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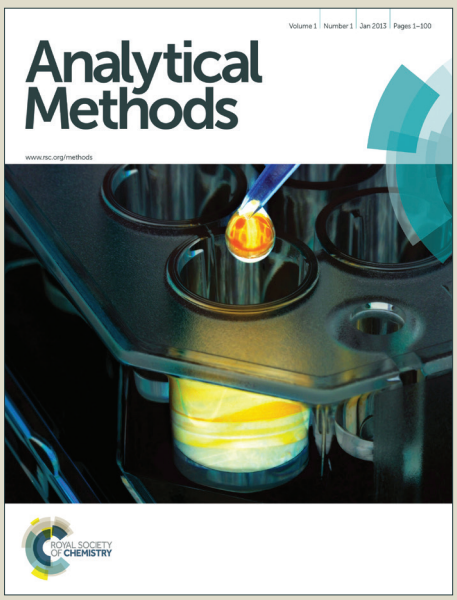

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Development and Validation of a Quantitative Method for Determination of Retigabine and its N-Acetyl Metabolite; Overcoming Challenges Associated with Circulating Labile N-Glucuronide Metabolites

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Abstract

Retigabine or ezogabine is an anticonvulsant approved for use in adjunctive treatment of partial epilepsy in adults. In humans, there is no evidence for cytochrome P450 catalyzed reactions; the drug is extensively metabolized primarily by N-glucuronidation reactions and acetylation to form a mono-N-acetylated metabolite. The majority of the drug-related material has been found to be excreted in the urine. The major challenge in developing a method for the quantitation of retigabine in human plasma or urine is minimizing the contribution from labile N-glucuronides, which are known to circulate at very high levels (approximately 25-fold) relative to parent. Degradation of these metabolites during sample handling and processing has been shown to lead to an increase in the concentrations of both retigabine and the N-acetyl metabolite; where this conversion is temperature, pH, and time dependent. Thus, it is important to consider these observations while developing a method for the accurate quantitation of retigabine from biological matrices in order to prevent the overestimation of both retigabine and its N-acetyl metabolite. Herein, we describe an extraction procedure to ensure accurate quantitation of retigabine and its N-acetyl metabolite from human plasma. This publication also provides specific recommendations for sample handling and storage of clinical samples prior to bioanalysis. The method was validated in human plasma over the concentration range of 5-2500 ng/mL for both analytes; the results from assay validation and incurred sample reproducibility demonstrate the method is rugged, precise, accurate, and well-suited for quantitative bioanalysis of both the drug and main metabolite.

Keywords: Retigabine, Ultra High Performance Liquid Chromatography Tandem Mass Spectrometry (UHPLC-MS/MS), N-glucuronides, Blood Stability, Sample Collection and Handling.

Abbreviations:

Retigabine, RTG; N-acetyl retigabine, NAMR; liquid liquid extraction, LLE; dried blood spots, DBS; higher limit of quantification, HLQ; lower limit of quantification, LLQ; ultra high performance liquid chromatography tandem mass spectrometry, UHPLC-MS/MS; N-glucuronides, N-Glu.

Introduction

Retigabine (INN, referred as RTG in this work) or ezogabine (USAN) is currently approved as an adjunctive treatment of refractory partial-onset seizures in adults; where efficacious doses range between 600 mg/day and 1,200 mg/day. Uncontrolled epilepsy results in a significant risk for increased morbidity and mortality. Among other antiepileptic drugs, RTG has a unique mechanism of action, serving as a potassium channel opener, by activating a certain family of voltage-gated potassium channels in the brain (allosteric modulator of KCNQ2-5).[1-3] RTG has shown to be an effective anticonvulsant in a broad range of epilepsy and seizure models[4-7] and thus far is the only compound which has demonstrated the ability to antagonize hyperexcitability in a concentration-dependent manner in two models of drug-resistant epilepsy.[6,7] In addition, RTG was shown to completely block epileptiform discharges in three models in human brain slices derived from patients with pharmaco-resistent epilepsy who underwent surgery for the treatment of intractable epilepsy.[8] In various randomized double-blind trials, RTG was shown to reduce seizure rates by up to 35%, compared to patients taking placebo.[9]

Although data collected from three, placebo-controlled studies in patients with partial onset seizures revealed efficacy increases with increasing dose, there was a 35%–50% between-patient variability and overlap in the individual predicted AUC values between dose levels (e.g., some subjects receiving 900 mg/day had higher AUC values than subjects receiving 1200 mg/day).[10] These data highlight the need to dose titrate RTG based upon individual patient response for efficacy and tolerability, not based on exposure. Throughout all approved does levels up to 1,200 mg/day, circulating Cmax concentrations were generally less than 2500 ng/mL.

The metabolism of RTG is remarkable and increases the bioanalytical complicity required to circumvent the unique metabolism profile. In humans, there is no evidence for cytochrome P450 catalyzed reactions; the drug is extensively metabolized primarily by N-glucuronidation reactions (N-Glu) and acetylation to form a mono-N-acetylated metabolite (NAMR; see Fig. 1).[11-12] The majority of the drug-related material has been found to be excreted in the urine as intact RTG/NAMR and as N-Glu metabolites (see Fig. 1). The metabolism of RTG in dogs is similar to that for humans, while in rats, more than 20 metabolites are produced by N-glucuronidation (predominate pathway), acetylation, ring closure reactions, and de-fluorination.[12] The major challenge in developing a quantitative method for the analysis of RTG in human plasma or urine is minimizing the contribution from labile N-Glu metabolites which are known to circulate at very high levels. Primary and secondary amino N-Glu (N- N-Glu 1 and N-Glu 2, respectively) metabolites have been reported for both RTG and NAMR.[11-13] The primary amino N-Glu 2 is the major glucuronide species in plasma of rats, dogs, and humans; while the secondary amino N-Glu 1has been detected only in rat bile and human liver slice and liver microsome in vitro assays.[12] Hiller et al., 1999 demonstrated a constant ratio between RTG and N-Glu metabolite in vivo in human and dog with the exposure of the combined N-Glu metabolites exceeding that for RTG by approximately 25-fold.[14] The authors suggested that enterohepatic circulation of RTG in these species is likely to be the result of reversible glucuronidation-deglucuronidation reactions.[14] In the dog, these N-Glu metabolites have been reported to easily convert back to the parent compounds; there is no reason to believe this conversion would be specific only to one species.[13] As this degradation would result in an overestimation of the concentration of RTG, appropriate

bioanalytical methods for any matrix should consider many factors to ensure accurate determination of RTG and NAMR.

In this publication, the stability of RTG, NAMR, and the N-Glu metabolites has been thoroughly investigated in plasma, blood, extraction conditions, UHPLC autosampler, and Dried Blood Spots (DBS) under a variety of conditions. As expected, their conversion was found to be time, matrix, temperature, and pH dependant. Appropriate extraction procedures from human plasma and urine were developed to minimize the contribution of these metabolites back to RTG and NAMR. Once stable conditions were identified and optimized; a suitable extraction and Ultra Performance Liquid Chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS) method in plasma was validated to assess precision and accuracy of the assay for the analysis of RTG and NAMR while minimizing the contribution from the N-Glu metabolites. This publication also provides specific recommendations for sample collection, handling, and storage of clinical samples prior to bioanalysis. The procedures described herein may also be applied to similar compounds with similarly labile N-Glu metabolites, particularly if they are found to circulate at relatively high levels compared to parent.

1. Experimental

1.1. Chemicals and reagents

RTG and NAMR were synthesized in-house by Chemical Development (GlaxoSmithKline, RTP, USA). [$^2H_4$]-RTG and [$^2H_4$]-NAMR were synthesized by Isotope Chemistry (GlaxoSmithKline, Stevenage, UK). Ahlstrom grade 226 BDS cards were obtained from ID Biological Systems (Greenville, NC, USA). Ammonium formate, acetonitrile, methanol, isopropanol, diethyl ether, ethyl acetate, ammonium hydroxide, ammonium acetate, ammonium bicarbonate, and tert-butyl methyl ether (MTBE) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Formic acid was purchased from Alfa Aesar (Ward Hill, MA, USA). Human whole blood, plasma, and urine were obtained from Bioreclamation Inc. (East Meadow, NY, USA). Control human plasma and whole blood were used in accordance with current GSK policies for collecting, obtaining, and using human biological samples in laboratory research.

1.2. Equipment

An Eppendorf 5810R centrifuge with a rotor capacity for four 96-well plates (Brinkmann Instrument, Westbury, NY, USA) was used for sample centrifugation. A Mettler UMX2 balance (Columbus, OH, USA) was used for compound weighing. A TomTec Quadra 3 SPE (Hamden, CT, USA) was used for liquid transfer. Arctic White LLC 96-well round 2 mL polypropylene plates and ArctiSeal silicone mats with PTFE film (Bethlehem, PA, USA) were used to extract analytes from plasma. One milliliter silanized glass vials along with 96-well plate covers (CapMat with Pre-Cut T/S Septa) from MicroLiter Analytical Supplies (Suwanee, GA, USA) were used for sample introduction to the UHPLC–MS/MS. An ACQUITY™ UPLC integrated

system from Waters (Milford, MA, USA) consisting of a sample manager combined with a sample organizer, capable of holding eight 96-deep well plates, and a binary solvent manager were used. A triple quadrupole mass spectrometer API-4000 (Applied Biosystems/MDS-Sciex, Concord, Ontario, Canada) was used.

