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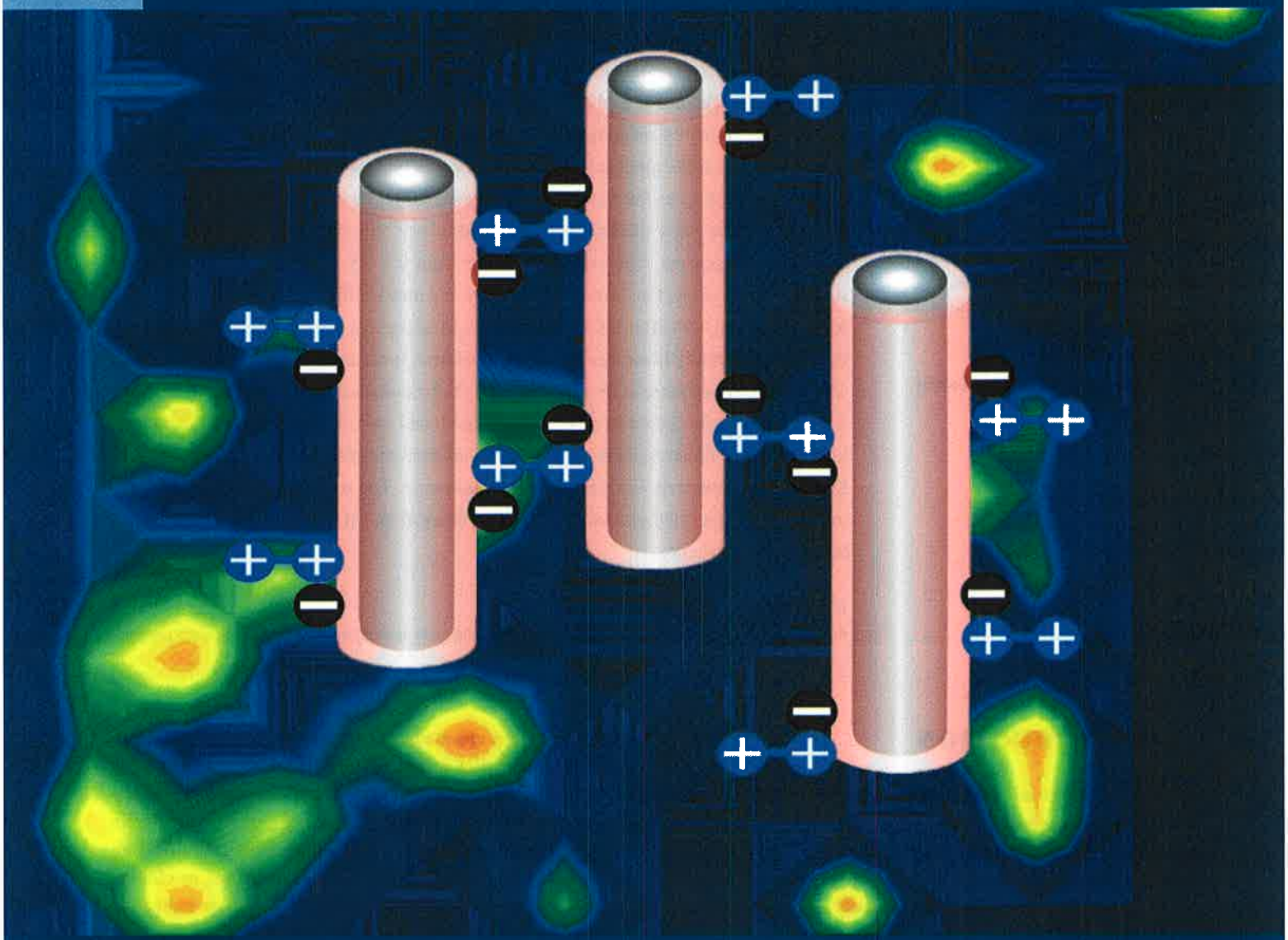

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RESEARCH ARTICLE

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Application of a micro plasma collection card for the detection of homocysteine by liquid chromatography with tandem mass spectrometry

Xiangmei Yuan[1]* | Youli Lu[2,3]* | Can Xiao[2,3] | Jianmin Zhu[2,3] | Wei Zhang[1] | Chen Yu[2,3] | Shuijun Li[2,3]

[1]School of Life Sciences, Shanghai University, Shanghai, P. R. China

[2]Shanghai Xuhui Central Hospital/ Zhongshan-Xuhui Hospital, Fudan University, Shanghai, P. R. China

[3]Shanghai Clinical Center, Chinese Academy of Science, Shanghai, P. R. China

Correspondence
Dr. Shuijun Li, Central Laboratory, Shanghai Xuhui Central Hospital/Zhongshan- Xuhui Hospital, Fudan University/Shanghai Clinical Center, 966 Middle Huaihai Road, Shanghai, 200031, P. R. China.
Email: sjli@scrc.ac.cn

*These authors contributed equally to this work.

