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# Development of a method for the determination of glycine in human cerebrospinal fluid using pre-column derivatization and LC–MS/MS

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ABSTRACT

An LC–MS/MS method using pre-column derivatization with phenylisothiocyanate (PITC) was developed to quantify glycine in human cerebrospinal fluid (CSF) and applied to the determination of glycine in human samples collected during clinical testing. The calibration curve range for the assay was 50–10,000 ng/mL and $^{13}C_2{}^{15}N$-glycine was used as an internal standard. Artificial CSF was used as a surrogate matrix for standards due to the presence of endogenous glycine in human CSF and this approach was validated with additional experiments involving either standard addition, or stable labeled glycine as an alternate calibration standard for endogenous glycine. Interday bias (% RE) and precision (% CV) were −4.2 and 12.3% at the LLOQ, and less than ±0.9 and 8.3% for higher concentrations, respectively. Glycine was stable in artificial CSF for at least 5 h at room temperature, 55 days at −70 °C (−60 to −80 °C range), and through three freeze–thaw cycles.



## 1. Introduction

Cerebrospinal fluid (CSF) is one of the most frequently characterized body fluids for the study of mental and neurological disorders. This reflects the expectation that the contents of CSF may be altered by changes that occur in the central nervous system due to disease processes. Because of these relationships, many methods for the analysis of CSF have focused on the characterization of disease biomarkers as a means of developing a better understanding of neurological disorders. These methods have ranged from the determination of individual biomarkers such as β-amyloid$_{1-42}$, tau protein [1], and visinin-like protein 1 (VLP-1) [2], to broader approaches involving the use of proteomic tools. The latter provide a comprehensive picture of the protein content of CSF, often through the use of electrophoretic gels [3,4].

One of the specific biomarker approaches that has shown some utility for the understanding of the disease processes involved in psychiatric disorders is the analysis of amino acids in CSF [5]. The determination of glycine has shown particular promise in this regard. Glycine is known to bind to the glycine-binding site of the NMDA (N-methyl-D-aspartate) receptor complex where it acts as a coagonist and positive modulator. Antagonists of glycine such as the street drug PCP (phencyclidine) that block the NMDA receptor, can induce schizophrenia-like symptoms [6], and so glycine has been investigated both as a biomarker for schizophrenia and as a potential pharmacologic agent for its treatment [7].

To understand the role of glycine as either a biomarker of disease or pharmacological intervention requires an accurate and precise method for its quantification in human CSF. Various methods for measuring glycine and other amino acids have been reported. These methods have usually required either pre-column or post-column derivatization, followed by the detection of the derivatized analyte using LC-UV, LC-fluorescence, or LC–MS/MS. Pre-column derivatizations are typically accomplished using reagents such as dansyl chloride [8], phenylisothiocyanate (PITC) [9–12], dabsyl chloride [13], O-phthalaldehyde (OPA) [14,15], 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA, Marfey's reagent) [16,17], and fluorenylmethyl chloroformate (FMOC) [18–20]. Post-column derivatizations have been accomplished using ninhydrin [21] or OPA [22] often using commercially available amino acid analyzers [23]. Fonteh et al. recently developed an LC–MS/MS compatible derivatization method for the simultaneous measurement of 75 free amino acids and dipeptides by making use of an EZ-Faast® amino acid analysis kit from Phenomenex [24].

The requirement for the method described here was that it be able to accurately quantify glycine in human CSF during clinical testing of a pharmacological treatment for schizophrenia. The method makes use of Edman's reagent (phenylisothiocyanate [PITC]) for the derivatization. The derivatization of glycine using PITC is shown in Fig. 1. Introduced by Edman in the 1950s, this method is attractive because the derivatization procedure can be

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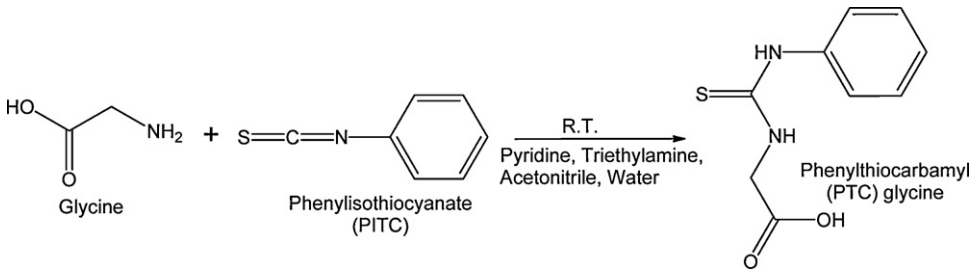


**Fig. 1.** Derivatization of glycine using phenylisothiocyanate (PITC) to form the phenylthiocarbamyl glycine (PTC-glycine).

performed at room temperature and is readily adaptable to a modern 96-well, LC–MS/MS based bioanalytical platform. The resulting phenylthiocarbamyl derivative (PTC-glycine) can be readily separated and quantified using standard reverse phase LC–MS/MS methodology. Because the method was intended for the determination of glycine in human CSF samples following administration of a drug expected to modulate glycine concentration, it was validated using a "fit for purpose" approach to meet most of the FDA criteria for bioanalytical methods validation [25]. During the course of method development, optimization and validation, additional experiments were also performed. These included the use of quadrupole time of flight mass spectrometry (Q-TOF-MS) for structural verification of the glycine derivatization product and also an interference peak present in biological samples. Also, because the method makes use of surrogate matrix, a strategy which has been successfully applied on many past occasions [26–28], experiments were conducted to verify the validity of this approach including standard addition and substitution of stable-labeled glycine as an alternate calibrant.