1.3. Stability of analytical stock solutions of RTG and NAMR

The stability of analytical stock solutions of RTG and NAMR in methanol at 1 mg/mL was investigated at ambient temperature and -80°C by diluting to an appropriate concentration and analyzing them by UHPLC-MS/MS. The mean analyte to internal standard peak area ratio of a stock solution at ambient temperature and -80°C was compared to that of a freshly prepared solution (in replicates of six).

1.4. Stability of RTG and NAMR in the presence of N-Glu

All efforts to synthesize the N-Glu metabolites of RTG and NAMR to suitable purity levels were unsuccessful due to stability issues associated with these metabolites. Therefore, plasma aliquots from healthy volunteers participating in a clinical study were pooled and anonymized for use in stability investigations of the N-Glu metabolites. The N-Glu of NAMR in this pooled plasma sample was at much lower concentration as compared to that for RTG N-Glu. Therefore, the main focus of this investigation was the N-Glu of RTG. The stability of the N-Glu in the pooled plasma sample was investigated using protein precipitation (PPT) as an extraction procedure. The stability was evaluated at 4ºC, 37ºC, and ambient temperature. To aliquots (in replicates of six) of 10 µL at different time points (0, 1, 2, 4, and 20h), 200 µL acetonitrile containing isotopically labelled internal standards was added

followed by vortex-mixing and centrifugation for approximately 5 min at 4ºC. One hundred microliters of the supernatant was diluted with 100 µL water and analyzed using UHPLC-MS/MS as described below. Additional plasma aliquots were used to investigate the stability of the N-Glu during storage in the autosampler. After PPT, an equal volume of the supernatant was diluted with an equal volume of a buffer solution (10 mM ammonium formate pH 3, 10 mM ammonium acetate pH 4 and native pH, or 10 mM ammonium bicarbonate native pH and pH 9). Two microliters of these mixtures were injected into the UHPLC-MS/MS after storage in the autosampler for 0, 0.75,1,2,4, and 20h at 4ºC.

The pooled plasma samples were spiked (20 µL to 1 mL) into fresh whole blood to assess the stability on the N-Glu in whole blood and DBS cards. The stability of the N-Glu in whole blood was performed in the same way as described for plasma samples using PPT. The stability of the N-Glu in DBS cards was investigated at ambient temperature and at -80°C for up to two weeks and the analysis was performed as follows. A 4 mm diameter paper disc was punched from the center of the Ahlstrom grade 226 DBS cards into the pre-washed 2 mL ArcticWhite 96-well polypropylene plate. Human control plasma sample (50 µL) was added to the 96-well plate containing DBS discs followed by vortex-mixing for approximately 30 min. The analytes were extracted from plasma using PPT as described above for the pooled plasma samples.

1.5. Potential overestimation of RTG and NAMR due to N-Glu conversion at -20℃ and -80℃

The samples were removed from the -80°C freezer, thawed, and pooled into two levels (low and high) based on the overall concentrations for the runs as previously determined. Aliquots of the pooled samples were extracted and analyzed (in replicates of six) with a freshly prepared duplicate standard curve and freshly prepared quality control (QC) samples to determine the initial concentration of the two pools (Day 0). The pooled incurred samples and QC samples were split into two aliquots (A and B). Aliquot A was stored at -80°C and aliquot B at -20°C. These aliquots were subsequently analyzed on Day 14.

1.6. Stability of RTG and NAMR in plasma and blood in the absence of N-Glu

The stability of RTG in spiked human plasma samples (in the absence of N-Glu) stored at ambient temperature was assessed at 15 and 2000 ng/mL (in replicates of six) by comparing the mean concentrations of samples extracted after storage for 24 hours against those of the samples extracted immediately upon thawing. The stability of NAMR stored at ambient temperature for 4h, 24h, and in an ice-water bath for 24h was also assessed in a similar way.

The stability of RTG and NAMR in spiked human blood samples stored in an ice-water bath and at 37°C (in the absence of N-Glu) was assessed at 15 and 2000 ng/mL (in replicates of six) by comparing the mean peak area ratio of samples extracted after storage for 4 hours against those of the samples extracted immediately upon spiking. The analytes were extracted from whole human blood samples (25 μL) by PPT using

200 µL of acetonitrile containing internal standard. Extracts were analyzed by the UHPLC-MS/MS method described in Sections 2.9 to 2.11. Differences were calculated between the peak area ratio of analyte to internal standard values obtained for fresh and stored samples.

Long term stability (LTS) of the analytes in human plasma were studied at 15, 200, and 2000 ng/mL (in replicates of six) at -80°C by comparing the mean concentrations of samples extracted after storage for 661 days.

1.7. Investigation on the stability of RTG and NAMR in human urine

To investigate the best treatment and storage conditions for RTG and its metabolites in human urine samples, the following three conditions were assessed during a clinical study where both blood (subsequently processed to plasma) and urine were collected:

1. Half a milliliter of urine was transferred to an appropriately labelled 1.4 mL polypropylene specimen container. This sample is referred as untreated sample.

2. A second aliquot was stabilized 1M Tris buffer with a ratio 9:1 (900 µL urine to100 µL 1M Tris buffer) in an appropriately labelled 1.4mL polypropylene specimen container.

3. The third urine aliquot was stabilized with 100 mM Tris buffer with a ratio of 1:49 (100 µL urine to 4900 µL 100 mM Tris buffer) in an appropriately labelled polypropylene specimen container.

Urine samples from three of the subjects were used to determine which of the three aliquots would show the most reproducible and accurate results for the analytes investigated. These samples were diluted with human control plasma in two steps to a final dilution factor of 1000 (including initial dilution) to allow the use of the validated plasma method for quatification. Due to visible precipitate observed in all tubes for aliquots 1 and 2 (untreated and treated with 1M Tris buffer samples), all samples were vortex- mixed well and immediately aliquoted in duplicate. Also, additional samples were analyzed similar way from these three treatment procedures or aliquots from three different subjects with and without centrifugation at approximately 3200×g for 5 min. All extracted samples were analyzed against a human plasma curve in duplicate and QC in replicates of six as described in Sections 2.9 - 2.11.

1.8. Preparation of calibration standards and QC samples

Analytical stock solutions of RTG, [$^2H_4$]-RTG, NAMR, and [$^2H_4$]-NAMR were individually prepared in methanol at a concentration of 1.0 mg/mL. Stock solutions were stored at -80ºC where the analytes are proven stable for at least 285 and 182 days for RTG and NAMR, respectively. Two separate sets of working solutions one for calibration standards (WS) and one for QC samples (WQ), containing both RTG and NAMR at concentrations of 50 µg/mL were prepared fresh at the day of analysis in 50/50 (v/v) acetonitrile/water using a serial dilution procedure.

The WS was used to make calibration standards in plasma using a serial dilution procedure at 2500, 2000, 1000, 500, 250, 100, 50, 25, 10, and 5 ng/mL for both RTG

and NAMR. The WQ was used to make QC samples in plasma at 10,000 (dilution QC, analyzed after 10-fold dilution), 2500, 2000, 200, 15, and 5 ng/mL for both RTG and NAMR. In the first validation run, freshly prepared QC samples were analyzed against freshly prepared calibration standards. For each subsequent validation run, six replicates of the thawed QC samples were analyzed against a freshly prepared standard curve.

1.9. Sample preparation

Approximately 0.5 mL diethyl ether was added to each well of the 2 mL ArcticWhite 96-well polypropylene plate. The plate was sealed with the ArctiSeal mat and vortex–mixed in an inverted position for approximately 3 min. Subsequently, the diethyl ether was discarded and the plate was left to dry in a chemical hood. This wash step was intended to remove any plastic residue from the plates and plate seals.

An aliquot of plasma sample (10 µL calibration standard, QC, incurred, or blank sample) was added to the washed 96-well plate followed by addition of 40 µL of human control plasma. A 25 µL aliquot of internal standard solution (200 ng/mL of $[^2H_4]$-RTG and $[^2H_4]$-NAMR in acetonitrile) was added to all wells with the exception of double blanks, which instead received 25 µL of acetonitrile only. One mL diethyl ether was added to all wells and vortex-mixed for approximately 15 min and centrifuged for approximately 5 min at approximately 3220xg. After vortex-mixing and centrifugation, the diethyl ether layer was transferred to a 2 mL polypropylene 96-well plate containing 1 mL silanized glass-inserts using a TomTec liquid handler and evaporated under a stream of nitrogen at 45 ºC (Note: extensive dry down should be avoided). The dried extracts were then reconstituted with 100 µL of 50/50 (v/v)

acetonitrile:10 mM ammonium bicarbonate (native pH, 7.9). Finally, the samples were vortex-mixed for approximately 1 min and centrifuged for approximately 1 min at approximately 3220xg prior to UHPLC analysis.

1.10. Chromatographic conditions

The analytical column was an ACQUITY UPLC™ C8, BEH, 2.1 mm x 50 mm, with 1.7 µm particle size from Waters Co (Milford, MA, USA). The column temperature was held at ambient temperature and the sample compartment was kept at 4ºC. Mobile phase A consisted of 10 mM ammonium formate (native pH) and mobile phase B was a mixture of acetonitrile/methanol 90/10 (v/v). The initial condition was 15% B until 0.5 min followed by a linear gradient ran from 15% B to 50% B until 1.2 min and held at 50% B until 1.9 min. Then, a linear gradient ran from 50% B to 95% B until 2.0 min and held at 95% B until 2.5 min to remove late eluting substances from the analytical column, after which the system was returned to the initial condition. The total run time, including sample loading was approximately 3.0 min and the flow rate was maintained at 0.6 mL/min throughout the run with the exception of the washing step (2.0-2.5 min) for which it was increase to 1.0 mL/min. A typical injection volume of 3 µL in a 10 µL loop (partial loop injection mode) was used. A solution of 20% acetonitrile in water was used as autosampler weak wash and mixture of acetonitrile/isopropanol/water (40/40/20; v/v/v) was used as strong wash.

