

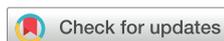
# Development of a UPLC-MS/MS method for the therapeutic monitoring of L-asparaginase

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This study aimed to develop a UPLC-MS/MS method for determining plasma levels of L-aspartic acid and L-asparagine and the activity of L-asparaginase. L-aspartic acid, L-asparagine, and L-aspartic acid-2,3,3-d<sub>3</sub> were extracted from human plasma by protein precipitation with sulfosalicylic acid (30%, v/v). The plasma samples were analyzed using an Imtakt Intrada amino acid analysis column with 25 mM ammonium formate and 0.5% formic acid in acetonitrile as the mobile phase with step gradient method at a flow rate of 0.5 mL/min. The injection volume was 5 µL, and the total run time was 15 min. Inter- and intra-batch accuracies (%) ranged from 96.62–106.0% for L-aspartic acid and 89.85–104.8%, for L-asparagine, and the coefficient of variation (CV%) did not exceed 7%. The validation results for L-aspartic acid and L-asparagine satisfied the specified criterion, however, the results for L-asparaginase activity assay showed a borderline validity. This study could be a foundation for further development of therapeutic drug monitoring systems using UPLC-MS/MS.

## Introduction

L-asparaginase is a therapeutic protein used to treat acute lymphoblastic leukemia (ALL). This enzyme hydrolyzes L-asparagine to L-aspartic acid and ammonia. Normal cells are able to synthesize L-asparagine using asparagine synthetase, thus normal cells are not greatly affected by L-asparaginase. Leukemic cells, however, have low activity or insufficient asparagine synthetase, L-asparagine starvation lead to DNA, RNA and protein synthesis[1] and apoptosis of leukemic cells is induced. [2,3] The purpose of L-asparaginase treatment is to obtain the maximum reduction of asparagine levels in patients.[4]

Activity of L-asparaginase inversely correlated with asparagine concentration and it was used as an assessment to estimate L-asparagine depletion.[4,5] The normal range of circulating asparagine was known as 40-80 µM.[4] Approximately less than 0.1–0.2 µM of asparagine concentration was considered an

asparagine depletion[4,6] although the critical cut-off of asparagine depletion for in vivo leukemic cell death is unknown,[1] and the determination of the levels were related to the lower limit of quantitation of the used methods. Otherwise, the L-asparaginase activity of 0.1 IU/mL was considered a target of L-asparaginase activity treatment for asparagine depletion from several clinical studies.[7-9] In a previous study with pegylated-L-asparaginase administration in adult ALL patients, 0.03 IU/mL was set as a cut-off value for the treatment outcome, and the survival rate of patients with greater than 0.03 IU/mL of L-asparaginase was higher than the patients with less than 0.03 IU/mL.[10]

Various factors affect the therapeutic effect and dose in individual patients including asparaginase formulation, inter-patient variability, antibody formation to asparaginase, and route of administration.[11] Adverse events including hypersensitivities also show large inter-individual variability.[12] Considering the various factors of drug response, therapeutic drug monitoring for L-asparaginase activity is clinically necessary. L-asparaginase activity was a positively correlated with clinical outcome. [10,13,14] Thus, analytical assessments for L-asparagine level and L-asparaginase activities are necessary for monitoring the therapeutic drug concentration.

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## Reviewer

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Several analytical methods for L-asparaginase have been reported using high-performance liquid chromatography (HPLC) with a UV detector, fluorescence detector, or enzyme-linked immunosorbent assay (ELISA).[15-17] Despite the high sensitivity and accuracy of UPLC-MS/MS methods, a lack of developed methods using UPLC-MS/MS have been reported. Thus, the current study aimed to develop an analytical method for quantifying L-aspartic acid and L-asparagine with a normal phase amino acid analysis column and for determining L-asparaginase activities in human plasma using UPLC-MS/MS, which would be applied to the monitoring of the therapeutic drug concentrations of L-asparagine level and L-asparaginase activities.

