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Short communication

Rapid and sensitive hydrophilic interaction chromatography/tandem mass spectrometry method for the determination of glycyl-sarcosine in cell homogenates

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ABSTRACT

A rapid, selective and sensitive hydrophilic interaction chromatography/tandem mass spectrometry (HILIC/MS/MS) was developed and validated for the determination of glycyl-sarcosine (Gly-Sar) in Caco-2 cell homogenates. After a simple protein precipitation with acetonitrile, the analyte was separated on a HILIC column and detected by a triple quadrupole mass spectrometry equipped with an electrospray ionization (ESI) source. The method was linear among the concentration range of 1–2000 ng/mL for Gly-Sar and the lower limit of quantification (LLOQ) was 1 ng/mL using as little as 50 μL of cell homogenates. The intra-day and inter-day relative standard deviations (RSD) were less than 15% and the relative errors (RE) were all within ±15%. The validated method was successfully employed in the study of Gly-Sar uptake inhibition in Caco-2 cells by valcytarabine, a potential substrate of the peptide transporter 1 (PEPT1).

1. Introduction

The intestinal peptide transporters play important roles in the absorption of dietary proteins and many peptidomimetic drugs. Numerous studies have suggested that di- and tripeptides as well as a variety of peptidomimetic agents are transported into the intestinal epithelial cells via the proton-coupled peptide transporter 1 (PEPT1) [1–3]. Caco-2 cell is a very popular *in vitro* model to evaluate the intestinal absorption and PEPT1-mediated drug transport. Glycyl-sarcosine (Gly-Sar, Fig. 1) is a hydrophilic dipeptide and is frequently used as a model substrate to study the transport mechanism of PEPT1 substrates in cell models. To determine the concentration of Gly-Sar rapidly, Gly-Sar was usually used in a radionuclide form, such as [^{3}H]Gly-Sar, [^{11}C]Gly-Sar and [^{14}C]Gly-Sar [4–6]. Not only was the radionuclide harmful to the human health, but also it was difficult to obtain the radionuclide because the use and disposal of radionuclide was strictly managed by the government in some countries. Therefore, it was desired to determine Gly-Sar in cell homogenates with a facile, rapid and sensitive quantitative liquid chromatographic method.

Up to date, there has been only one report on the determination of Gly-Sar in cell homogenates using a LC–LC/MS method. In this method, the analytes were separated with a LC–LC method and the sample pretreatment procedure was very complicated [7]. In the present study, we attempted to develop a facile hydrophilic interaction chromatography coupled with tandem mass spectrometry (HILIC–MS/MS) method to determine Gly-Sar in Caco-2 cell homogenates. The validated results showed that this method was rapid, selective and sensitive to determine Gly-Sar in cell homogenates. The lower limit of quantification (LLOQ) was 1 ng/mL with a simple protein precipitation procedure using as little as 50 μL of cell homogenates. Finally, it was successfully applied to Gly-Sar uptake inhibition study after Caco-2 cell was incubated with 10 μM Gly-Sar along with various concentrations (0.1–20 mM) of valcytarabine, a potential substrate of PEPT1 for 15 min.

2. Experimental

2.1. Chemicals and reagents

Gly-Sar (>99.0% purity) was purchased from Sigma (St. Louis, MO, USA). Isoniazid (internal standard, I.S. 99.7% purity) was a

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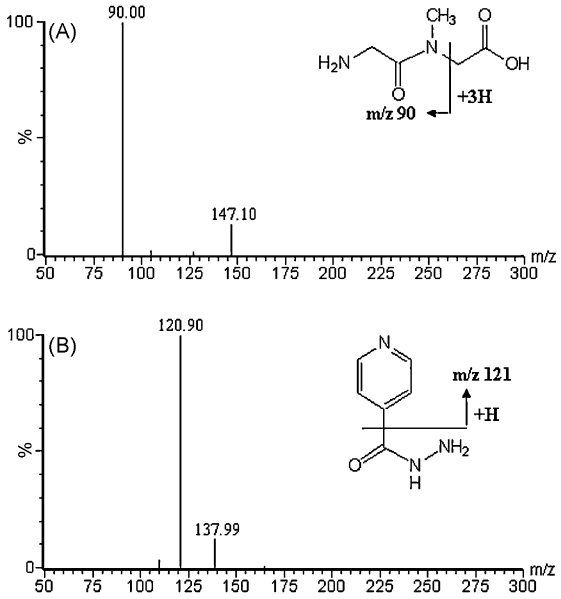