## 2. Experimental

### 2.1. Materials and reagents

Glycine (ReagentPlus®, ≥99%) was obtained from Sigma Aldrich (St. Louis, MO, USA). $^{13}C_2{}^{15}N$-glycine (≥98%) was obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Methanol, acetonitrile, isopropanol, and water (HPLC grade) were obtained from either J.T. Baker (Phillipsburg NJ, USA) or Sigma Aldrich (St. Louis, MO, USA). Formic acid (A.C.S. reagent grade) and ammonium formate (HPLC Grade) were obtained from Sigma Aldrich, Inc. (St. Louis, MO, USA). Acetic acid (USP Grade) and magnesium chloride (USP Grade) were supplied by J.T. Baker (Phillipsburg, NJ, USA). Sodium chloride (USP Grade), potassium chloride (A.C.S. Grade), calcium chloride (USP. Grade), sodium phosphate dibasic heptahydrate (A.C.S. Grade), and sodium phosphate monobasic monohydrate (USP Grade) were supplied by Mallinckrodt (Hazelwood, MO). Triethylamine (99%) was obtained from Riedel-de Haen (Seelze, Germany). DriSolv® Pyridine (>99.9%) was obtained from EMD Chemicals Inc. (Gibbstown, NJ, USA). Phenylisothiocyanate was obtained from Pierce Chemical (Rockford, IL, USA). Human cerebrospinal fluid used for measurement of glycine in healthy subjects was obtained from Bioreclamation Inc. (East Meadow, NY, USA) and PrecisionMed Human Biological Samples (San Diego, CA, USA) via lumbar puncture. CSF donor subjects were categorized as healthy based on self-reported information provided on a health certification questionnaire, and the findings from a physical examination. All patients gave consent for samples to be used for research purposes.

Artificial cerebrospinal fluid was prepared according to a procedure from DURECT Corporation (Cupertino, CA) [29], by dissolving 17.32 g sodium chloride, 0.448 g potassium chloride, 0.412 g calcium chloride, and 0.326 g magnesium chloride in 1 L of water (solution A). Solution B was prepared by dissolving 0.428 g sodium phosphate dibasic heptahydrate and 0.054 g sodium phosphate monobasic monohydrate in 1 L water Solutions A and B were then mixed in equal proportions. The PITC "coupling solution" was prepared according to the protocol detailed in the package insert from Pierce Chemical [30], by mixing 20-mL acetonitrile, 10-mL pyridine, 4-mL triethylamine, and 6-mL water in a glass storage container and stored at room temperature prior to use.

### 2.2. Instrumentation

The LC–MS/MS system consisted of a Shimadzu system SCL-10A vp controller, two Shimadzu LC20AD pumps (Columbia, MO, USA), and an API-5000 triple quadrupole mass spectrometer with a TurboIonSpray® interface (Applied Biosystems PE Sciex, Foster City, CA, USA). Data were collected and processed using Analyst® software (version 1.4.1; Applied Biosystems). Samples were introduced into the LC–MS/MS system with a Leap CTC PAL Autosampler (Carrboro, NC, USA).

### 2.3. Chromatographic conditions

Extracted samples were separated by reversed-phase liquid chromatography on a Waters (Milford, MA, USA) Atlantis C18, 3.9 mm × 150 mm (3 μm) column. The mobile phases were 10 mM ammonium formate in water containing 0.00175% formic acid (mobile phase A) and 10 mM ammonium formate in methanol containing 0.00175% formic acid (mobile phase B). A gradient at a flow rate of 300 μL/min was used such that mobile phase B was maintained at 20% for the first 0.3 min, then ramped to 95% over 2.7 min and maintained at 95% for 4.0 min, followed by a reduction to 20% over 2.0 min and maintained at 20% for 1.0 min for column re-equilibration.

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

Two primary stock solutions (1 mg/mL) of glycine reference standard were separately prepared in 50/50 (v/v) methanol/water, and stored at room temperature. One 1 mg/mL stock solution was used to prepare spiking calibration standards and the other 1 mg/mL stock solution was used to prepare spiking stock solutions for quality control QC preparation. Glycine quality control (QC) stock solutions were prepared at concentrations of 5000, 20,000, and 100,000 ng/mL in 50/50 (v/v) methanol/water. Glycine calibration standards were prepared at concentrations of 50, 100, 250, 500, 1000, 2000, 5000, and 10,000 ng/mL in 50/50 (v/v) methanol/water. Lower limit of quantitation (LLOQ), low, middle, and high quality control (QC) samples containing 50, 150, 1500, and 8500 ng/mL glycine were prepared by diluting the appropriate volume of QC spiking stock solution to 5 mL with artificial CSF. Following prepa-

ration, 0.1-mL aliquots of quality control samples were transferred to separate 1.8-mL cryovials (NUNC No. 368632), capped and stored at −70 °C (−60 to −80 °C range). These quality control samples were used for validation of the method and analysis of human CSF samples. $^{13}C_2{}^{15}N$-glycine working internal standard was prepared at a concentration of 250 ng/mL in 50/50 (v/v) methanol/water, and stored at room temperature.

### 2.5. MS/MS detection

Derivatized glycine and derivatized internal standard ($^{13}C_2{}^{15}N$-glycine) were dissolved and diluted in 50/50 (v/v) methanol/water. The compounds were separately infused via a connection tee into the mobile phase flow of an API-5000 mass spectrometer using a syringe pump (Harvard Apparatus, Holliston, MA, USA). The mobile phase was set at a composition of 50/50 mobile phase A and B pumping at flow rate of 300 μL/min. Precursor ions for glycine and $^{13}C_2{}^{15}N$-glycine were identified in mass spectra using the TurboIonSpray® source operated in the positive ionization mode. The Quantitative Optimization Component of Analyst® (Applied Biosystems) was used for automatic optimization of the MS and MS/MS parameters. The ESI spray voltage was +5000 V, the source temperature was 425 °C, the curtain, collision, nebulizer, and auxiliary gas settings were 50, 10, 50 and 50, respectively (all unitless settings). The ion transitions selected for MS/MS detection were $m/z$ 211.1 → 136.1 and $m/z$ 214.1 → 136.1 for derivatized glycine and derivatized $^{13}C_2{}^{15}N$-glycine (IS), respectively. Declustering potential (DP), collision energy (CE), and collision cell exit (CXP) potentials were 51, 25 and 20 V, respectively. Q1 mass spectra for derivatized glycine and $^{13}C_2{}^{15}N$-glycine are shown in Fig. 2.