1.11. Mass spectrometric conditions

An API-4000 mass spectrometer with a TurboIonspray interface (TIS) was operated in the positive ionization mode. The instrument was optimized for RTG,

[$^2H_4$]-RTG, NAMR, and [$^2H_4$]-NAMR by infusing a 10 ng/mL solution in acetonitrile/10 mM ammonium formate (native pH, 50/50 v/v) at 0.6 mL/min through an Agilent pump 1100 series (Palo Alto, CA, USA) directly connected to the mass spectrometer. The MRM transitions of m/z 304→230, m/z 308→234, m/z 274→232, and m/z 278→236 were chosen for RTG, [$^2H_4$]-RTG, NAMR, and [$^2H_4$]-NAMR, respectively. Two MRM periods were used to acquire more data points across the peaks. The first period (approximately 1.5 min) was used for monitoring the MRM transitions of NAMR and [$^2H_4$]-NAMR while the second for RTG and [$^2H_4$]-RTG, respectively. Dwell times of 200 msec were used for NAMR and its internal standard, and 150 msec for RTG and its internal standard. The optimized mass spectrometric conditions included the following MS conditions: TIS source temperature, 650ºC; TIS voltage, 5000 V; curtain gas, 30 psi (nitrogen); nebulizer gas (GS1), 80 psi (zero air); turbo gas (GS2), 80 psi (zero air). The collision energy values were 24 eV for NAMR and [$^2H_4$]-NAMR and 28 eV for RTG and [$^2H_4$]-RTG.

1.12. Data analysis

MS data were acquired and processed (integrated) using the proprietary software application Analyst™ (Version 1.4.2, Applied Biosystems/MDS-Sciex, Canada). Calibration plots of analyte/internal standard peak area ratio versus RTG and NAMR concentrations were constructed and a weighted $1/x^2$ linear regression was used. Concentrations of RTG and NAMR in validation samples were determined from the appropriate calibration line and used to calculate the bias and precision of the method with an in-house LIMS (Study Management System, SMS2000, and version 2.3, GlaxoSmithKline).

2. Results and Discussion

2.1. General objective

The objective was to develop a rugged, sensitive, and relatively high-throughput UHPLC-MS/MS method allowing simultaneous determination of RTG and NAMR from human plasma with a minimum contribution from the N-Glu metabolites. In addition, the method would be used for determination of RTG exposure in paediatric studies and therefore required a relatively small blood volume to be collected.

2.2. Stability of analytical stock solutions of RTG and NAMR

RTG is a non-hygroscopic white to slightly colored powder with very poor solubility in water. The solubility in methanol is much better and therefore methanolic solutions of both RTG and NAMR were used to prepare analytical stock solutions. The stability of RTG and NAMR at a concentration of 1mg/mL was evaluated at ambient conditions and -80°C at different time periods. The stability of RTG and NAMR in methanol at 1 mg/mL was assessed during method validation by diluting to an appropriate concentration and analyzing by UHPLC-MS/MS. The mean analyte to internal standard peak area ratio of stored stock solution was compared to that of a freshly prepared solution (in replicates of six). The difference was less than 5%, and indicates that RTG and NAMR are stable in analytical solutions of methanol stored at ambient conditions for up to 22h. However, prolonged storage (greater than one week) of these solutions under ambient conditions resulted in significant degradation of the analytes, and in the presence of light, these solutions were observed to generate a slight purple hue.

Therefore, it was decided to prepare analytical stock solutions of both RTG and NAMR in amber vials and the storage at -80°C. Under these conditions, the stability of these analytes was proven to be stable for at least 285 and 182 days for RTG and NAMR, respectively.

2.3. Challenges associated with N-Glu metabolites

The major challenge in developing a method for the bioanalysis of RTG in human plasma is minimizing the contribution from labile N-Glu metabolites (see Fig. 1), where these metabolites are known to circulate at very high levels relative to parent. The UHPLC-MS/MS response of RTG N-Glu in plasma has been shown to be approximately 25-fold of that for RTG; thus, a very minor degradation of the N-Glu metabolites would have a large impact on the quantitation of RTG. Therefore, it is important to minimize the impact of the N-Glu during sample storage, handling, and bioanalysis to prevent overestimation of actual circulating RTG levels. The N-Glu metabolite of NAMR was also detected, albeit at much lower concentrations. Therefore, the main focus of this investigation was around the stability of the RTG N-Glu; optimal conditions for this species would also likely be the same for the NAMR N-Glu.

The first goal was to thoroughly investigate the stability of the RTG N-Glu in various matrices (plasma, blood, and buffer solutions at different pH; see Fig. 2-4). For this purpose, plasma aliquots from individual subjects from a clinical study containing high concentration of RTG N-Glu were pooled and anonymized and used in the investigation. Although N-Glu 2 is the predominate circulating species[12], this method did not have the specificity to monitor only this species as both N-Glu

metabolites have same molecular weight and were not chromatographically resolved. The major impact on the RTG N-Glu stability was found to be temperature and pH. The N-Glu was found to be stable at 4ºC in human plasma for at least 24h (see Fig. 2), while approximately, two thirds of the N-Glu was found to convert back to RTG at 37ºC over this same time period. Not surprisingly, the instability of the RTG N-Glu was more pronounced in human blood (see Fig. 3). An interesting observation was the linear elimination of the N-Glu in human blood at 37ºC for up to 6h. *It is therefore our recommendation that blood samples from clinical studies should be handled on wet ice prior to plasma isolation, and that separation should be performed immediately after sample collection and immediately frozen*.

Other sources of instability were observed, and were correlated with processed extract as well as on-column instability. The on-column degradation from RTG N-Glu back to parent was observed to occur at high temperatures (e.g. 65°C) and when acidic mobile phase was used (see Fig. 4 and 5); this degradation can be seen in the increase in the baseline of RTG MRM transition and peak fronting – panel 5B. Therefore, the column temperature was kept at ambient conditions and a neutral mobile phase was used (see Fig. 5 panel D). Furthermore, there was a concern about possible N-Glu degradation during storage of the samples in the autosampler during the bioanalytical run; therefore sample extraction conditions to remove the N-Glu metabolites from the matrix using liquid liquid extraction (LLE) were investigated.

A LLE procedure was developed which extracted both RTG and NAMR from plasma, leaving the labile N-Glu metabolites in the plasma phase. Initial experiments comparing the amount of N-Glu from two plasma aliquots (with and without LLE)

suggested that the amount of N-Glu extracted using LLE was less than 1%. Several extraction solvents were evaluated and a remarkable behaviour was noticed for NAMR when solvents such as MTBE and ethyl acetate were used for LLE extraction. Three peaks for NAMR were observed when these solvents were evaluated (see Fig. 6); and it is suspected the peak splitting is likely related to migration of the acetyl group to neighbouring amino functional groups. This phenomenon was not observed following use of diethyl ether as the LLE solvent.

Finally, an experiment was designed to assess the stability of N-Glu metabolites in the reconstitution solution (see Fig. 4). In this investigation, the reconstitution solution consisted in a mixture of acetonitrile:buffer (pH ranging from 3 to 9). As expected, the results showed that the N-Glu rapidly degrades to RTG at pH 3 and was stable at basic pH greater than 7.9 (see Fig. 4). There were no differences in the results between reconstitution solutions at pH 7.9 and 9. The mixture of acetonitrile:10 mM ammonium bicarbonate (pH 7.9) was chosen since no pH adjustment is required. *Based on the N-Glu levels known to circulate, it is our recommendation that plasma samples should be subjected to LLE extraction (or SPE[13]) to remove N-glu metabolites in order to prevent conversation back to parent during bioanalytical processing.*

2.4. Analysis of RTG and NAMR DBS samples

DBS technology has proven to be an alternative to liquid matrix sampling technique with many preclinical and clinical applications, particularly when low sample volumes are desired.[15-20] Analyte stability in blood for many compounds, under ambient storage conditions, has shown to be equivalent to that of liquid samples

stored at -20°C or below. It was hypothesized that the RTG N-Glu might be stable within DBS, therefore the suitability of this approach was investigated, and was sought to be applied to a planned paediatric study.[21]

A few challenges were faced during the development of a DBS method for RTG and NAMR in human DBS samples. In the first attempt, the GE Whatman DMPK A (FTA) DBS cards were evaluated and soon abandoned as chemical derivatization was detected between the analytes and one of the card's additives; the primary amine groups on both RTG and NAMR were found to be covalently modified. To mitigate this derivitization with DMPK A cards, the chemically untreated Ahlstrom grade 226 BDS cards were then selected for the method. Commonly used solvents for DBS, such acetonitrile, methanol, water, or a mixture of all, were investigated to optimize the analyte extraction procedure from the cards.