Fig. 1. Product ion mass spectra of $[M+H]^+$ ions of (A) Gly-Sar and (B) isoniazid.

gift from Shenyang Hongqi Pharm. Co. (Shenyang, China). Caco-2 cells were purchased from the American Tissue Culture Collection (Manassas, VA, USA). Blank cell homogenates (final concentration of protein 200 μg/mL) was prepared by sonicating cell samples. Acetonitrile (HPLC-grade) was purchased from Fisher Scientific (Pittsburgh, PA, USA). Formic acid (FA) was purchased from Concord Chemical (Tianjin, China). Water was obtained using an EASYPURE® II RF/UV ultrapure water system (Barnstead International Corp., USA).

2.2. Instrumentation

A Waters ACQUITY TQD system was employed for the determination of Gly-Sar and Isoniazid, which consisted of an ACQUITY UPLC system (Waters Corp., Milford, MA, USA) with cooling autosampler and column oven, and an ACQUITY triple-quadrupole tandem mass spectrometer with an electrospray ionization (ESI) interface (Waters Corp., Milford, MA, USA). All data were acquired and processed by MassLynx 4.1 software with QuanLynx program (Waters Corp., Milford, MA, USA).

2.3. HILIC/MS/MS conditions

The chromatographic separation was performed on an ACQUITY UPLC BEH HILIC column (50 mm × 2.1 mm, 1.7 μm, Waters Corp., Milford, MA, USA). A gradient elution program was conducted with the mobile phase A (water containing 0.1% formic acid), and the mobile phase B (acetonitrile) as follows: 0 (10% A), 2.3 min (90% A), 4.3 min (10% A), 6.5 min (10% A) and finished at 6.5 min. The flow rate was 0.2 mL/min and column temperature was 22 °C. From 1.3 to 3.0 min the eluent was injected into the detector, the remainder was diverted to waste. Injection wash solvents were acetonitrile–water–0.1% formic acid (90:10:0.1, v/v/v) and acetonitrile–water–0.1% formic acid (10:90:0.1, v/v/v) for weak and strong wash, respectively. The MS ionization source conditions were as follows capillary voltage 3.5 kV, cone voltage 20 V, source temperature 110 °C and desolvation temperature 380 °C. The optimized collision energy (CE) was 11 and 14 eV for Gly-Sar and isoniazid, respectively. Nitrogen gas was used as cone and desolvation gas with a flow rate of 50 and 500 L/h, respectively. Argon was used as collision gas at a pressure of approximately 3.35×10^{-3} mbar. MRM was employed for quantitation based on the transitions of protonated molecular ions of Gly-Sar at m/z 147 → 90 and Isoniazid at m/z 138 → 121. The scan time was set at 0.2 s per transition.

2.4. Preparation of standard and quality control samples

A series of standard solution of Gly-Sar at the concentration of 1, 2, 10, 50, 100, 500, 1000 and 2000 ng/mL were prepared with cell homogenates. The working solution of internal standard (4.0 μg/mL of Isoniazid) was prepared with water. Calibration samples were prepared by spiking 50 μL of blank cell homogenates with 50 μL of standard solution and 50 μL of internal standard solution. The quality control (QC) samples at low, medium, high concentration levels were prepared in the similar way. The nominal concentrations of Gly-Sar in QC samples were 2, 500, and 1600 ng/mL, respectively.

2.5. Sample preparation

A 50 μL of internal standard solution (4.0 μg/mL) was added to 50 μL of cell homogenate, followed by 50 μL of water. The sample was vortexed for 1 min and then deproteinized with 400 μL of acetonitrile. The sample mixture was centrifuged at 2500 × *g* for 10 min to remove the protein precipitate. The supernatant was transferred to an autosampler vial at 4 °C, and injected (5 μL) into HILIC/MS/MS system.

2.6. Method validation

The method was validated for selectivity, linearity, accuracy, precision, matrix effect, extraction recovery and stability. Selectivity was assessed by comparing chromatograms of six different batches of blank cell homogenate with the corresponding spiked cell homogenate.

The calibration curve was obtained by plotting the peak-area ratio (y) of Gly-Sar to I.S. against the concentration (x) of Gly-Sar using weighted ($1/x^2$) least-square linear regression, which was expressed in the form of $y = A + Bx$. The linearity was assessed in duplicate on three consecutive days.