### 2.6. Structural confirmation studies using UPLC and Q-TOF-MS

High resolution mass spectrometry was used to confirm the structure of derivatized glycine and a potential chromatographic interference peak (see Section 3.1) using an acquity ultrapressure liquid chromatograph (UPLC) coupled to a premier quadrupole time-of-flight mass spectrometer (Q-TOF-MS), both from Waters Corporation (Milford, MA, USA). UPLC separations were performed using a 1.8 μm 2.1 × 100 mm HSS T3 column with a gradient elution at a flow rate of 400 μL/min. Solvents A and B consisted of $H_2O$ and acetonitrile, respectively, both containing 0.1% formic acid. The following gradient program was applied: 0–2 min, 5% B; 2–8 min, 5% B to 45% B; 8–9 min, 45% B to 95% B; 9–10.5 min, 95% B; 10.5–11 min, 95% B to 5% B; 11–13 min, 5% B. High resolution MS data were collected on the Q-TOF-MS in the positive ion, V mode using nitrogen and argon as API and collision gas, respectively. Leucine enkephalin ($[M+1]^+ = 556.2771$) was used for the lock mass. Two functions were employed for the detection: $m/z$ 100–900 full scan and MS/MS of $m/z$ 211.055 using a collision energy ramp 15–35 V. All data were processed with MassLynx V4.1 (Waters Corporation).

### 2.7. Preparation and derivatization of standards, QC's and samples

CSF samples were thawed at room temperature and then vortex mixed. For preparation of standards, 20 μL of working standard solution (Section 2.4) was mixed with 20 μL of artificial CSF in separate 1.2-mL costar tubes (Corning, NY, USA). For preparation of CSF samples, QC's and blank artificial CSF, 20 μL each of CSF, QC's and blank artificial CSF were mixed with 20 μL 50/50 (v/v) methanol/water in separate 1.2-mL costar tubes. A 40 μL aliquot of 250 ng/mL working internal standard solution (Section 2.4) was added to each of the tubes containing the samples, standards, quality controls and blank artificial CSF and the tubes were vortex mixed.

The PITC derivatization was based on the manufacturer's recommended protocol [30] and adapted to the 96-well format. All tubes were dried down under heated nitrogen at 30 °C (approximately 30 min; first dry down step). To each tube, 100 μL of coupling solution (Section 2.1) was added and vortex mixed for approximately 1 min. All tubes were then dried down under heated nitrogen at 30 °C (approximately 20 min; second dry down step). An additional 100 μL of coupling solution was added to each tube and vortex mixed for approximately 1 min and then 10 μL of PITC was added and vortex mixed for approximately 5 s. The derivatization reactions were left to proceed at room temperature for 30 min and then the tubes were vortex mixed for 5 s and dried down under heated nitrogen at 30 °C (approximately 60 min; third dry down step). Dried samples, standards, quality controls, and blank artificial CSF were reconstituted in 200 μL of 1:2 mobile phase A/mobile phase B and vortex mixed for approximately 1 min. The sample was then diluted a second time by taking 40 μL of this reconstituted solution and adding it to a 96-well autosampler plate to which 160 μL of 1:2 mobile phase A/mobile phase B was added and vortex mixed. This second dilution was necessary to prevent MS/MS saturation at the highest concentrations of the standard curve. The 96-well autosampler plate was capped with a silicone cover (Varian, Lake Forest, CA, USA) and transferred to an autosampler tray for injection (10 μL) onto the LC–MS/MS system.

### 2.8. Method validation

#### 2.8.1. Lower limit of quantitation (LLOQ) and calibration curves

Calibrants were analyzed in duplicate at the beginning and end of three separately prepared analytical runs. Calibration curves were derived from the peak area ratios (glycine/internal standard) using $1/x^2$ weighted linear least-squares regression of the area ratio versus the nominal concentration of the corresponding standard. The regression equation from the calibration standards was used to back calculate the measured concentration for each standard, quality control, and patient sample.

#### 2.8.2. Accuracy and precision

Three analytical runs on separate days were used to evaluate accuracy and precision. In each analytical run, QC samples at concentrations of 50 (LLOQ), 150, 1500, and 8500 ng/mL were analyzed ($n = 6$). The high QC (8500 ng/mL) was also diluted 2- and 5-fold with artificial CSF for use as a dilution QC to verify the validity of diluting CSF samples. The assay intra- and interday accuracy (% RE) and precision (% CV) were calculated from the measured QC concentrations.

#### 2.8.3. Standard addition experiments

Two standard addition experiments were used to evaluate the use of artificial CSF as a surrogate matrix. The endogenous glycine concentration of a single lot of human CSF (Bioreclamation Inc., East Meadow, NY, USA) was determined by analyzing six replicate samples using the method described above, and this lot was then used for two standard addition experiments. For the first experiment, 20 μL of the characterized human CSF was pipetted into each of six Costar tubes followed by the addition of 20 μL of a 500 ng/mL working calibration standard. The concentration of glycine in these replicate samples was then determined using the derivatization method (artificial CSF used for calibration standards and QCs). For the second experiment, 480 μL of the characterized human CSF was pipetted into an Eppendorf tube followed by the addition of 20 μL of a 10,000 ng/mL calibration standard and mixed. 20 μL aliquots were then pipetted into each of six costar tubes followed by 20 μL of 50/50 (v/v) methanol/water. The concentration of glycine in these