While three consecutive precision and accuracy validation runs were successful, these were completed using only in vitro derived spiked samples, not actual incurred samples containing the metabolites. The suitably of DBS and ambient storage for this compound also needed to include an investigation around the stability of the N-Glu metabolites. Therefore the stability of RTG N-Glu was investigated in DBS samples stored under both ambient storage conditions and at -80°C. Even though, the N-Glu metabolites were stable after storage at -80°C for 14 days, extensive conversion back to RTG was observed following storage under ambient conditions over the same time period.[21] In light of these data, there was no advantage afforded through the use of DBS sampling and storage, stability was only assured following storage of the DBS under frozen conditions. Therefore, DBS as a sampling and storage mechanism was

discontinued and appropriate frozen storage and processing conditions were identified and validated in plasma to minimize ex-vivo conversion of these liable metabolites.

2.5. UHPLC-MS/MS method validation of wet plasma

Learning experiences from method development were taken into consideration for the design of the method for accurate extraction and analysis of both RTG and NAMR from plasma and a successful method validation was performed. The method was validated according to GSK departmental working practices and international regulatory expectations, and the following parameters were assessed:

- Selectivity, sensitivity and linearity
- Bias and precision
- Recovery and matrix effects
- Stability in processed extracted samples at 4°C
- The ability to dilute samples above the HLQ
- Stability in methanol at ambient temperature and -80°C
- Stability in human plasma at ambient temperature
- Stability in human whole blood in an ice water bath and at 37°C
- The effect of freeze-thaw from -80°C to ambient temperature

All of the parameters evaluated met GSK's SOP acceptance criteria for both analytes and the validation was considered successful; however considering the unique metabolic profile of retigabine, incurred samples were also evaluated to assess the accuracy, reproducibility, and robustness of the method. Therefore, stability information generated from pooled clinical samples is the most relevant to ensure accurate evaluation of exposure of RTG in biological matrices.

During method development, significant non specific binding issues were noted for both RTG and NAMR when plastic vials or untreated glass tubes, particularly at low concentrations and in the absence of plasma proteins; this issue was circumvented through the use silanized glass inserts.

2.6. Stability of RTG and NAMR in blood and plasma in the absence of N-Glu metabolites

RTG (in the absence of N-Glu) spiked in human plasma stored at ambient temperature for 24h was found to be stable; with a percent difference of -0.7% and -2.5% at 15 and 2000 ng/mL (in replicates of six), respectively. Similarly, the stability of NAMR in spiked human plasma samples stored under ambient temperature was also assessed at 15 and 2000 ng/mL (in replicates of six). The percent difference for NAMR stored at ambient temperature for 24h was greater than 15% (-11.3% and -18.4% at 15 and 2000 ng/mL, respectively) for one of the concentration levels evaluated and indicates that NAMR is not stable in human plasma under the aforementioned conditions. The percent difference for NAMR stored at ambient temperature for 4h and in an ice water bath for 24h was less than 15% (-3.2% (15 ng/mL) and -2.6% (2000 ng/mL) at ambient temperature and -1.3% (15 ng/mL) and -2.7% (2000 ng/mL) in an ice water bath) and indicates that NAMR is stable in human plasma stored at ambient temperature for up to 4h and in an ice water for up to 24h.

The stability of RTG and NAMR in spiked human whole blood samples stored in an ice water bath and at 37°C was assessed at 15 and 2000 ng/mL (in replicates of 6) by comparing the mean peak area ratio of samples extracted after storage for 4h against those of the samples extracted immediately upon spiking. Differences were

calculated between the peak area ratio of analyte to internal standard values obtained for fresh and stored samples. For RTG, the percent difference was less than 15% (-3.5% (15 ng/mL) and-1.5% (2000 ng/mL) in the water ice bath and 3.5% (15 ng/mL) and -13.7% (2000 ng/mL) at 37°C). For NAMR, the percent difference was less than 15% (actually -4.5% (15 ng/mL) and -9.5% (2000 ng/mL) in the water ice bath and -9.1% (15 ng/mL) and -14.4% (2000 ng/mL) at 37°C). The results indicate that RTG and NAMR are stable in human blood stored in an ice water bath and at 37°C for at least 4h in the absence of N-Glu, however, it is unlikely that the N-Glu metabolites would demonstrate similar stability.

The long term stability of RTG and NAMR in plasma in the absence of N-Glu was also generated by analysis of QC containing both analytes at three concentration levels (15, 200, and 2000 ng/mL) after storage at -80°C for 661 days. Fresh QC samples at the same concentration levels were prepared and analyzed to ensure the accuracy of the calibration curve. All results met SOP acceptance criteria indicating that RTG and NAMR are stable in plasma samples at -80°C for 661 days in the absence of N-Glu metabolites.

2.7. Potential overestimation of RTG and NAMR due to N-Glu conversion at -20℃ and -80℃

As RTG and NAMR N-Glu metabolites have previously been shown to convert back to the corresponding parent and the rate of degradation being temperature dependant, the potential exists for overestimation of RTG and NAMR as a result of N- Glu conversion during storage at -20°C or -80°C. The main objective of this

investigation was to determine the maximum time for storage at -20°C to facilitate utilization of clinical study facilities for sample collection where access to-80°C storage may not be feasible. Incurred study samples were removed from -80°C storage, thawed and pooled into two levels (low and high) based on the previously determined concentrations. The pooled incurred samples were split into two aliquots (A and B). Aliquot A was stored at -80°C and aliquot B at -20°C. These aliquots were analyzed at Day 0 and subsequently at Day 14 (N=6). The percent difference (Day 14 vs Day 0) at -20°C storage was 9.0% and 14.6% for RTG and 2.0% and 3.7% for NAMR at the low and high concentrations, respectively (See Fig 7). At -80°C storage the percent difference was 2.4% and 7.8% for RTG and -1.3% and 0.9% for NAMR at the low and high concentrations, respectively (See Fig. 7). The percent difference for RTG was markedly greater at -20°C and indicates that study samples must be stored at -80°C for long time durations. The percent difference for NAMR is less marked, but still apparent and indicates a stability concern and suggests that study samples should be stored at -80°C. *Based on the percent differences listed above it is proposed that samples for analysis of RTG and NAMR be stored at -20℃ for no more than 7 days, prior to analysis or transferred to -80℃.* This will ensure that accurate concentrations of RTG will be determined following temporary storage at -20°C.

Incurred sample reanalysis (ISR) was performed for all clinical studies using the validated method; and the results confirmed that the method and storage conditions described herein are suitable for an accurate determination of RTG and NAMR. A plot showing ISR results summary for a representative clinical study where study samples were reanalyzed up to 14 days later is presented in Fig. 8. It is worth

highlighting that after storage and reanalysis for 14 days there is no trend observed in the data that suggests instability under these storage and assay conditions.

2.8. Selectivity and linearity

The characteristic precursor $[M+H]^+$ to product ion transitions m/z 274→232, m/z 278 →236, m/z 304→230, and m/z 308→234 are consistent with the structures of the NAMR, $[^2H_4]$-NAMR, RTG, and $[^2H_4]$-RTG, respectively. The selectivity of the method was established by the analysis of control human plasma samples from 6 individual volunteers. UHPLC-MS/MS chromatograms of the blanks and QC samples were visually examined and compared for chromatographic integrity and potential interferences. Representative UHPLC-MS/MS chromatograms of RTG and NAMR of a blank sample, QC samples at the low limit of quantification (LOQ) and high limit of quantitation (HLQ), and the internal standards are shown in Fig. 9 and 10, respectively. No unacceptable interferences at the retention times of RTG, NAMR or the internal standards were observed. Post-column infusion experiments were also performed to investigate potential ion suppression effects from endogenous blood interferences on the MRM transitions of the investigated analytes. For this purpose, a control human plasma sample was processed as described in the section for sample preparation. Two µL of the extracted sample was injected into the UHPLC system with a continuous post–column infusion at 20 µL/min of a solution containing 10 ng/mL NAMR, $[^2H_4]$-NAMR, RTG, and $[^2H_4]$-RTG. No evidence of ion suppression was observed at the retention times for the investigated analytes and their corresponding internal standards.

The linearity of the method was evaluated by analyzing ten calibration standards in duplicate over the nominal concentration ranges of 5-2500 ng/mL for both analytes. The correlation coefficients obtained using $1/x^2$ weighted linear regressions were better than 0.9989 for RTG and 0.9993 for NAMR.

2.9. Bias and precision

The maximum bias observed for RTG and NAMR in the validation QC samples was -5.2% and -6.8%, respectively (see Table 1). The maximum within-run precision values observed were 4.2% for RTG and 6.1% for NAMR. The maximum between-run precision values observed were 3.2% for RTG and 1.7 for NAMR. As defined by the lower and upper QC concentrations possessing acceptable accuracy and precision, the validated range of the method based on 10 μL of EDTA human plasma is 5-2500 ng/mL for the analysis of RTG and NAMR.

2.10. Stability in processed extracted samples in the absence of N-Glu metabolites

The stability of RTG and NAMR in processed extracted samples was assessed by re-injecting a validation run after storage at 4°C for 48h, against freshly prepared calibration standards. The accuracy, precision and sensitivity of these samples were found to be acceptable on re-injection, indicating that the processed samples were stable when stored at 4°C and storage of clinical samples prior to bioanalysis for at least 48h. While these samples were in vitro prepared and did not include the presence of the N-Glu metabolites, it is important to note that these species are removed in the preceding LLE and the pH of the samples is optimal (Fig 4).