Generally, it is recommended that blood samples for homocysteine detection should be centrifuged immediately to separate plasma in order to avoid continuous synthesis by blood cells. The use of a micro plasma collection card may improve sample stability and result accuracy by offering automatic and instant plasma separation. We compare a micro plasma collection method with routine wet plasma to explore applications of the dried plasma spots for homocysteine determination by using liquid chromatography with tandem mass spectrometry. The method was validated for both dried plasma spots and wet plasma. The assay was linear from 0.5–45 μmol/L with good precisions and accuracies. The extraction recovery and matrix effect for dried plasma spots were >97% and 0.98 after internal standard normalization, respectively. It was reproducible for retaining homocysteine in dried plasma spots and kept stable for 30 days. The plasma conversion factor was 7.77 ± 0.7% by calculating the ratio of homocysteine concentration between dried plasma spots and wet plasma (n = 165). Neither hematocrit nor homocysteine concentration affected the plasma conversion factor as long as the hematocrit was within the normal range. The results support the clinical usefulness of the dried plasma spots as a convenient and stable biological matrix for testing homocysteine.

KEYWORDS
dried plasma spots, homocysteine, liquid chromatography with tandem mass spectrometry, micro plasma collection card

1 | INTRODUCTION

Homocysteine is an intermediary metabolite formed during the metabolism of methionine to cysteine. It can be irreversibly catabolized by trans-sulfuration to cysteine or re-methylated to methionine [1]. It is generally accepted as an independent risk factor for atherosclerosis and cardiovascular disease because elevated homocysteine levels may lead to vascular endothelial injury [2]. Furthermore, dysregulation of homocysteine metabolism is also associated with kidney disease, neural tube defects, Alzheimer's disease dementia and osteoporosis [3–7]. Accurate and reliable quantification of homocysteine in plasma samples is essential in clinical practice to detect hyperhomocysteinemia after an ischaemic event or to monitor a possible adjunctive risk factor in higher risk patients [8]. LC–MS/MS is a widely used technique for homocysteine determination with excellent sensitivity and specificity [9–11]. Reliable LC–MS/MS measurements can

Article Related Abbreviations: Σ%RE, cumulative percentage relative error; DBS, dried blood spot; DPS, dried plasma spot; fHcys, free homocysteine; HCT, haematocrit; IS, internal standard; LLOQ, lower limit of quantitation; LoA, limits of agreement; tHcys, total homocysteine

be acquired using small plasma volumes, straightforward sample preparation and short run times [12].

In plasma, homocysteine exists both in oxidized forms including those bound to proteins or thiols and in a reduced form (free homocysteine, fHcys) [13–15]. Storage of plasma may lead to changes in the distribution of thiols, increases in oxidized forms of homocysteine and decreases in fHcys levels. Thus, the determination of total homocysteine (tHcys) is preferred, provided the oxidized homocysteine in plasma is treated with reductants to convert them to tHcys [16]. Similarly, a time- and temperature-dependent release of fHcys from erythrocytes leads to an artificial increase of tHcys in the plasma [17,18]. Thus, it is necessary to separate erythrocytes from blood as soon as possible after blood drawing.

Dried blood spot (DBS) sampling is used to detect homocysteine, especially in newborn screening [19,20]. This usually requires a small volume of whole blood collected on a card without the need for venipuncture and centrifugation, which makes storage and transportation more convenient and simplifies sample preparation [21–23]. However, DBS sample variation or severe alterations in the haematocrit (HCT) may influence the concentration of homocysteine [24,25]. Inconsistent spotting also hampers its wider application in clinical practice. In this manuscript, a commercially available micro plasma collection card was used as a sampling device that could automatically separate blood cells from plasma instantly after blood drawing [26]. Compared to DBS, a constant volume of plasma was collected on the dried plasma spot (DPS) regardless of blood volume and HCT value [26,27]. This technique was first reported by Li et al. using a filter-based DPS card for the quantitative determination of guanfacine in whole blood [28]. It offers an alternative to the direct detection of homocysteine in wet plasma or DBS [29]. In our study, a validation procedure was performed to evaluate the clinical applicability of DPS in homocysteine determination by an established LC–MS/MS method [12]. A plasma conversion formula was used to convert the concentration of homocysteine from DPS to wet plasma [30].

2 | MATERIALS AND METHODS

2.1 | Chemicals, reagents and materials

DL-homocysteine (purity 98%) and trichloroacetic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). The internal standard (IS) DL-homocystine-3,3,3′,3′,4,4,4′,4′-D8 (purity 98%) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). The reducing agent 1,4-dithiothreitol was obtained from Sinopharm Chemical Reagent (Shanghai, China). Formic acid (HPLC grade) was purchased from Anpel Laboratory Technologies Company, Shanghai, China. Methanol (HPLC grade) was purchased from Merck (Darmstadt, Germany). Deionized water was made by a Milli-Q system (Millipore, MA, USA). All other reagents were of analytical grade. Noviplex™ Plasma Prep Cards, packaged by the manufacturer in plastic bags with a desiccant pack, were supplied by Beijing Bio Biotech (Manufactured by Novilytic, West Lafayette, IN, USA).

2.2 | Preparation of the DPS and wet plasma

Blood samples (K_2EDTA anticoagulated) were collected by intravenous puncture from inpatients of Shanghai Xuhui Central Hospital. Each sample was divided into three aliquots, one for the detection of HCT, another for the preparation of DPS, and the last for the preparation of wet plasma. All processes were completed within 2 h. HCT was determined for each sample by fluorescence staining and flow cytometry using a Sysmex XT-4000i Hematology Analyzer (Sysmex, Japan). The DPS card was prepared by dropping approximately 40 μL of whole blood onto the test area of the micro plasma collection card and waited until the control spot turned red. After 3 min, the top layer of the test card was peeled off. The lower layer was dried at an ambient temperature for at least 15 min and then preserved in the original plastic bag packaging with a desiccant pack. The remaining whole blood was centrifuged (4000 rpm for 5 min) within 30 min to separate plasma. Figure 1 shows the workflow of DPS preparation and LC–MS/MS analysis.