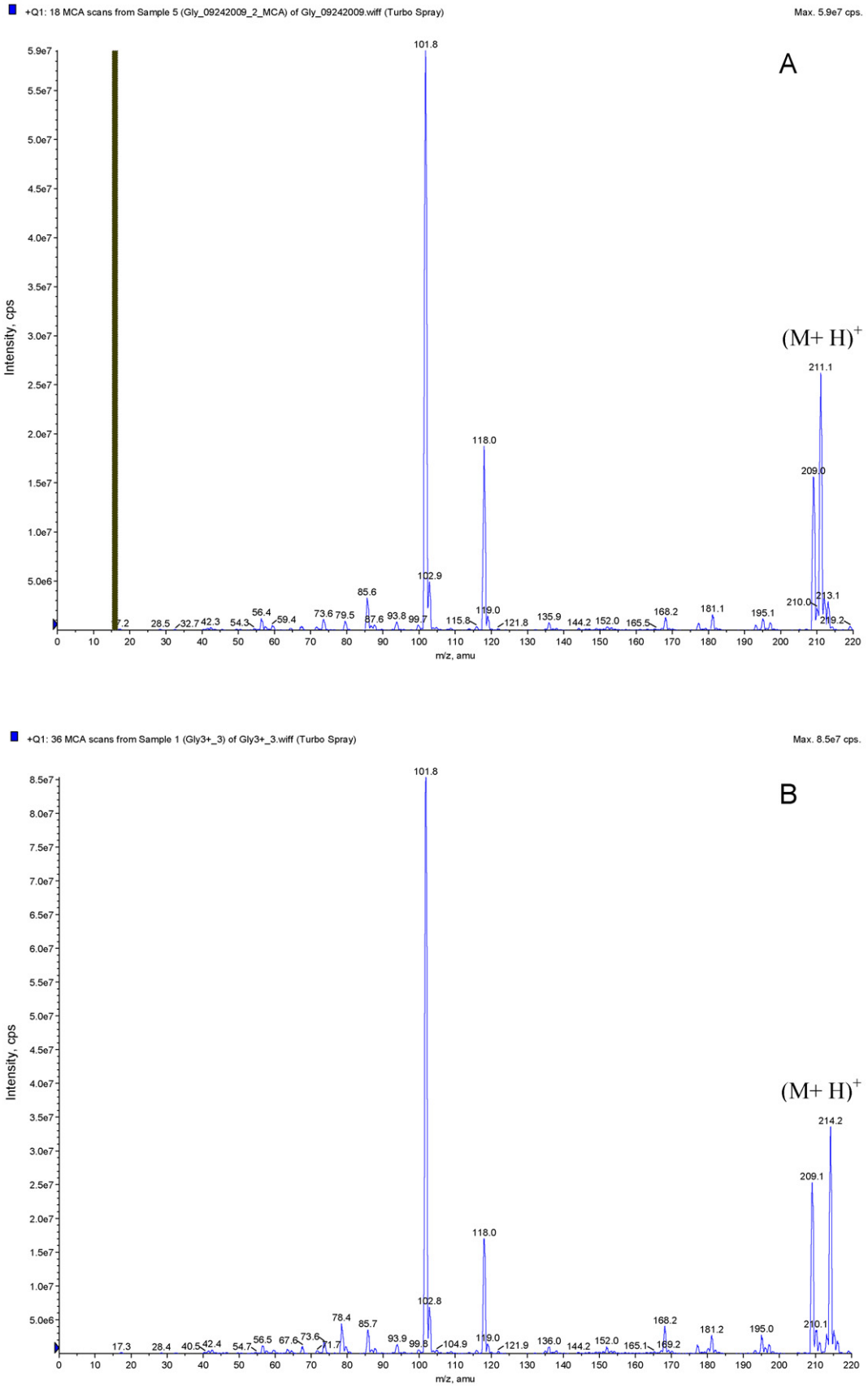


**Fig. 2.** Q1 ion spectra $(M+H)^+$ for: (A) phenylthiocarbamyl glycine ($m/z$ 211) and (B) phenylthiocarbamyl $^{13}C_2{}^{15}N$-glycine ($m/z$ 214).

replicate samples was then measured (artificial CSF used for calibration standards and QCs).

*2.8.4. Stability*

To evaluate freeze/thaw stability in the surrogate biomatrix, low and high ($n = 6$) QC samples at concentrations of 150 and 8500 ng/mL were subjected to three freeze–thaw cycles in which the samples were maintained at room temperature for at least 1 h and then at −70 °C (−60 to −80 °C range) for at least 12 h in each cycle. The samples were then analyzed using freshly made calibration standards. To evaluate bench top stability, quality control samples ($n = 6$ at low and high QC concentrations) were left on the bench top at room temperature for 5 h prior to extraction. To determine the stability of extracted samples stored in the autosampler tray, calibration standards ($n = 2$ at each concentration) and QC samples ($n = 6$ at each concentration) were extracted and analyzed. They were re-injected after storage in the autosampler tray at approximately 6 °C for 84 h. Long-term stability was evaluated using QC samples ($n = 6$ at low and high QC concentrations) stored in the freezer at −70 °C (−60 to −80 °C range) for 55 days prior to analysis. Additional sample stability was performed in human CSF on incurred samples after storing samples at −70 °C (−60 to −80 °C range) for 56 days (Section 3.5).

To evaluate the stability of the glycine stock solutions prepared in 50:50 MeOH/water, solutions stored at room temperature were compared to solutions made fresh on the day of analysis. Peak areas for derivatized glycine were compared.

