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Determination of glycine in biofluid by hydrophilic interaction chromatography coupled with tandem mass spectrometry and its application to the quantification of glycine released by embryonal carcinoma stem cells

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ABSTRACT

Because glycine plays a prominent role in living creatures, an accurate and precise quantitative analysis method for the compound is needed. Herein, a new approach to analyze glycine by hydrophilic interaction chromatography (HILIC) coupled with electrospray ionization tandem mass spectrometry (ESI-MS/MS) was developed. This method avoids the use of derivatization and/or ion-pairing reagents. N-methyl-D-aspartate (NMDA) is used as the internal standard (IS). The mobile phase for the isocratic elution consisted of 10 mM ammonium formate in acetonitrile–water (70:30, v/v, adjusted to pH 2.8 with formic acid), and a flow rate of 250 μL/min was used. Two microliters of sample was injected for analysis. The signal was monitored in the positive multiple reaction monitoring (MRM) mode. The total run time was 5 min. The dynamic range was 40–2000 ng/mL for glycine in the biological matrix. The LLOQ (lower limit of quantification) of this method was 40 ng/mL (80 pg on column). The validated method was applied to determine the dynamic release of glycine from P19 embryonal carcinoma stem cells (ECSCs). Glycine spontaneously released from the ECSCs into the intercellular space gradually increased from 331.02 ± 60.36 ng/mL at 2 min in the beginning to 963.52 ± 283.80 ng/mL at 60 min and 948.27 ± 235.09 ng/mL at 120 min, finally reaching a plateau, indicating that ECSCs consecutively release glycine until achieving equilibration between the release and the reuptake of the compound; on the contrary, the negative control NIH/3T3 embryonic fibroblast cells did not release glycine. This finding will help to improve our understanding of the novel effects of neurotransmitters, including glycine, on non-neural systems.

1. Introduction

Glycine is one of the fundamental amino acids. Mutations that lead to the replacement of glycine by other amino acids may result in the malfunction of some proteins, leading to diseases such as osteogenesis imperfecta and Ehlers–Danlos syndrome [1,2]. In addition to acting as an intermediate and building block of proteins in general metabolism, glycine is a major inhibitory neurotransmitter that targets glycine receptors and a co-agonist that activates the excitatory N-methyl-D-aspartate (NMDA) receptor, along with glutamate, the endogenous agonist of the receptor in the central nervous system [3]. Glycinergic neurons synthesize glycine mainly from serine using the enzyme serine hydroxymethyltransferase (SHMT) and then package it into presynaptic vesicles via the vesicular inhibitory amino acid transporter (VIAAT) before release into the synaptic cleft. By binding to the strychnine-sensitive receptor (GlyR), the released glycine opens the receptor-integrated chloride channel, causing an inhibitory postsynaptic potential. Glycine is also indispensable for the opening of the ion channel of the NMDA receptor, which contains a strychnine-insensitive glycine-binding site. Released glycine can be taken up into cells by its specific transporter (GLYT), terminating the glycinergic signal through the sequestration of glycine from the cleft. Recently, glycine signaling has been discovered in non-neural cells. By regulating cell proliferation, differentiation, migration and cytokine production, glycine modulates immune cells, endothelial cells and macroglial cells. In the case of renal cells, hepatocytes and endothelial cells, glycine protects them from ischemic cell death [4,5]. While investigating the components of the non-neural neurotransmitter system, our group found that undifferentiated P19 embryonal carcinoma stem cells (ECSCs) express SHMT, VIAAT and GLYT1 (unpublished data).