In this study, patient samples were collected for the method validation as well as for investigating plasma conversion factor of the DPS and its influencing factors. The use of patient samples were in accordance with the policies of informed consent and acquired the ethical approval of the Ethics Committee of Shanghai Xuhui Central Hospital.

2.3 | Calibration standards

Standard stock solutions of homocysteine and IS were separately prepared at concentrations of 1 mg/mL in water. Calibration standards were used to determine homocysteine concentration in the DPS and wet plasma samples. The final concentrations of the calibrators were 0.5, 1, 5, 15, 30 and 45 μmol/L in diluent. The diluent solution was a substitute of matrix containing a very low level of endogenous homocysteine (< 0.1 μmol/L) that was obtained by precipitating pooled human plasma (tHcys concentration no >10 μmol/L) with two volumes of acetonitrile and then diluting the supernatant three times with water. The signal of homocysteine in diluent solution was monitored at each run, with intensity is less than 20% of LLOQ. Calibration samples were analysed in sextuplicate, and calibration curves were obtained by relating peak area ratios of homocysteine/IS to homocysteine concentrations. Homoscedasticity of calibration data was tested at 95% confidence level. The regression was selected based

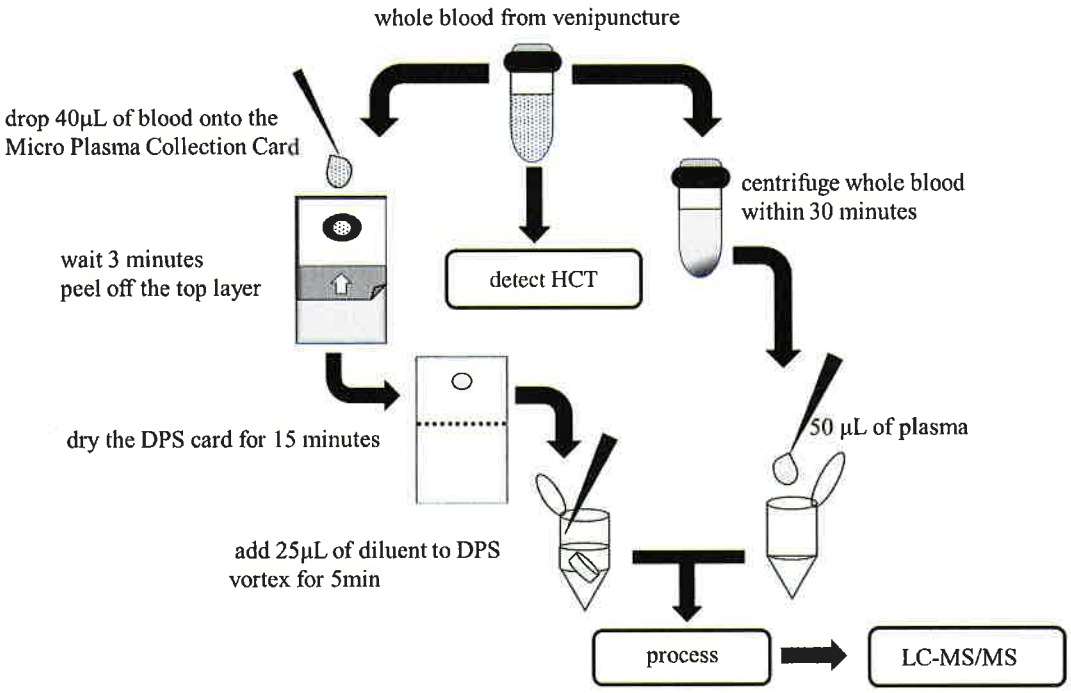

FIGURE 1 Workflow of DPS preparation and sample analysis. Blood drawn from venipuncture was used to prepare DPS and wet plasma as well as detecting hematocrit

on the coefficient of correlation (r) and in the cumulative percentage relative error (Σ%RE) [31,32].

2.4 | Samples processing and LC–MS/MS analysis

Calibration standards and wet plasma were processed as previously described with a slight modification [12]. In brief, 50 μL of samples was mixed with an equivalent volume of the IS solution (5 μmol/L, homocystine-D8) and reducing agent (46 mg/mL 1,4-dithiothreitol). After reduction for 30 min at ambient temperature, samples were precipitated by 50 μL of 15% trichloroacetic acid and centrifuged for 5 min at 16,000 × g. The supernatant was injected and analysed by LC–MS/MS.

After being taken out from the lower layer of the card, DPS was extracted with 25 μL of diluent, and vortexed for 5 min, then added 25 μL mixtures of IS, reducing agent and trichloroacetic acid and vortexed again for 1 min. The following steps were in accordance with the wet plasma process.