## 3. Results and discussion

### 3.1. LLOQ and standard curves

The standard curves were validated over the concentration range of 50–10,000 ng/mL from three separately prepared analytical runs on different days. Although lower concentrations were easily detected, this curve range was chosen based on the predicted concentrations of glycine in human CSF samples. Average correlation coefficient ($r^2$) for the three curves was 0.99. The interday accuracy (% RE) and precision (% CV) for glycine calibration standards are presented in Table 1. The % RE of the mean back calculated concentrations of the standards from the theoretical concentrations were between −3.6 and 4.0%. The lower limit of quantitation (LLOQ), defined as the lowest concentration that could be measured with a precision better than 20% CV and accuracy within ±20% of the theoretical concentration, was 50 ng/mL. Example chromatograms of derivatized blank artificial CSF, the LLOQ standard, internal standard, human CSF are shown in Fig. 3. An interfering peak corresponding to the retention time of PTC-glycine was present in derivatized artificial CSF and it consistently represented approximately 30% of the LLOQ peak area in all blanks, standards, QC's and unknown samples. This interfering peak appeared to be an artifact of the derivatization procedure because it was also present in derivatized distilled water. Its presence was not observed to have a significant impact on the assay based on the fact that it was present in all samples at a constant level, and the assay demonstrated a consistent ability to accurately and precisely quantitate glycine in QC's at the LLOQ.

PTC-glycine carryover was also evaluated by analyzing blank artificial CSF following the ULOQ standard ($n = 5$). Carryover was calculated as any peak observed that was greater than the constant amount of interference peak observed. No significant carryover originating from the assay (≤5% of the mean LLOQ response) was observed. The small peaks that were observed in blank artificial CSF seemed to be solely originating from the derivatization procedure as discussed earlier. No increase in peak area was observed following injection of ULOQ samples.

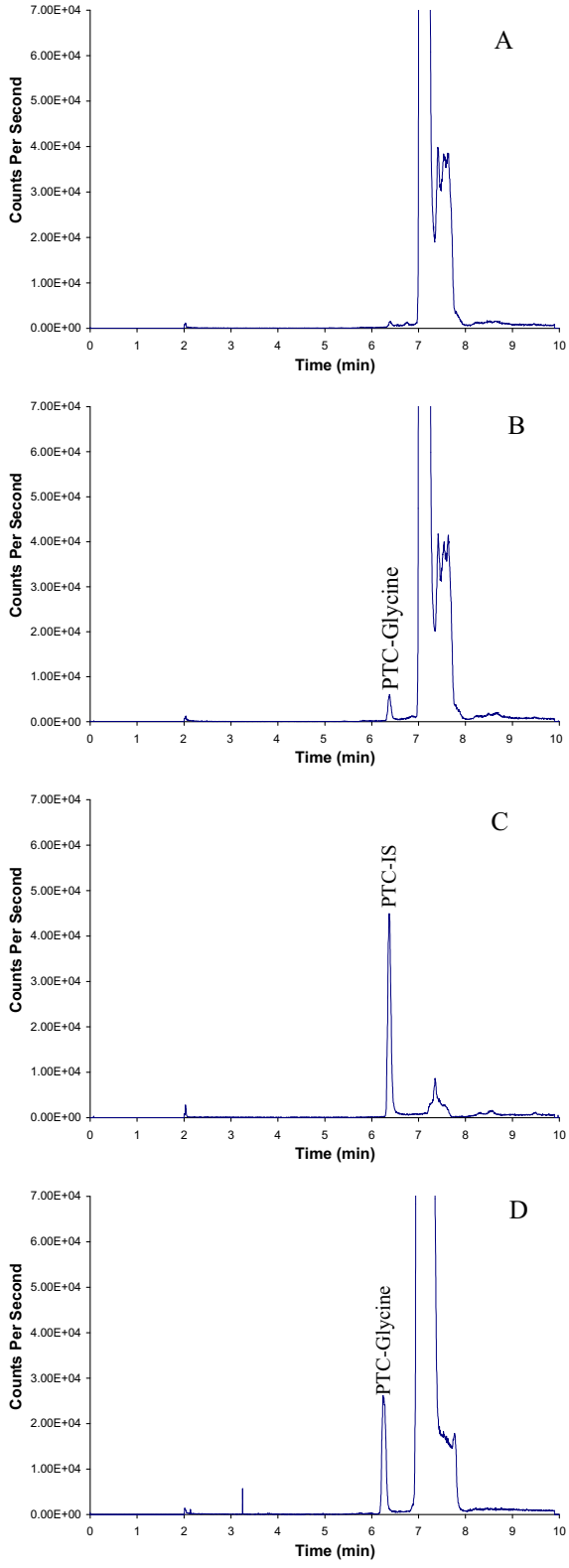


**Fig. 3.** LC–MS/MS chromatograms for PITC derivatized samples: (A) blank artificial CSF, (B) glycine in artificial CSF (50 ng/mL), (C) $^{13}C_2{}^{15}N$-glycine internal standard (250 ng/mL), (D) human CSF (glycine concentration = 365 ng/mL).

### 3.2. Characterizing the blank interference peak using UPLC and Q-TOF mass spectrometry

The concentration of the interfering peak observed in blank samples was too small to provide meaningful structural information

**Table 1**
Interday precision and accuracy of glycine calibration standards.

| Day | Statistic | Standard concentration (ng/mL) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 50 | 100 | 250 | 500 | 1000 | 2000 | 5000 | 10,000 |
| 1 | | 55.7 | 99.6 | 245 | 487 | 1030 | 1970 | 4840 | 10,200 |
| | | 46.0 | 95.4 | 248 | 475 | 1030 | 2040 | 5150 | 10,400 |
| 2 | | 47.0 | 98.0 | 241 | 476 | 1050 | 1940 | 4930 | 9930 |
| | | 53.3 | 104.0 | 244 | 488 | 1040 | 2090 | 4960 | 10,300 |
| 3 | | 49.7 | 103.0 | 244 | 478 | 1030 | 2000 | 4880 | 9720 |
| | | 51.2 | 96.5 | 240 | 489 | 1060 | 2090 | 5000 | 10,400 |
| Days 1–3 | Mean | 50.5 | 99.4 | 244 | 482 | 1040 | 2020 | 4960 | 10,200 |
| | % CV ($n=6$) | 7.3 | 3.5 | 1.2 | 1.3 | 1.2 | 3.1 | 2.2 | 2.7 |
| | % RE ($n=6$) | 1.0 | −0.6 | −2.4 | −3.6 | 4.0 | 1.0 | −0.8 | 2.0 |