## Methods

### Reagents

Structures of L-aspartic acid, L-asparagine, and L-aspartic acid-2,3,3-d<sub>3</sub> (internal standard) are presented in Figure 1. L-aspartic acid, L-asparagine, L-aspartic acid-2,3,3-d<sub>3</sub>, acetonitrile, methanol, and hydrogen chloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). Formic acid was purchased from Duksan (Korea). All reagents were of HPLC grade or extra-pure grade. Deionized water was obtained by the Millipore Milli-Q system at 18.2 M $\Omega$  (Bedford, USA).

### Preparation of working solution, quality control samples, and calibration curve standards

L-aspartic acid and L-asparagine stock solutions (20 mM) were dissolved in 1% formic acid solution. The working solution also was prepared using 1% formic acid solution; the mixture was diluted to 19.5, 39.1, 78.1, 156.3, 312.5, 625, and 1250  $\mu$ M, and plasma was spiked at a ratio of 9:1. The internal standard (L-aspartic acid-2,3,3-d<sub>3</sub>) was prepared in 0.1 N hydrogen chloride solution and was diluted in the same manner for the curve sample at 1 mM. All working solutions were stored at 4°C until analysis.

Deionized water was used for preparing quality control (QC) and calibration standards. Because amino acids such as L-aspartic acid, L-asparagine, and L-glutamine are endogenous substrates, they interfere with accurate analysis. Thus, deionized water was used as the surrogate matrix to quantify the levels of L-aspartic acid and L-asparagine in this study. Low concentration (LoQC), medium concentration (MeQC), and high concentration (HiQC) QC samples were prepared by adding 10  $\mu$ L of the working solution to 90  $\mu$ L of deionized water (LoQC, MeQC, and HiQC were 5, 20, and 80  $\mu$ M, respectively). The L-aspartic acid and L-asparagine working solutions were diluted with deionized water for the calibration standards (final concentrations were 1.95, 3.91, 7.81, 15.63, 31.25, 62.5, and 125  $\mu$ M, respectively).

### UPLC-MS/MS conditions

The instruments consisted of an Acquity UPLC (Waters, MA, USA) coupled with a XEVO TQ-MS triple quadrupole tandem mass spectrometer (Waters, MA, USA). Amino acid separation was conducted on an Intradra amino acid analysis column (100  $\times$  3 mm, 3  $\mu$ m, Imtakt Corporation, Kyoto, Japan). For the mobile phase, 25 mM ammonium formate (solvent a) and acetonitrile with 0.5% formic acid (solvent b) were used with the gradient method. The autosampler and column oven temperature were maintained at 10°C and 40°C, respectively. Mass detection was performed in positive ion mode for all the analytes. Multiple reaction monitoring (MRM) mode was used for quantification, m/z 134.0  $\rightarrow$  88.03 for L-aspartic acid, m/z 133.02  $\rightarrow$  73.99 for L-asparagine, and m/z 137.02  $\rightarrow$  90.96 for the IS (L-aspartic acid-2,3,3-d<sub>3</sub>) (Fig. 1). All the results were processed by Masslynx version 4.1 (Waters, MA, USA).