The LC–MS/MS system was composed of an API 4000 tandem mass spectrometer (Applied Biosystems, USA) and a Shimadzu LC-20AD liquid chromatography instrument (Shimadzu, Japan) and was controlled by Analyst 1.6.2 software (Applied Biosystems, USA). The mobile phase consisted of 0.02% formic acid in methanol and 0.02% formic acid in water (10:90, v/v). Isocratic elution was carried out at a flow rate of 0.35 mL/min. Chromatographic separation was performed using the Capcell C18 MGIII analytical column (5 μm, 2.0 mm × 100 mm, Shiseido, Japan) and was maintained at ambient temperature (23–25°C). The injection volume was 10 μL.

Ion source was ESI operated in positive mode. Ion source voltage was 5 kV and temperature was maintained at 550°C. The nitrogen ion source gas 1 and gas 2 were both 60 psi. The nitrogen curtain gas was 30 psi, while the collision gas was 8 psi. Quantitative analysis of ion pairs (Q1 to Q3 transition, m/z), which were detected by a multiple reaction monitoring scan, was used for homocysteine (136.3 to 90.1 m/z) and IS (140.3 to 94.1 m/z). The optimized mass spectrometry parameters for declustering potential, collision cell exit potential, collision energy, and entrance potential were 60, 10, 25 and 15 volts, respectively.

2.5 | Recovery and matrix effect

The experiments for evaluating extraction recovery and matrix effects from DPS samples were conducted according to a previous study [33] by using blood from six individuals. Four types of samples were used in these experiments. Sample set A was made by adding 5 μL of the mixed solution of homocysteine (1 or 20 μmol/L) and IS (5 μmol/L) onto DPS samples and dried prior to extraction; sample set B represented the non-spiked corresponding DPS samples that were directly extracted; sample set C were prepared by spiking the neat standard (IS had been reduced before) into the supernatant after DPS extraction; and sample set D were acquired by spiking the neat standard (IS

had been reduced before) into an equivalent volume of solvent. The extraction recovery and matrix effect values for homocysteine were calculated from the absolute peak areas as, *recovery (%) = (A−B)/(C−B)* × 100; and *matrix effect (%) = (C−B)/D* × 100. The values for IS were calculated as *recovery (%) = A/C* × 100; and *matrix effect (%) = C/D* × 100. The same experiments were carried out to evaluate the extraction recovery and matrix effects from wet plasma.

2.6 | Accuracy and precision

Accuracy and precision were determined by replicate analysis of wet plasma and DPS samples spiked with known amounts of homocysteine. For the former, 50 µL of the pooled plasma was spiked with 5 µL of working solutions to give a final homocysteine concentration of 0.5, 3, 20, 40 and 160 µmol/L. For the latter, the DPS from six individuals were extracted, and the extracted solutions were pooled and spiked to give a final concentration of 0.5, 1, 5 and 20 µmol/L. Intra- and inter-assay accuracy were evaluated by assaying samples from three continuous analytical runs. For processing wet plasma samples with homocysteine concentration of 160 µmol/L, 50 µL of samples mixed with 200 µL of the IS solution, 200 µL of the reducing agent. After reduction, samples were precipitated by 200 µL of 15% trichloroacetic acid before analysed. The obtained concentration was multiplied by dilution factor (4) to calculate the actual concentration.

2.7 | Stability

Blood samples from six patients were used to make DPS samples. Both DPS and the remaining blood samples were stored at ambient temperature. The homocysteine stability in DPS and blood samples was measured through time.

2.8 | Determination of plasma conversion factor

Generally, a fixed plasma volume is absorbed by the DPS card, indicating that the analyte content in DPS could be converted to wet plasma concentration if we know the plasma volume absorbed by DPS. In order to measure this volume, the blood samples from six subjects were used to make DPS samples. The plasma volume deposited in the DPS was determined by weighing the card before and after the collection. The linear regression equation was acquired by weighing the wet plasma volume of 1, 2, 5, 10, and 50 µL separately: $y = 0.0011x + 0.0001$, $r = 0.9999$ ($n = 6$) where x represented plasma volume (µL) and y represented plasma mass (g). The plasma volume absorbed by DPS was calculated by this equation.

However, a fixed plasma volume might not be useful. Usually, the conversion factor is concentration ratio rather than volume ratio. It has been reported that the conversion factor is usually compound-dependent and should be determined [30]. In our study, the plasma conversion factor was calculated by the following formula: *Plasma conversion factor (%) = Concentration of compound in DPS/Concentration of compound in wet plasma* × 100. Blood samples from 84 patients with HCT ranging from 25.7 to 65.4% were used to determine the plasma conversion factor. A total of 165 samples was included to investigate the difference and correlation of homocysteine concentration between DPS and wet plasma.

Samples with homocysteine concentrations >45 µmol/L were retested after dilution as above. The obtained concentration was multiplied by dilution factor to calculate the actual concentration.

2.9 | Statistical analysis

Data were processed using Microsoft Excel 2010 (Microsoft, Bellevue, USA) and MedCalc Version 15.2.2 (MedCalc Software, Mariakerke, Belgium), and are presented as the mean ± SD, unless otherwise stated.

