibration curve parameters with standard errors, regression coefficients and LLOQ's of acrylamide, glycidamide and antipyrine ($n = 3$).

| Compound | Linear range (μg/mL) | Regression parameters | | $R^2$ | LLOQ (μg/mL) |
|---|---|---|---|---|---|
| | | Slope ± S.T.D. error | Intercept ± S.T.D. error | | |
| Acrylamide | 0.5–20 | 0.6183 ± 0.0044 | −0.0192 ± 0.0046 | 0.999 | 0.5 |
| Glycidamide | 0.5–20 | 0.0354 ± 0.0009 | −0.0024 ± 0.0010 | 0.991 | 0.5 |
| Antipyrine | 5.0–200 | 0.0056 ± 0.0001 | −7.2498 ± 0.7895 | 0.999 | 5.0 |

syringe pump and a post-column T-piece as reported elsewhere [25]. The results indicated the absence of significant ion suppression at the retention time of the analytes (Fig. 3).

### 3.3. Linearity

The eight-point calibration plots obtained by $1/x$ weighted linear regression with the equation $y = bx + c$ were highly linear over the range of 0.5–20 μg/mL of AA or GA and 5–200 μg/mL of AP with the correlation coefficients of 0.999, 0.991 and 0.999, respectively. The calibration curve parameters with standard errors and regression coefficients are summarized in Table 1. Deviation of calibration standards from their nominal concentrations were less than 15% in all studied calibration levels. The LLOQ of AA and GA with acceptable accuracy and precision (<15% R.S.D.) was 0.5 μg/mL representing 125 pg injected on the column and LLOQ of AP was 5 μg/mL. We found our LLOQ to be in line with the published LLOQ values determined by LC–MS/MS for AA analysis in food (1–5 ng/mL or 2–200 pg/injection) [11] or GA analysis in biological matrices (0.1 μM; 670 pg/injection) [16] and to be about 16–80 times more sensitive than LLOQ values found by HPLC-UV methods (0.1 μg/mL for AA or 2 ng/injection and 0.5 μg/mL for GA or 10 ng/injection) [17]. Since the method was developed and optimized for the known concentration range, there was no need for optimization of sensitivity. Expressed as signal to noise value, peak intensities at LLOQ were 70, 20 and 1000 for AA, GA and AP, respectively, made in perfusion medium and/or tissue homogenate.

### 3.4. Precision, accuracy and recovery

The precision and accuracy of all QC samples shown in Table 2 were within the acceptable range [24]. Similar precisions and accuracies were obtained by Barber et al. [17] and Twaddle et al. [16]. The method was accurate and precise between runs and within individual runs at each level for all the analytes. The recovery of AA and AP were 100% and GA more than 90% showing that the recovery of the analytes was consistent, precise and reproducible which is comparable to the other published methods [16,17].

### 3.5. Stability

There was no significant degradation of AA, GA or AP after three freeze–thaw cycles (at least 24 h interval between each cycle) in comparison with freshly prepared samples (mean difference 4%). The short-term temperature stability also showed no significant degradation of any of the three analytes in perfusion medium or tissue homogenate after 4 h storage at room temperature (mean difference +4%). The AA, GA and AP concentrations in 14 and 30 days of long-term stability samples were within the range of −20 to +10%. The stock solution of AA, GA and AP in water was stable for 30 days when stored at −20 °C and kept at room temperature for 6 h after thawing. Post-preparation samples were found to be stable during 24-h storage at 10 °C in the autosampler.

### 3.6. Application

The bioanalytical method described was developed for the study of transplacental transfer of AA and GA with AP in full-term human placentas. Also, the putative accumulation of AA, GA and AP in placental tissue after perfusions is of interest and will be analyzed with this method. When analyzing the unknown samples from two pilot perfusions by LC–MS/MS the quality control samples and standard curve analyzed showed good reproducibility, accuracy and linearity. In the pilot perfusions, AA, GA and AP were found not only in maternal but also in fetal samples as well as in placental tissue (Figs. 2 and 4). The concentrations of AP in the pilot experiment were confirmed with a validated HPLC-UV method developed for the analysis of AP in perfusion medium. The concentrations of AA, GA and AP measured in maternal and fetal circulations were above the LLOQ of the method and all the measured concentrations were within the range of the method. Thus, the method was applicable

**Table 2**
Intra-day and inter-day precision[a] and accuracy[b] for acrylamide, glycidamide and antipyrine in perfusion medium.