using the API-5000, so the interference peak was concentrated and then characterized after developing an approximately equivalent chromatographic separation on a UPLC-Q-TOF mass spectrometer. A glycine-free solution consisting of 10 mL water/MeOH (1:1) was processed through the described derivatization procedure, concentrated to approximately 300 μL and then injected onto the UPLC. This was then compared to a glycine standard (10,000 ng/mL) derivatized and analyzed using the same method. Extracted ion chromatograms from both samples showed that the interfering peak had the same retention time as PTC-glycine and that the accurate mass and tandem mass spectra of the interfering peak were also identical to the corresponding full scan and MS/MS spectra from the derivatized glycine standard (Fig. 4). This provided evidence that the interference was probably due to PTC-glycine and not some other unknown co-eluting component. Additional experiments indicated that the interference peak was not detected as an impurity in any of the pre-derivatization components in the reaction including acetonitrile, pyridine, triethylamine, water, or phenylisothiocyanate, suggesting that PTC-glycine found in the blank samples was formed as a result of the derivatization procedure. The combined data supported the conclusion that the source of the PTC-glycine interference in blank samples was probably due to the derivatization of glycine present as a small contaminant in one or more of the reaction components or generally as an environmental contaminant. Switching to new reagents also did not seem to have a significant effect on the size of this contaminant peak.

### 3.3. Intraday and interday accuracy and precision from QC samples

Intraday and interday accuracy and precision results from QC samples are shown in Table 2. The intraday % CV was between 1.1 and 19.8%, and the interday % CV was between 2.0 and 12.3%. The interday mean accuracy (% RE) was between −0.9 and 8.1% including the LLOQ (50 ng/mL). The intraday % CV of the assay with respect to the 2-fold and 5-fold dilution QC's was 2% and the intraday % RE was between 4.7 and 8.1%, which indicated that a 2-fold and a 5-fold dilution would be appropriate for samples expected to exceed the 10,000 ng/mL upper limit of quantitation (ULOQ).

### 3.4. Validation of the use of artificial CSF by standard addition

Human CSF typically contains endogenous concentrations of glycine which would interfere with its use as a blank matrix in a bioanalytical assay. It is also comparatively difficult to obtain from humans in the volumes necessary for validation and conducting assays on a routine basis. Based on these limitations, the use of artificial CSF as the blank matrix was validated by determining the accuracy and precision of quantifying glycine spiked into human CSF in a standard addition experiment. Six replicates of a single lot of human CSF were assayed using the method described in this report to establish a baseline glycine concentration (678 ng/mL).