2.11. Recovery and matrix effects

The recovery of RTG and NAMR from human plasma samples spiked at 15, 200 and 2000 ng/mL was assessed by comparing the individual analyte/internal standard peak area ratios of the extracted samples to the mean analyte peak area ratio of the blank extracts of human plasma spiked at the same concentration after extraction. The recovery was greater than or equal to 37.0% for RTG and 47.3% for NAMR at all concentrations; and while this recovery is relatively low, the LLQ was adequate for study support and the method met or exceeded all acceptance criteria (see Fig. 9 and 10). The precision was less than 15% at all concentrations and the recovery is therefore acceptable. The effects of matrix components on the UHPLC-MS/MS response of RTG and NAMR in six individual lots of human plasma was assessed at three different concentrations (15, 200, and 2000 ng/mL) by comparing the analyte responses of blank extracts of human plasma spiked after extraction, with the response of matrix free samples spiked at the same concentrations after extraction. The precision of the calculated matrix effect values between the different lots of plasma was less than 15% at all concentrations and is therefore acceptable.

2.12. Matrix dilution

The ability to dilute samples containing RTG and NAMR at concentrations above the HLQ was demonstrated by performing a 10-fold dilution of human plasma QC sample spiked at 10,000 ng/mL for both analytes. A 10 µL aliquot of this extract was diluted with 90 µL human control plasma. A 10 µL aliquot of this solution was further extracted as described in the extraction procedure. Concentrations of RTG and NAMR in these samples were determined and corrected for the dilution factor. The

bias and precision values were less than 15%, indicating that a 10-fold dilution of the extracts of human plasma samples containing RTG and NAMR up to 4 times the HLQ is acceptable.

2.13. Investigation on the stability of RTG and NAMR in human urine

The majority of the RTG-related material has previously been determined to be excreted in the urine as intact RTG/NAMR or as the corresponding N-Glu metabolites. Urinary and renal adverse events such as proteinuria or urinalysis has been reported with higher frequency in patients receiving RTG as compared to those receiving placebo (17% versus 13%),[22] as a result, an investigation was conducted around these findings.[23] The researchers concluded that urinalysis findings in the study resulted from inappropriate urine handling techniques and/or ex vivo degradation of renally excreted RTG and related materials, resulting in urine turbidity and discoloration that interfered with the automated analysis procedures performed by the central laboratory used in the study.[23]

A follow up investigation ensued in order to address potential issues with urine collection and identify appropriate stabilizers to ensure accurate determination of RTG and NAMR in urine samples. Urine samples from a separate clinical study were collected into three tubes: 1) untreated, 2) stabilized 1M Tris buffer with a ratio 9:1, and 3) stabilized with 100 mM Tris buffer with a ratio of 1:49. A subset of the urine samples were analyzed and compared; samples were thawed and diluted with human control plasma to a final dilution factor of 1000 (including initial dilution) to allow the use of the validated plasma method. Evidence of drug precipitation in the form of solid precipitate was observed in all tubes for aliquots 1 and 2 (untreated and treated

with 1M Tris buffer samples) and was attributed to poor solubility of RTG in urine. To achieve reproducible results, all samples were vortex- mixed well and immediately aliquoted in duplicate. The results show an increase of RTG concentration in untreated samples as compared to stabilized with 100 mM Tris buffer with a ratio of 1:49 (see Fig. 11). This increase was attributed to degradation of N-Glu metabolites in urine generating RTG.

Additional samples from all three treatment procedures and three different subjects were analyzed with and without centrifugation at approximately 3200×g for 5 min; the intent was to separate precipitated drug or drug-related material. The supernatant was diluted in two steps to achieve a dilution factor of 1000 fold and analyzed against a human plasma curve in duplicate and QC in replicates of six as described in Section 2.9. RTG concentration decreased significantly for the untreated and 1 M treated aliquots (see Fig. 11) after centrifugation and it is most likely related to poor solubility of RTG in urine. The procedure where urine was treated with 100 mM Tris buffer with a ratio of 1:49 demonstrated the smallest difference between spun and unspun results indicating that this procedure is the most appropriate for urine collection in RTG clinical studies (see Fig. 11). The results for NAMR were similar across all treatments and did not significantly change with centrifugation.

2.14. Proposed procedure for blood, plasma and urine handling

Due to concerns over the high concentrations of the labile N-glu metabolites, a secondary objective of this publication is to provide clear guidance on the key aspects of sample handling and storage of clinical samples prior to bioanalysis. Therefore after collection, blood samples must be handled on wet-ice prior to plasma isolation;

and this step should be completed as soon as possible at 4ºC. Shipments of frozen plasma samples must use dried ice, preceded and followed by storage at -80°C. Additionally, bioanalysis should be performed as soon as possible following sample receipt, and ISR soon after. In our laboratory, samples were typically assayed within 2 weeks.

For clinical sites lacking the ability to store plasma samples at -80°C it is recommended that samples for analysis of RTG and NAMR be stored at -20°C for no more than 7 days. *Storage of plasma samples at refrigerated temperatures is not recommended*. The authors also recommend stabilizing urine samples with 100 mM Tris buffer with a ratio of 1:49 and freezing them immediately at -80°C prior to bioanalysis.

Sample storage information/paperwork for several diagnostic laboratories, suggests that plasma samples can be stored at ambient conditions or refrigerated (4°C) conditions for up to two weeks. However these stability were most certainly generated in the absence of the N-glu metabolites; our data generated on incurred samples demonstrates that under these conditions there will be substantial conversion of the N-Glu metabolites back to RTG and/or NAMR, resulting in an artificially high concentration of parent and not representative of the drug levels which were circulating at the time of sample collection!

Conclusions

In summary, an accurate and precise plasma microsampling UHPLC-MS/MS assay was successfully developed and employed to support several RTG clinical trials. This method was designed to stabilize the N-Glu metabolites, and minimize their degradation during sample handling, manipulation, and bioanalysis. The results from assay validation revealed the method to be rugged, accurate, precise and suitable for accurate quantitative measurements of RTG and NAMR in the presence of high circulating concentrations of labile N-Glu metabolites. In the end, LLE on the plasma samples was used to remove the majority of the N-Glu metabolites, and the LC method was optimized to prevent N-Glu degradation of the remaining metabolites (autosampler temperature at 4°C, neutral reconstitution solution and mobile phase). The method employs just 10 µL plasma which allows its application to paediatric studies, however requires sample storage at -80°C and rapid bioanalysis of study samples.

Due to the overlap in exposure between dosages and the lack of a direct pharmacokinetic/pharmacodynamic correlation between drug levels and seizure frequency, GSK **does not** recommend determination of drug levels. However, if drug levels are determined the appropriate precautions and methods must be applied. If they are not followed and artificially high drug levels are reported, there is the risk that the physician may reduce the dose resulting in loss of seizure control for the patient. *Due to the unique and complex metabolic profile of RTG, this manuscript stresses the importance of understanding the metabolic profile of the analyte of interest and the role of incurred samples in method development and validation.*

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Ethical conduct of research

This study was funded by GlaxoSmithkline (GSK). Prior to submission, the manuscript underwent institutional review and approval. The study was conducted according to the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved. All authors are current employees and shareholders of GSK.

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Table 1: Bias, precision and mean validation sample concentrations for RTG and NAMR in human plasma

Nominal Concentration	RTG					NAMR				
	5	**15**	**200**	**2000**	**2500**	**5**	**15**	**200**	**2000**	**2500**
RUN 1										
Mean	5.08	15.28	201.29	1956.81	2443.02	4.87	15.36	204.34	2069.06	2590.67
Precision (%CV)	4.2	2.1	1.6	2.6	2.0	5.6	4.9	0.9	1.3	1.8
Bias %	1.5	1.8	0.6	-2.2	-2.3	-2.5	2.4	2.2	3.5	3.6
RUN 2										
Mean	4.74	14.98	206.80	2006.73	2400.85	4.90	15.01	203.39	2031.06	2523.55
Precision (%CV)	1.3	2.7	0.9	1.8	2.0	4.5	3.8	1.5	0.4	1.2
Bias %	-5.2	-0.1	3.4	0.3	-4.0	-2.0	0.1	1.7	1.6	0.9
RUN 3										
Mean	4.87	15.40	204.96	1986.78	2449.44	4.66	14.94	202.99	2069.76	2608.91
Precision (%CV)	3.3	2.9	1.9	1.3	2.1	6.1	4.6	1.4	0.9	1.8
Bias %	-2.5	2.6	2.5	-0.7	-2.0	-6.8	-0.4	1.5	3.5	4.4
Overall Statistics										
Mean	4.90	15.22	204.35	1983.44	2431.10	4.81	15.10	203.57	2056.62	2574.38
Bias(%)	-2.0	1.5	2.2	-0.8	-2.8	-3.8	0.7	1.8	2.8	3.0
Between-run precision (%)	3.2	0.9	1.2	1.0	0.7	1.7	Negligible		1.0	1.6

Legends for Figures

Figure 1. **In vivo Metabolic Pathway of RTG catalized by N-Acetyl Transferases and UGTs.**

Figure 2. **Stability of N-Glu expressed as the area ratio N-Glu/RTG in human plasma during storage at 4°C (■), ambient temperature (▲), and 37°C (▼).**

Figure 3. **Stability of N-Glu expressed as the area ratio N-Glu/RTG in whole human blood during storage at 4°C (●), ambient temperature (■), and 37°C (▼).**

