MAJOR PAPER

1H NMR Spectroscopic Quantification of Plasma Metabolites in Dialysate during Hemodialysis

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We used 1H nuclear magnetic resonance (NMR) spectroscopy to assess metabolic responses in patients undergoing hemodialysis (HD). We collected 71 samples of plasma and dialysate from 10 patients before, during, and after HD. We used the dialysate as a possible substitute for blood plasma to quantify small metabolites by 1H NMR. We confirmed TSP (sodium 3-(trimethylsilyl) propionate 2, 2, 3, 3-d4) as a reference of NMR intensity in dialysate. We examined TSP sensitivities in various dialysate spectra and the correlation between signal intensities and added quantities of TSP. We used integrations of signal areas on 1H NMR spectra of plasma and dialysate to quantify concentrations of creatinine, lactate, alanine, and valine and calculate their ratios between plasma and dialysate. The ratios of metabolites in plasma to dialysate were 3.2 ± 0.4 (creatinine), 3.6 ± 0.5 (valine), 3.8 ± 0.7 (alanine), and 4.0 ± 0.8 (lactate) mM (mean ± standard deviation [SD]). The broader distributions of ratios in levels of lactate and alanine suggested their de novo production during the HD session. Estimation of blood metabolite levels using dialysate is useful for quantitative analysis of metabolic status in blood during HD.

Keywords: alanine, creatinine, lactate, metabolomics, valine

Introduction

In our previous study to determine levels of metabolites in plasma samples before and after hemodialysis (HD), 1H nuclear magnetic resonance (NMR) metabolomics demonstrated increased levels of lactate after HD in some patients, which suggests impaired energy metabolism.1

In the present study, we used 1H NMR spectroscopy to measure levels of metabolites and simultaneously monitor behaviors of metabolites in plasma and dialysate during HD. We also tried to estimate the concentration ratios of metabolites in plasma to dialysate. In dialysate, the low-molecular-weight metabolites in plasma filtered out by membrane can be stably quantitated by 1H NMR metabolomics without interference by proteins that are problematic in plasma.2 Moreover, anemia in patients undergoing HD limits collections of blood samples, but dialysate is sufficient to collect samples during HD. Determining levels of metabolites in dialysate may allow estimation of plasma levels for more convenient quantitative analysis of metabolites in the body.

We have shown that TSP (sodium 3-(trimethylsilyl) propionate 2, 2, 3, 3-d4) is a poor intensity reference in plasma because of the adsorption to plasma proteins.3 Recent high performance membranes remove not only small sized molecules but also middle-sized ones,4 such as β2-microglobulin and others.5 These middle sized molecules may also adsorb TSP, resulting in an appropriate intensity marker.

We have examined the feasibility of using of TSP as a quantitative standard of dialysate and the relationship of metabolite concentrations between blood and dialysate during HD.
Materials and Methods

Patients

The ethics committee of Koujinkai Hospital (Koujinkai Hemodialysis Clinic, Miyagi, Japan) approved the study protocol, and we obtained written consent from all patients. We enrolled 10 patients undergoing hemodialysis (3 men; aged 66 ± 5 years old; HD duration, 25 ± 11 years, range 6 to 36 years). Patients underwent dialysis using type IV membranes for 4 hours 3 times a week. Six patients used citrate buffer (0.7 mM), and 4 patients used acetate buffer (8 mM), both of which contain glucose (8 to 12 mM). The flow rates were 500 mL/min for dialysate and 215 ± 5 mL/min for blood. The Kt/V values expressing dialysis adequacy were 1.62 ± 0.19, estimated by the single-pool model.6

Sample collection and preparation

Table 1 summarizes sample collections and preparations for NMR in the following 5 experiments. Experiment 2 used samples collected from 10 patients 15 min after the start of HD and in Experiment 5, samples collected at various times during HD. Experiments 1, 3, and 4 used samples from one female patient (60 years old, duration of HD, 29 years). In Experiment 3, the sample was divided into 5 parts, and TSP was added to each of the 5 in different concentrations.

Venous blood samples were collected into lithium heparin tubes, and dialysate samples were transferred into standard plastic tubes. Samples were stored at −25°C and thawed at room temperature immediately before use. For NMR measurement, to 200 μL of plasma samples with 400 μL of the so-called NMR cocktail (saline, NaN3 and 50 μL D2O) were added TSP and calcium formate, and to dialysate samples of 550 μL were added D2O 50 μL and TSP or calcium formate (Table 1). Ten minutes after centrifugation of the sample at 13,000 rpm, we transferred the supernatant into a 5-mm NMR tube.

Quantitative NMR spectroscopy of plasma and dialysate

We recorded single-pulse 1H NMR spectra using a 600-MHz NMR spectrometer (ECA600, JEOL Ltd., Tokyo, Japan). We suppressed the water signal by a presaturation and set the flip angle to 90°. Sixty four scans were collected for 64 K data points with a spectrum width of 9 kHz. Repetition times (TR) were set at 37.4 s for plasma and 42.4 s for dialysate because the T1 of the formate signal (8.47 ppm) in plasma was 6.5 s and that of dialysate was 7.8 s.