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The prominent role of glycine in living creatures warrants an accurate and precise quantitative analysis method for the compound. However, in contrast to the neurotransmitters glutamate and gamma-aminobutyric acid (GABA), there have been few reports to date on the quantitative determination of glycine in biological samples with sensitive and simple detection methods. The most commonly used methods to measure glycine in biological fluids are based on high-performance liquid chromatography with either electrochemical detection (HPLC-ECD) [6] or fluorescence detection (HPLC-FD) [7]. In these methods, derivatization of glycine is necessary due to its high polarity, low volatility and molecular structure that lacks either a chromophore or fluorophore. Derivatization also results in derivatized impurities, which complicate the analysis. Moreover, these detection methods lack specificity because analytes are identified by retention time. In comparison to the ECD and FD, mass spectrometry (MS) provides better sensitivity and specificity because analytes are identified by both retention time and molecular mass. Therefore, mass spectrometry coupled with capillary electrophoresis (CE) or gas chromatography (GC) has been employed in the quantification of glycine [8,9]. Tandem mass spectrometry (MS/MS) further improves specificity and sensitivity because the specific dissociation pathways of analytes are based on their molecular structure in MS/MS detection. Glycine is a polar molecule, leading to poor retention in traditional reversed-phase columns during the LC–MS/MS analysis. For this reason, ion-pairing reagents and derivatization reagents have often been used in the analysis of glycine by LC–MS/MS [10–12]. Ion-pairing reagents such as tridecafluoroheptanoic acid decrease the ionization efficiency of glycine in electrospray ionization (ESI) due to limited volatility and lead to compromised detection sensitivity. Derivatization reagents such as propyl chloroformate (PrCl) and 9-fluorenylmethyl chloroformate (FMOC) can increase the chromatographic separation and retention on traditional reversed-phase columns; however, complicated and time-consuming sample pretreatment procedures hinder their application in LC–MS/MS analysis.

Herein, a new approach to analyze glycine by hydrophilic interaction chromatography (HILIC) coupled with electrospray ionization tandem mass spectrometry (ESI-MS/MS) is reported, avoiding the usage of derivatization and ion-pairing reagents. This method features the direct injection of samples into an HPLC system, with no sample pretreatment.

The discovery of the non-neural glycinergic transmission components, namely the synthase SHMT, the vesicular transporter VIAAT and the cell membrane transporter GLYT1, by a biological method in the ECSCs is novel, but whether the transmitter glycine itself can be released by the cells and what the dynamic features of the release encompass remain unknown. To determine this, the established and validated analytic method was applied to delineate the characteristics of glycine released into the intercellular space by the cells. It was shown that the ECSCs spontaneously released glycine whereas the negative control NIH/3T3 embryonic fibroblast cells did not.

2. Experimental

2.1. Materials

Glycine, the internal standard (IS) N-methyl-D-aspartic acid (NMDA), and all chemicals used for the preparation of the Krebs-HEPES buffer (KHB, pH 7.4) were purchased from Sigma–Aldrich (St. Louis, MO, USA). The KHB contains: 135 mM NaCl, 5 mM KCl, 0.6 mM $MgSO_4$, 2.5 mM $CaCl_2{\cdot}2H_2O$, 1.3 mM NaH_2PO_4, 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 0.2 mM ascorbic acid and 6 mM glucose. HPLC-grade acetonitrile (ACN), water and formic acid were purchased from MERCK (Darmstadt, Germany). Ammonium formate (A.R.) was purchased from Shanghai Chemical Reagent Co., Ltd. (Shanghai, China). Cell lines of P19 and NIH/3T3 were provide by the Cell Bank of the Chinese Academy of Sciences.

2.2. Preparation of standard stock solutions and working solutions

Standard stock solutions of glycine and IS were prepared as free forms at a concentration of 1.0 mg/mL in HPLC-grade water. All stock standard solutions were stored at −20 °C. The glycine stock solution was stepwise diluted with appropriate amounts of KHB to yield working solutions at 6 different concentrations. The internal standard working solutions were obtained by diluting the stock solutions with acetonitrile.

2.3. Preparation of calibration standards and quality control (QC) samples

Calibration standards were prepared at concentrations of 40, 100, 200, 400, 1000 and 2000 ng/mL by diluting a fixed amount of stock solution of glycine in KHB in tubes. QC samples were prepared in a similar way at concentrations of 100, 400 and 1600 ng/mL. All calibration standards and all QC samples were stored at −70 °C.