Figure 4. **Increase in RTG as a result of the degradation of N-Glu in acetonitrile/10 mM ammonium formate pH 3 (●), acetonitrile/10 mM ammonium acetate pH 4 (■), acetonitrile/10 mM ammonium acetate native pH (▲) acetonitrile/10 mM ammonium bicarbonate pH 7.9 (▼), acetonitrile/10 mM ammonium bicarbonate pH 9 (♦) in the autosampler at 4°C.**

Figure 5. **On column N-Glu Degradation of a Pooled Plasma Sample after PPT (A and B) or Liquid-Liquid Extraction (LLE; C) under Acidic and at Neutral pH and Ambient Conditions (D). The arrow in panel B highlights the on-column conversion from the N-Glu metabolites to RTG (shown as peak fronting); however the arrow in panels C and D indicate the lack of this conversion under these specified conditions.**

Figure 6. **Chromatographic peak splitting for NAMR the internal standard (NAMR-IS) when using LLE solvents such as MTBE and ethyl acetate.**

Figure 7. **Increase of RTG and NAMR concentrations due to N-Glu conversion at -20°C and -80°C.**

Figure 8. **ISR summary results for determination of RTG and its metabolite NAMR from a clinical study reanalyzed 14 days after original analysis.**

Figure 9. **Chromatograms of RTG from blank human plasma (A), plasma spiked with 5 ng/mL (B), 2500 ng/mL (C) and the internal standard (D).**

Figure 10. **Chromatograms for NAMR from blank human plasma (A), plasma spiked with 5 ng/mL (B), 2500 ng/mL (C) and the internal standard (D).**

Figure 11. **Investigation on the stability of RTG and NAMR in human urine to determine the most appropriate procedure for urine collection, storage and stabilization. Different colors represent different subjects.**

Figure 1. In vivo Metabolic Pathway of RTG catalized by N-Acetyl Transferases and UGTs.

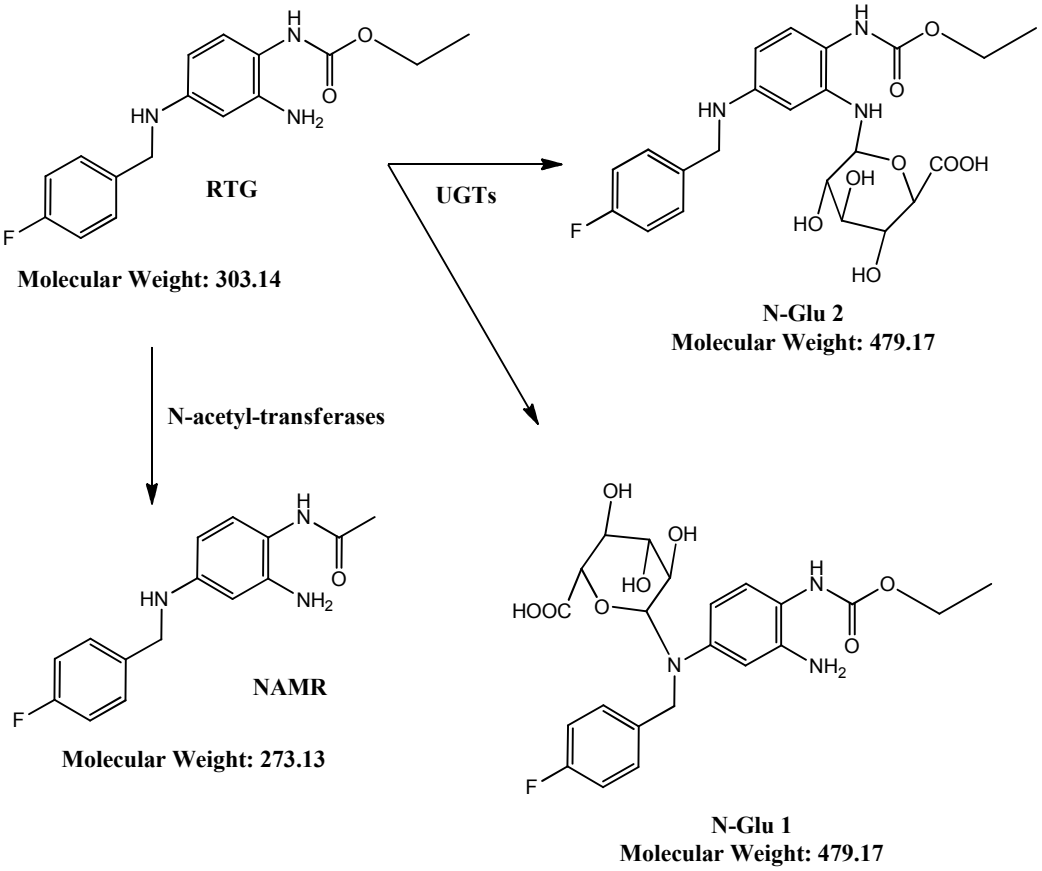
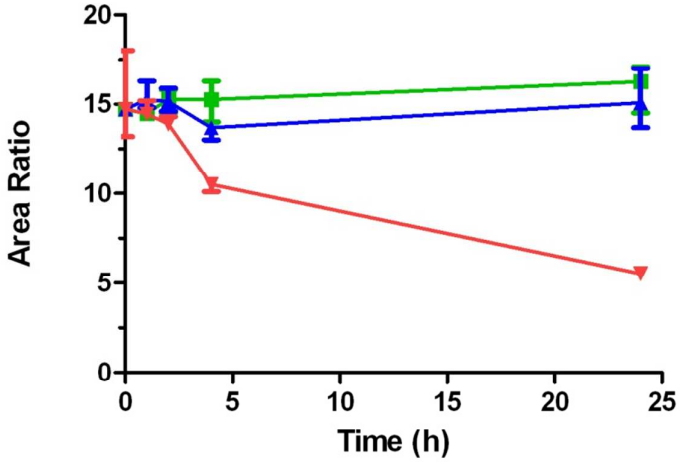


Figure 2. Stability of N-Glu expressed as the area ratio N-Glu/RTG in human plasma during storage at 4°C (■), ambient temperature (▲), and 37°C (▼).



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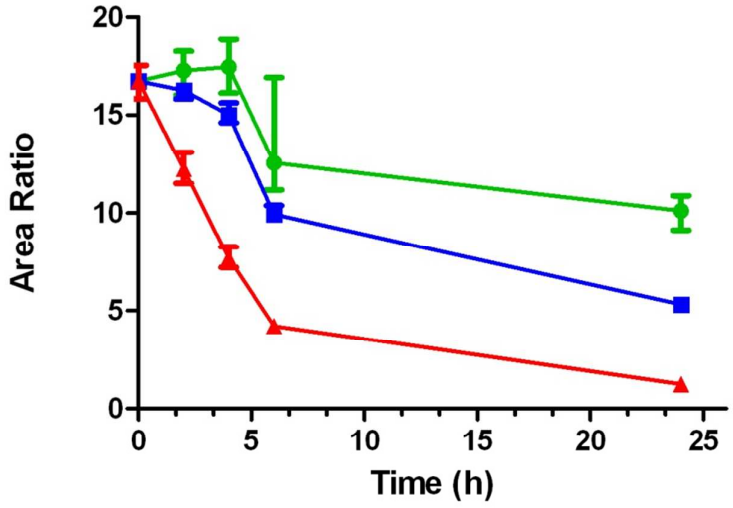

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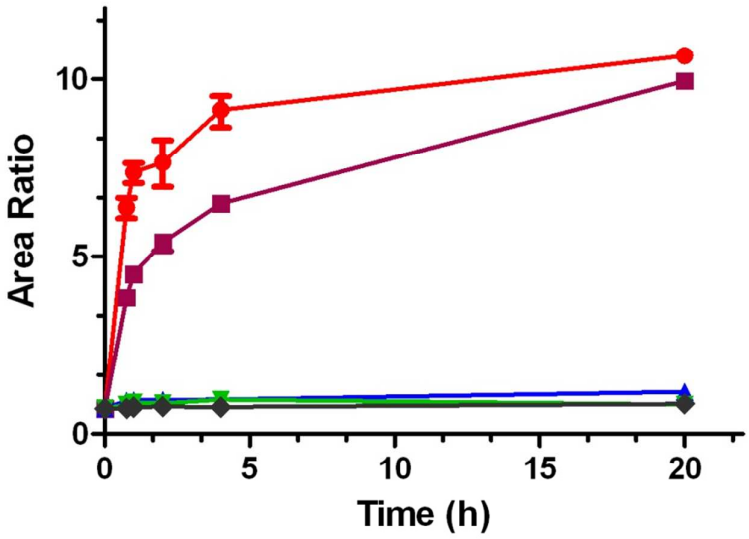