Quantification of signals on the spectra

Free induction decays were processed using Alice2 for Windows software (V. 6.0, JEOL Ltd.). Metabolites in plasma were quantified by integrations of signal areas against calcium formate as a standard. The present method was verified in plasma, previously.3 In dialysate spectra, quantification of peak was referenced to calcium formate in Experiments 1 to 3 and to TSP in Experiment 4 and 5.

Results

Experiment 1: Representative spectra

Figure 1 shows one-dimensional (1D) spectra of plasma and dialysate from a representative patient 60 min after the start of HD using citrate dialysate buffer. The high density of albumin gives the plasma spectrum a curved baseline; thick signals are lipids, and sharp signals are small metabolites and

<table>
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<th>Experiments</th>
<th>Results</th>
<th>No. of patients</th>
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<th>(final conc. in sample) mM</th>
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Table 1. Sample collection and preparations for 5 experiments

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include high creatinine peaks characteristic of patients with kidney disease. Signal assignments are presented elsewhere. The dialysate spectrum appears to contain no appreciable lipids and proteins. Peak integrations ensure analytical confidence because of the flat baseline, and every small metabolite can be quantified by an adequate internal standard. As shown, signals of citrate and glucose, components of dialysate buffer, are distinct in the dialysate spectrum. To both plasma and dialysate samples 0.5 mM TSP and 1 mM calcium formate were added. The area of signal of calcium formate was adjusted to be equal for both spectra; the smaller TSP intensity signal shown in the plasma spectrum indicate heavy adsorption of TSP to interfering molecules in plasma.

Presence of some sharp signals in only the dialysate suggests they are hidden by a macromolecular mixture in plasma. For example, subtle peaks of pyruvate and acetoacetate are detected clearly on the dialysate spectrum but not on the plasma spectrum.

**Experiment 2: TSP sensitivities in various patients**

Dialysate samples were collected 15 min from the start of HD sessions because more molecules flow into the dialysate earlier in HD. We prepared the samples from 10 patients for NMR measurement by adding calcium formate and TSP as delineated in Table 1. Figure 2 shows TSP signal sensitivities calculated with calcium formate as a quantitation standard. We examined the samples of 10 patients with various plasma levels of albumin (Fig. 2a) and \( \beta_2 \)-microglobulin (Fig. 2b). The results show constant values of TSP signals values among patients. The squares of correlation were \( R^2 = 0.01 \) for albumin concentrations and 0.03 for \( \beta_2 \)-microglobulin concentrations.

**Experiment 3: TSP linearity in addition**

We obtained one dialysate sample 15 min after the beginning of HD from a patient with relatively higher plasma levels of albumin (3.9 g/dL) and \( \beta_2 \)-microglobulin (32 mg/L) and divided the sample into 5 tubes. We then added TSP to each of the 5 tubes to final concentrations of 0.1, 0.2, 0.3, 0.5, and 0.7 mM, and added to give equal final concentration of 1 mM of the calcium formate. On each of the 5 spectra, TSP signal was quantified by its peak integral with calcium formate as a quantitation standard. Figure 3 shows the correlation between detected TSP signal intensities versus the added...
Fig. 2. TSP (sodium 3-(trimethylsilyl) propionate 2, 2, 3, 3-\textsuperscript{d}_4) signal sensitivities in various dialysates. We examined TSP signal sensitivities with calcium formate as a quantitation standard on the spectra of dialysate in 10 patients with various plasma levels of albumin and \(\beta_2\)-microglobulin (\(\beta_2\)-MG). Dialysate samples were collected 15 min after beginning of hemodialysis sessions.

Fig. 3. Correlation between concentrations of TSP (sodium 3-(trimethylsilyl) propionate 2, 2, 3, 3-\textsuperscript{d}_4) added and the TSP signal areas calculated on dialysate spectra. On the 5 spectra, the TSP signal was quantified with the calcium formate as a quantitation standard.

Fig. 4. A sample time course of creatinine concentrations calculated on the spectra of plasma and dialysate. ○'s are plasma levels (left axis) and □'s are dialysate levels (right axis) of creatinine. Before and after hemodialysis (HD), indicate time 0 and 4 hr from the start of HD, respectively, when the values of only plasma levels of creatinine are depicted. The broken line shows plasma levels of creatinine. The ratios of levels of creatinine between plasma and dialysate at one, 2, and 3 hr after initiation of HD were the same 3.5.

Experiment 4: A time course of creatinine

Figure 4 shows the quantification of a time course of creatinine. Before and after HD, we calculated the values on the plasma spectra, and we calculated concentrations on the plasma and dialysate spectra at 3 other intermediate points of 1, 2, and 3 hours after beginning of HD. Near the begin-
Table 2. Statistics of the ratio histograms for 4 metabolites between plasma and dialysate. The respective 30 spectra of plasma and dialysate, indicated as N, were used. CVs, coefficients of variation.