2.4. Liquid chromatography–mass spectrometry

LC was performed on an Agilent 1200 HPLC system (Agilent Technologies, CA, USA), and separation was carried out at 30 °C using a Merck ZIC-HILIC column (2.1 mm × 100 mm, 3 μm; Merck Sequant, Umea, Sweden). Isocratic elution was performed. The mobile phase consisted of acetonitrile and water (70:30, v/v, the aqueous phase contained 10 mM ammonium formate and was adjusted to pH 2.8 with formic acid). The flow rate was set at 250 μL/min, and the injection volume was 2 μL. The HPLC system was coupled to an Agilent 6410 triple quadrupole mass spectrometer (Agilent Technologies, CA, USA) using multiple reaction monitoring (MRM). The eluent from the first 1.5 min during the run was diverted to waste to avoid potential contamination and ion suppression in the ion source. An electrospray ionization interface (ESI) in positive ionization mode was used. The ESI source parameters were set as follows: nebulizer gas (N_2) flow, rate 10 L/min; gas temperature, 350 °C; capillary voltage, 4000 V; and nebulizer pressure, 45 psi. MRM mode was used to quantify glycine (transition: *m/z* 76.1 $[M+H]^+ \rightarrow$ 30.1, fragmentor 50 V, collision energy 15 eV) and IS (transition: *m/z* 148 $[M+H]^+ \rightarrow$ 88, fragmentor 60 V, collision energy 15 eV). The analytic data were processed using the MassHunter software package (Agilent Technologies, CA, USA) containing qualitative and quantitative analysis modules.

2.5. Sample preparation procedure

One hundred microliters of sample was mixed with 100 μL acetonitrile containing IS at a concentration of 500 ng/mL. Two microliters of the mixtures was analyzed by LC–MS/MS.

2.6. Method validation

The selectivity was assessed using blank KHB with and without glycine and IS. Calibration curves were constructed using 6 standards in the range of 40–2000 ng/mL. The linearity of the relationship between the peak area ratio and the concentration was demonstrated by the correlation coefficient (R) obtained at the weighing factor of $1/x$. The concentrations of the QC samples were calculated according to the equation of the calibration curve. Intra-day and inter-day precision and accuracy were evaluated by

assaying five replicates of each spiked QC sample at the low, middle and high concentrations on three separate days. Accuracy was expressed as relative error (RE). Precision was expressed as relative standard deviation (RSD). The lower limit of quantification (LLOQ) was defined as the lowest concentration in the calibration curve. The matrix effects were calculated using the response of the glycine–KHB solution divided by that of the glycine–water solution at equivalent concentrations. The stability was investigated at three levels under the following conditions: (1) autosampler stability at room temperature for 24 h, (2) long-term stability at −70 °C for 30 days, and (3) freeze/thaw stability after three freeze/thaw cycles at −70 °C.

2.7. Determination of glycine release from ECSCs and NIH/3T3 cells

P19 ECSCs and NIH/3T3 cells were cultured in cell culture dishes (10 cm diameter) in complete medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% GlutaMAX and 10% fetal bovine serum (FBS). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2/95% air.

After one day of plating on the culture dishes, the attached cells were washed 3 times with 10 mL KHB. Afterwards, the cells were incubated in 8 mL of fresh KHB or potassium chloride enriched-KHB (PCE-KHB, with 50 mM KCl). For the time course experiments, the supernatants (120 μL) comprising the glycine released by the cells were removed for analysis at time points of 2, 3, 5, 10, 15, 30, 60 and 120 min after the fresh KHB was added. An equal volume of fresh KHB or PCE-KHB was added back into the corresponding culture dishes after each sampling. In addition to collection of the supernatants for the analysis of glycine released into the intercellular space by the cells, intracellular glycine was also detected. The cells were lyzed with 5 mL lysate solution (ACN:H_2O = 80:20, v/v) for 20 min to liberate the intracellular glycine for the determination of the glycine remained within the cells. Three independent tests were conducted in duplicate. All samples were stored at −70 °C prior to analysis.