### Method validation

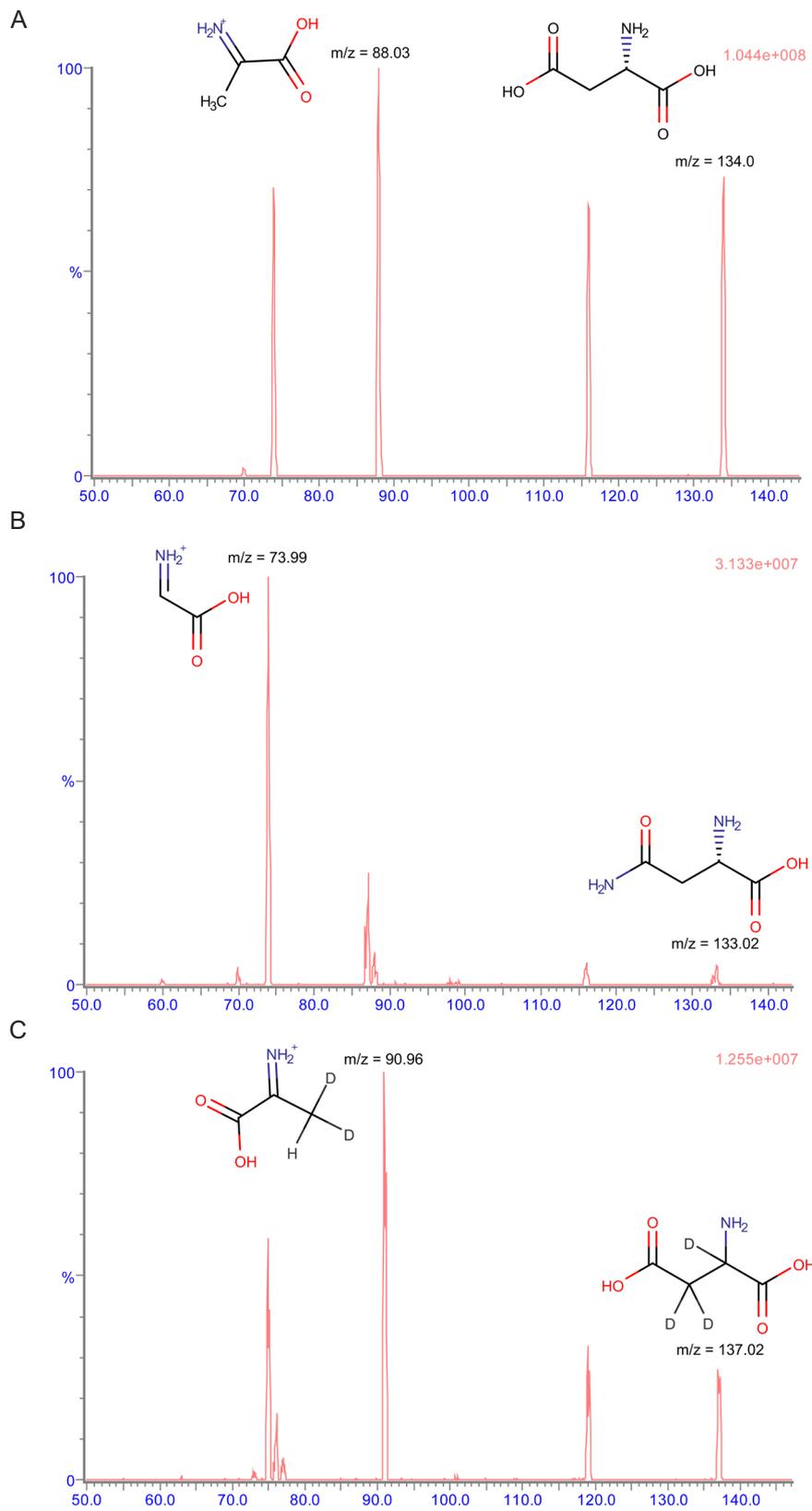
The validity of developed method was confirmed based on "Guideline on Bioanalytical Method Validation" of the Ministry of Food and Drug Safety (MFDS) and "Guidance for Industry Bioanalytical Method Validation" of the U.S Food and Drug Administration (FDA).

Linearity was assessed at an L-aspartic acid and L-asparagine concentration range of 1.95–125  $\mu$ M in the surrogate matrix. A calibration curve weighting factor (1/x<sup>2</sup>) was used to assess whether the correlation coefficient (r) was greater than 0.997 and the coefficient of determination (r<sup>2</sup>) was greater than 0.995.

The accuracy and precision were assessed to measure the LLOQ (lower limit of quantification, 1.95  $\mu$ M), LoQC (5  $\mu$ M), MeQC (20  $\mu$ M), and HiQC (80  $\mu$ M) in five consecutive samples (intra-batch) and three different batches (inter-batch). Accuracy should be within  $\pm$  20% for LLOQ and  $\pm$  15% for other QC samples. Precision should be within the coefficient of variation (CV, %) of less than 15% for other QC samples and less than 20% for LLOQ. Sensitivity was accepted when the signal-to-noise (S/N) was greater than 10 in the LLOQ sample.