3 | RESULTS AND DISCUSSION

3.1 | Linearity, recovery, matrix effect, accuracy, and precision

The chromatographic behaviour of homocysteine in the DPS samples and in wet plasma was consistent, and no obvious interference was observed in the DPS samples (Figure 2). The lower limit of quantitation (LLOQ) in diluent was established at 0.5 µmol/L with signal to noise (S/N) of 40 (Figure 2B). The LOD (S/N ≥ 3) was at 0.10 and 0.19 µmol/L in DPS and wet plasma respectively, which was estimated by the real samples (Figure 2C and D). Calibration data presented significant heteroscedasticity (F = 1583.66; F_{tab} (5, 5; 0.95) = 5.05) and several weighting factors were evaluated. Among them, $1/x^2$ presented the smallest Σ%RE, of 18.18% (Σ%RE of unweighted regression was 107.4%) and was used for all quantitative measurements. The calibration curve was linear in the range of 0.5–45 µmol/L. The linear equation for homocysteine was $y = (0.0428 \pm 0.0012)\ x + (0.000255 \pm 8e\text{-}005)$ ($R^2 = 0.9989 \pm 0.0004$).

Table 1 summarizes the extraction recovery and matrix effects for DPS and wet plasma. The recovery of homocysteine from DPS and wet plasma samples were 77–83.5 and 68–70.2%, respectively, and nearly 97% after IS normalization. During method development, we tried a larger extraction volume (50 µL) which showed a little higher recovery than 25 µL. While considering the response of LLOQ sample (see Figure 2B), we chose 25 µL as the optimal extraction volume. It had a good reproducibility and the signal to noise ratio

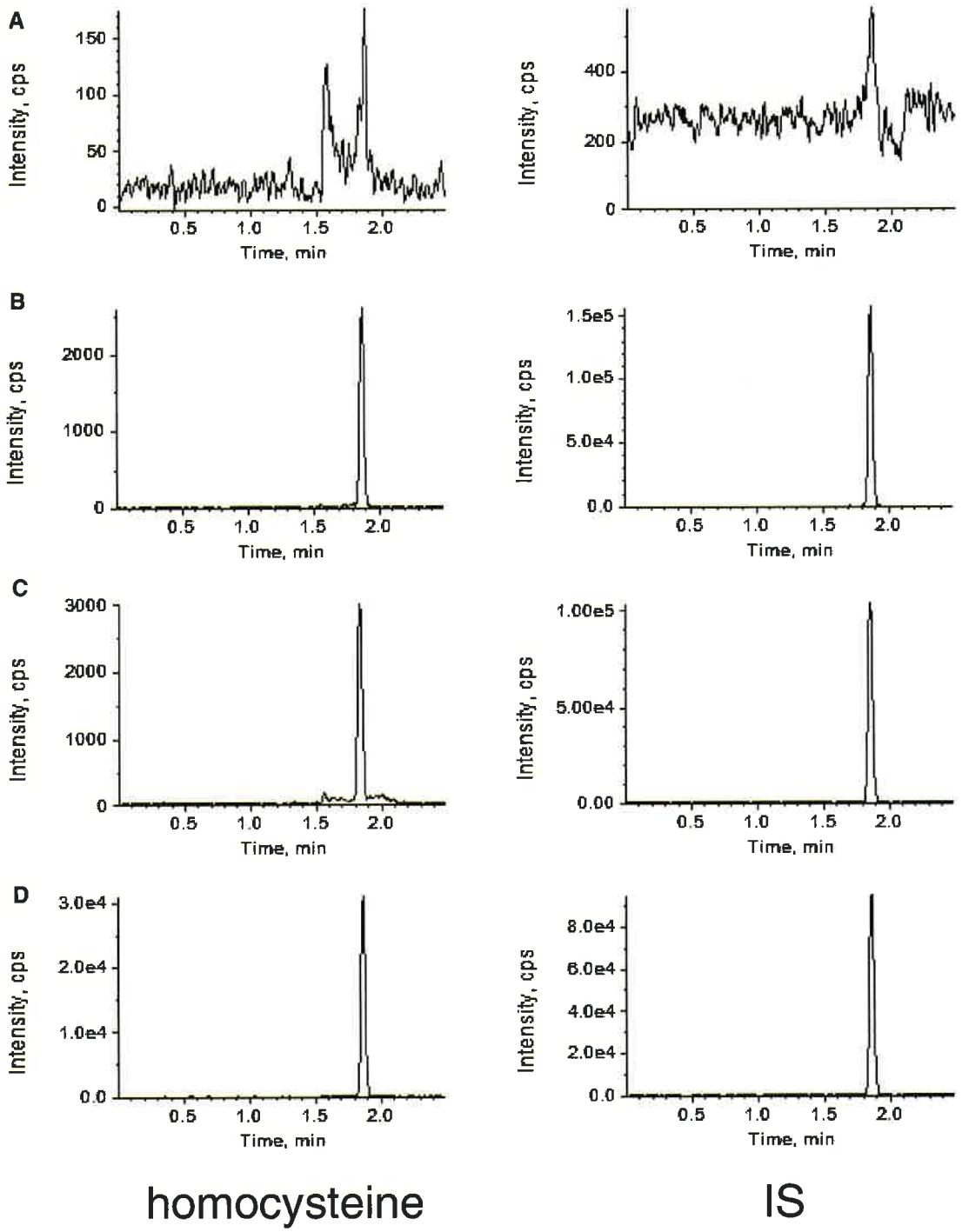

FIGURE 2 Representative chromatograms of homocysteine (*m/z* 136.3 to 90.1) and isotope IS (*m/z* 140.3 to 94.1). (A) Diluent used as substitute of matrix. (B) LLOQ sample prepared by the diluent. (C) DPS (1.02 μmol/L) and (D) wet plasma samples (12.6 μmol/L) were from a same individual

of LLOQ sample was enough for quanification. The matrix effects of homocysteine from DPS and wet plasma samples were in the range of 0.670–0.731 and 0.677–0.688, respectively. After IS normalization, the relative matrix effects were nearly 1. Therefore, the results of both DPS and wet plasma showed that the procedures for the sample processing did not significantly introduce any relative matrix effects to the measurement of homocysteine.