3. Results and discussion

3.1. Optimization of chromatography and mass spectrometry conditions

NMDA, an analog of glycine, was selected as the IS due to its similar physicochemical properties and mass spectrometric behavior with glycine. Compared with the negative mode, positive ESI generates a higher response in the present acidic elution system. As an amino acid, glycine offers ionizable amine functional groups, which are protonated under the acidic elution system. The protonated glycine ion ($[M+H]^+$ 76.1) was selected for further fragmentation. The product ions and other mass parameters were obtained automatically using the MassHunter optimizer software (Version B.03.01, Agilent Technologies, CA, USA). The product mass spectra of glycine and IS are shown in Fig. 1.

The selection of the proper HPLC column is important in the detection of glycine using LC–MS/MS. Because glycine is a small, polar and amphoteric molecule that is difficult to retain in conventional reserved-phase columns, ion pairing reagents or derivatization procedures are typically utilized to increase the retention time. However, most ion pairing reagents are not volatile and inhibit ionization, and the derivatization procedures involve complicated pretreatment and tedious work. Therefore, in our study, a HILIC column, which is suitable for the retention and separation of high polar and hydrophilic compounds, was selected to separate glycine from the interferents. The retentivity and

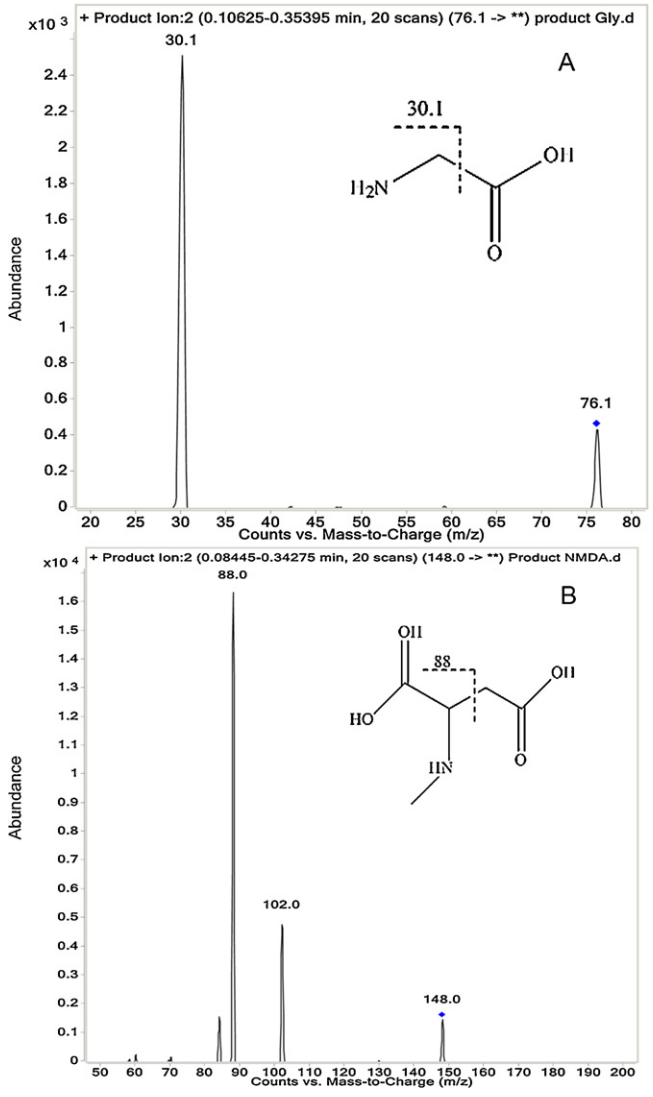

Fig. 1. Collision-induced dissociation mass spectra of glycine (A) and IS (B).