Carry-over was assessed by injecting a double blank sample 5 times after ULOQ (upper limit of quantitation) sample injection and comparing whether the peak areas of analytes and internal standard in double blank samples were greater than 20% of the LLOQ and 5% of the IS.

Sample stability was assessed under various conditions. Stock stability (sample incubated at room temperature (18–21°C) for 24 h), freeze-thaw stability (three freeze-thaw cycles at -80°C and ambient temperature), processed sample stability (stored in an autosampler at 10°C), and short-term stability (at 18–21°C for 15 h) were assessed. To evaluate re-injection reproducibility, three LoQC and HiQC samples of the previous day and three newly prepared LoQC and HiQC samples were measured and compared. Freeze-thaw stability, short-term stability, processed sample stability, and re-injection stability were calculated. All stability test results were calculated by comparing with freshly



**Figure 1.** Product ion spectra and fragmentation patterns of (A) L-aspartic acid, (B) L-asparagine, and (C) L-aspartic acid-2,3,3-d<sub>3</sub> (IS) by collision-induced dissociation of the corresponding parent ion.

prepared QC samples. The CV was calculated by following the formula for CV of all the stability samples should be within 15% in the LoQC and HiQC sample.

### L-asparaginase activity assay

The L-asparaginase activity assay was developed based on the previous study by Nath et al. [15] L-asparaginase stock solution (2,000 U/mL) was prepared by completely dissolving Leunase® (10,000 U) in 5 mL of Trizma-HCl (pH 7.4) buffer. L-asparaginase stock solution was serially diluted to a final concentration of 0.1, 0.2, 0.5, 1, 5, 10 and 15 U/mL using drug-free blank plasma. Twenty microliters of L-asparaginase standard samples (0.1, 0.2, 0.5, 1, 5, 10 and 15 U/mL) or blank plasma were added to each polypropylene tube, and 100 µL of 100 mM L-asparagine was added. After incubation at 37°C for 10 minutes, samples were immediately transferred to an ice bath, 250 µL of 30% sulfosalicylic acid (SSA) was added, and the solution was vortexed. Deionized water (250 µL) and 100 µL IS were added and vortexed. Non-incubated standard and blank samples were prepared in the same manner, but 30% sulfosalicylic acid was added as soon as the L-asparagine standard sample or blank plasma was transferred, followed by incubation at room temperature. Finally, the mixture was centrifuged at 13,000 rpm for 5 minutes and 4 µL of the supernatant was injected into the UPLC-MS/MS. The L-aspartic acid produce rate was calculated by dividing the L-aspartic acid concentration difference in non-incubation samples and incubation samples by incubation time using the following formula:

$$\text{L-aspartic acid produce rate } (\mu\text{M}/\text{min}) = \frac{(\text{L-aspartic acid concentration of incubated sample}) - (\text{L-aspartic acid concentration of non-incubated sample})}{\text{incubation time (10 min)}}$$

The activity of L-asparaginase was assessed from the production rate of L-aspartic acid using a standard curve of standard L-asparaginase samples (Fig. 3). The standard curve was fitted by Curve expert version 2.6.3 (Hyams Development, USA).

## Results

### Method validation

The calibration standard presented linearity in the range of seven different concentration points. The range of correlation coefficient and coefficient of determination for L-aspartic acid calibration standard were 0.9993–0.9998 and 0.9987–0.9989, respectively, and those for L-asparagine calibration standard were 0.9981–0.9989 and 0.9963–0.9978, respectively. The results showed sufficient linearity in the L-aspartic acid and L-asparagine concentration ranges of 1.95–125 µM for all the validation batches.