Intra- and inter-day precision (the relative standard deviation, RSD) and accuracy (the relative error, RE) were determined by analysis of low (2 ng/mL), medium (500 ng/mL), and high (1600 ng/mL) QC samples ($n = 6$) on three different days.

Matrix effects (ME) were evaluated by comparing the peak areas of analytes in the post-extraction spiked blank cell homogenate (A) at low and high concentration levels with those of corresponding standard solutions (B). The absolute matrix effect was calculated as follows: ME = A/B × 100%. The ME value of 100% indicates that no matrix effect was observed. The value >100% indicates ion enhancement, and <100% is ion suppression.

The extraction recovery was investigated by comparing the mean peak areas of six extracted samples with those of spike-after-extraction samples. To prepare the spike-after-extraction samples, blank cell homogenate was processed according to the sample preparation procedure described above. The supernatant was added with the appropriate standard solutions of Gly-Sar at concentrations corresponding to the final concentration of the extracted samples. The recovery of I.S. was also investigated.

Stability at low and high concentration levels during three freeze–thaw cycles (−80 to 22 °C), on storage in room temperature for 4 h and on storage in polypropylene tubes at −80 °C for 30 days was evaluated. The stability of extracted samples in autosampler vials at 4 °C for 24 h was also evaluated. These analysis results were compared with those obtained from the freshly prepared samples. The analytes were considered stable in the samples when 85–115% of the initial concentrations were found.

2.7. Application of method

Valcytarabine, 5′-*O*-L-valinyl ester prodrug of cytarabine, was a potential substrate of PEPT1. To assess the interaction of valcytarabine with PEPT1, the inhibitory effect of valcytarabine on Gly-Sar uptake by Caco-2 cells was examined. Caco-2 cells were seeded at a density of 1×10^5 cells/cm^2 in 24-well plastic cluster trays and used 15 days after seeding. Caco-2 cells were incubated with 10 μM Gly-Sar along with various prodrug concentrations (0.1–20 mM) for 15 min. The cells were washed three times with ice-cold water, and were transferred to a 1.5-mL tube. The cells were homogenized in 0.3 mL of water by sonicating. The homogenates were centrifuged at $2500 \times g$ for 10 min, and the supernatants were collected and frozen at −80 °C until analysis. Protein concentration in the supernatant was determined with the Coomassie Brilliant Blue assay using a bovine serum albumin as standard. The concentrations of Gly-Sar were determined with HILIC/MS/MS.

3. Results and discussion

3.1. Method development

Gly-Sar had an amino group and a carboxyl group in the molecular structure. It has a stronger mass response under the positive ionization mode than the negative mode. It was the same case for hydrophilic isoniazid. In addition, ESI source provided a better response than APCI source for the two analytes. The product spectrum of the $[M+H]^+$ of Gly-Sar was dependent on the collision energy. The major fragment ion *m/z* 90 was formed at higher collision energy (11 eV) (Fig. 1), and the most abundant ion *m/z* 130 was formed at lower collision energy (6 eV). The transition of *m/z* 147 → 90 gave a higher signal-to-noise (S/N) ratio and better response than that of *m/z* 147 → 130 during the analysis of the spiked cell homogenate samples. As a result, the transition of *m/z* 147 → 90 was selected for MRM analysis. The precursor ion of isoniazid was determined to be the $[M+H]^+$ ion at *m/z* 138; the most intense fragment ion was *m/z* 121 formed by loss of neutral NH_3, which was chosen for MRM analysis.

Sufficient chromatographic retention in the quantitative determination of hydrophilic drug components in biological samples using mass spectrometric detection is highly recommended, in order to avoid possible interferences from drug-related biotransformation products or ionization suppression due to co-eluted endogenous substances [8]. A C_{18} column (ACQUITY UPLC BEH C_{18}, 50 mm × 2.1 mm, 1.7 μm) did not retain Gly-Sar because of the high polarity of the analyte, but a hydrophilic interaction column (ACQUITY UPLC BEH HILIC, 50 mm × 2.1 mm, 1.7 μm) elicited a suitable retention. Additionally, the internal standard, isoniazid, is also a very hydrophilic cationic solute, and has a similar hydrophilic interaction chromatographic behavior to the dipeptide Gly-Sar. The mobile phase was composed of acetonitrile–water (0.1% formic acid) with gradient elution and the presence of a small amount of formic acid was able to improve the detection response of the analytes in the positive ion mode.