selectivity of HILIC columns can also vary due to the polar stationary phase bonding to different functional groups. In our study, two commercially available columns with different polar stationary phases were tested: Atlantis HILIC Silica column (2.1 mm × 100 mm, 3 μm; Waters, MA, USA) and ZIC-HILIC column (2.1 mm × 100 mm, 3 μm; Merck, Umea, Sweden). The Atlantis HILIC Silica column uses an unmodified silica phase, and the ZIC-HILIC column is zwitterionic with sulfobetaine groups covalently attached to the silica surface. Preliminary testing indicated that glycine more strongly retained on the ZIC-HILIC than on the HILIC Silica column under acidic conditions. This difference might be attributed to an ion-exchange or electrostatic interaction between the negatively charged carboxylic acid and positively charged amino group of the glycine and the negatively charged sulfonate and positively quaternary ammonium of the sulfobetaine group. The pK_a values of glycine are 2.35 and 9.78, which means that glycine mainly exists as a dipole ion and cation in an acidic mobile phase (pH ~3). The scheme of electrostatic interactions is shown as Fig. 2.

In the HILIC mode, the retention of glycine increased with an increase in the acetonitrile proportion. Increasing the amount of acetonitrile enhanced the ionization and sensitivity, but a high proportion of acetonitrile (85%) yielded bad peak shape and long retention time. To balance the peak shape, total run time and

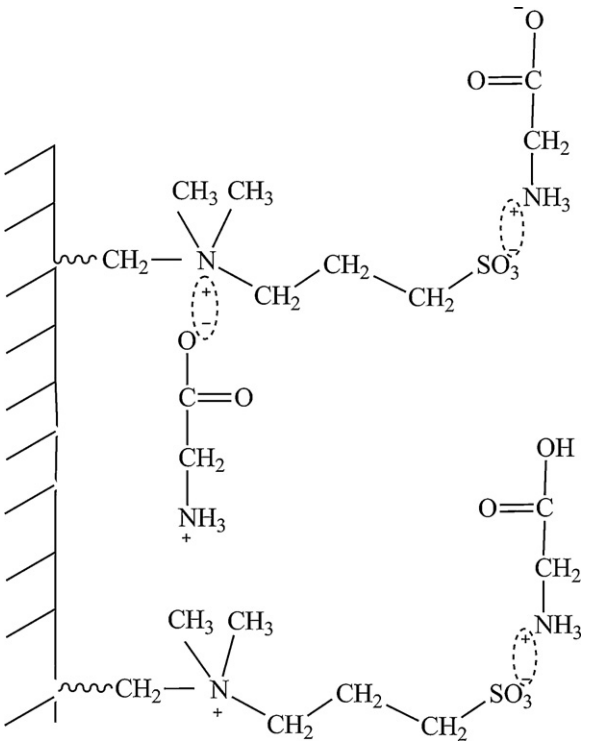

Fig. 2. The ion exchange mechanism of glycine interacting with the stationary phase on the ZIC-HILIC column.

sensitivity, the proportion of mobile phase was optimized and finally set at 70:30 (ACN:H_2O, v/v). Good sensitivity and peak shape and a relatively short run time were achieved with this chromatographic condition.

Then, we investigated the effect of buffer pH on glycine retention. Glycine is an amphoteric compound with an acidic (carboxyl group) and a basic group (amino group), so the mobile phase pH should have significant impact on the retention and selectivity in the HILIC due to the variation in solute (glycine) ionization corresponding to different pH values. In our study, the effect of the mobile phase pH on ZIC-HILIC was investigated by changing the pH of the aqueous buffer solutions before the addition of acetonitrile. Considering the buffering range (2.8–4.8) of the ammonium formate solution, the pH of buffer salt solutions (10 mM ammonium formate, pH ~6.5) was adjusted with formic acid to pH 4.8, 3.8 and 2.8. Further decreases in the pH are not compatible with the ZIC-HILIC column. The retention of glycine on ZIC-HILIC remained unchanged in the above mentioned pH range. We selected a pH value of 2.8 under which the greatest abundance was obtained due to the highest ionization efficiency.