The intra- and inter-day accuracy ranged from 96.62–106.0% and 97.66–105.4%, respectively, and precision ranged from 0.28–2.64% and 1.58–3.86%, respectively for L-aspartic acid. For L-asparagine, the intra- and inter-day accuracy ranged from 89.85–104.8% and 93.98–103.5%, respectively, and precision

**Table 1.** Accuracy and precision of validation QC samples for L-aspartic acid and L-asparagine

Compound			Concentration (µM)			
			1.95	5	20	80
L-aspartic acid	Accuracy (%)	Batch 1	98.97	106.0	101.2	96.69
		Batch 2	102.8	106.0	101.2	96.69
		Batch 3	96.62	104.2	99.63	99.60
		Inter-batch	99.45	105.4	100.7	97.66
	Precision (CV, %)	Batch 1	0.28	1.01	1.76	0.51
		Batch 2	2.05	1.01	1.76	0.51
		Batch 3	0.29	2.38	2.64	0.89
		Inter-batch	3.86	2.08	1.58	1.58
L-asparagine	Accuracy (%)	Batch 1	93.54	104.8	103.9	97.71
		Batch 2	89.85	104.8	103.9	97.71
		Batch 3	98.56	100.8	100.2	97.55
		Inter-batch	93.98	103.5	102.7	97.65
	Precision (CV, %)	Batch 1	0.30	3.47	2.08	2.58
		Batch 2	5.64	3.47	2.08	2.58
		Batch 3	0.28	2.59	4.09	1.76
		Inter-batch	6.46	3.17	2.17	2.17

QC, quality control; CV, coefficient of variance.

ranged from 0.28–5.645 and 2.17–6.46%, respectively (Table 1).

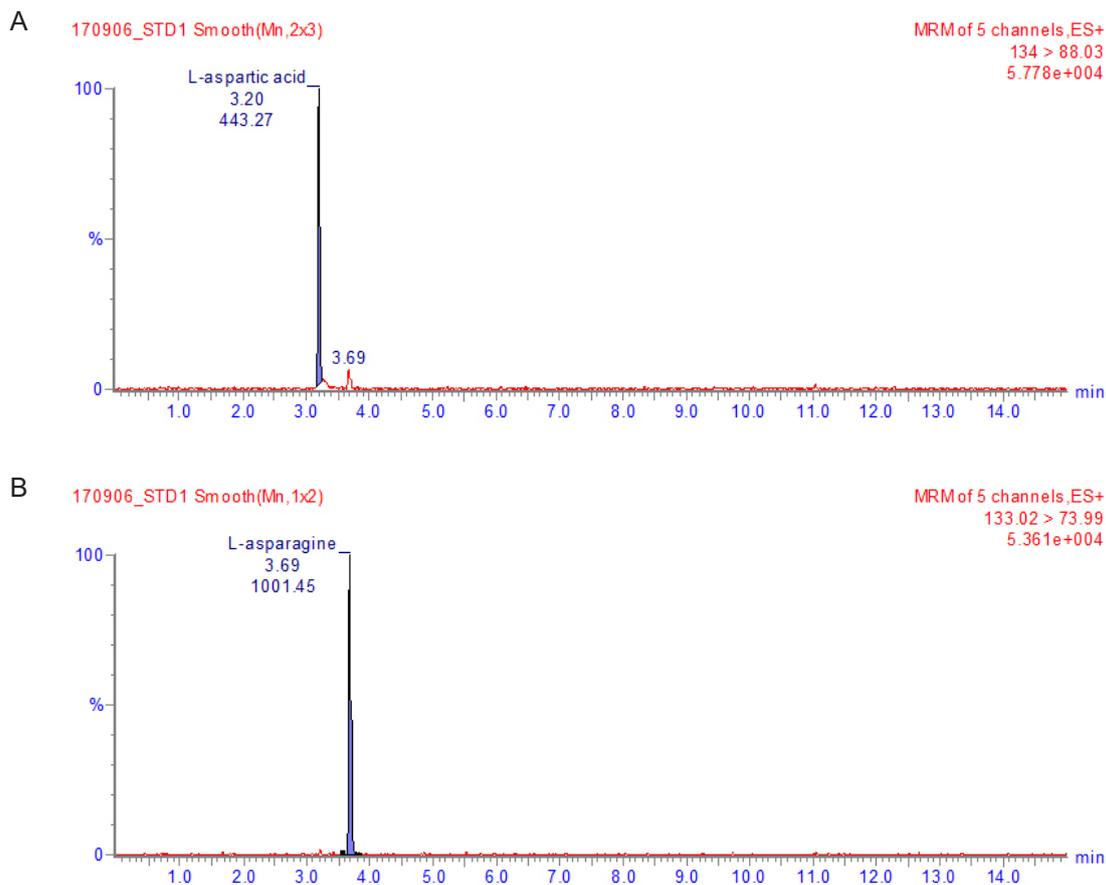
The chromatograms for the LLOQ sample were presented in Figure 2. The signal-to-noise ratios in the LLOQ sample were 443.27 (Fig. 2A) and 1001.45 (Fig. 2B) for L-aspartic acid and L-asparagine, respectively. Five-replicate double blank samples (deionized water) were injected after injecting the ULOQ sample (125  $\mu$ M) to assess carry-over. As a result, an interference