The precision and accuracy of homocysteine measurements in wet plasma and DPS were investigated by spike-recovery experiment which is listed in Table 2. Inter- and intra-assay precisions (%CV) for DPS and wet plasma spiked at four or

TABLE 1 Recovery and matrix effect of homocysteine from DPS and wet plasma samples. ($n = 6$)

Species	Spiked concentration (μmol/L)	Recovery (%)			Matrix effect		
		Homocysteine	IS	IS-normalization	Homocysteine	IS	IS-normalization
DPS	1	83.5 ± 8.9	85.6 ± 3.0	97.5	0.670 ± 0.071	0.683 ± 0.047	0.981
	20	77.0 ± 6.4	78.9 ± 2.5	97.6	0.73[illegible] ± 0.051	0.736 ± 0.057	0.994
Wet plasma	3	70.2 ± 6.1	72.7 ± 8.3	96.6	0.67[illegible] ± 0.055	0.700 ± 0.088	0.967
	40	68.0 ± 6.4	69.9 ± 2.4	97.3	0.68[illegible] ± 0.063	0.709 ± 0.083	0.970

TABLE 2 Intra- and interassay accuracy and precision of wet plasma and DPS samples

Species	Endogenous level (μmol/L)	Spiked concentration (μmol/L)	Intra-assay ($n = 6$)		Inter-assay ($n = 18$)	
			Accuracy (%)	Precision (%CV)	Accuracy (%)	Precision (%CV)
Wet Plasma	15.2 ± 1.1	0.5	101.5	5.3	102.5	5.4
		3	101.3	2.6	98.5	5.0
		20	101.0	3.1	104.6	4.8
		40	98.4	2.2	96.0	3.4
		160	98.9	1.1	97.7	3.1
DPS	1.0 ± 0.1	0.5	95.7	3.5	91.2	3.3
		1	98.7	2.9	93.5	9.9
		5	91.4	2.1	87.8	7.6
		20	98.7	1.6	97.1	4.4

five concentration levels were both <10% whereas inter- and intra-assay accuracies were 87.8–104.6% and 91.4–101.5%, respectively.

3.2 | Reproducibility of the DPS

The preparation of DPS was quick and easy. Plasma penetrated the membrane and reached saturation within 3 min (data not shown). The reproducibility of DPS was determined by assaying six replicates from the same individual ($n = 3$), followed by calculating the percent coefficient of variation (%CV) for each set of replicates. The %CV did not exceed 3.7% when the levels of homocysteine on DPS were at 1.73 ± 0.04, 2.04 ± 0.07 and 8.13 ± 0.28 μmol/L ($n = 6$). These results showed that it is repeatable of sampling by DPS card for determining the concentration of homocysteine in plasma.

3.3 | Stability

When blood was placed at ambient temperature, the plasma concentration of homocysteine increased by 32.2, 49.7, and 108% after 12, 24, and 48 h, respectively. It kept stable in DPS for 30 days compared to those in the whole blood (from six individuals, see Figure 3). The results indicated that DPS could be stored and transported for a longer time than wet plasma at ambient temperature before homocysteine analysis.

3.4 | Plasma conversion factor and influence of HCT

The plasma volume deposited in the DPS card was 2.75 ± 0.15 μL by volumetric weighing. Plasma volume ratio, which was calculated from the ratio of absorbed plasma volume on the DPS to 50 μL of wet plasma volume, was 5.5 ± 0.3%. However, in our study, the plasma conversion factor, which was calculated from the ratio of homocysteine concentration, was 7.77 ± 0.7% (Figure 4A, the data were acquired from the samples of the patients whose HCT were in the range of 32.5–54%, $n = 75$). It seems impossible that the concentration in DPS was higher than that in wet plasma.

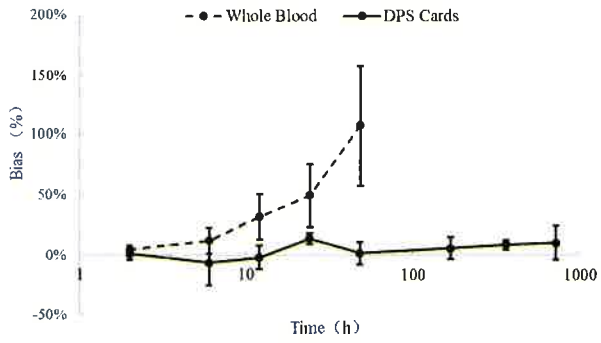

FIGURE 3 Homocysteine stability in DPS and wet plasma at ambient temprature. The concentration of homocysteine increased by 108% after 48 h in whole blood and kept stable in DPS card for 30 days. Data were obtained from six individuals