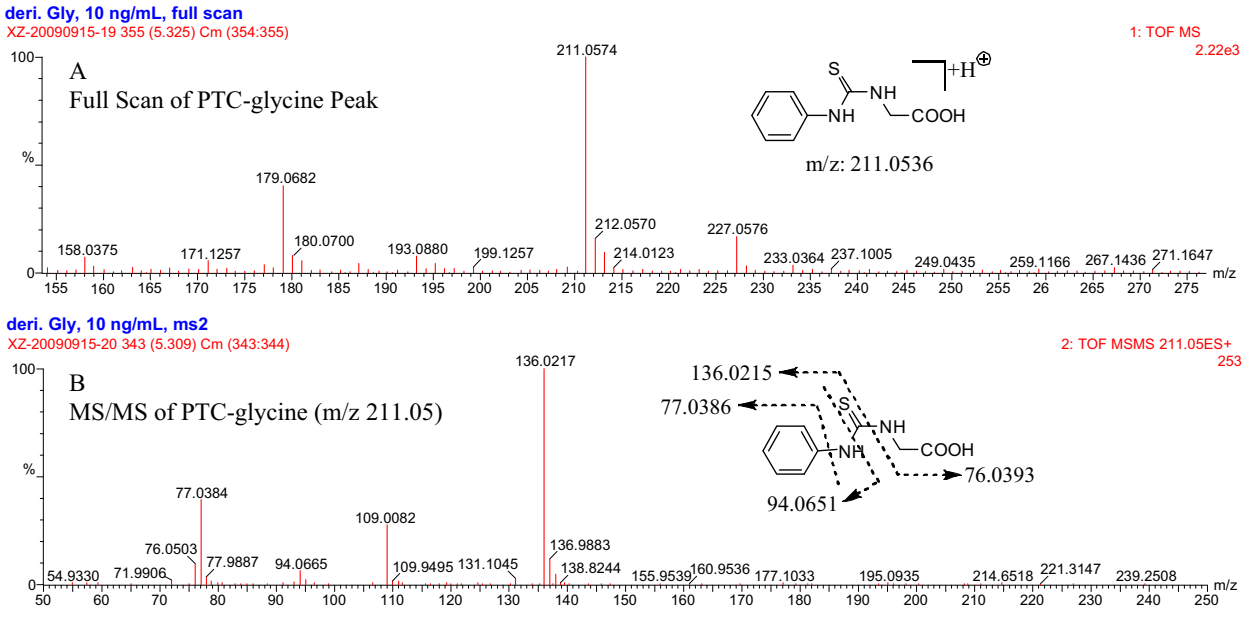


**Fig. 4.** Quadrupole time of flight mass spectra for the PTC-glycine peak: (A) full scan (B) MS/MS ($m/z$ 211.05).

**Table 2**
Intraday and interday accuracy and precision of glycine quality control samples.

| Day | Statistic | Quality control concentration (ng/mL) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 50 | 150 | 1500 | 8500 | 8500 (2-fold dil.) | 8500 (5-fold dil.) |
| 1 | Intraday mean ($n$ = 6) | 53.5 | 155 | 1460 | 8690 | 8900 | 9190 |
| | % CV | 19.8 | 2.3 | 10.1 | 1.1 | 2.0 | 2.0 |
| | % RE | 7.0 | 3.3 | −2.7 | 2.2 | 4.7 | 8.1 |
| 2 | Intraday mean ($n$ = 6) | 53.1 | 157 | 1640 | 8760 | | |
| | % CV | 6.4 | 3.4 | 1.5 | 4.2 | | |
| | % RE | 6.2 | 4.7 | 9.3 | 3.1 | | |
| 3 | Intraday mean ($n$ = 6) | 49.6 | 142 | 1450 | 7820 | | |
| | % CV | 3.7 | 3.7 | 3.3 | 5.8 | | |
| | % RE | −0.8 | −5.3 | −3.3 | −8.0 | | |
| | Interday mean | 52.1 | 151 | 1510 | 8420 | 8900 | 9190 |
| | % CV | 12.3 | 5.3 | 8.3 | 6.4 | 2.0 | 2.0 |
| | % RE | 4.2 | 0.7 | 0.7 | −0.9 | 4.7 | 8.1 |
| | $n$ | 18 | 18 | 18 | 18 | 6 | 6 |

**Table 3**
Standard addition of glycine into human cerebrospinal fluid.

| Sample | Measured conc. (ng/mL) | Average conc. (% CV) | Expected conc. | Bias (%)[a] | Average bias (%) |
|---|---|---|---|---|---|
| Human CSF lot 236314 | 654<br>675<br>668<br>687<br>664<br>722 | 678<br>(3.5) | N/A | N/A | N/A |
| Expt. 1[b] | 1240<br>1250<br>1230<br>1220<br>1230<br>1250 | 1237<br>(1.0) | 1178 | 5.0<br>5.8<br>4.2<br>3.4<br>4.2<br>5.8 | 4.7 |
| Expt. 2[c] | 1110<br>1150<br>1120<br>1210<br>1140<br>1130 | 1143<br>(3.1) | 1051 | 5.3<br>8.6<br>6.2<br>13.1<br>7.8<br>7.0 | 8.0 |

[a] (Measured conc. – predicted conc.)/actual conc.) * 100.
[b] 10 ng of glycine (20 μL of 500 ng/mL working calibration standard) was added to a 20 μL sample of Human CSF (Lot 236314). Sample was analyzed as a calibration standard. Expected concentration: 1178 ng/mL.
[c] 200 ng of glycine (20 μL 10,000 ng/mL working calibration standard) was added to 480 μL of Human CSF (Lot 236314). A 20 μL aliquot was analyzed as a sample. Expected concentration = 1051 ng/mL.

This characterized human CSF was then used for two subsequent spiking experiments. Additional glycine was added to this lot using two independent dilution procedures to result in nominal total mean glycine concentrations of either 1178 ng/mL or 1050 ng/mL. The samples were then analyzed using the method described in this report and the measured mean concentrations of glycine were 1237 ng/mL and 1143 ng/mL (4.7 and 8.0% bias of expected), respectively. The individual results of the spiking experiments are shown in Table 3. This low degree of bias supported the use of calibration curves generated in artificial CSF for the determination of glycine in human CSF.

### 3.5. Stability

Stability of glycine in artificial CSF samples during multiple freeze–thaw cycles, after incubation at room temperature, after autosampler storage, and after long-term storage at −70 °C (−60 to −80 °C range) is summarized in Table 4. Glycine appears to be stable

**Table 4**
Stability of glycine.

| Nominal conc. (ng/mL) | Room temperature for 5 h | | | −70 °C (−60 to −80 °C range) for 55 days | | |
|---|---|---|---|---|---|---|
| | Determined mean (ng/mL; $n$ = 6) | Mean RE (%, $n$ = 6) | CV (%, $n$ = 6) | Determined mean (ng/mL; $n$ = 6) | Mean RE (%, $n$ = 6) | CV (%, $n$ = 6) |
| 150 | 160 | 6.7 | 2.3 | 146 | −2.7 | 5.9 |
| 8500 | 8490 | −0.1 | 0.9 | 8980 | 5.6 | 1.4 |
| | Three freeze–thaw cycles | | | Autosampler storage (8–12 °C) for 84 h | | |
| | Determined mean (ng/mL; $n$ = 6) | Mean RE (%, $n$ = 6) | CV (%, $n$ = 6) | Determined mean (ng/mL; $n$ = 6) | Mean RE (%, $n$ = 6) | CV (%, $n$ = 6) |
| 150 | 160 | 6.7 | 2.2 | 154 | 2.7 | 4.5 |
| 8500 | 8470 | −0.04 | 2.2 | 8600 | 1.2 | 3.8 |

**Table 5**
Low and high glycine concentrations in human CSF following 56 days storage at −70 °C (−60 °C to −80 °C range).

| CSF sample conc. | Average conc. Day 1 (ng/mL) | Average conc. Day 56 (ng/mL) | Bias (%)[a] |
|---|---|---|---|
| Low | 325 | 323 | −0.6 |
| High | 1943 | 1883 | −3.1 |

[a] Bias (%) ((Avg. run 2 − Avg. run 1) * 100)/((Avg. run 2 + Avg. run 1) * 0.5).

in artificial CSF through three freeze–thaw cycles (% CV was 2.2% and accuracy within 7% of nominal) and was stable after storage on the bench-top at room temperature for 5 h prior to extraction (% CV was ≤2.3% and accuracy within 7% of nominal). The % CV was ≤4.5% and accuracy was within 2.7% of nominal after extracted glycine QC samples were stored in the autosampler tray at 6 °C for 84 h. Long-term stability was evaluated after glycine was stored at −70 °C (−60 to −80 °C range) for 55 days and analyzed against freshly prepared calibration curves. The % CV was ≤5.9% and accuracy within 5.6% of nominal. These data suggest that glycine was stable in artificial CSF for at least 55 days under this storage condition. A 1 mg/mL solution of glycine in 50:50 MeOH/water was shown to be stable for at least 6 days when stored at room temperature. The LLOQ (50 ng/mL) and ULOQ (10,000 ng/mL) in 50:50 MeOH/water is stable for at least 51 days at room temperature. The stability of glycine in human CSF at low and high concentrations of glycine was also evaluated. Glycine appears to be stable in human CSF for at least 56 days at −70 °C (−60 to −80 °C range). The results are summarized in Table 5.