3.2. Method validation

Representative chromatograms of a blank, a blank cell homogenates spiked with Gly-Sar (1 ng/mL) and isoniazid (4 μg/mL), a studied sample are shown in Fig. 2. No interferences from endogenous substances with the analyte and the internal standard were detected.

The matrix effects calculated were in the range of 92.9–108.3%, which were within the accepted limits. The typical equation of the calibration curves was as follows: $y = 0.600 + 1.361x$ ($r = 0.9991$),

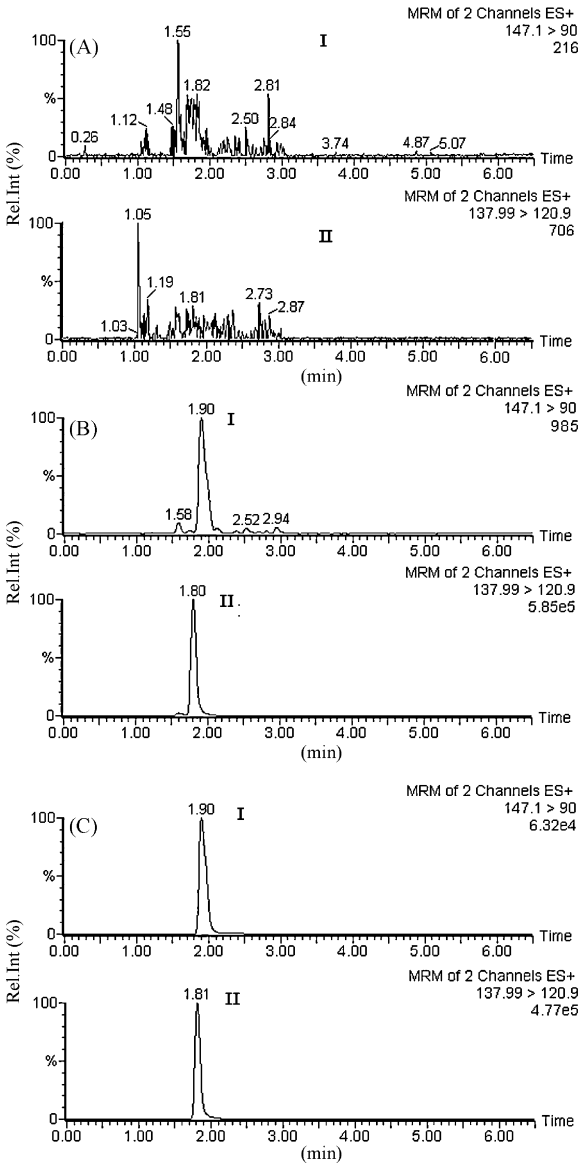

Fig. 2. Representative MRM chromatograms of Gly-Sar (I), isoniazid (I.S., II) in cell homogenates: (A) a blank Caco-2 cells homogenates sample; (B) a blank Caco-2 cells homogenates sample spiked with Gly-Sar (1 ng/mL), isoniazid (4 μg/mL); (C) Caco-2 cells homogenates sample containing 235 ng/mL of Gly-Sar after Caco-2 cells were incubated with 10 μM Gly-Sar along with valcytarabine (2 mM) for 15 min.

where *y* represents the peak area ratio of Gly-Sar to IS and *x* represents the concentration of Gly-Sar in cell homogenates. The correlation coefficient (*r*) exceeded 0.99, showing a good linearity among the concentration range of 1–2000 ng/mL. The lower limit of quantification (LLOQ) was 1 ng/mL for Gly-Sar, which was sensitive enough to determine the low concentration of Gly-Sar in Caco-2 cell homogenates. The intra- and inter-RSD were both less than 15% and the RE were within ±8.2% at LLOQ level, which were within the accepted limits.

Table 1 summarizes the intra- and inter-day precision and accuracy for Gly-Sar in QC samples. The intra- and inter-day RSD were measured to be below 7.1%, and the relative errors were from −5.6% to 2.0%. All the values were within the accepted ranges of ±15% and the method was accurate and precise.

The mean extraction recoveries were 97.8 ± 3.9%, 95.6 ± 4.2%, and 93.1 ± 5.0% for Gly-Sar at 2, 500, and 1600 ng/mL, respectively. The mean recovery of the internal standard was 93.8 ±4.3%.