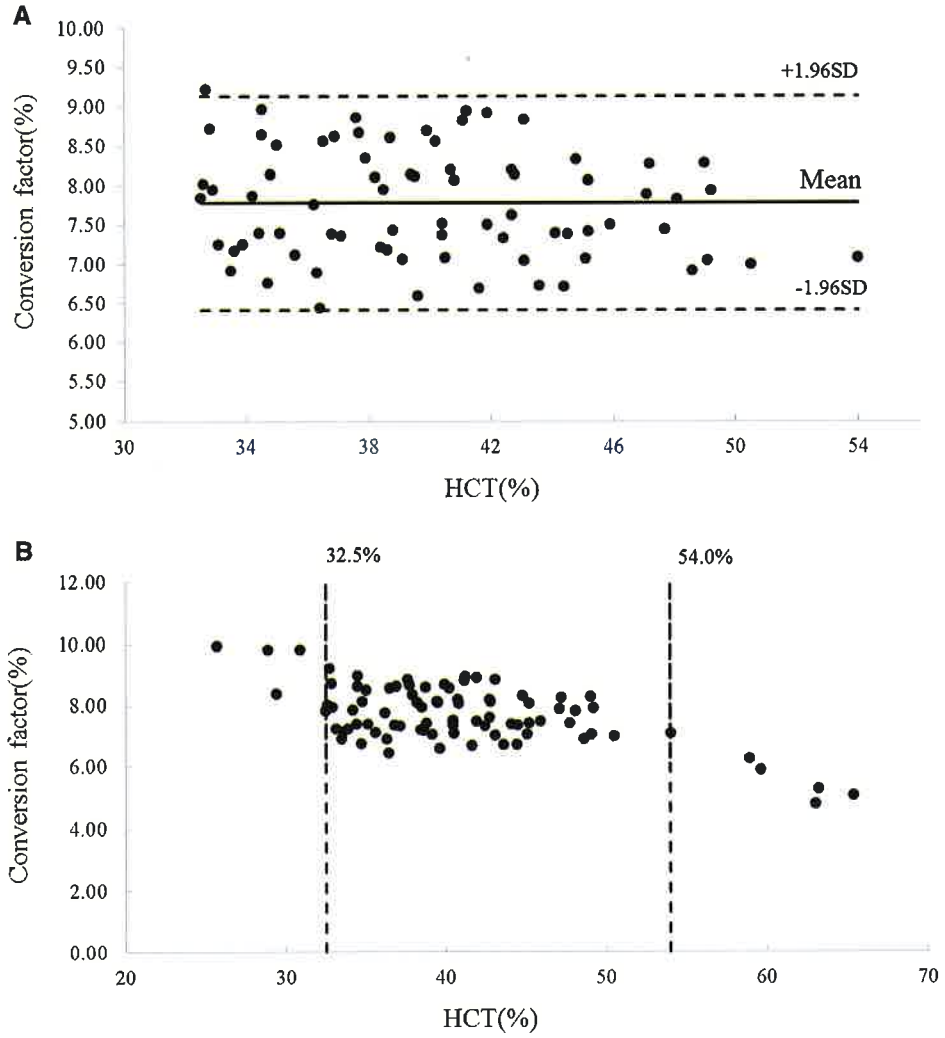

FIGURE 4 Influence of HCT on plasma conversion factor of homocystein. (A) Homocystein plasma conversion factor in the normal range of HCT values (7.77 ± 0.7%, HCT: 32.5–54%, $n = 75$) (B) The distribution of homocystein plasma conversion factor in the abnormal range of HCT values (HCT: 25.7–65.4%, $n = 84$)

Maybe there are more factors are not identified contributing to the volume in DPS card. This was reasonable for the preparation of DPS needs to wait for 3 min before the upper layer was peeled out. During this time, water in the plasma will volatilize (the DPS card was dried within 15 min at room temperature). Besides, the conversion factor is lower sometimes as we had observed on other compounds (data not shown). We also cannot exclude the possibility that recovery of homocysteine from wet plasma is lower.

Previous research indicates that HCT must not be ignored when preparing DPS for steroids determinations [30]. In our research, it was shown that the plasma conversion factor of homocysteine could be ignored when HCT ranged from 32.5 to 54% (Figure 4B), which covers the normal ranges of HCT in humans: 38.0–50.8 and 33.5–45.0% in adult Chinese men and women, respectively [34]. However, when HCT was out of the normal range, the plasma conversion factors of homocysteine could increase or decrease significantly. The possible reason is that HCT might influence the viscosity of the blood, thus affecting the volume absorbed by DPS card [35].

3.5 | Homocysteine concentration in human blood

Plasma conversion factor can be used to convert the compound level on DPS into wet plasma, which was practical for clinical application. The conversion formula was: *Concentration of compound in wet plasma = Concentration of compound in DPS /Plasma conversion factor.* Bland-Altman plot with 95% limits of agreement (LoA) showed that the difference of homocysteine concentration measured by wet plasma and DPS was 0.4% (95% LoA-14.3–15.0%). Only 4/165 (2.4%) of values fell outside the limits of the