### 3.6. Incurred sample reanalysis

It is currently a requirement by the FDA that bioanalytical methods used in support of clinical trials demonstrate incurred sample reproducibility (ISR) [31] and so an abbreviated ISR experiment was included as part of the validation activities performed for this assay. Ten lots of CSF were collected from non-dosed subjects and were then assayed on two separate days. The data are shown in Table 6. A bias of ≤10.3% on replicate samples analyzed on different days indicated the ability to reproducibly analyzed glycine from human CSF.

### 3.7. Alternate use of stable-labeled glycine for calibrants

One alternative considered for these experiments, which would have allowed human CSF to be used as the control matrix, was the use of stable-labeled glycine for the calibration standards. This is based on the nearly identical physical and chemical properties of labeled and unlabeled compounds, and the ability to mass spectrometrically distinguish between them. Experiments using this approach were conducted with human CSF as the control matrix, and $^{13}C_2$-glycine and $^{13}C_2{}^{15}N\,D_2$-glycine as calibration and internal standards, respectively. Glycine concentrations were determined in ten different lots of human CSF with this approach and then compared with those obtained from the same ten lots of CSF assayed using the validated assay (artificial CSF matrix, native glycine as the calibration standard, and $^{3}C_2{}^{15}N$-glycine as internal standard). Results of this comparison are shown in Table 7. Bias between the two approaches varied from −4.9 to +16.5%. These results demonstrated an additional method for calibration using genuine human CSF, as well as further confirming results obtained through the use of artificial CSF. Despite the feasibility of using stabled-labeled glycine as a calibrant, this approach was eventually not applied to the analysis of clinical samples as the use of artificial CSF was proven to be adequate.

**Table 6**
Incurred sample reanalysis.

| Human CSF lot # | Concentration (ng/mL) | | | |
|---|---|---|---|---|
| | Run 1 | Run 2 | Average | Bias (%) |
| 1 | 344 | 315 | 330 | −8.80 |
| 2 | 370 | 351 | 361 | −5.27 |
| 3 | 347 | 313 | 330 | −10.30 |
| 4 | 343 | 336 | 340 | −2.06 |
| 5 | 262 | 264 | 263 | 0.76 |
| 6 | 385 | 362 | 374 | −6.16 |
| 7 | 315 | 324 | 320 | 2.82 |
| 8 | 555 | 518 | 537 | −6.90 |
| 9 | 379 | 374 | 377 | −1.33 |
| 10 | 309 | 305 | 307 | −1.30 |

**Table 7**
Glycine concentrations determined from ten CSF samples using artificial versus human CSF.

| Human CSF lot | Avg. glycine conc. ($n$ = 2) Artificial CSF (ng/mL) | Glycine conc. Human CSF (ng/mL) | Difference (%) |
|---|---|---|---|
| 1 | 330 | 328 | −4.9 |
| 2 | 361 | 390 | 5.4 |
| 3 | 330 | 336 | −3.3 |
| 4 | 340 | 358 | 4.2 |
| 5 | 263 | 283 | 7.6 |
| 6 | 374 | 389 | 1.1 |
| 7 | 320 | 328 | 4.1 |
| 8 | 537 | 577 | 4.0 |
| 9 | 377 | 423 | 11.0 |
| 10 | 307 | 365 | 16.5 |

**Table 8**
Diurnal variation of glycine in human CSF.

| Subject | Sample time | Description | Conc. (ng/mL) |
|---|---|---|---|
| | 7:20 am | Not fasted | 359 |
| | 1:10 pm | Not fasted | 347 |
| 1 | 7:21 pm | Fasted | 320 |
| | 1:05 am | Fasted | 284 |
| | 6:55 am | Fasted | 314 |
| | 8:10 am | Not fasted | 351 |
| | 1:25 pm | Not fasted | 352 |
| 2 | 7:35 pm | Fasted | 317 |
| | 1:25 am | Fasted | 281 |
| | 7:25 am | Fasted | 301 |
| | 8:45 am | Not fasted | 488 |
| | 1:55 pm | Not fasted | 372 |
| 3 | 8:03 pm | Fasted | 315 |
| | 1:55 am | Fasted | 311 |
| | 7:50 am | Fasted | 298 |

### 3.8. Human sample analysis

The described method for the determination of glycine was successfully applied to various human CSF samples. In the experiment shown here, glycine concentrations were quantified in CSF samples obtained from three healthy human subjects throughout the day in order to determine natural variations due to sleep or fasting. Data are shown in Table 8 and indicate that glycine concentrations in CSF changed little throughout the day, with a slight increase after eating.

## 4. Conclusions

An LC–MS/MS method for the determination of glycine in human cerebrospinal fluid using pre-column derivatization has been developed and validated on a fit-for-purpose basis. The assay was demonstrated to be applicable to the determination of glycine over the range of 50–10,000 ng/mL with an upper limit of quantitation that can be extended to 50,000 ng/mL with sample dilution.

The method was applied successfully to the analysis of human CSF samples obtained from healthy human subjects.

## Conflict of interest statement

All co-authors were in the employment of Amgen Inc. during the duration of the research detailed in this report. No additional financial or personal conflicts of interest have been identified that could have inappropriately influenced the work submitted.

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