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<th>Ala</th>
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<tr>
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<tr>
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</tr>
<tr>
<td>CV</td>
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<td>0.18</td>
<td>0.20</td>
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Fig. 5. (a) Histogram of the concentration ratios for the sum of the 4 metabolites (creatinine, valine, alanine and lactate) between plasma and dialysate. Triangle in the histogram depicts the mean that is 3.5. The standard deviation (SD) of the histogram is 0.5. (b) Histograms of ratios of concentrations between plasma and dialysate for creatinine, valine, alanine, and lactate. Triangle in each histogram depicts the mean.

Experiment 5: Quantifications of metabolites on the spectra

We collected 10 samples of plasma and dialysate from 10 patients simultaneously at every 3 points of 1, 2, and 3 hr after the beginning of HD. We measured a total of 30 plasma and 30 dialysate spectra on which we quantified areas of creatinine, alanine, valine, and lactate signals.

On the 30 pair spectra of plasma and dialysate, we then calculated the ratios of each of the 4 metabolites in plasma to dialysate (a total of 120 ratios calculated). Figure 5(a) shows histograms of the ratios; the mean ratio was 3.5, the same as described in Experiment 4. However, rather than a single sharp peak, there were wide distributions with tails toward higher values. We then separately examined the histogram of each metabolite. Figure 5(b) and Table 2 summarize the histogram analysis. The mean and standard deviation (SD) of concentration ratios of creatinine was 3.2 ± 0.4, the smallest of the 4 molecules. The ratio histogram of lactate concentration indicated a higher value of 4.0 ± 0.8 and a broad distribution with tails toward higher values. The ratio of valine was 3.6 ± 0.5, and that of alanine was 3.8 ± 0.7.

Discussion

Dialysate for HD analysis is collected noninva-
sively from patients, and its collection over the course of an HD session provides more detailed information regarding metabolites than that obtained by plasma measurement before or after HD. The use of dialysate also offers accurate quantitation of all small metabolites by NMR. On the other hand, mixed relaxation effect, such as from inhomogeneous magnetic field, chemical exchange, and cross relaxation, has prevented acquisition of quantitative profiles of small molecules using NMR pulse techniques for diminishing macromolecular signals, such as the CPMG (Carr–Purcell–Meiboom–Gill) spectrum of plasma.8

Our experiments showed no appreciable interaction of TSP with molecules in dialysate, which demonstrates TSP to be an excellent internal standard. Formate, the only good quantitation standard when plasma is used, should not be used as a standard in dialysate because it may be a biomarker related to kidney disease.9,10 However, we have demonstrated dialysate as a suitable substitute for plasma with the concentration ratios of respective metabolites, and to give information-rich data.

As Fig. 5(b) shows, the creatinine ratio histogram demonstrated single-peak distribution. Creatinine, a representative marker of HD therapy, behaves following a single-pool model without production in the body during HD.6 On the other hand, the mean values of the ratio histograms of the other 3 metabolites were larger than that of creatinine; which indicated their production from the body during HD. Table 2 also shows coefficients of variation (CVs) in ratio concentrations, which ranged from 0.14 for creatinine and 0.2 for lactate. In our results, the CV of 14% for creatinine was comparable value to routinely available biochemical analysis for measuring blood creatinine. Thus, the level of creatinine in dialysate was evaluated to be a good substitute to that level in plasma. The same is for valine, which had the same CV value as creatinine. The values of 20% for lactate and 18% for alanine indicated greater variations in concentration ratios than those for creatinine and valine.

In several cases, we have found drastic increases in levels of lactate in dialysate during HD (data not shown), which suggested de novo production by the body. HD therapies rapidly remove electrolytes, water, and small molecules including bioactive necessities as well as uremic toxins from the blood. In response, there is presumably some reaction to maintain homeostasis in the blood, that may require the production of some metabolites from the body, such as that shown by lactate, alanine, and valine. These responses contribute to the distribution of these metabolites toward higher values in the ratio histograms in Fig. 5(b) and larger values of their means in Table 2.

Our study has several limitations. We did not consider other major metabolites of glucose, citrate, and acetate because they were contained in original dialysate buffers as described in Material and Method. The ratios of metabolite concentrations between plasma and dialysate estimated here actually depend on the conditions of HD, such as the rate of blood flow or water removal. We collected samples in regular HD sessions, when patients were in stable condition.

In regular HD sessions, we could assess plasma levels of metabolites by NMR measurement of dialysate within 20% errors. Dialysate could be a first choice for easy and noninvasive measurement by NMR, with direct measurement in plasma in each HD condition in the case of some abnormal data. Further time course investigation of these metabolite behaviors during HD will provide important information for monitoring therapy and diagnosing each patient’s metabolic status.

Conclusion

NMR measurement of dialysate has shown its suitability as a substitute for plasma when TSP is used as a concentration standard and quantification of metabolites in plasma is adapted by adequate ratios dependent upon the metabolites. The metabolite profiles in dialysate over the course of an HD session will offer important information regarding metabolic status of patients undergoing hemodialysis.

Acknowledgements

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References

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