3.2. Method validation

The method was validated with respect to selectivity, linearity, accuracy, precision, LLOQ, matrix effect and stability. The chromatograms of the blank matrix showed no endogenous interference at the retention time of glycine and IS. The representative MRM chromatograms of the blank matrix (KHB), the blank matrix spiked with glycine and IS, and the supernatant sampled at 3 min after P19 cells were cultured by KHB are shown in Fig. 3. The calibration curve exhibited a good linear correlation over the range of 40–2000 ng/mL ($y = 0.0161x + 0.0326$, $R^2 = 0.999$) with a weighting factor of $1/x$. The LOD (limit of detection) of the method was 10 ng/mL (signal-to-noise of 3), and the LLOQ was 40 ng/mL (signal-to-noise > 10) with acceptable precision and accuracy (RE and RSD within 20%). The data for the accuracy and precision of the

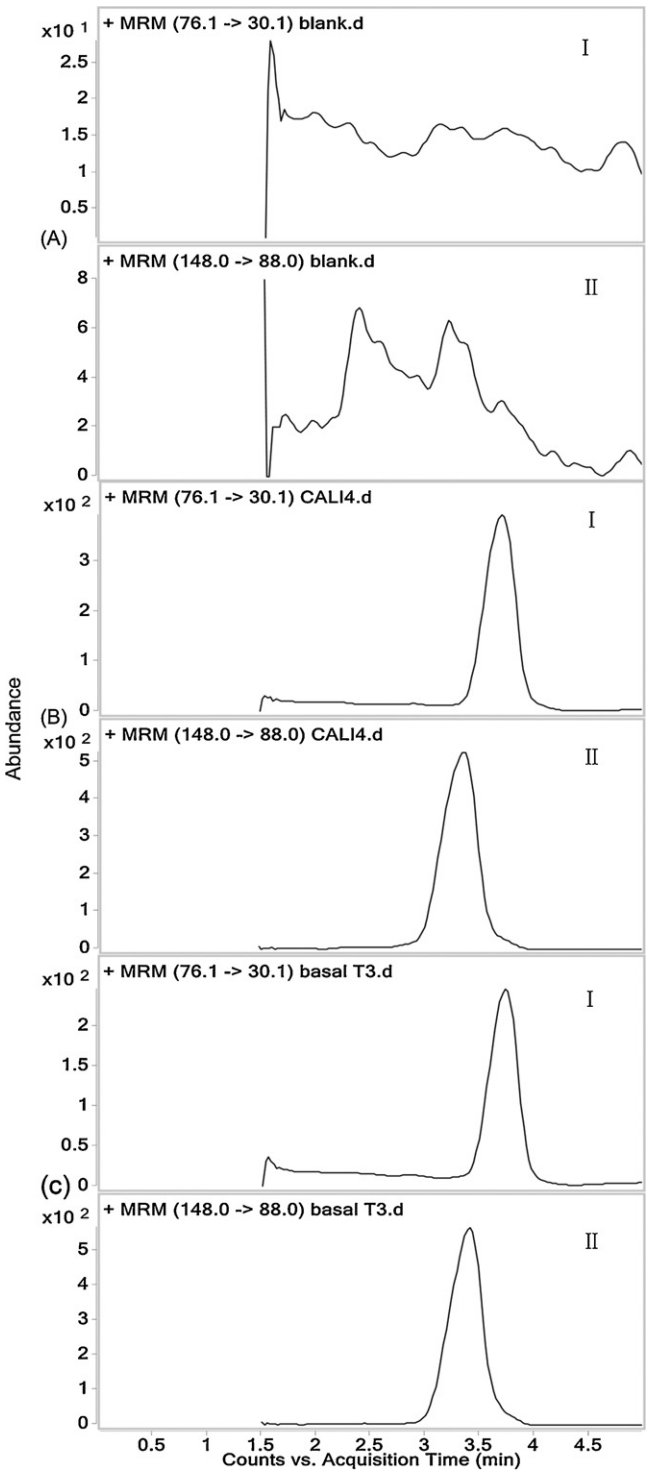

Fig. 3. Representative MRM chromatograms of (A) a blank KHB sample (non-spiked), (B) a blank KHB sample spiked with glycine (400 ng/mL), (C) a sample obtained at 3 min after the ECSCs were cultured in KHB in place of the old complete medium. Peak I refers to glycine and peak II refers to IS.