peak was not found in the double blank sample.

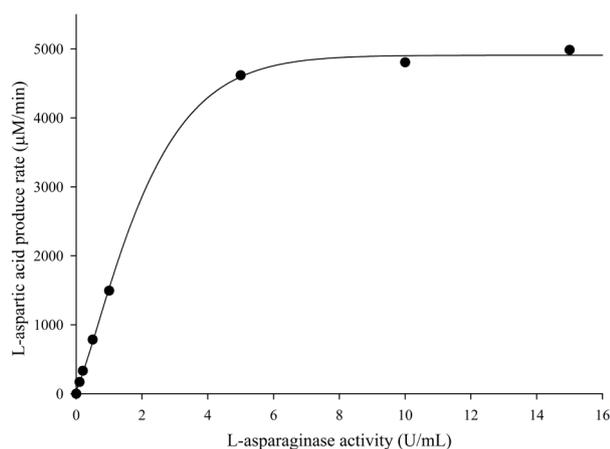
Stock solution short term stability, short-term stability, freeze-thaw stability, processed sample stability, and re-injection stability were evaluated to assess sample stability. Stock solutions of L-aspartic acid, L-asparagine, and internal standards were stable at room temperature for 24 hours. Processed samples were also stable at room temperature for 15 hours. The re-injection was

**Table 2.** Summary of stability for L-aspartic acid and L-asparagine

Compound	Item	Stability at 5 $\mu$ M (%)	Stability at 80 $\mu$ M (%)
L-aspartic acid	Re-inject	2.24	0.64
	Processed sample	0	-0.73
	Freeze-thaw	1.69	1.66
	Short-term	1.06	-0.58
	Stock short-term	-11.6	0.04
L-asparagine	Re-inject	0.87	-1.85
	Processed sample	1.44	-4.60
	Freeze-thaw	0.79	1.99
	Short-term	-0.07	-3.18
	Stock short-term	-3.83	0.61



**Figure 2.** The chromatogram of (A) L-aspartic acid and (B) L-asparagine in lower limit of quantification (LLOQ) sample (LLOQ = 1.95  $\mu$ M).



**Figure 3.** The standard curve of L-asparaginase activity assay.

compared with the results obtained by three-replicate analyses of LoQC and HiQC samples using the samples analyzed the day before (Test) and fresh samples (Reference) (Table 2).

### L-asparaginase activity assay

The developed analytical method for L-aspartic acid and L-asparagine was applied to determine those levels for the L-asparaginase activity. The ranges of accuracy for LoQC, MeQC and HiQC were 91.46-120.12%, 89.36-143.8% and 97.24-146.8% in quantitation of plasma samples for L-asparaginase activity. The L-asparaginase concentration-L-aspartic acid produce rate (L-asparaginase activity) profile is presented in Figure 3. Among the QC samples for the activity assay, 66.7% of QC samples met the criteria, while more than 50% of QC samples per level met the criteria.