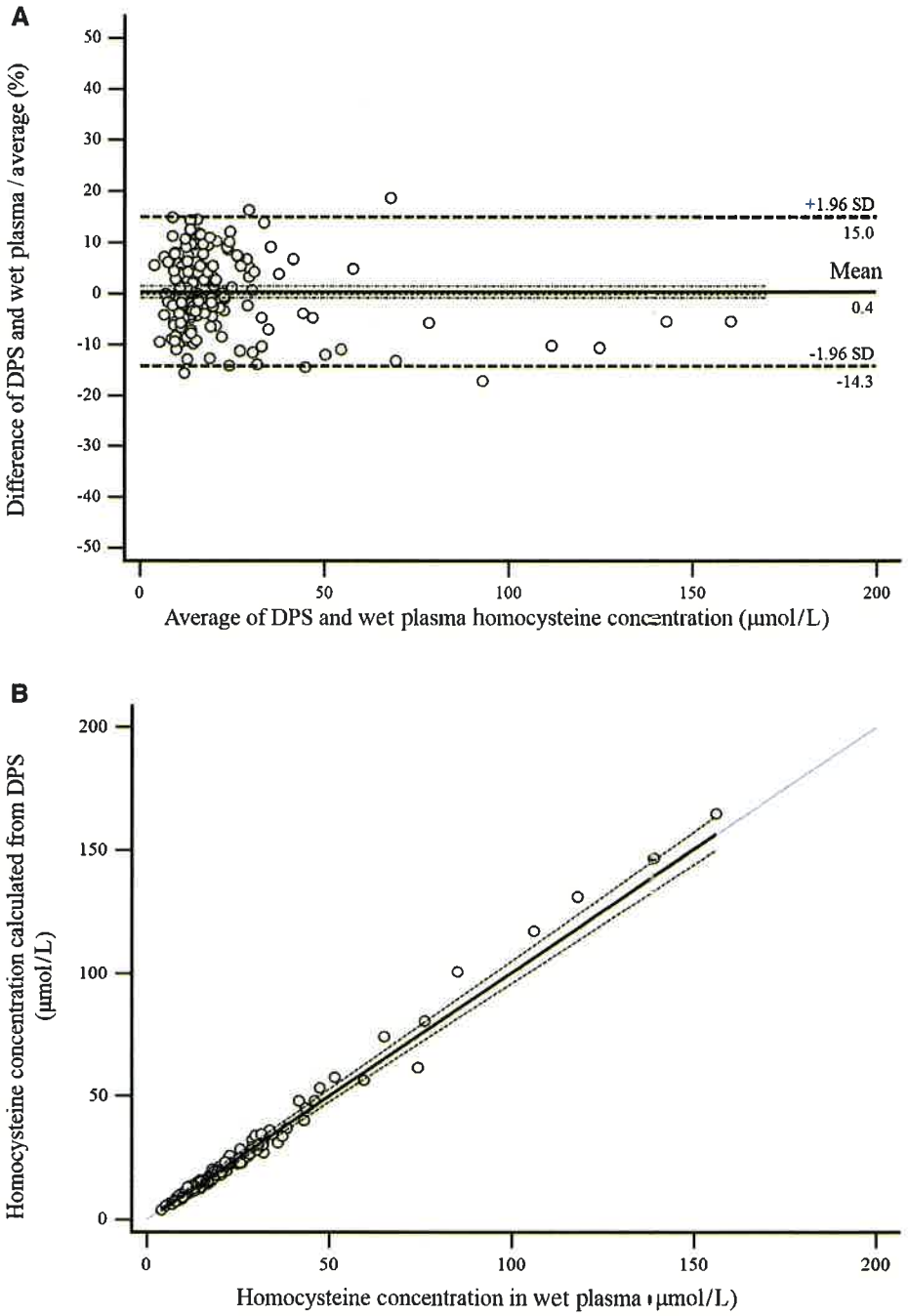

FIGURE 5 Comparison of DPS to wet plasma results of homocysteine. Method comparison consisted of 165 residual plasma samples. (A) Bland-Altman plot of differences. (B) Passing-Bablok regression

agreement (Figure 5A), and fell outside the total error of homocysteine of 15.48%, taking into account the Westgard criteria. Passing-Bablok regression showed no significant linear bias ($P = 0.56$), no constant bias and no proportional bias (Figure 5B), where $y = -0.06$ (95%CI-0.63–0.48) + 1.01 (95%CI 0.97–1.05) x. Hence, the homocysteine concentrations calculated from the DPS showed an excellent correlation with those measured in wet plasma when homocysteine concentrations were <156 µmol/L.

A recent study reported a linear accumulation of cortisol concentrations in EDTA plasma in DPS sampling devices, but the researchers used an external spiking method [30]. In this study, we used real patient samples and the results showed a good consistency in a wide range of concentrations, from normal to medical decision levels. The plasma conversion factor might also be influenced by other compound-dependent factors such as extraction solvent, anticoagulant, and adsorption affinity with plasma or red blood cells. In our study,

only the influence of HCT and compound concentration were investigated.

4 | CONCLUDING REMARKS

In this study, the DPS samples demonstrated acceptable accuracy and precision during quantitative determination of homocysteine by LC–MS/MS. Fixed and trace volumes of plasma samples were absorbed by the DPS card, which offered convenient sample collection and preparation. DPS also displayed obvious advantage in stability compared with wet plasma. Compound-dependent conversion factor was used to convert homocysteine concentration on the DPS to the final plasma level without being influenced by HCT and compound concentration. The described micro plasma collection technique may provide a useful alternative to wet blood or DBS for clinical diagnosis.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

ORCID

Shuijun Li http://orcid.org/0000-0002-8414-9632

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