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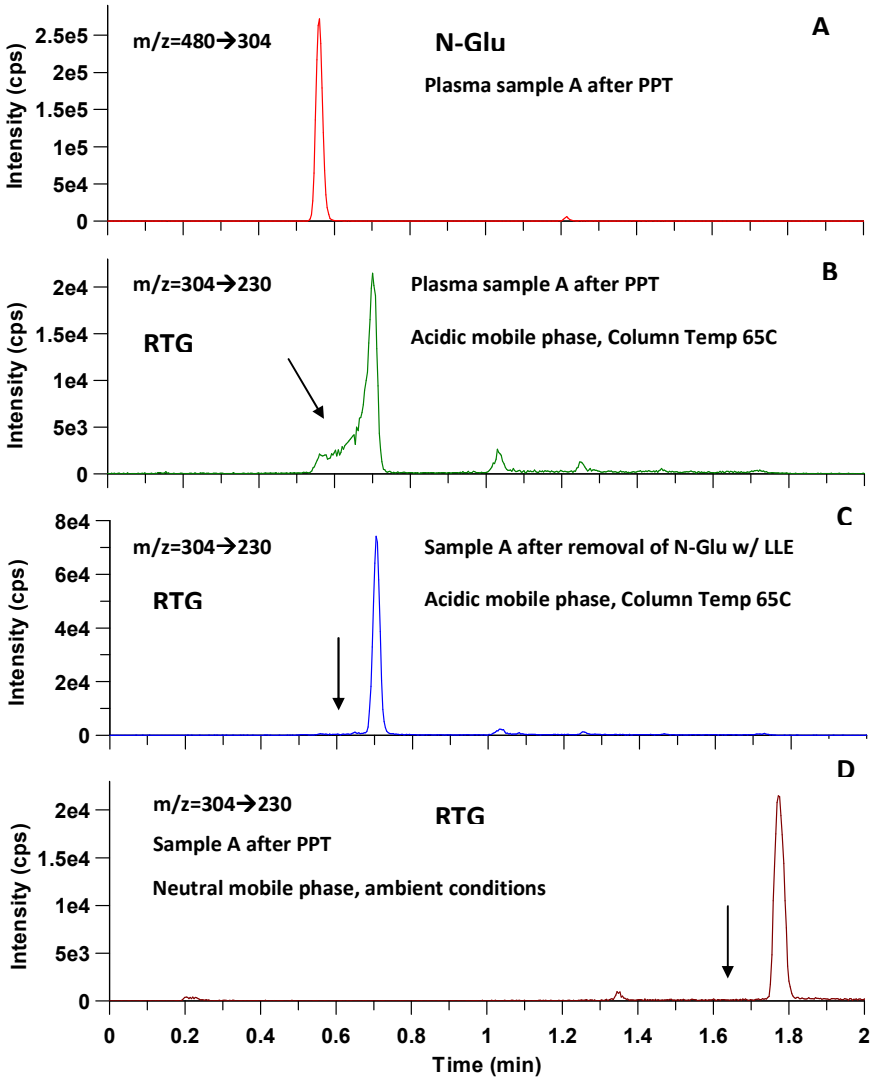

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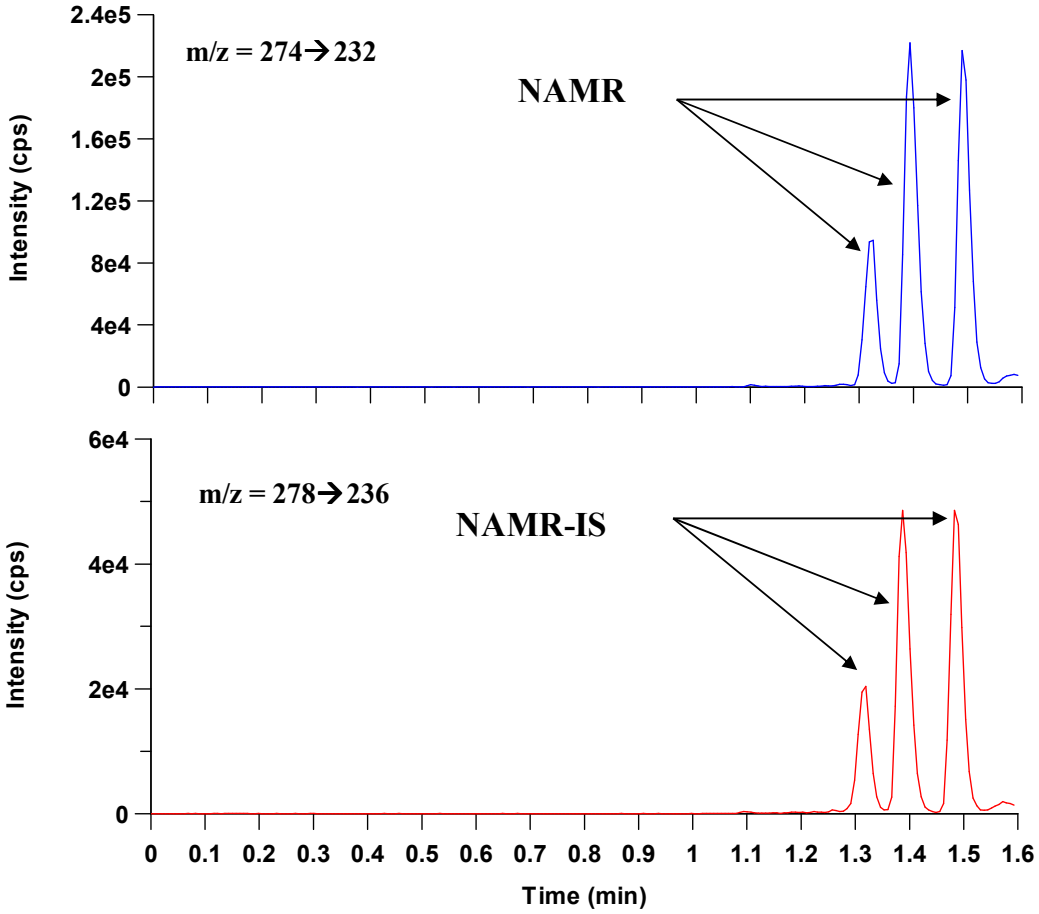

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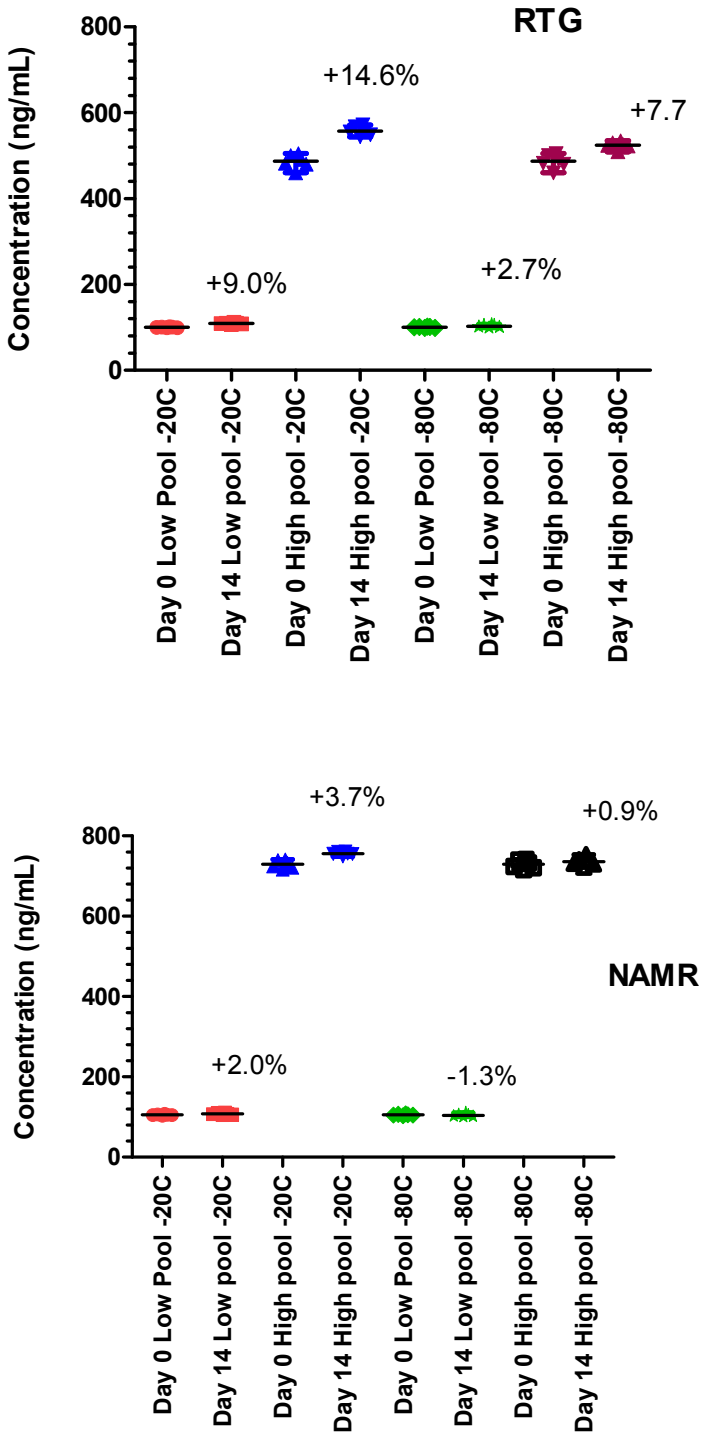