intra- and inter-assay are summarized in Table 1. The matrix effect was 92.03 ± 5.87% at the low QC concentration, 97.76 ± 1.46% at the middle concentration, and 91.24 ± 1.01% at the high concentration, which indicates that there was no obvious ion suppression under these conditions.

The results for the stability evaluation of the glycine in KHB samples are summarized in Table 2. Glycine was stable in KHB after the autosampler condition for 24 h (recovery was in the range

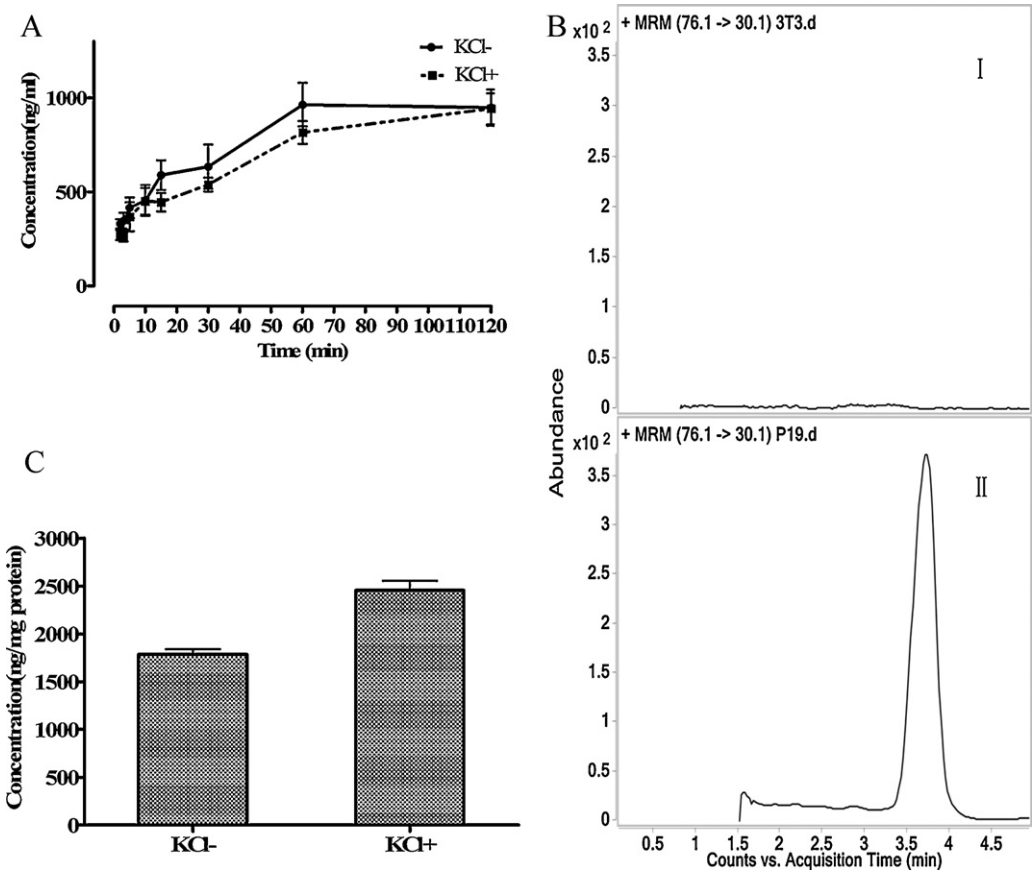

Fig. 4. (A) Time course of the glycine level in the supernatants of the ECSCs with and without KCl introduction ($n = 3$ in duplicate). (B) Representative MRM chromatograms of the supernatants of the NIH/3T3 embryonic fibroblast cells (I) and P19 embryonal carcinoma stem cells (II). (C) Glycine level in the lysates of the cells at the end point of sampling with and without KCl introduction ($n = 3$ in duplicate).