### Discussion

An analytical method for L-aspartic acid and L-asparagine without any derivatization was developed using a UPLC-MS/MS. An amino acid column and a deionized water as surrogate matrix were used. L-aspartic acid and L-asparagine are endogenous amino acids and baseline levels could influence the evaluation of the L-asparaginase response. In order to minimize endogenous background interference, several alternative methods, such as background subtraction, removal of analytes, or use of surrogate or artificial matrices could be applied for the analysis.[18-20] The range of calibration was 1.95–125 µM. In this method, the inter- and intra-batch accuracies (%) ranged from 96.62–106.0% for L-aspartic acid and 89.85–104.8%, for L-asparagine, and the coefficient of variation (CV%) did not exceed 7%. For L-asparaginase activity, the total calibration ranges were 0.1–15 U/mL. In the current study, while the analysis methods for L-aspartic acid and L-asparagine levels met the validation criterion, a borderline validity was obtained when assay for determining L-asparaginase activities. The criteria of QC samples were more than 67% of QC samples should be within

± 15% of the nominal, and more than 50% of QC samples per level should be within ± 15% of their nominal.[21] In the study, among the QC samples, 66.7% of QC samples met the criteria, while more than 50% of QC samples per level met the criteria.

The results, especially the calibration ranges in the study, were considered similar to those of previous studies. Previously, the high-performance of liquid chromatography (HPLC) method [15,22] or microplate reader-based method[16,23] was used for pharmacokinetic assessment or therapeutic drug monitoring for L-asparaginase or pegylated L-asparaginase. J. Boos and colleagues used HPLC method to determine the amino acids of L-aspartic acid, L-asparagine, L-glutamic acid, and L-glutamine which were related to the reaction of L-asparaginase. From the quantitation of the amino acids, L-asparaginase activity was calculated in ALL children. The detection limit of amino acid was 0.1 µM and the range of calibration was 0.1–100 µM. The inter-assay and intra-assay coefficients of variations were approximately 10% and 4% with 0.1 µM of asparagine.[4] On the other hand, C. Lanvers et al. determined the L-asparaginase activities using the indooxine method and the Nesslerization method. The total calibration ranges were 2.5–1,250 U/L. The inter-day and intra-day accuracies of three L-asparaginase of ASNASE-*E.coli* HAP-A-1-3, PEG-ASNASE-*E.coli* HAP-A-1-3, and ASNASE-*E.chrysanthemi* were both within 15%. And overall recoveries were approximately 101±9.92%.[24]

The calibration range of L-asparagine was 1.95–125 µM, while the clinical cut-off of L-asparagine depletion was known as 0.1–0.2 µM.[4,6] However, the reference range of normal circulating L-asparagine was 40–80 µM and the baseline level of L-asparagine in the patients was 38.7 µM.[25] Thus, this method could be applied to evaluate the L-asparagine levels in the patients with L-asparaginase administration. This study could be a foundation for further development of therapeutic drug monitoring systems using UPLC-MS/MS. Further studies with ALL patients with adequate sample size and overall evaluations including glutamate and glutamine levels should be conducted, which are known to be related the L-asparaginase activities. [1,4] With the well-developed analytical methods, individual L-asparagine and L-aspartic acid levels would be checked and individual L-asparaginase dosing regimens might be modified in ALL patients after L-asparaginase administrations.

In conclusion, a UPLC-MS/MS method was developed for quantifying L-aspartic acid and L-asparagine in human plasma. The method was applied to determine L-asparaginase activity, however, a borderline validity was obtained. Further evaluation of L-asparaginase activity using UPLC-MS/MS is needed for before the therapeutic application of this method.

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## Conflict of interest

- Authors: All authors declare no competing financial interests.
- Reviewers: Nothing to declare
- Editors: Nothing to declare

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