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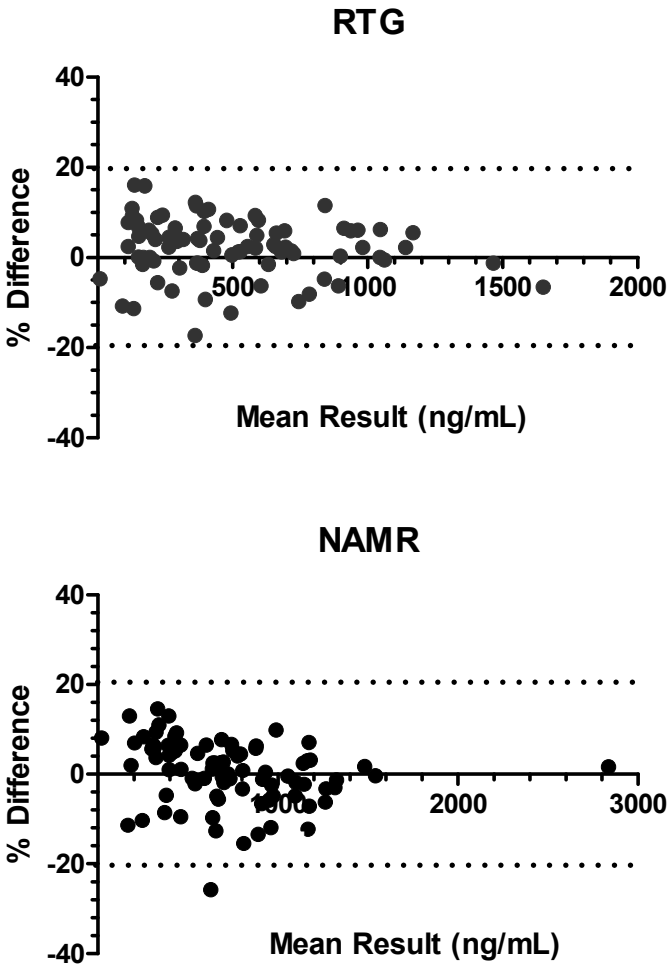

Figure 9: Chromatograms of RTG from blank human plasma (A), plasma spiked with 5 ng/mL (B), 2500 ng/mL (C) and the internal standard (D).

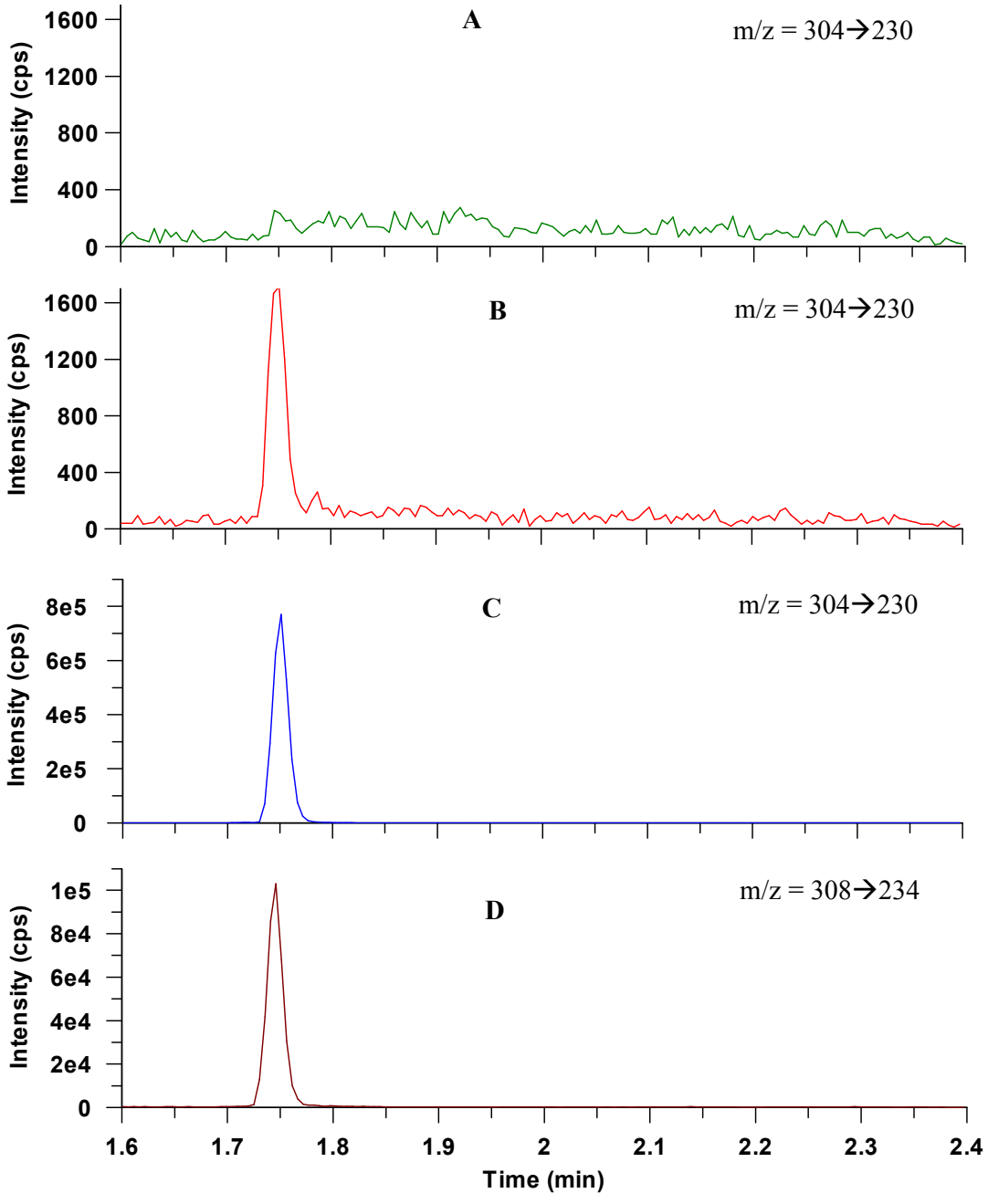

Figure 10: Chromatograms for NAMR from blank human plasma (A), plasma spiked with 5 ng/mL (B), 2500 ng/mL (C) and the internal standard (D).

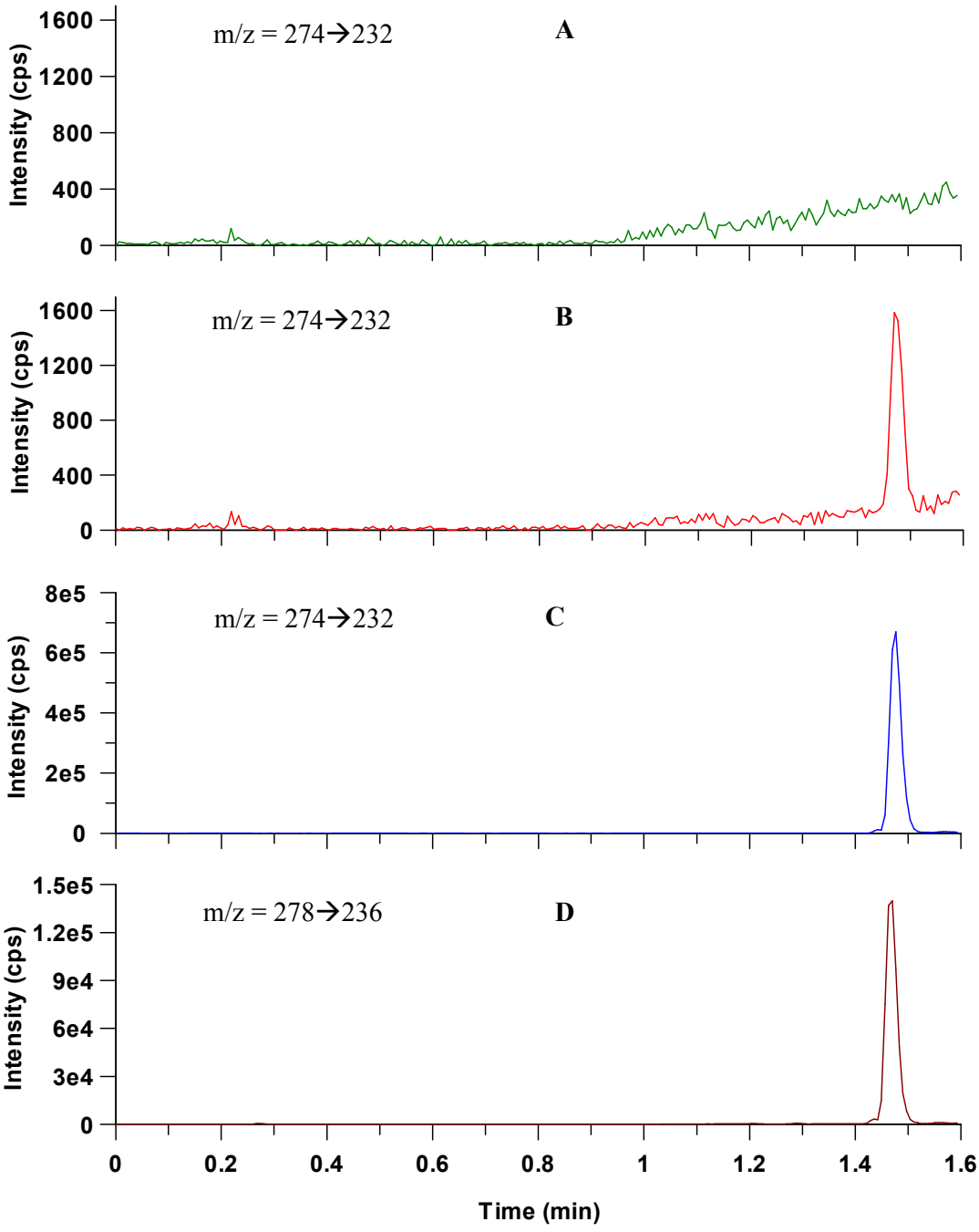

Fig 11. Investigation on the stability of RTG and NAMR in human urine to determine the most appropriate procedure for urine collection, storage and stabilization. Different colors represent different subjects.