| Analytes | Nominal concentration (μg/mL) | Intra-day precision and accuracy | | | Inter-day precision | |
|---|---|---|---|---|---|---|
| | | Mean ($n = 5$) (μg/mL) | R.S.D. (%) | Mean accuracy (%) | Mean ($n = 3$ days) (μg/mL) | R.S.D. (%) |
| Acrylamide | 0.5 | 0.5 | 1.1 | 98 | 0.52[c] | 3.3 |
| | 1 | 1.0 | 2.2 | 102 | 1.0 | 4.8 |
| | 4 | 3.9 | 1.2 | 97 | 4.4 | 15 |
| | 15 | 15 | 0.5 | 98 | 15 | 7.5 |
| Glycidamide | 0.5 | 0.6 | 3.5 | 114 | 0.56[c] | 4.9 |
| | 1 | 1.0 | 3.4 | 103 | 1.0 | 6.8 |
| | 4 | 3.7 | 3.3 | 93 | 4.2 | 12 |
| | 15 | 14 | 1.9 | 95 | 14 | 8.5 |
| Antipyrine | 10 | 9.5 | 18 | 95 | 9.5 | 14 |
| | 40 | 38 | 2.6 | 95 | 41 | 11 |
| | 150 | 141 | 1.6 | 94 | 144 | 8.5 |

[a] Precision ≤ 15% R.S.D., for LLOQ ≤ 20% R.S.D.
[b] Deviation from calculated content ≤ 15% (LLOQ ≤ 20%).
[c] $n = 2$ days.

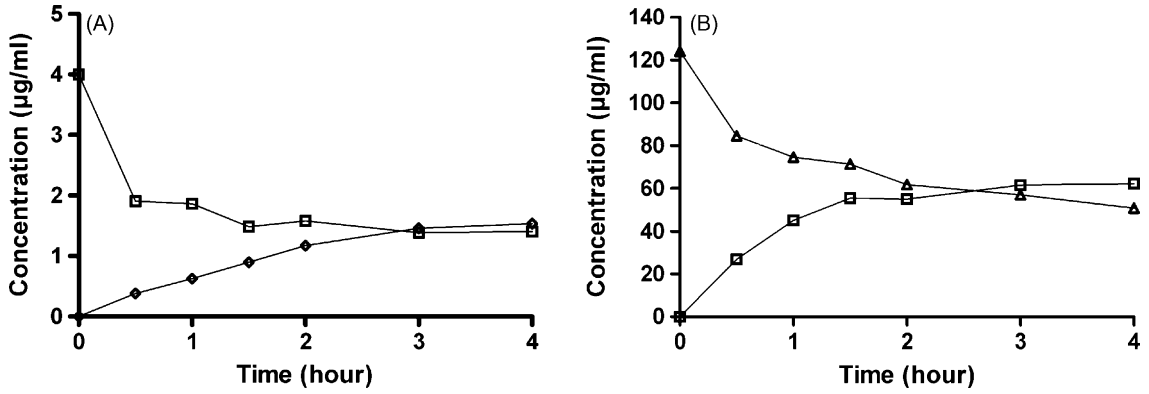


**Fig. 4.** Transfer of acrylamide (A) and antipyrine (B) through the human placenta during the 4 h placental perfusion in one pilot study. The concentrations in maternal (triangles) and fetal (squares) circulations in one perfusion with 4 μg/mL of acrylamide (A) and 100 μg/mL of antipyrine (B).

for the measurement of AA, GA and AP concentrations obtained from placental perfusion.

## 4. Conclusions

AA and GA were initially analyzed by HPLC-UV, because the sensitivity of the UV was reported to be adequate for our purpose according to a study with rat plasma [17]. However, sufficient specificity was gained only by LC–MS/MS due to the high background signal of perfusion medium in UV detection. Additionally, in our LC–MS/MS method there are no sample preparation steps which may induce loss of analytes during sample preparation [17]. The developed LC–MS/MS method was also fast, increasing the sample throughput, the sample preparation was straightforward and the method was suitable for measuring AA, GA and AP simultaneously both in perfusion medium and in placental tissue. The sensitivity of the method was 0.1 μg/mL for AA and GA and thus it can well be used to detect concentrations in maternal and fetal circulations after perfusing placentas with 5 or 10 μg/mL of AA or GA. Additionally, the one-step protein precipitation as the sample preparation for LC–MS/MS gave a sufficient recovery. The method was accurate, and precise in intra-day and inter-day tests fulfilling the following acceptance criteria from FDA guidelines: the R.S.D. was not more than 15% except for LLOQ, for which it did not exceed ≤20%. Also, the accuracy determined as the deviation from nominal value was not more than 15% except for LLOQ, for which it did not exceed 20%. The long-term stability of analytes in perfusion medium stored in −20 °C for 30 days was good, enabling to run samples from several perfusions at the same time. Also, the post-preparative stability was good enabling the use of an autosampler for over-night analysis. According to our knowledge, this is the first study where AA, GA and AP can be analyzed simultaneously by LC–MS/MS and which is applicable to analysis of perfusion medium and tissue from human placental perfusion.

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