Table 1
Accuracy and precision for the analysis of Gly-Sar in Caco-2 cell homogenates.

Concentration (ng/mL)		RSD/% (n = 18)		Relative error (%)
Added	Found (mean)	Intra-day	Inter-day	
2	2.04	3.5	7.1	2.0
500	472	6.7	3.8	−5.6
1600	1631	3.9	5.1	1.9

Table 2
Stability data of Gly-Sar in Caco-2 cell homogenates under different conditions (n = 3).

Storage conditions	Added	Found	RSD (%)	RE (%)
Benchtop for 4 h at 23 °C	2	1.96	6.5	−2.0
	1600	1580	4.6	−1.3
Three freeze/thaw cycles	2	2.09	2.8	4.5
	1600	1630	6.3	1.9
Autosampler rack for 24 h at 4 °C	2	2.10	5.7	5.0
	1600	1526	3.8	−4.6
Freezing for 30 days at −80 °C	2	2.16	7.1	8.1
	1600	1634	3.5	2.1

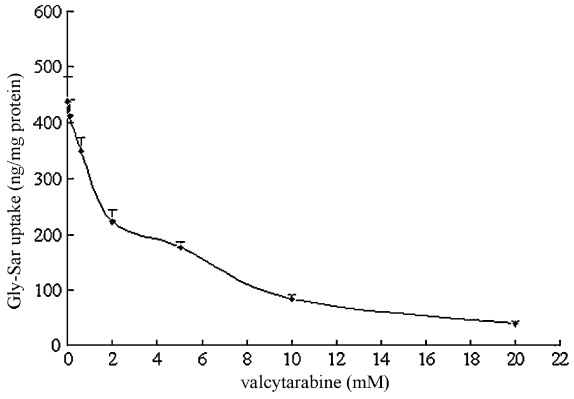

Fig. 3. Effect of valcytarabine on Gly-Sar uptake in Caco-2 cells when Caco-2 cells were incubated with 10 μM Gly-Sar along with various concentrations (0.1–20 mM) of valcytarabine for 15 min.

The stability result of Gly-Sar was summarized in Table 2, showing Gly-Sar was stable in cell homogenates under the storage condition described above.

3.3. Application of the method

The HILIC/MS/MS method was successfully applied to determine the concentration of Gly-Sar in cell homogenates after Caco-2 cells were incubated with 10 μM Gly-Sar along with various concentrations (0.1–20 mM) of valcytarabine for 15 min. The uptake of Gly-Sar was normalized by the cellular protein content of each well. The uptake of Gly-Sar by Caco-2 cells in the presence of valcytarabine is shown in Fig. 3. It is clear that Gly-Sar uptake was inhibited by valcytarabine in a concentration-dependent manner, and valcytarabine competed with Gly-Sar to interact with PEPT1.

4. Conclusion

The present study developed and validated a rapid, specific, and sensitive hydrophilic interaction chromatography/tandem mass spectrometry (HILIC/MS/MS) method for the determination of Gly-Sar in cell homogenates. A HILIC column elicited a suitable retention for the highly polar dipeptide Gly-Sar, and it is very convenient for the analysis of large numbers of cell samples due to simple plasma pretreatment and short analysis time. It has been successfully applied to the inhibition uptake of Gly-Sar in Caco-2 cells by a potential PEPT1 substrate, valcytarabine.

References

[1] H. Daniel, Annu. Rev. Physiol. 66 (2004) 361.
[2] K. Inui, T. Terada, Membrane Transporters as Drug Targets, Academic/Plenum Publishers, New York, 1999, p. 269.
[3] T. Terada, K. Inui, Am. J. Physiol. 273 (1997) 706.
[4] C.P. Landowski, X.Q. Song, P.L. Lorenzi, J.M. Hilfinger, G.L. Amidon, Pharm. Res. 22 (2005) 1510.
[5] N.B. Nabulsi, D.E. Smith, M.R. Kilbourn, Bioorgan. Med. Chem. 3 (2005) 2993.
[6] A. Guo, P. Hu, P.V. Balimane, F.H. Leibach, P.J. Sinko, J. Pharm. Exp. Ther. 289 (1999) 448.
[7] W.S. Putnam, L. Pan, K. Tsutsui, L. Takahashi, L.Z. Benet, Pharm. Res. 19 (2002) 27.
[8] Y.S. Hsieh, C.J. Duncan, M. Liu, J. Chromatogr. B 854 (2007) 8.