Table 1
The accuracy and precision of intra- and inter-assay ($n = 5$).

	Nominal concentration (ng/mL)	Mean calculated concentration (ng/mL)	RSD (%)	RE (%)
Intra-day	100	107.68	12.42	7.78
	400	395.60	1.78	−1.11
	1600	1644.87	1.38	2.80
Inter-day	100	100.42	11.90	0.42
	400	376.86	5.36	−5.79
	1600	1553.53	4.83	−2.90

of 93.84–94.83%), after storage at −70 °C for 30 days (recovery was in the range of 98.25–105.69%), and after three freeze–thaw cycles (recovery was in the range of 97.08–103.66%). Therefore, the method was evaluated as credible and robust under the experiment conditions.

Table 2
The stability of glycine in KHB samples ($n = 5$).

	Recovery (%)		
	Low	Middle	High
Autosampler condition for 24 h	93.84	94.66	94.83
30 days in KHB below 70 °C	105.69	98.25	104.56
Freeze–thaw 3 times	103.66	97.08	99.78

3.3. Application to the detection of glycine released by ECSCs

The dynamic release of glycine from ECSCs, with or without the high-concentration-potassium treatment after the washout of endogenous glycine in the old cell culture medium, was detected using the established analytic method. The glycine that remained in the cell lysates was also determined. The analysis of lysates agreed with the chromatography and mass spectrometry method described above, despite the distinct matrix. The feasibility was validated in terms of matrix effect, accuracy, and precision. The P19 cells were tested to see if they would react to high-concentration potassium, which can depolarize the excitable cells and stimulate neurotransmitter release [13–15]. After washout of the endogenous glycine in the old cell culture medium, the spontaneous release of glycine from the ECSCs into the fresh KHB gradually increased along the time course, from 331.02 ± 60.36 ng/mL at 2 min in the beginning to 963.52 ± 283.80 ng/mL at 60 min and 948.27 ± 235.09 ng/mL at 120 min, reaching a plateau (Fig. 4A). This indicates that ECSCs consecutively release glycine until achieving equilibration between the release and the reuptake of the compound. In contrast, glycine release from the NIH/3T3 fibroblast cells could not be detected (Fig. 4B-I represents the MRM chromatogram of the supernatant of NIH/3T3 cells, compared with that of the P19 cells as shown in Fig. 4B-II), indicating that the glycine release is specific to the ECSCs. Non-neural cells have been reported to be capable of releasing neurotransmitters such as acetylcholine, GABA and glutamate [16–18]. The fact that ECSCs spontaneously release glycine will improve the understanding of the novel effects of neurotransmitters, including glycine, on non-neural systems.

A high-concentration of potassium evoked no additional glycine release, suggesting the non-excitable characteristic of the cells (Fig. 4A). The glycine concentrations in the lysates were 1784.76 ± 138.21 ng/mg protein and 2455.17 ± 225.22 ng/mg protein for ECSCs with and without the high-concentration-potassium treatment, respectively (Fig. 4C). Less glycine was released into the intercellular space and more remained in the intracellular compartment under the treatment of high-concentration of potassium; this phenomenon needs to be further investigated.

4. Conclusions

A new approach to the analysis of glycine that avoids the usage of derivatization and ion-pairing reagents by employing hydrophilic interaction chromatography (HILIC) coupled with electrospray ionization tandem mass spectrometry (ESI-MS/MS) was developed. This method features the direct injection of samples into an HPLC system with no sample pretreatment and provides enough simplicity, sensibility, precision and accuracy to detect glycine in a high-salt biofluid. The method was applied to determine the dynamic release of glycine into the intercellular space from ECSCs. With minor changes, it may also be used for other biological matrix samples (such as those from microdialysis).

Acknowledgements

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